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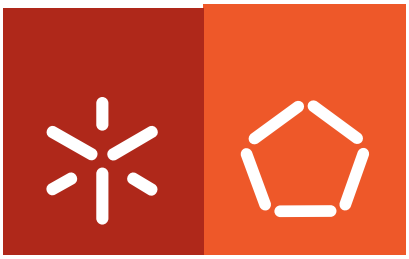

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Mycobiota and aflatoxigenic profile of Portuguese almonds and chestnuts from production to commercialisation

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Chemical and Biological Engineering

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Assinatura:

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RESEARCH LEADING TO THE MOLECULAR BIOLOGY STUDIES USED FOR THE AUTHENTICATION OF STRAINS RECEIVED FUNDING FROM THE EUROPEAN COMMUNITY'S SEVENTH FRAMEWORK PROGRAM (FP7, 2007/2013), RESEARCH INFRASTRUCTURES ACTION, UNDER GRANT AGREEMENT NO. FP7-228310 (EMBARC PROJECT).

ABSTRACT

Aflatoxin (AF) contamination of nuts is an increasing concern to the consumer's health. Portugal is a big producer of almonds and chestnuts, but there is no scientific knowledge on the safety of those nuts. AFs B_1, B_2, G_1 and G_2 are produced mainly by some species of *Aspergillus* belonging to section *Flavi*, which is composed of a large number of very closely related species. While these species are difficult to differentiate morphologically and even genetically, they differ in a characteristic that is of paramount importance for food safety, as only some are responsible for the production of the highly toxigenic AFs. Taxonomy and species identification are therefore subject of great interest, and the establishment of schemes for species and for aflatoxigenic strains identification that are simultaneously accurate, sensitive, robust and expedite is mandatory.

This work had three major goals: the first was to provide knowledge on the general mycobiota, aflatoxigenic fungi and AF contamination of Portuguese almonds and chestnuts, and its evolution throughout the various stages of production (field, storage and processing). For this matter, 45 chestnut samples were collected from orchards from Trás-os-Montes. Forty-seven almond samples were collected in Trás-os-Montes at different stages of production: field, storage and processing. All fungi belonging to genus *Aspergillus* were isolated and identified to the section level, and all isolates belonging to section *Flavi* were further tested for their aflatoxigenic ability. Fungi representative of other genera were identified to the genus level. Almond samples were tested for AF contamination.

The mycobiota of almonds and chestnut was found to vary in terms of both matrix and stage of production. Chestnuts were mainly contaminated with the genera *Fusarium*, *Cladosporium*, *Alternaria* and *Penicillium*, and the genus *Aspergillus* was only rarely found, whereas almonds were more contaminated with *Aspergillus*. No *Aspergillus* section *Flavi* were isolated from chestnuts. In almonds, *Fusarium*, *Cladosporium*, *Alternaria* and *Penicillium* decreased from field to the end of processing, whereas *Aspergillus* increased significantly, including those from section *Flavi*. In total, 352 fungi belonging to section *Flavi* were isolated from Portuguese almonds, of which 231 isolates (66%) were aflatoxigenic. Even so, only one sample from storage was found to be contaminated with AFs (4.97 µg/kg) at a level below the maximum levels recently imposed by the Commission Regulation (EU) No 165/2010.

The second goal of this work was to characterise and identify the isolates of *Aspergillus* section *Flavi* by applying a polyphasic approach including classic phenotypic and molecular methods as well as the innovative technology protein spectral analysis Matrix-Assisted Laser Desorption/Ionisation-Time of Flight Intact-Cell Mass Spectrometry (MALDI-TOF ICMS), and to devise accurate and sensitive schemes for species identification. For the morphological analysis, fungi were cultured on different media and were characterised for several macro and micro morphological features. Morphological analysis was complemented with biochemical analyses, which consisted of determining the extrolite profiles relative to AFs and cyclopiazonic acid. A group of selected isolates was identified molecularly based on the sequencing of the ITS region and partial calmodulin gene. Spectral analysis was made by MALDI-TOF ICMS to obtain spectra of protein masses. Dendrograms of relatedness were obtained for each set of data and used to compare sensitivity and accurateness of the different approaches.

From the preliminary morphological analysis, three morphotypes were identified: as "*A. flavus* morphotype" (36.4% of the isolates), "*A. parasiticus* morphotype" (55.4%), and "*A. tamarii* morphotype" (8.2%). The 3 morphotypes were then divided into 9 phenotypes based on their extrolite profile. Genotypic and spectral analyses clustered the selected isolates into the same 3 groups created by morphological analysis. Furthermore, all sets of data, including the morphological complemented with extrolite profile, were able to further resolve the isolates into

more restrictive clusters. They all positioned two of the 9 phenotypes in two unidentified terminal clades closely related to *A. parasiticus*.
The third goal was to test a molecular method based on multiplex PCR and RT-PCR for the ability to differentiate aflatoxigenic and non-aflatoxigenic isolates. Two genes of the AF biosynthetic pathway, *aflD* (= *nor1*) and *aflQ* (= *ord1*= *ordA*), were tested for presence and expression (by PCR and RT-PCR, respectively). The presence of both genes did not correlate with aflatoxigenicity. In terms of gene expression, *aflD* was not considered a good marker for differentiating aflatoxigenic from non-aflatoxigenic isolates, but *aflQ* showed a good correlation between expression and AF-production ability.
In conclusion, Portuguese almonds and chestnuts seem to be generally safe in terms of AF contamination. Nevertheless, the majority of the isolates of *Aspergillus* section *Flavi* obtained from Portuguese almonds was found to be aflatoxigenic, which may constitute a problem in terms of food safety if storage and processing conditions are not effectively controlled. At present, these conditions seem to be guaranteed, since only one almond sample was found to be contaminated. At the species identification level, good agreement was obtained between the 3 methods of analysis since they all generated similar dendrograms with concordant strain clustering. Morphological analysis has shown sensitive and reliable as a preliminary method for species identification only when complemented with the extrolite profile. The calmodulin gene showed to be more robust and reliable as genomic marker for this group of fungi than the ITS region, providing good DNA barcoding potential. MALDI-TOF ICMS results confirmed that this technique is highly reliable for fungal identification, and is faster and less expensive in terms of labour and consumables when compared with other biological techniques, which is essential whenever there is a paucity of characters for defining many fungal species and when high numbers of isolates are involved. Expression analysis of the *aflQ* gene seems to be a good method for the differentiation of aflatoxigenic and non-aflatoxigenic isolates.

KEYWORDS

Almonds, chestnuts, aflatoxins, aflatoxigenic fungi, *Aspergillus* section *Flavi*, MALDI-TOF ICMS.

SUMÁRIO

A contaminação com aflatoxinas (AFs) dos frutos de casca rija é um problema com interesse crescente no que respeita à saúde do consumidor. A castanha e a amêndoa são produtos agrícolas de elevado interesse económico para Portugal, no entanto não existe conhecimento científico quanto à sua segurança em termos de AFs. As AFs B_1, B_2, G_1 e G_2 são micotoxinas de elevado grau toxigénico, e são produzidas por algumas espécies de *Aspergillus* secção *Flavi*. Esta secção integra um elevado número de espécies muito próximas, tanto ao nível morfológico como molecular, mas que diferem numa característica de elevado interesse para a segurança alimentar - a sua capacidade para produzir AFs. Por esta razão, a taxonomia e identificação de espécies desta secção revestem-se de grande interesse, pelo que o estabelecimento de esquemas de identificação simultaneamente precisos, sensíveis, robustos e expeditos é imperioso.

O presente trabalho teve três objectivos principais. O primeiro objectivo foi obter informação sobre a incidência de fungos filamentosos, com particular incidência sobre os fungos produtores de AFs, e sobre a contaminação com AFs das amêndoas e castanhas portuguesas, e sua evolução ao longo das várias fases de produção. Neste sentido, foram analisadas 45 amostras de castanha colhidas em soutos de Trás-os-Montes e 47 amostras de amêndoa de Trás-os-Montes e Algarve colhidas em diferentes fases de produção (campo, armazenamento e processamento). Todos os fungos do género *Aspergillus* foram isolados e identificados até à secção, e todos os fungos pertencentes à secção *Flavi* foram identificados até à espécie e caracterizados quanto à sua capacidade aflatoxigénica. Fungos representativos de outros géneros foram identificados apenas até ao género. As amostras de amêndoa foram ainda analisadas quanto à contaminação com AFs. A micobiota das amêndoas e castanhas variou em termos de matriz e de fase de produção. As castanhas mostraram contaminação dominada pelos géneros *Fusarium*, *Cladosporium*, *Alternaria* e *Penicillium*, sendo o género *Aspergillus* encontrado com pouca frequência, enquanto nas amêndoas o género *Aspergillus* foi detectado com elevada incidência. Não foi isolado qualquer fungo da secção *Flavi* de castanhas. Nas amêndoas, os géneros *Fusarium*, *Cladosporium*, *Alternaria* e *Penicillium* diminuiram progressivamente desde o campo até ao final do processamento, enquanto a incidência de *Aspergillus*, incluindo a secção *Flavi*, aumentou. No total das amostras de amêndoa foram isolados 352 fungos pertencentes à secção *Flavi*, dos quais 66% eram aflatoxigénicos. No entanto, apenas foi identificada uma amostra contaminada com níveis detectáveis de AFs (4,97 µg/kg), mas inferiores aos níveis máximos impostos pelo Regulamento (CE) Nº 165/2010 da Comissão Europeia.

O segundo objectivo do presente trabalho foi caracterizar e identificar os isolamentos de *Aspergillus* secção *Flavi* através de uma abordagem polifásica incluindo a caracterização fenotípica clássica e molecular, assim como a inovadora tecnologia de análise espectral de proteínas *Matrix-Assisted Laser Desorption/Ionisation-Time of Flight Intact-Cell Mass Spectrometry* (MALDI-TOF ICMS), e delinear esquemas robustos e simultaneamente sensíveis de identificação de espécies. A análise macro e micromorfológica em diferentes condições de cultura foi complementada com a análise de extrólitos, nomeadamente AFs e ácido ciclopiazónico (CPA). Um grupo de 24 fungos foi identificado molecularmente pela análise de sequências da região ITS e do gene da calmodulina. A análise espectral por MALDI-TOF ICMS foi aplicada a 69 fungos. Foi construído um dendrograma de similaridade para cada um dos grupos de dados e os resultados foram comparados em termos de precisão, robustez e sensibilidade.

Na análise morfológica preliminar foram identificados três morfotipos distintos designados "morfotipo *A. flavus*" (36,4% dos isolamentos), "morfotipo *A. parasiticus*" (55,4%), e "morfotipo *A. tamarii*" (8,2%). Estes morfotipos foram posteriormente divididos em nove fenótipos, com base no seu perfil de extrólitos. As análises genotípica e espectral criaram três grupos (clades)

correspondentes aos obtidos na análise morfológica e posicionaram dois dos nove fenótipos em clades terminais relativos a *taxa* não identificados.
O terceiro objectivo deste trabalho foi testar um método baseado em PCR multiplex e RT-PCR para diferenciação de estirpes aflatoxigénicas e não-aflatoxigénicas. Para tal, foram seleccionados dois genes da cadeia biossintética das AFs, *aflD* (= *nor1*) e *aflQ* (= *ord1= ordA*), para os quais a presença e expressão foram testadas por PCR e RT-PCR, respectivamente. A presença de ambos os genes não se correlacionou com a capacidade aflatoxigénica dos indivíduos testados. Em termos de expressão, apenas o gene *aflQ* mostrou boa correlação com a produção de AFs, tendo sido considerado um bom marcador molecular da capacidade aflatoxigénica.
Em conclusão, as castanhas e amêndoas com origem em Portugal parecem possuir boa qualidade em termos de contaminação com AFs. No entanto, a maioria dos isolamentos de *Aspergillus* secção *Flavi* provou ser aflatoxigénica, o que pode constituir um problema de segurança alimentar para as amêndoas, caso as condições de armazenamento e processamento não sejam devidamente controladas.
Em termos de identificação de espécies, foi obtido um nível de concordância elevado entre as diferentes abordagens usadas. A análise morfológica mostrou-se um método fiável e sensível para identificação preliminar dos isolamentos apenas se complementada com a análise de extrólitos. O gene da calmodulina mostrou-se mais robusto e sensível do que a região ITS, demonstrando maior potencial como marcador molecular. Os resultados obtidos por MALDI-TOF ICMS confirmaram que esta técnica é altamente fiável na identificação de *Aspergillus* secção *Flavi*, tendo como principal vantagem o facto de ser significativamente menos dispendioso, tanto em termos de tempo como de consumíveis, quando comparado com as restantes metodologias. Esta técnica reveste-se de elevada importância nos casos em que estão envolvidos numerosos espécimens com elevada proximidade taxonómica.
Relativamente à diferenciação de isolamentos aflatoxigénicos e não-aflatoxigénicos, a análise da expressão do gene *aflQ* sob condições indutoras da produção de AFs mostrou ser um método fiável.

PALAVRAS-CHAVE

Amêndoas, castanhas, aflatoxinas, fungos aflatoxigénicos, *Aspergillus* secção *Flavi*, MALDI-TOF ICMS.

To my supervisors, Professor Nelson Lima and Dr. Armando Venâncio, for accepting me as their student, and for all the advice, support, and guidance throughout this work.

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To all of those who, directly or indirectly, friendly or scientifically, willingly or involuntarily, helped this work to come to a good end. I will not name them. They will know.

Last of all, but not at all the least, to my loving family, and especially my sweet, sweet Isabel and my precious Henrique, who laughed and cried with me (almost) every day of this journey. They are my strength. This is also theirs.

This thesis is structured in 7 Chapters:

Chapter 1 introduces general aspects such as the background and scope of this work, as well as the motivation which led to the proposal of the theme, and attempts to integrate the various aspects developed throughout the thesis. The chapter is completed by the presentation of the aims of the work.

Chapter 2 is a Literature Review which aims at introducing the theoretical fundamentals and state of the art of the aspects more directly related to the theme. The span of the thesis is revealed in this chapter, where three major themes, corresponding to the three major goals of the work, are exposed: i) the fungal and mycotoxin contamination of food commodities, with special emphasis on AFs and aflatoxigenic fungi on nuts; ii) the molecular aspects of aflatoxin production and the ways of efficiently differentiating potentially hazardous fungi within section *Flavi*; and iii) the taxonomy of *Aspergillus* section *Flavi*, to which the most significant aflatoxigenic fungi belong, with focus on the various taxonomic problems and approaches used to elucidate them.

Chapter 3 describes the materials and methods used to perform this work. This chapter is divided in sections corresponding to the three major themes previously exposed: methods for the characterisation of mycobiota and AFs of almonds and chestnuts are addressed in section 1; methods used in the process of differentiating aflatoxigenic from non-aflatoxigenic fungi are described in section 2; and section 3 describes the methods for the characterisation and identification of isolates of *Aspergillus* section *Flavi*.

Chapter 4 presents the main results obtained in the various stages of the work and the subsequent analysis, and **Chapter 5** discusses those results. Both these chapters are divided in the same three sections described for Chapter 3.

Chapter 6 summarises the main conclusions withdrawn from the work, and exposes perspectives and suggestions for future work.

Chapter 7 encloses the references used to support the study.

As a supplement to the body of the work, 3 appendices give additional information.

Appendix 1 shows the details on the statistical analysis. **Appendix 2** lists all *Aspergillus* section *Flavi* isolates used in this study, with a fully detailed list of the corresponding

characteristics. **Appendix 3** includes molecular dendrograms that complement and reinforce those presented in the text.

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1 GENERAL INTRODUCTION

1.1 Framework

Since the discovery of aflatoxins in the 1960's and subsequent recognition that mycotoxins are of significant health concern to both humans and animals, interest was gradually developed for mycotoxins in various foods and feed. In the last years, concerted attempts have been made to examine the level of contamination of foods and the potential for effective control of mycotoxins from entering the human and animal food chain, especially in cereals, nuts and wine. Because controlling the occurrence of mycotoxins in finished products is practically impossible, regulatory bodies are continuously assessing the occurrence of mycotoxins throughout the various stages of production, from the farm to the fork. This type of information is important for the development of Decision Support Systems for predicting the level of risk in a particular product of a particular geographic origin, and the data obtained are used for the establishment of regulatory levels and of control schemes at import.

So, information on the key components of fungal and mycotoxin contamination in the food commodities is mandatory for the various stages of production. Because fungal contamination and mycotoxin production vary greatly with the environmental conditions in which they develop, pre-harvest conditions, post-harvest storage, transport and processing are all important stages in the food chain which need to be monitored. The knowledge on the fungal population incidence and diversity and on their mycotoxigenic potential is an indication of what the safety of the products might be, given different production, storage and processing conditions.

Aflatoxins are a group of mutagenic, teratogenic and immunosuppressive mycotoxins that include the most widely studied aflatoxins B_1, B_2, G_1 and G_2. Aflatoxin B_1 is considered the most carcinogenic compounds naturally produced. These mycotoxins are produced as secondary metabolites mostly by some species belonging to *Aspergillus* section *Flavi* when growing on a variety of food products. Tree nuts are among the commodities with moderate to high risk of aflatoxin contamination, since they are generally produced under environmental conditions which also favour aflatoxigenic fungal growth and toxin production.

Within the genus *Aspergillus*, section *Flavi* is one of the most significant, and is one of the best studied among fungi. This section is composed of a large number of very closely related species and is usually divided in two groups of species. One includes the aflatoxigenic species *A. flavus*, *A. parasiticus* and *A. nomius*, which cause serious problems in agricultural commodities, and the other one includes the non-aflatoxigenic species *A. oryzae*, *A. sojae* and *A. tamarii*, traditionally used for production of fermented foods. Even though these species differ so greatly on their physiological abilities, they are difficult to differentiate morphologically and even genetically. As a consequence, taxonomy and species identification have been subject of great interest for scientists aiming to clarify the species concept and limits within the section.

The establishment of schemes for species identification and for the rapid differentiation of aflatoxigenic and non-aflatoxigenic strains that are simultaneously accurate, sensitive, robust and expedite is mandatory. At present, reliable identification schemes of section *Flavi* typically imply the analysis of a wide variety of morphological, biochemical and molecular traits. But none of the methods alone has been accepted as flawless in recognising species and, as a result, polyphasic approaches are progressively given more emphasis in taxonomic decision-making. Recently, Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Intact Cell Mass Spectrometry (MALDI-TOF ICMS) has been used to generate spectra of protein masses which result in a taxon specific fingerprint. This technique has already shown high potentialities to discriminate very closely related taxa, but has rarely been used in fungal species identification, either on its one or as part of a polyphasic scheme of identification.

1.2 Motivation

Almonds and chestnuts are of great economic and social impact in Portugal, as they constitute the main income of rural populations especially from the portuguese northeast. Portugal has a typical Mediterranean climate, generally characterised by long periods of high temperatures and moderate to scarce rainfall. Almonds are extremely dry nuts produced under highly stressfull environmental conditions, in regions where the maturation and harvest period corresponds to a hot and dry summer. On the other hand, chestnuts are produced under more cold and humid conditions. Under such conditions, tree nuts, and

mostly almonds, are known to be targets of infection for a variety of fungi that can induce spoilage or produce toxic metabolites, and they have been associated with aflatoxigenic fungi and aflatoxin contamination more than with other known mycotoxins. However, and contrasting to what happens for other producing countries, there is no scientific knowledge on the fungal incidence and aflatoxigenic safety of nuts originating from Portugal.

The intention of this work was thus to gather information on the mycobiota associated with Portuguese almonds and chestnuts from the field to the end of processing, as well as on the environmental and processing conditions. By collating the different sets of information, we intended to determine the real as well as the potential mycotoxigenic profile of those commodities, especially in what concerns aflatoxins.

The work plan also included the development of a method which could easily and consistently differentiate aflatoxigenic from non-aflatoxigenic strains of *Aspergillus* section *Flavi*. The first approach was to characterise all the isolates belonging to *Aspergillus* section *Flavi* in terms of morphology and extrolite profile (including their aflatoxigenic ability), and from thereafter try to identify them to the species level. But, as more and more isolates were being obtained, we were confronted with the extreme difficulty of identifying them to the species level based solely on classic phenotypic features. At the same time, the Micoteca da Universidade do Minho (MUM) acquired the knowledge and the technology of identifying microorganisms by MALDI-TOF MS. This technology had already been vastly and successfully applied to the identification and characterisation of bacteria, but its application to fungi was still insipid. Since we had, at this time point of the work, gathered a significant number of section *Flavi* isolates and they were well characterised at the classic phenotypic level, the work was thereafter directed to assess the ability and reliability of MALDI-TOF MS for the identification of closely related isolates of section *Flavi* as well as for the differentiation of aflatoxigenic and non-aflatoxigenic isolates.

1.3 Aims of the Work

The work developed under the frame of this Thesis had three major goals:

i) To provide knowledge on the general mycobiota, aflatoxigenic fungi and aflatoxin contamination of Portuguese almonds and chestnuts, and on its evolution throughout the various stages of production (field, storage and processing).

ii) To test methods capable of differentiating aflatoxigenic from non-aflatoxigenic isolates of *Aspergillus* section *Flavi*.

iii) To characterise and identify the isolates of *Aspergillus* section *Flavi* by applying a polyphasic approach including classic phenotypic and molecular methods as well as the innovative technology of protein spectral analysis Matrix-Assisted Laser Desorption/Ionisation-Time of Flight Intact-Cell Mass Spectrometry (MALDI-TOF ICMS), and to devise accurate and sensitive schemes for species identification.

2 LITERATURE REVIEW

2.1 Mycobiota and mycotoxins of almonds and chestnuts

2.1.1 General mycology and mycotoxicology of foods

Foods, whether they are raw or processed, are usually contaminated with fungal spores or even mycelium fragments from the environment. As a result of their growth, several kinds of food spoilage may develop: off-flavours, toxin, decolorisation, rotting, loss of nutritional quality, and formation of pathogenic or allergenic propagules.

In fungi, as in all eukaryotes, essential metabolites are produced from intermediate metabolic pathways like glycolysis and the citric acid cycle. Secondary metabolism, on the other hand, removes products from intermediate metabolic pathways when growth is temporarily restricted, which are then directed to highly specific biosynthetic pathways. One of the most important groups of such metabolites is mycotoxins. Mycotoxins are generally defined as low-molecular-weight natural products produced as secondary metabolites by filamentous microfungi, which are toxic to vertebrates in low concentrations (Bennett & Klich, 2003; Paterson & Lima, 2010). These metabolites constitute a chemically, as well as toxigenically, heterogeneous assemblage produced by a wide variety of fungi from different precursors and pathways, being that these are often specific for individual genera, species, or even strains (Frisvad, 1989). It is not clear what the role of these metabolites is in nature. In general, the most commonly accepted idea is that mycotoxin-producing fungi are better protected against other organisms sharing the same trophic niche, but other theories have been raised. Several studies (reviewed in Reverberi et al., 2010) have implied the biosynthesis of mycotoxins in fungal protection against oxidative stress and insect mycophagy, as well as on the reduction of host chemical defences against fungal attack.

Mycotoxins have long been associated with food consumption, but the term mycotoxin was only coined in 1962 after a huge problem in England, in which approximately 100,000 turkeys died. This mysterious turkey X disease, as it was then called, was later linked to a peanut meal contaminated with secondary metabolites from *Aspergillus flavus* – the aflatoxins (van der Zieden et al., 1962). The mycotoxin term was

then extended to a number of previously known fungal toxins (e.g. the ergot alkaloids), some compounds that had originally been isolated as antibiotics (e.g. patulin), and a number of new secondary metabolites revealed in screens targeted at mycotoxin discovery (e.g. ochratoxin A - OTA) (Bennett & Klich, 2003).

Depending on the definition used, and recognising that most fungal toxins occur in families of chemically related metabolites, hundreds of compounds are now recognised as mycotoxins, of which only a reduced number regularly receives attention as threats to human and animal health (Paterson & Lima, 2010). AFs have been the most widely studied mycotoxins, but fumonisins, OTA, patulin, zearalenone, trichothecenes and citrinin have been subject of increased interest in the last years.

Mycotoxins occur mostly in temperate and tropical regions of the world, depending on the fungal species. Major food commodities affected are cereals, nuts, dried fruit, coffee, cocoa, spices, oil seeds, dried peas, beans and fleshy fruits, particularly apples. Mycotoxins may also be found in fruit juices, beer and wine resulting from the use of contaminated cereals and fruits in their production. They can also enter the human food chain via meat or other animal products such as eggs, milk and cheese as the result of livestock eating contaminated feed (CAST, 2003; Filtenborg et al., 2004; Venâncio & Paterson, 2007; Reddy et al., 2009).

Mycotoxins are produced by an array of diverse fungal species that are generally saprophytic and opportunistic, weak pathogens. Most of the mycotoxins which are considered important food contaminants are produced primarily by three genera of fungi, namely *Aspergillus*, *Penicillium* and *Fusarium* (CAST, 2003; Filtenborg et al., 2004; Frisvad & Thrane, 2004; Venâncio & Paterson, 2007; Reddy et al., 2009; Paterson & Lima, 2010). The genus *Aspergillus* represents a large group of fungi that occupies very diverse ecological niches. Although members are distributed worldwide, *Aspergillus* spp. appear most abundant between latitudes 26º to 35º north or south of the equator (Klich, 2002b). Thus, these fungi are more common in subtropical and warm temperate climates. Generally regarded as saprophytes, *Aspergillus* spp. grow on a large number of substrates and are very important in nutrient cycling. Their ability to thrive in high temperatures and with relatively low available water makes them well suited to colonise a number of grain and nut crops. Mycotoxins associated with *Aspergillus* species include AFs, ochratoxins, versicolorins, sterigmatocystin, gliotoxin, citrinin, CPA, patulin, citreoviridin,

cyclopiazonic acid, penicillic acid and tremorgenic mycotoxins (CAST, 2003; Frisvad & Thrane, 2004). More recently, fumonisins have been added to this group after they have been confirmed to be produced by *Aspergillus* section *Nigri* (Nielsen et al., 2009).

Members of the genus *Penicillium* generally grow and can produce mycotoxins over a wider range of temperatures than those of the genus *Aspergillus*, but are not generally as adapted to hot and dry conditions, being more abundant in temperate climates. Some of the most important secondary metabolites produced by these fungi are common to *Aspergillus*: OTA, citrinin, patulin, penicillic acid, penitrem A, cyclopiazonic acid.

Fusarium is a large complex genus with species adapted to a wide range of habitats. They are worldwide in distribution and many are important plant pathogens. However, many species are soil borne and exist as saprophytes, and few are significant mycotoxin producers. Some of the most important mycotoxins related to this genus are trichothecenes, zearalenone and fumonisins.

There is a vast literature on fungi and mycotoxins associated with different types of foods and food commodities, and numerous reviews have been published (e.g. Moss, 1998; Bennett & Klich, 2003; CAST, 2003; Magan, 2006; Murphy et al., 2006; Do & Choi, 2007; Venâncio & Paterson, 2007). The present work will be focusing on the study of mycobiota and mycotoxins in Portuguese almonds and chestnuts, with special focus on contamination by aflatoxigenic fungi and AFs.

2.1.2 Ecophysiology of foodborne fungi and mycotoxin production

Despite all efforts to control fungal contamination, toxigenic fungi are ubiquitous in nature and occur regularly in worldwide food supplies. Fungal growth and mycotoxin production only occur under favourable conditions, which vary for each species depending on adaptability. Food intrinsic parameters associated to extrinsic factors are responsible for the spectrum of contaminating and dominating mycobiota. This is mostly related to the physiology of fungi and their adaptation to the different matrices and environmental conditions. Filtenborg et al. (2004) refer that normally less than 10 species are present in a given food commodity, and only 1 to 3 dominate and are responsible for spoilage. Before a contaminated sample is analysed for mycotoxins, it is important to know which mycotoxins are likely to be present. Since the production of mycotoxins is often species

specific, the knowledge of which mycotoxins are likely to be present can be achieved by an accurate identification of the contaminating fungi.

Knowledge on food intrinsic and extrinsic parameters and on the ecophysiological characteristics of fungi complemented with evidence on the composition and succession of the mycobiota in commodities throughout production chain is an important step towards the prediction of possible mycotoxin contamination. The most important factors that influence growth and mycotoxin production are environmental temperature, substrate water activity (a_W), relative humidity, gas composition, substrate composition, inoculum concentrations, microbial interactions and mechanical or insect damage (Gqaleni et al., 1997; Guynot et al., 2003; Giorni et al., 2008). In particular, it is the interaction between some or all of these factors that determines whether contamination increases and mycotoxins are produced. Interactions between available water and temperature are fundamental because they represent the two-dimensional niche in which fungi may be able to germinate, grow and actively compete for the allocation of the available resources (Marin et al., 1998; Samapundo et al., 2007a, 2007b). It is also generally well agreed that, in contrast to bacterial growth, a_W is the most significant factor controlling fungal growth (Sautour et al., 2002; Samapundo et al., 2007b).

Fungi contaminating food commodities are traditionally divided into two groups, field fungi and storage fungi. Field fungi are typically those that can grow at moisture contents in equilibrium with relative humidity of 70 to 90% and temperatures around 20 to 25 ºC. These fungi usually require $a_W > 0.85$ for active growth, and grow optimally at a_W near 0.99. *Alternaria*, *Cladosporium*, *Fusarium*, and *Helminthosporium* are all traditionally classified as field fungi. On the other hand, storage fungi are generally adapted to lower humidity levels and higher temperatures. Fungi like *Aspergillus* and *Penicillium* are major representatives of this group. The minimal necessary a_W for most *Aspergillus* and *Penicillium* species is 0.75-0.85, and they generally grow optimally at a_W 0.93-0.98. *Aspergillus* requires a_W as low as 0.73 for active growth, whereas *Penicillium* needs at least 0.78-0.80 (Rosso & Robinson, 2001; Filtenborg et al., 2004; Magan, 2006). Furthermore, *Aspergillus* spp. are generally more adapted to temperatures of 30-40 ºC, whereas *Penicillium* spp grow optimally at 25-30 ºC (Filtenborg et al., 2004).

The classification as field or storage fungi has been based on studies done in temperate climates (Christensen, 1974; in CAST, 2003). However, under warm, humid

subtropical or tropical climates — or even in temperate climates in which the growing season is unusually hot and dry — species of *Aspergillus* and *Penicillium* can infect seeds early in the field. Perhaps the best example of a species that can infect seeds both in the field and in storage is *A. flavus*. In temperate climates, the fungus is predominantly a storage fungus, but in some regions of the world grains and nuts are more likely to be colonised during pre-harvest than in storage. Many species of *Fusarium*, as well as some species of *Penicillium*, also infect grain in the field as well as in storage.

As a consequence of the different ecophysiological adaptation of fungi, mycobiota naturally contaminating food commodities follows a typical succession since the early days of development in the field until the end of storage. *Fusarium*, *Cladosporium* and *Alternaria* are typically the predominant field contaminants, and they establish before harvest, and *Penicillium* and *Aspergillus* tend to predominate during storage. In fact, it is not uncommon to find studies reporting high frequencies of *Fusarium* isolation in field samples, and a trend toward its decrease during storage accompanied by the gradual increase in frequencies of *Aspergillus* and *Penicillium* (e.g. da Silva et al., 2000; Adebajo & Popoola, 2003; Atehnkeng et al., 2008; Nakai et al., 2008; Sanchez-Hervas et al., 2008). In dry products (a_W = 0.65-0.75), only extreme xerophilic filamentous fungi like *A. restrictus* and *Eurotium* species are able to grow (Filtenborg et al., 2004).

Because few fungi grow at a_W < 0.70, fungal growth can be prevented by drying agricultural products to a_W below 0.65 and keeping it under this level. However, if the moisture of the stored product increases due to microbial or insect activity, moisture migration or increase in environmental relative humidity, other fungal species besides extreme xerophiles begin to grow. For example, growth of *A. restrictus* begins at a_W of 0.70 or slightly higher, the *Eurotium* group begins at a_W 0.80 to 0.85, and many *Penicillium* and *Aspergillus* species begin to grow at a_W above 0.85. *A. flavus*, for example, dominates at a_W of 0.94, and *A. ochraceus* and *A. niger* are dominant at a_W of 0.98 (Marin et al., 1998).

2.1.3 Almonds and chestnuts in Portugal and in the World

2.1.3.1 Almonds

Almond tree, *Prunus dulcis* (Miller) D.A. Webb, synonym *Amygdalus communis* L., is a cultivated tree originating from wild trees from Central Asia, which is currently dispersed throughout the world. The almond tree is adapted to dry and hot climates, and that is the reason why it is mainly established in Mediterranean countries (Portugal, Spain, Italy, France) and others with similar climatic characteristics, like USA (specifically California), Australia, South Africa, Chile and Argentina (Monteiro et al., 2003).

The almond is not a true nut, but a drupe, which consists of an outer dehiscent hull (exocarp) and a hard shell (endocarp) with the edible seed (kernel) inside, involved by a brown seed coat (Monteiro et al., 2003). The almond kernel is a very nutritious seed, with extremely low water content (4 to 6%), and high levels of protein (18%), fat (54%) and carbohydrates (20%) (Wareing et al., 2000). Fat content is almost exclusively constituted by unsaturated fatty acids (Sathe et al., 2008; Celik et al., 2010), and the most important sugars are saccharose and raffinose (Barreira et al., 2010). It is also rich in a wide variety of minerals like calcium, potassium, iron and phosphorus (Wareing et al., 2000).

The almond is harvested when it reaches complete dryness, during late August and September, usually by mechanical shaking of the tree or by shaking the fruits with a wood stick. The fruits are collected immediately after fall and dried for a few weeks or months until they reach water content of less than 6% (Monteiro et al., 2003). In this way, almonds are considered the fruits which withstand the longest storage periods without visible depreciation, if adequate environmental conditions (mainly humidity) are maintained (Monteiro et al., 2003). As such, almonds' major problem in term of biological infestation is insect damage while still on the tree, and significant fungal contamination is usually associated with insect-damaged fruits (Schade et al., 1975; Schatzki & Ong, 2001; Campbell et al., 2003; Whitaker et al., 2010).

Portugal is the eighteenth country in the list of producing countries, and is responsible for only 0.5% of worldwide production (Figure 2.1). The major producer is, by far, the USA, representing more than 50% of worldwide production, with yields 3 times higher than the world average. The other most important countries, Spain and Italy, are

from the Mediterranean basin, like Portugal. National production has been suffering tremendous reduction of production and yield in the last decade. Throughout a period of 5 years, from 2001 to 2005, and even if the area of production was maintained near 38,000 hectares, the production suffered a two-fold reduction, from 27,000 ton to 13,800 ton, with yields reducing from 891 to 263 kg/ha (INE, 2005). The yield is, in fact, much lower than the world average, and is only one-tenth of the American yield. International trade balance pends for exportation of in-shell fruits for Europe (mainly Spain) and importation of shelled fruits from Spain and California, USA. Portugal imports six times more than it exports (1,600 ton vs. 260 ton; 6 million euros vs. 900,000 euros).

In Portugal, the northeast region of Trás-os-Montes is the major national producer of almonds. Even with low yields, the culture represents significant cultural and economic incomes for local populations, since, under the traditional culturing methods, no major inputs are made other than harvesting. The region integrates one almond Protected Denomination of Origin (Denominação de Origem Protegida, D.O.P), D.O.P. Douro. In 2005, Trás-os-Montes almond represented 60% of the national almond area (approximately 22,800 ha) and 90% of the national production (12,000 tons) (INE, 2005). Yields for Trás-os-Montes production are around 0.5 Ton/ha, which represents 144% of the national yield. The rest of the national almond is produced in the south region of Algarve, with yields five-fold lower. These numbers reflect mostly the progressive abandonment of the culture in the south of the country.

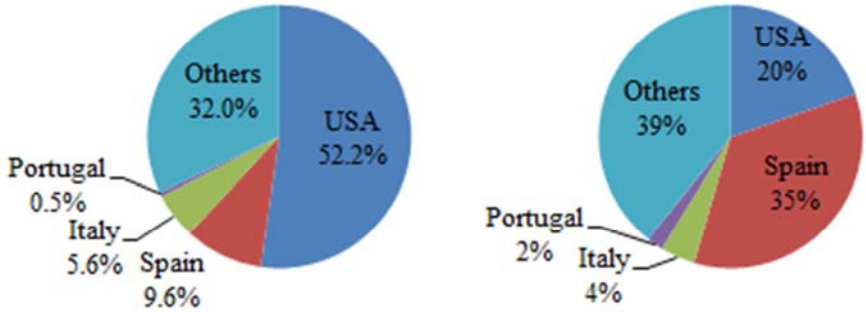

Figure 2.1 Worldwide almond production (A) and area of production (B) of the 3 major producers and Portugal, for the year 2008 (http://faostat.fao.org, accessed 16.07.2010).

2.1.3.2 Chestnuts

Chestnuts are produced by a wide variety of species from the genus *Castanea*. These species divide into three major groups that have specific geographical distribution: *C. crenata* and *C. molissima* predominate in Asia and produce the Asian (Japanese and Chinese) chestnuts; *C. dentata* produces American chestnuts in North America, and *C. sativa* produces the European chestnuts, also known as sweet chestnuts. *C. sativa* is adapted to regions with humid and temperate to cold climate, and does not withstand long hot and dry periods (Serrano et al., 2001).

European chestnuts are the most consumed chestnuts because of their interesting nutritional characteristics. They are rich in carbohydrates (around 40%), mostly starch, and present minerals, vitamins and appreciable levels of fiber, but low amounts of protein (2−4%) and, unlike typical nuts, low amounts of fat (1.5−5%) (Wareing et al., 2000; Barreira et al., 2009). They are also an interesting source of essential fatty acids (Barreira et al., 2009).

Chestnuts have approximately 50% water content (Wareing et al., 2000; Barreira et al., 2009), and for that reason they are typical seasonal fruits that maintain their optimal commercial quality, turgescence and health for only a brief period when compared to other nuts. One of the major difficulties is the high perishability of the product. The major factors in post-harvest depreciation are moulding or rotting caused by fungi and the larval development of insects (Wells & Payne, 1975). Fungal infections often start in the larval galleries of insects (Wells & Payne, 1975), and many nuts become infected on the ground before picking. Some moulds are considered endophytes that colonise the fruits at various stages during their development but do not cause any symptoms of disease until after fruit fall (Washington et al., 1997, 1998). In Portugal, the traditional method of harvest is to allow the nuts to fall to the ground, and then harvest them manually or mechanically, with vacuum equipment. Interception of chestnuts in nets either on the ground or suspended above the ground is made in Italy and France mainly to ease and accelerate chestnut collection, but it is not a usual harvest method in Portugal.

Portugal is the fourth country in terms of worldwide European chestnut production (Figure 2.2). In 2004, chestnut culture in Trás-os-Montes occupied 85% of the national area devoted to chestnut production, and corresponded to 84% of the national production.

The region integrates three chestnut D.O.P. - Terra Fria, Padrela and Soutos da Lapa. It is the fruit with major significance in the portuguese import/export balance, with ratios of 1:6 (INE, 2005). There are no known statistics for processed chestnuts, but it is known that most of it is exported already processed.

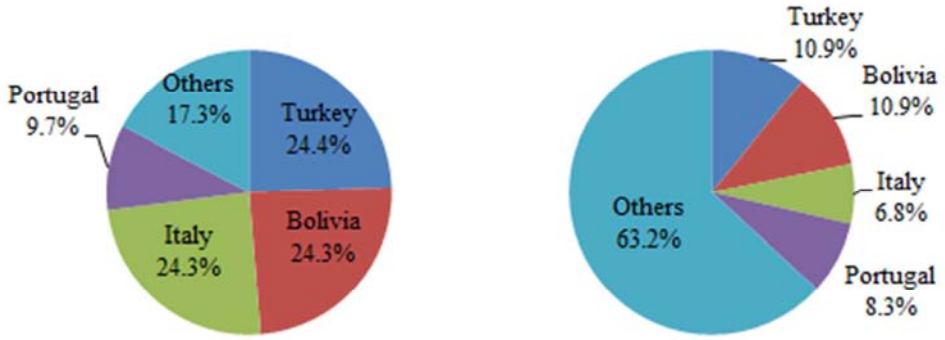

Figure 2.2 Worldwide chestnut production (A) and area of production (B) of the 3 major producers and Portugal, for the year 2008 (data refer to sweet chestnut only) (http://faostat.fao.org, accessed 16.07.2010).

2.1.4 Fungi and mycotoxins associated with tree nuts

Some genera, like *Botrytis*, *Cladosporium* and *Rhizopus*, are major spoilage fungi in a variety of nuts, but they are not known to produce significant mycotoxins. On the other hand, *Aspergillus*, *Fusarium* and *Penicillium* include species capable of producing a wide range of mycotoxins (Pitt & Hocking, 1997). These fungi have generally been reported as dominant contaminants in various kinds of nuts, like almonds and chestnuts (Wells & Payne, 1975; King et al., 1983; King & Schade, 1986; Jimenez et al., 1991; Abdel-Gawad & Zohri, 1993; Teviotdale & Hendricks, 1994; Bayman et al., 2002; Overy et al., 2003; Jermini et al., 2006; Khosravi et al., 2007), but also pistachios, peanuts, walnuts, hazelnuts and Brazil nuts (Abdel-Gawad & Zohri, 1993; Freire et al., 2000; Bayman et al., 2002; Khosravi et al., 2007; Sieber et al., 2007; Gonçalez et al., 2008; Nakai et al., 2008; Singh & Shukla, 2008). One thing that these studies remark is that there is a different dominating mycobiota in each type of nut. Also, the mycobiota is found to vary widely depending on the conditions and stage of production, storage and processing, which is related not only to technological issues but also to the geographic location from where nuts originate.

As mentioned, almonds are mainly produced in California, USA, and these fruits play an important role in that country's markets. For that reason, Californian almonds have been largely studied and represent the majority of the studies on fungal and mycotoxin contamination in this type of nut. Fungal contamination of almonds is almost always dominated by genera *Aspergillus*, *Penicillium*, *Rhizopus* and *Eurotium*, independently of the stage of production (field or storage). In fact, the typical field fungi are usually considered minor contaminants in this type of nut even before harvest, due mainly to the dryness of the substrate and to the environmental conditions (usually extremely hot and dry) at the end of maturation. Sections *Flavi* and *Nigri* are the predominant aspergilla, with other sections being present only rarely. It can generally be observed that *Eurotium* spp. and *Aspergillus* sections *Flavi* and *Nigri* seem to evolve in a positive way from field to storage/market (Purcell et al., 1980; Bayman et al., 2002). None of these studies refer to mycotoxin contamination, but fungal evolution makes it clear that, if adequate storage conditions are not guaranteed, AFs and OTA are potential threats to these nuts. Almonds have not been a frequent subject of survey for mycotoxins other than AFs, but Zaied et al. (2010) have searched for OTA in almonds from Tunisian markets and found contamination with 61 µg/kg, proving that this may be a potential risk for almonds.

Fewer studies have been devoted to determining fungal contamination of chestnuts, and none has analysed chestnuts originating from the 4 biggest producing countries, Turkey, Bolivia, Italy and Portugal. In fact, some of those studies are relative to marketed chestnuts with unknown origin. Reports on chestnuts marketed (origin not reported) in cold and humid countries, like Canada (Overy et al., 2003) and Switzerland (Sieber et al., 2007) refer to contaminations strongly dominated by *Penicillium* spp., with *Aspergillus* spp. being of no significance, while studies from drier and warmer regions, like Georgia, USA (Wells & Payne, 1975) and Ar'Ar, Saudi Arabia (Abdel-Gawad & Zohri, 1993) report important incidences of *Aspergillus* (sections *Wentii*, *Flavi* and, to a lesser extent, *Nigri*).

Table 2.1 summarises the results of fungal surveys on almonds and chestnuts from various studies.

Table 2.1 General fungal contamination of almonds and chestnuts (genus *Eurotium* is included as the teleomorph of *Aspergillus glaucus* group).

Reference	Geographic region	Stage of production	Treatments	Most frequent fungi (incidence)	*Aspergillus* identified (incidence)
Almonds					
Phillips et al., 1979	California, USA	Field-collected	Non-disinfected kernel	*Aspergillus* (100%) *Eurotium* (30%) *Penicillium* (27%) *Rhizopus* (19%)	Sect. *Nigri* (99%) Sect. *Flavi* (60%)
			Surface-sterilised shell	*Aspergillus* (35%) *Alternaria* (19%) *Rhizopus* (8.5%) *Eurotium* (3%)	Sect. *Nigri* (35%) Sect. *Flavi* (0.4%)
Purcell et al., 1980	California, USA	Field-collected	Non-disinfected kernel	*Aspergillus* (60%) *Alternaria/ Ulocladium* (40%) *Eurotium* (20%) *Penicillium* (15%) *Rhizopus* (5%)	Sect. *Nigri* (60%) Sect. *Flavi* (20%) Sect. *Circumdati* (15%) Sect. *Wentii* (10%)
			Surface-sterilised shell	*Alternaria/ Ulocladium* (60%) *Aspergillus* (35%) *Eurotium* (15%) *Rhizopus* (10%) *Penicillium* (5%)	Sect. *Nigri* (30%) Sect. *Circumdati* (3%) Sect. *Flavi* (1%) Sect. *Wentii* (1%)
		Storage	Non-disinfected kernel	*Aspergillus* (80%) *Eurotium* (35%) *Alternaria/ Ulocladium* (20%) *Penicillium* (5%) *Rhizopus* (2%)	Sect. *Nigri* (80%) Sect. *Flavi* (25%) Sect. *Circumdati* (15%) Sect. *Wentii* (10%)
			Surface-sterilised shell	*Aspergillus* (45%) *Alternaria/ Ulocladium* (30%) *Eurotium* (20%) *Rhizopus* (10%) *Penicillium* (1%)	Sect. *Nigri* (45%) Sect. *Circumdati* (2%)
Jimenez et al., 1991	Spain	Market-bought, roasted	Surface-sterilised	*Penicillium* (27%), *Aspergillus* (> 25%) *Eurotium* (9.7%) *Rhizopus* (9.7%)	*A. flavus* (24.7%) *A. niger* (20.6%)
Abdel-Gawad & Zohri, 1993	Saudi Arabia	Market-bought	Surface-sterilised	*Aspergillus* (100%) *Rhizopus* (100%) *Penicillium* (80%) *Eurotium* (60%) *Fusarium* (20%)	*A. niger* (100%) *A. flavus* (100%) *A. japonicus* (60%) *A. ochraceus* (60%) *A. ustus* (40%) *A. terreus* (40%) *A. sydowii* (40%) *A. versicolor* (20%) *A. tamarii* (20%)

n.d.: not detected
n.r.: not reported

(continues)

Table 2.1 (continued)

Reference	Geographic region	Stage of production	Treatments	Most frequent fungi (incidence)	*Aspergillus* identified (incidence)
Teviotdale & Hendricks, 1994	California, USA	Field-collected	Non-disinfected (CFU counts)	Yeasts *Cladosporium* *Penicillium* *Aspergillus*	n.r.
Bayman et al., 2002	California, USA	Field-collected	Non-disinfected	*Penicillium* (91%) *Aspergillus* (> 22%) *Rhizopus* (13%)	*A. niger* (22%) *A. melleus* (6%) *A. ochraceus* (6%) *A. flavus* (3%) *A. fumigatus* (3%)
			Surface-sterilised	*Rhizopus* (17%) *Aspergillus* (6%) *Penicillium* (3%)	*A. niger* (6%) *A. nidulans* (2%)
		Market-bought	Non-disinfected	*Aspergillus* (> 60%) *Rhizopus* (51%) *Penicillium* (4%)	*A. niger* (60%) *A. flavus* (4%) *A. nidulans* (3%) *A. fumigatus* (1%) *A. tamarii* (1%)
			Surface-sterilised	*Rhizopus* (79%) *Aspergillus* (> 26%) *Penicillium* (6%)	*A. niger* (26%) *A. ochraceus* (4%) *A. melleus* (4%) *A. flavus* (19%) *A. nidulans* (1%) *A. tamarii* (1%)
Khosravi et al., 2007	Iran	Market-bought	Surface-sterilised	Yeasts (60%) *Aspergillus* (20%) *Penicillium* (20%)	n.r.
Chestnuts					
Wells & Payne, 1975	Georgia, USA	Field-collected	Surface-sterilised	*Penicillium* (40.7%) *Rhizopus* (17.5%) *Alternaria* (17.2%) *Aspergillus* (16.8%) *Fusarium* (6.4%)	*A. wentii* *A. flavus* *A. oryzae* *A. niger*
Abdel-Gawad & Zohri, 1993	Saudi Arabia	Market-bought	Surface-sterilised	*Aspergillus* (100%) *Rhizopus* (100%) *Penicillium* (100%) *Eurotium* (100%) *Fusarium* (60%)	*A. flavus* (100%) *A. niger* (100%) *A. fumigatus* (80%) *A. parasiticus* (80%) *A. sydowii* (60%) *A. versicolor* (40%) *A. wentii* (40%) *A. terreus* (20%) *A. tamarii* (20%)
Overy et al., 2003	Canada	Market-bought	Surface-sterilised	*Penicillium* (67.1%) *Aspergillus* (2.0%) *Trichoderma* (0.3%)	*A. ochraceus* (2.0%) *A. japonicas* (0.3%)
Jermini et al., 2006	Switzerland	Market-bought	Water-soaked	*Ciboria bastiana* *Penicillium* *Mucor hiemalis*	n.d.

n.d.: not detected
n.r.: not reported

2.1.5 Aflatoxins

2.1.5.1 Aflatoxins as a health threat to humans and animals

AFs are a group of difuranocoumarin derivatives consisting of 5 heterocycles that occur in several chemical forms. The four major AFs are AFB_1, AFB_2, AFG_1 and AFG_2 (Figure 2.3), and they are named based on their fluorescence under UV light (B for blue and G for green) and relative chromatographic mobility during thin-layer chromatography. AFB_1 is considered the most potent natural carcinogen known and is usually the major AF produced by aflatoxigenic strains. It is therefore the best studied. Numerous other AFs have been described, especially as mammalian biotransformation products of the major metabolites (Bennett & Klich, 2003). One such example is AFM_1, the predominant metabolite of AFB_1 in milk from lactating humans and animals that consume AFB_1-contaminated food or feed.

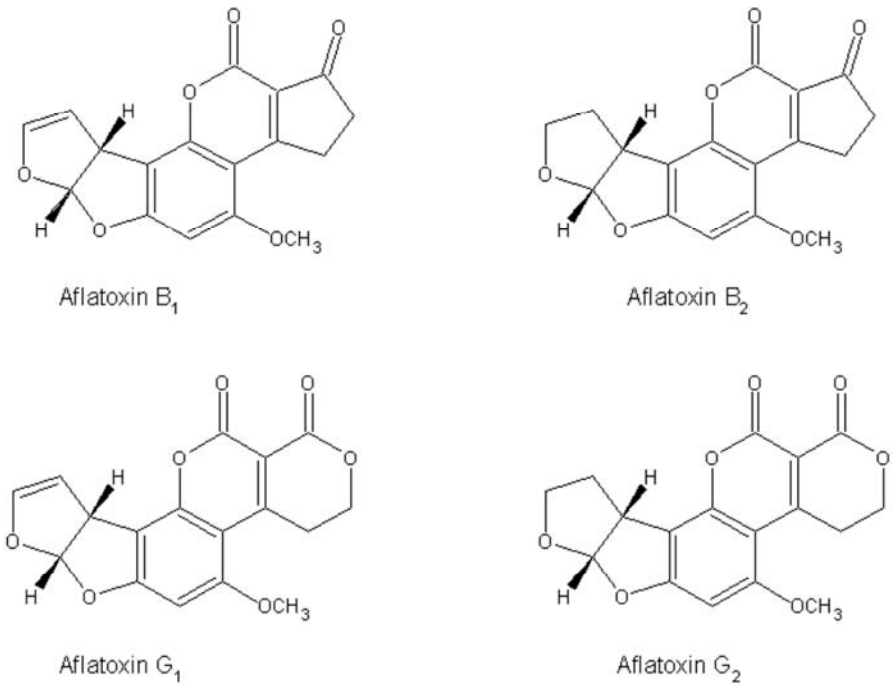

Figure 2.3 Chemical structure of the four major AFs (from: Reiter et al., 2009).

AFB_1 is metabolised by the liver through the cytochrome P450 enzyme system to the major carcinogenic metabolite AFB_1-8,9-epoxide (AFBO), or to less mutagenic forms such as AFM_1, AFQ_1, or AFP_1 (Shimada & Guengerich, 1989; Crespi et al., 1991). There are several pathways that AFBO can take, resulting in cancer, toxicity, and AFBO excretion. The exo-form of AFBO readily binds to cellular macromolecules including genetic material (proteins and DNA), to form adducts. It is the formation of DNA-adducts that leads to gene mutations and cancer.

AFs are associated with both toxicity and carcinogenicity in human and animal populations. The diseases caused by AF consumption are called aflatoxicoses. Acute aflatoxicosis occurs when moderate to high levels of AFs are consumed. Acute episodes of disease symptoms may include haemorrhage, acute liver damage, oedema, alteration in digestion, absorption and/or metabolism of nutrients, and may result in death (Varga et al., 2009). Chronic aflatoxicosis results in cancer, immune suppression, and other "slow" pathological conditions.

There are substantial differences in species susceptibility. Moreover, within a given species, the magnitude of the response is influenced by age, sex, weight, diet, exposure to infectious agents, and the presence of other mycotoxins and pharmacologically active substances. LD50 for AFB_1 ranges from 0.5 mg/kg for the adult dog to 10.2 mg/kg for the hamster (Moss, 1996). For humans, LD50 probably falls in the middle of the range (Moss, 1998). Because of the differences in AF susceptibility in test animals, it has been difficult to extrapolate the possible effects of AFs to humans, but according to the International Agency for Research on Cancer (IARC), there is sufficient evidence for carcinogenicity of naturally occurring mixtures of AFs, mixtures of AFB_1, AFG_1 and AFM_1, and of AFB_1 alone, limited evidence for AFB_2 and inadequate evidence for AFG_2 and AFM_1 (IARC, 2002). Exposure to AFs in the diet is considered an important risk factor for the development of primary hepatocellular carcinoma, particularly in individuals already exposed to other liver pathologies such as hepatitis B (Henry et al., 2002). Several studies have linked liver cancer incidence to estimated AF consumption in the diet (Li et al., 2001). The results of these studies have not been entirely consistent, and quantification of lifetime individual exposure to AF is extremely difficult. The incidence of liver cancer varies widely from country to country, but it is one of the most common cancers in China,

the Philippines, Thailand and many African countries (Bennett & Klich, 2003), where contaminated maize and rice are the major dietary constituents.

Also, acute toxicity of AFs in humans has been observed, even if rarely. Acute aflatoxicosis epidemics occurred in India in 1974, due to the consumption of maize heavily contaminated with AF (Krishnamachari et al., 1975). More than 100 people died. Also, three cases of acute aflatoxicosis occurred in Kenya in 1981 (Ngindu et al., 1982), in 2004 and in 2005, causing more than 150 deaths (CDC, 2004; Azziz-Baumgartner et al., 2005; Lewis et al., 2005; Probst et al., 2007).

2.1.5.2 Risk assessment and Regulatory issues

AFs have been found to contaminate many crops frequently at nanogram levels, although occasionally they can be found at levels of tens to hundreds of ng/g. Commodities with a high risk of AF contamination include peanuts, corn, cottonseed, Brazil nuts, pistachios, spices, figs and copra. Commodities with an intermediate risk of AF contamination include almonds, pecans, and raisins. Walnuts, soybeans, beans, pulses, cassava, grain sorghum, millet, wheat, oats, barley, and rice seem to be less susceptible to AF contamination (CAST, 2003).

Because controlling the occurrence of mycotoxins in finished products is practically impossible, regulatory bodies are continuously assessing the levels of acceptable exposure to humans by using a risk assessment process to establish tolerable daily intakes of selected mycotoxins. Monitoring programs assessing the occurrence of mycotoxins along with available toxicological data are used to make an assessment of exposure-risk to humans or animals. The result is the establishment of regulatory levels for selected mycotoxins where sufficient information has been obtained.

Risk assessment is based on the hazard or toxicity of a mycotoxin and the expected degree of exposure of individuals or populations. The hazard of mycotoxins to individuals is probably more or less the same all over the world, except for those populations, e.g. from Shanghai, Thailand, China, Gambia, Taiwan, with high levels of hepatitis B infection, for whom AF potency is significantly enhanced (Henry et al., 2002; CAST, 2003). On the other hand, exposure is not the same worldwide, because of different levels of contamination as well as dietary habits in the various parts of the world. AFs prevail in less

developed tropical and subtropical countries where climate and storage conditions are favourable to fungal growth and toxin production. Furthermore, populations from those countries rely extensively on some of those crops which have been found more susceptible to AF, mostly grains.

Worldwide regulations exist for mycotoxins and generally are based on toxicological data, occurrence and distribution, and epidemiological data. In Europe, current regulations are based mostly on scientific opinions of authoritative bodies, as the Joint Expert Committee on Food Additives of the United Nations (JECFA - a scientific advisory body of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO)) and the European Food Safety Authority (EFSA). The EFSA is an independent body of the European Commission (EC), established in 2002, and charged, among other tasks, with the development of risk assessments on issues of concern in the food and feed supply. EFSA publishes its risk assessments in the form of scientific opinions which form the main scientific basis for the preparation of EU regulations. Another important EU activity is SCOOP (Scientific Cooperation on Questions relating to Food), funded by the European Commission, and targeted to make the best estimates of intake of contaminants by EU inhabitants. The objectives of SCOOP activity is to provide the scientific basis for evaluation and management of risk to public health arising from dietary exposure to mycotoxins, taking into account recently available data on occurrence and consumption. Special emphasis is placed on evaluation of dietary intake of mycotoxins in each of the EU member states and in high-risk sub-groups of the population.

For the mycotoxins currently considered most significant, JECFA has evaluated their hazard in several sessions (see review by van Egmond et al., 2007). In 2001, a JECFA session was devoted to mycotoxins. Reports resulting from this session provided detailed insight into the process of risk assessment of mycotoxins (FAO, 2001, 2002). The reports addressed several concerns about the mycotoxins considered - their properties and metabolism, toxicological studies, and final risk evaluation.

In the early days of mycotoxin regulations, control measures focused mainly on AFs. They were established by industrialised countries, and limits often had an advisory or guideline character. Over the years, the number of countries with known specific mycotoxin regulations has increased from 33 in 1981 (Schuller et al., 1983) to 100 in 2003

(FAO, 2004), with specific limits being established for many food and feed commodities and products for 13 different mycotoxins or groups of mycotoxins.

Until the late 1990's, setting of mycotoxin regulations was mostly a national concern. As a consequence, tolerated levels of mycotoxins varied widely between countries. The Task Force Report of the Council for Agricultural Science and Technology (CAST), USA, collated information on almost 80 countries all over the world and, in 1997, for the specific case of AFs, tolerated levels varied from zero (undetectable) to 1000 µg/kg (CAST, 2003). Preferably, regulations should be harmonised with those in other countries with which trade contacts exist. Unnecessarily strict regulative actions make it difficult for importing countries to obtain supplies of essential commodities such as food grains and animal feedstuffs. Also, exporting countries may have difficulty finding markets for their products. For example, stringent regulations for AFs in the EU (EC, 2006) make it difficult for some countries to export food commodities and feed for their European trading partners. As a consequence, several economic communities, e.g. EU, Mercado Cómun del Sur (MERCOSUR), Australia and New Zealand, have been developing efforts during the last decade in order to harmonise their mycotoxin regulations, thus overruling existing national regulations.

In an attempt for harmonisation, EFSA has recently (March 2007) published an opinion on the potential increase in the risk to consumer health of a possible increase in current maximum levels for AFs in almonds, hazelnuts, pistachios, and derived products (http://www.efsa.europa.eu, accessed 15.07.2010). The panel concluded that changing the maximum levels for total AFs in almonds, hazelnuts, and pistachios from 4 to 8 or 10 µg/kg would have minor effects on estimates of dietary exposure and cancer risks. As a consequence, EC recently adopted legislation changing their AFs regulatory limits (EC, 2010a) and sampling plans (EC, 2010b) for tree nuts to more closely conform to that developed by the Codex Committee on Contaminants in Foods (CCCF) and adopted by the Codex Alimentarius Commission (CAC) in July 2008 (CCCF, 2008). The Codex AF sampling plan for tree nuts (almonds, pistachios, and hazelnuts) requires that two 10 kg samples both test less than 10 µg/kg for total AFs (AFT) to accept the lot. The EU adopted the Codex plan, but added an AFB_1 limit of 8 µg/kg. As a result, an almond lot requires two 10 kg samples to each test less than both limits (8 µg/kg AFB_1 and 10 µg/kg AFT) for

the lot to be accepted into the food chain. This revision still does not harmonise with USA regulations, which determine maximum levels for total AFs of 20 µg/kg.

EU food and feed imports are informed in part through the EU's Rapid Alert System for Food and Feed (RASFF). The RASFF is a tool used to exchange information on potential risks entering the food and feed system at any point in the EU, so that all EU member states may be alerted to take the appropriate measures to assure food and feed safety (Wu, 2008). In 2009, RASFF reported a total of 669 alerts or notifications for mycotoxins, of which 95% were for AFs, mostly from nuts, nut products and seeds (638, 81%) (EC, 2010c). A significant part of these notifications (42%) were for peanuts from Argentina, China, USA, Brazil, Egypt and South Africa. Pistachio nuts from Iran, Turkey and USA originated 136 notifications (21%), 63 notifications (9.9%) on hazelnuts nearly all from Turkey, 55 notifications (8.6%) on almonds mainly from USA and a few from Australia, and 7 notifications (1%) on Brazil nuts from Brazil and Bolivia. The remaining notifications were on figs (10%), spices (3.6%), cereals (2%) and feed (1.4%).

Although this is circumstantial evidence, it reflects market realities and conforms to the position of the industry groups, in which peanut, almond and pistachio producers are greatly affected by the economic impact of AF contamination, whereas others (e.g. walnut producers) are primarily concerned with spoilage microorganisms such as *Rhizopus*, *Penicillium* and *A. niger*.

2.1.6 Aflatoxigenic fungi and aflatoxins in tree nuts

2.1.6.1 Aflatoxigenic species

All known aflatoxigenic species belong to genus *Aspergillus*. Currently, 14 species have been identified as having the ability to produce at least one of the four major naturally occurring AFs. Nine of them belong to section *Flavi*, being *A. flavus* and *A. parasiticus* the most significant and widespread. *A. flavus* populations have been found to be extremely diverse in terms of toxigenicity, and only about 40% of known isolates produce AFs (Frisvad et al., 2006b). The species has been divided into two morphotypes depending on the size of sclerotia, L-type strains producing large sclerotia (> 400 µm) and S-type strains producing microsclerotia (< 400 µm; Cotty, 1989). S-type strains are usually associated

with the production of large amounts of AFBs (S_B) or, more atypically, AFBs and AFGs (S_{BG}). Some of these atypical S_{BG} strains have been recently ascribed to the new aflatoxigenic species *A. parvisclerotigenus* (Frisvad et al., 2005) and *A. minisclerotigenes* (Pildain et al., 2008).

A. parasiticus strains are more uniform in their toxigenic abilities: they are usually strongly aflatoxigenic, producing both AFBs and AFGs. Non-aflatoxigenic strains have rarely been reported (Horn et al., 1996; Tran-Dinh et al., 1999; Vaamonde et al., 2003; Razzaghi-Abyaneh et al., 2006). Recently, a new species closely related to *A. parasiticus*, *A. arachidicola*, has been described (Pildain et al., 2008). *A. nomius* is also strongly aflatoxigenic, having an aflatoxigenic profile similar to *A. parasiticus* (Kurtzman et al., 1987). Other aflatoxigenic species of this section have been identified: *A. pseudotamarii* (Ito et al., 2001), a close relative of the non-aflatoxigenic species *A. tamarii*, and *A. bombycis* (Peterson et al., 2001), closely related to *A. nomius*.

Outside section *Flavi*, five species have also been identified as aflatoxigenic, but, to our knowledge, they have not been implicated in food contamination. Two species belong to section *Ochraceorosei*, *A. ochraceoroseus* and *A. rambellii* (Frisvad et al., 2005), and three belong to section *Nidulantes*, *Emericella astellata* (Frisvad et al., 2004), *E. olivicola* (Zalar et al., 2008) and *E. venezuelensis* (Frisvad & Samson, 2004). Numerous other species have been incorrectly indicated as aflatoxigenic, mainly as a result of wrong identification or strain contamination. Frisvad et al. (2006a) have collected a list of those species, which include *A. flavo-fuscus, A. glaucus, A. niger, A. oryzae, A. ostianus, A. sulphureus, A. tamarii, A. terreus, A. terricola, A. wentii, Emericella nidulans* (as *A. nidulans), E. rugulosa* (as *A. rugulosus*), *Eurotium chevalieri, E. repens, E. rubrum, Mucor mucedo, Penicillium citrinum, P. citromyces, P. digitatum, P. frequentans, P. expansum, P. glaucum, P. puberulum, P. variabile, Rhizopus* sp. and the bacterium *Streptomyces* sp.

2.1.6.2 Biodiversity and biogeography of aflatoxigenic species

Regarding the distribution and economic importance of aflatoxigenic species, only species belonging to section *Flavi* have been found to be of significance in foods and food commodities. From those, *A. flavus* and *A. parasiticus* remain the most important and representative aflatoxigenic species occurring naturally in food commodities all over the

world. For many years, researchers did not separate *A. flavus* from *A. parasiticus* in field studies, which led to some confusion regarding their distribution. Even today, as new species are constantly being described, some doubts remain on the true identification of some isolates that phenotypically resemble the most common species. As a matter of consequence, biodiversity and biogeography of aflatoxigenic species needs to be regarded with caution.

Incidence of the aflatoxigenic species varies with crop and geographic location. *A. flavus* is the most commonly reported species and has been isolated from soils and cultures in all of the major biomes (Klich, 2002b). Although *A. flavus* may be found in all climatic zones, it is isolated relatively more frequently in warm temperate zones (latitudes 26–35º) than in tropical or cooler temperate zones, and is quite uncommon in latitudes above 45º (Klich, 2002b). It is therefore not surprising that chronic AF problems are associated with crops in latitudes below 35º and are generally not a major problem in crops raised in Europe (Klich, 2007). The atypical S_B and S_{BG} strains have been identified from Australia, Thailand, West Africa and Argentina (Saito & Tsuruta, 1993; Cotty & Cardwell, 1999; Geiser et al., 2000; Vaamonde et al., 2003; Pildain et al., 2008). In North America, only S_B strains have been reported (Cotty & Cardwell, 1999; Horn & Dorner, 1999).

The species is widespread in a variety of foods, but is mostly found in oil seeds, nuts, cereals and dried fruits. Also, the highly variable aflatoxigenic profile of *A. flavus* populations seems to be dependent as much on the geographic origin as on the substrate. For instance, isolates from peanuts seem to be predominantly aflatoxigenic (70-100% of all isolates) and in proportions significantly higher than in other crops, independently of the geographic origin (Joffe, 1969; Schroeder & Boller, 1973; Lisker et al., 1993; Barros et al., 2003, 2005; Vaamonde et al., 2003; Pildain et al., 2004; Nakai et al., 2008). Also, Brazil nuts (Arrus et al., 2005a), white sultanas and dried figs (Iamanaka et al., 2007) have been associated with extremely high proportions of toxigenic isolates. On the other hand, populations from crops like maize, wheat, coffee beans and cotton have proportions of aflatoxigenic isolates that range from 5 to 50% (Cotty, 1997; Wicklow et al., 1998; Vaamonde et al., 2003; Razzaghi-Abyaneh et al., 2006; Atehnkeng et al., 2008). No studies on almonds were found referring to the proportions of section *Flavi* species in this substrate.

A. parasiticus is apparently less widespread in nature, and it seems to be more adapted to survival in the soil and less dependent on crop infection than *A. flavus* (Horn, 2007). As a matter of fact, this species is generally isolated quite rarely from the majority of foods and was found to be important only in soils and underground foods like peanuts (Klich, 2002b; Vaamonde et al., 2003; Horn, 2005). Also, it has been reported to be geographically restricted to USA, South America and Australia (Frisvad et al., 2006b), and to be rare in Southeast Asia (Pitt et al., 1993).

Aflatoxigenic species other than *A. flavus* and *A. parasiticus* appear to be of minor importance to agriculture. *A. nomius* has rarely been identified in survey studies from agricultural soils and commodities (Fiebelman et al., 1998; Ito et al., 1998; Abbas et al., 2005; Razzaghi-Abyaneh et al., 2006; Ehrlich et al., 2007; Johnsson et al., 2008; Olsen et al., 2008). The species has been isolated from diverse regions, but is still considered rare. Yet, this can be an artefact resulting from its strong resemblance with *A. flavus*, since recent data indicate that *A. nomius* may be a major contributor to AF contamination of Brazil nuts (Johnsson et al., 2008; Olsen et al., 2008). The significance of the recently described species needs further investigation in terms of food and geographic distribution, but they are probably less significant from the point of view of AF contamination of foods and feeds. They have been reported rarely and with restricted distribution. *A. arachidicola* and *A. minisclerotigenes* have been reported from an Argentinian uncultivated peanut plant (Pildain et al., 2008), whereas *A. pseudotamarii* and *A. caelatus* have been isolated from tea fields in Japan (Ito & Goto, 1994; Ito et al., 1999).

The differences in aflatoxigenic fungi community structure are reflected in the relative abundance of AFBs and AFGs in crops produced in various regions (Cotty, 1997). Furthermore, the average AF-producing potential of fungal communities varies with geography with some regions having communities with greater AF-producing potentials and, as a result, crops grown in those regions are more vulnerable to contamination (Cotty, 1997; Jaime-Garcia & Cotty, 2006). For instance, geographical divergence in the strongly aflatoxigenic S-strains incidence has been associated with increased crop AF content (Jaime-Garcia & Cotty, 2006), and are presumed to be responsible for the major contamination problems occurring in Africa. Also, regions and cultures where *A. parasiticus* is more abundant are usually associated with higher levels of contamination with AFGs than those richer in *A. flavus*.

The factors responsible for the toxigenic profile of *A. flavus* populations in a region or substrate are not fully understood. It has been suggested that modern agricultural management practices may create unique ecological niches which select toxigenic fungi and that the extent of these selective forces influences the relative proportion of toxigenic and atoxigenic strains in a given area (Bilgrami et al., 1981). Bilgrami et al. (1988) and Horn & Dorner (2001) suggest that adverse environmental conditions like those usually found in nature (by competition with other microorganisms and by exposure to stressful conditions), have a stabilising effect on AF production ability and other wild-type characters in *A. flavus*, and that they are lost in nutritionally rich environments. Perrone et al. (2007) further suggest that, since section *Flavi* isolates are essentially saprophytic, polyketide metabolites like AFs may increase fungal survival in soil, but that such benefit may be unnecessary in carbon-rich environments, where the ability to produce AFs could be a vestigial function. Adaptation of *A. flavus* to certain crops, namely the carbon-rich ones, is perhaps conducive to gene loss, since many of the isolates incapable of AF production have multiple mutations in their AF gene cluster (Chang et al., 2005).

Also, in *A. parasiticus*, when normal development is thwarted, the resulting isolate permanently loses some of its normal development functions, and loss of AF production is usually related to loss of conidia formation (Guzman-de-Pena & Ruiz-Herrara, 1997; Kale et al., 2003; Wilkinson et al., 2004). Maybe the defects in *A. parasiticus* isolates are too severe and turn them unviable, thus justifying the fact that almost all isolates are aflatoxigenic. These theories may be supported by the fact that surveys from soil or from soil-growing crops like peanuts usually render higher incidences of *A. parasiticus* and aflatoxigenic *A. flavus* than surveys on crops that do not contact directly with soil, like maize and almonds (Wicklow et al., 1998; Barros et al., 2003, 2005). Also, the levels of *A. parasiticus* are usually higher in soil than would be expected from levels present in the corresponding culture (Horn et al., 1995; Doster et al., 1996).

2.1.6.3 Ecophysiology of aflatoxigenic fungi and aflatoxin production

Growth of aflatoxigenic fungi and the biosynthesis of AFs is strongly dependent on growth conditions such as substrate composition, pH, a_W, temperature or modified atmospheres. Depending on the particular combination of external growth parameters the

biosynthesis of AFs can either be completely inhibited, albeit normal growth is still possible, or the biosynthesis pathway can be fully activated. Knowledge about these relationships enables an assessment of which parameter combinations can control fungal growth and AF biosynthesis or which are conducive to AF production.

Environmental conditions and water availability

Although some geographical variation might be attributed to isolation and divergence (Cotty & Cardwell, 1999), responses of AF producers to climate are important influences (Cotty, 1997; Cardwell & Cotty, 2002). AF producing fungi are native to warm arid, semi-arid, and tropical regions with changes in climate resulting in large fluctuations in the quantity of AF producers (Bock et al., 2004). These fungi compete poorly under cool conditions and the quantity of *A. flavus* in cool areas (temperature minima < 20 °C) is low compared to warmer regions (temperature minima > 25 °C) where aflatoxigenic fungi are common throughout soils, air, and on crop surfaces. Hence, crops grown in warm climates have greater likelihood of infection by AF producers.

Similarly to what has been described for fungi in general, temperature and the equilibrium environmental relative humidity/substrate a_W are the factors that more strongly affect growth of aflatoxigenic fungi and AF production. Several in vitro studies have been developed on the determination of cardinal temperatures and a_W for fungal growth and AF production. *A. flavus* and *A. parasiticus* can grow over a wide range of temperatures (12 to 48 ºC), but growth is maximised in the range 25 ºC - 37 ºC (Pitt & Hocking, 1997; Marin et al., 1998; Sautour et al., 2002; O'Brian et al., 2007; Samapundo et al., 2007a, 2007b; Schmidt-Heydt et al., 2009). Reported values for optimal a_W for *A. flavus* and *A. parasiticus* growth vary from 0.97 to 0.99 (Gibson et al., 1994; Marin et al., 1998; Sautour et al., 2002), but growth has been reported for minimum a_W near 0.80, for temperatures around 30 ºC (Pitt & Miscamble, 1995; Rosso & Robinson, 2001; Samapundo et al., 2007a, 2007b; Johnsson et al., 2008).

Usually, AFs are produced under temperature and a_W ranges that are not as wide as those found for growth. AF production has been determined to occur at temperatures between 20 and 37 ºC, and for $a_W > 0.85$, but they were found to be optimally produced at 28-30 ºC and $a_W > 0.95$ (Gqaleni et al., 1997; O'Brian et al., 2007; Schmidt-Heydt et al., 2009).

AF contamination of crops can be divided into two distinct phases, with fungal infection of the developing crop in the first phase and increases in contamination after maturation in the second phase. Although episodes of contamination are often attributed to one phase or the other (e.g. due to poor post-harvest handling or associated with insect damage in the field), both phases contribute to many contamination events. Developing crops are frequently very resistant to infection by *A. flavus* and subsequent AF contamination due to natural mechanisms of resistance that are onset as response to fungal attack. But when crops are exposed to stressful environmental conditions such as drought and high temperatures significant infections usually occur (Bircan et al., 2008).

The roles of high temperature and drought in plant contamination are being progressively elucidated. On the one side, these factors directly affect the physiology of the plants and the mechanisms of resistance, leaving them particularly susceptible to fungal infection and AF production. For instance, drought stress induces a great increase of amino acids production in plants, mostly proline, and proline has been reported to stimulate AF production (see below). Also, production of phytoalexins (antimicrobial compounds produced by plants in response to fungal attack), which have been shown to inhibit spore germination and hyphal extension of *A. flavus* in immature (high a_W) peanuts (Wotton & Strange, 1985), is inhibited by drought stress (Wotton & Strange, 1987; Dorner et al., 1989; Strange & Rao, 1994). Another possibility for the higher contamination of crops under stress is that the fungi that normally compete with *A. flavus* do not grow as readily under these conditions, giving *A. flavus* a competitive advantage due to its xerotolerant nature, even compared with other *Aspergillus* species (Klich, 2007).

The second phase of contamination may occur at any time from crop maturation until consumption. During this period, a_W of nuts is usually sufficiently low (< 0.70) to inhibit fungal growth and AF production. In a study of peanut contamination from sowing to harvest, Gonçalez et al. (2008) only found aflatoxigenic fungi and AF contamination in pods at full maturity. *A. flavus* and *A. parasiticus* were not isolated in the stages of maturity with the highest water contents (a_W > 0.98), thus confirming that natural resistance against fungal invasion is only effective at a_W > 0.97, and is lost at the final stages of maturity, when a_W decreases to values below 0.95 (Dorner et al., 1989). In theory, nuts are most susceptible to AF contamination when a_W is between 0.95 and 0.85. Above 0.95, AF production is inhibited by host resistance, and below 0.85 that effect is the result

of fungal growth restriction due to reduced water availability. So, adequate conditions of relative humidity during storage must be effective at maintaining substrate a_W below at least 0.85. If, on the other hand, crops are exposed to conditions of higher humidity, dry seeds develop water content conducive to contamination (Adebajo & Popoola, 2003; Johnsson et al., 2008; Riba et al., 2010). As an example, Johnsson et al. (2008) tested the effect of storing Brazil nuts under different a_W conditions, and detected that both levels of the inoculated fungus and toxin levels increased significantly with time in samples stored at $a_W > 0.85$, but not in samples stored at a_W below that. When growth has started, colony counts and AFs will accumulate as long as a_W and nutrient availability allow it. Growth will cease after drying of the nuts but fungal spores and AFs that have been formed earlier will remain. That is a possible explanation for the finding of AF contamination in stored products with $a_W < 0.70$ (da Silva et al., 2000; Gonçalez et al., 2008).

Nutritional requirements

Many plants and substrates support growth and AF production by aflatoxigenic moulds, but AFs are most frequently associated with high-carbohydrate and high-fat food and feed like ground nuts and derived products, almonds, pistachios, Brazil nuts, maize, rice, figs, cotton seed and spices. In fact, some *Aspergillus* species, mainly *A. flavus* and related species, seem to hold a unique position in the fungal world. They primarily obtain the resources needed for growth in a saprophytic mode and, thus, retain the ability to secrete a large diversity of hydrolases to help access nutrients. The most significant hydrolytic proteins associated with *Aspergillus* section *Flavi* are proteases, lipases, amylases and pectinases (Mellon et al., 2007). Lipases play a prominent hydrolytic role in *A. flavus* metabolism when triglycerides are used as a carbon source. In particular, the *lipA* gene, encoding a lipase in *A. flavus*, has been suggested to promote AF formation in lipid-rich environments, for its putative role in capturing carbon nutrients from lipid sources (Yu et al., 2003). Furthermore, fatty acids are involved in one of the first enzymatic reactions of the aflatoxin pathway (reviewed in Yabe & Nakajima, 2004). The effects of lipids on fungal growth and AF production have been studied in *A. flavus* and *A. parasiticus* by many researchers. Mellon et al. (2000) determined that *A. flavus* growth was enhanced on media with triglycerides as a sole carbon source. In addition, AF production levels were 800-fold higher in lipid-rich substrate than in the same substrate extracted from

lipids. Fanelli et al. (1983) had already demonstrated that lipophilic epoxy fatty acids stimulated AF production in toxigenic fungi. Ergosterol oxidation was also found to induce both fungal growth and AF production (de Luca et al., 1995).

It is also known that carbon and nitrogen sources play a vital role in the regulation of AF production (reviewed by Payne & Brown 1998; Wilkinson et al., 2007; Senyuva et al., 2008). Simple sugars such as glucose, saccharose, maltose and galactose induce AF production, while more complex nutrients such as peptone, lactose, oleic acid and starch do not. These findings support the fact that fat-rich nuts like almonds, pistachios and peanuts are more frequently involved in AF contamination than starch-rich chestnuts. The types and concentrations of the nitrogen source in the substrate are equally critical to fungal growth and to subsequent AF production. It has been reported that the amino acids proline and cystine stimulates AF production more than cysteine, asparagine, tryptophan or methionine when *A. flavus* and *A. parasiticus* are grown in culture.

Interaction with the host plant

In vitro experiments with chemical components of tree nuts have shown a significant difference in the ability of different tree nuts to support AF production. Walnuts were found to contain a series of chemicals (phenols, naphtoquinones, tannins, plumbagin) with potent effects against AF biosynthesis (Campbell et al., 2003; Fukuda et al., 2003; Mahoney & Molineux, 2004). These compounds were found in the seed coat, not the nutmeat itself, and were found only as traces in almonds. On the other hand, various chemical compounds with some sort of biological activity shown to occur in high concentrations in almonds, like triterpenoids, phenolics and sterols (Mahoney & Molineux, 2004), failed to show significant anti-aflatoxigenic activity. Studies on this matter have not been developed in almonds as intensely as in walnuts, but they will need to be addressed, since it is a known fact that, in almonds, AFs usually accumulate in the seed coat, and removing this pellicle usually reduces AF in the nutmeat to undetectable levels.

Besides differences between crops, there are also varietal differences within each crop. Various authors detected differences (significant in some cases) between varieties of walnuts and almonds in terms of chemical composition as well as resistance to *A. flavus* and AF accumulation (Gradziel & Wang, 1994; Gradziel et al., 2000; Dicenta et al., 2002; Mahoney et al., 2003; Mahoney & Molineux, 2004). Dicenta et al. (2002) tested 40 almond

cultivars from different geographic origins and, even if at different levels, all showed susceptibility to *A. flavus*. They concluded that there is no relationship between geographic origin of the cultivars and degree of susceptibility. However, cultivars from California have soft shell, whereas those traditionally cultivated in Europe have hard shell. This difference has been correlated with AF contamination (Gradziel & Wang, 1994), where soft-shell cultivars showed higher susceptibility. This factor, among others (mostly environmental), may constitute the reason why European almonds are seldom contaminated with AFs. However, European producers (and particularly those from the Portuguese northeast region) are currently converting their orchards into soft cultivars which give higher yields and are easier to process. This and the climatic changes in the Mediterranean basin into more arid and hot can lead to a change in this paradigm in the near future (Paterson & Lima, 2009).

The speed at which phytoalexins accumulate after challenge and the concentrations reached are influenced not only by environmental conditions but also by genotype of the plant, and resistant cultivars have been found to accumulate more phytoalexins than the susceptible ones (Strange & Rao, 1994).

Physical barriers and damage

A. flavus is a weak plant pathogen which seems to lack the ability to penetrate the shell of nuts (Dickman et al., 1986), being that entry into the edible kernel usually depends on breaks caused by abrasions or insects. As a consequence, AFs are rarely found in kernels with intact hulls (Sommer et al., 1986; Arrus et al., 2005). Insect-feeding damage is a major factor leading to pre-harvest fungal infection of nut kernels of almond, and subsequent AF contamination. Wounds to the protective layers surrounding nut kernels (hull, shell, seadcoat) provide avenues for infection by wind-borne spores of aflatoxigenic fungi (Doster & Michailides, 1995, 1999; Schatzki & Ong, 2001; Campbell et al., 2003).

The majority of studies on mechanisms of AF contamination in tree nuts are dedicated to pistachio (Dickman et al., 1986; Sommer et al., 1986; Doster & Michailides, 1995, 1999; Mahoney & Rodriguez, 1996; Mahoney & Molyneux, 1998; Campbell et al., 2003). Less is known for other nuts, particularly almonds (Phillips et al., 1979; Schatzki & Ong, 2000, 2001; Campbell et al., 2003) and chestnuts. In a study on pistachio from California, Mahoney & Rodriguez (1996) detected that AF was not produced in pistachio

shells despite of high shell colonisation by *A. flavus*, and lower levels of contamination were detected on in-shell kernels than on shelled kernels. In pistachios, high levels of AFs are associated with early-splitting shells and insect-damaged kernels, in which the kernel can be exposed to fungal spores (Sommer et al., 1986; Mahoney & Rodriguez, 1996; Campbell et al., 2003). In almonds, the maturation process is different, and hull-splitting exposes the hard shell, which remains closed, giving access to insects but usually not directly to fungi (Schatzki & Ong, 2001). Either way, insect-damaged nuts are usually excluded from downstream processing through visual or mechanical sorting, based on external evident damage or decolorisation typical of fungal contamination, thus reducing the incidence of contaminated nuts in the final product.

Alternate routes of infection may occur during development of the kernel or through natural breaches which take place as the kernel matures, mainly through the stem-end of the nut when the fruit is still soft (Campbell et al., 2003). At this stage, the kernel is vulnerable to being pierced by sucking-insects common to pistachio and almonds. This route presents a problem, since there are no evident external signs of damage to the nut, making it difficult to remove such nuts from the processing stream.

Mahoney & Rodriguez (1996) observed that the seed coat matrix of pistachio appears to be more conducive to adhesion and/or germination of *A. flavus* spores than the shell, but the waxy cuticular layer below the seed coat that exists in tree nuts seems to be an effective barrier to *A. flavus* infection. They analysed pistachios with and without seed coat (but with the cuticular layer intact), and detected that none of the blanched nuts (without seed coat) suffered *A. flavus* colonisation or AF contamination, whereas 62% of the kernels with seed coat were colonised by the fungus and 44% were contaminated with the toxin after 10 days of inoculation. There are no reports on the differential analysis of the AF content of the shell, nutmeat and seed coat of almonds, but there is a general recognition that removing the seed coat from contaminated kernels is a practice that greatly reduces AF contamination. Even if pistachios and almonds differ significantly in their maturation process (Schatzki & Ong, 2000), we can assume that the physical and chemical barriers between the outer shell and the nutmeat might have a similar effect on fungal growth in both pistachios and almonds.

A major reservoir of aflatoxigenic *Aspergillus* spores is in the orchard litter surrounding tree nuts (Campbell et al., 2003). In general, almond fruits are harvested by

shaking them from the tree immediately prior to collection. It is not known whether there is direct infection of nuts while still on the tree, but the infected litter may contribute to increase the probability of wounded nuts being infected by fungal spores. Also, spores can adhere to fallen fruits and infect them during storage, even in non-damaged nuts, if proper post-harvest handling and storage conditions are not guaranteed. For other nuts that are allowed to fall and stay on the ground for days or weeks, like chestnuts, contamination and infection can potentially occur during that stage.

Interaction with other microorganisms

Antagonism between aflatoxigenic fungi and other microorganisms have been reported in several studies. Phillips et al. (1979) analysed pre-harvest almonds naturally and artificially infected with various fungi and found that most of the fungi, and mostly *Rhizopus* and *Eurotium* species, reduced the colonisation of kernels by *A. flavus* and *A. parasiticus*. Also in almonds, Joffe (1969) observed that fungi with higher a_W requirements had stronger antagonistic effect over *A. flavus* than those fungi usually associated with dry foods. It is not completely understood if competition is due to antagonistic effects or to the physical and chemical environment being more adapted to ones than to the others. Other studies have shown a positive correlation between *A. flavus* and *A. niger* (Doster et al., 1996; Bayman et al., 2002). This association is probably due to the fact that sections *Flavi* and *Nigri* share common habitats and ecophysiological characteristics (Rosso & Robinson, 2001; Esteban et al., 2006; Magan, 2006; Klich, 2007), so conditions that favour one of these fungi probably favour the other. An alternative explanation is that infection by one species makes the fruit more susceptible to the other (Bayman et al., 2002).

Several in vitro studies have shown that the presence of other filamentous fungi, namely *A. niger*, *Rhizopus* spp., *Trichoderma* spp. and *Penicillium* spp., among others, can significantly reduce AF accumulation, by AF production inhibition and/or by AF degradation (Wicklow et al., 1980; Mislivec et al., 1988; Doster et al., 1996; Aziz & Shahin, 1997; Calistru et al., 1997; La Pena et al., 2004). A number of bacteria (*Bacillus* spp., *Pseudomonas* spp., *Ralstonia* spp. and *Burkholderia* spp.; Palumbo et al., 2006; Mohadmadipour et al., 2009) and saprophytic yeasts (*Pichia* spp., *Candida* spp. and *Kluyveromyces* spp.; Hua et al., 1999; La Penna et al., 2004) have also been shown to possess some kind of effect on *A. flavus* growth and AF production.

It has also been shown that non-aflatoxigenic *A. flavus* have an effect of competitive exclusion towards aflatoxigenic isolates (Cotty & Bayman, 1993; Cotty, 1994).

2.1.6.4 Control measures

In contrast to many crops, tree nuts undergo minimal or very light processing, such as blanching, and the majority of the crop is traded and consumed as whole or shelled nuts. Any subsequent processing, such as incorporation into baked goods, is performed by the buyer or ultimate consumer after AF analysis has been performed. There is thus little opportunity to reduce AF levels by artificial means and natural methods must therefore be found. Preventing AF accumulation in crops can be achieved by either controlling the fungus or controlling AF production, via the use of any of several measures alone or in combination, in pre- or post-harvest stages of production (Campbell et al., 2003; Cleveland et al., 2003; Munkvold, 2003; Strosnider et al., 2006).

Pre-harvest control measures

The general strategy for pre-harvest AF control methods is to alter the conditions under which the crop is grown so that infection is avoided. Any management practice to maximise plant performance and decrease plant stress will decrease AF contamination (Payne, 1998). The most immediate cultural measures include tillage practices, fertilization regimes, crop rotation, proper plant density, planting date, and irrigation (Wu et al., 2008). None of these apply to the cultural practice of tree nuts, and these measures have mostly been applied to annual crops such as maize and peanuts. Since AF contamination in almonds has been strongly associated with damage inflicted by specific insects (Schade et al., 1975; Schatzki & Ong, 2001; Campbell et al., 2003; Whitaker et al., 2010), insect control could also be an effective control measure. Also, AF is a bigger problem in plants under stress, and can be reduced by lowering plant stress, mainly by irrigation in drought periods. Because irrigation and the application of fungicides and insecticides in almond and chestnut orchards is not in equation, cultural strategies are probably reduced to harvesting under dry conditions and immediately after nuts have fallen from the tree.

The nature of tree nut harvesting and processing, which involves considerable potential for spreading of fungal spores and AFs throughout the lots, mandates that the

most effective method of control would be to prevent AF formation by the nuts themselves, through the use of cultivars more resistant to fungal infection or with natural products in their composition which confer natural resistance to fungal infection and/or AF production.

Because chemical control procedures for mycotoxin contamination are not economically feasible for most crops, interest exists for developing effective biocontrol agents to decrease mycotoxin contamination. Efforts on the biological control of aflatoxigenic strains of *A. flavus* and related species are in progress and involve the use of atoxigenic strains as biocompetitors of toxigenic strains (competitive exclusion; Cotty, 1994; Cotty & Bhatnagar, 1994). Inoculating crops with atoxigenic *A. flavus* strains has been found to effectively inhibit AF production in fields of cotton (Cotty, 1994) and corn (Abbas et al., 2009). Wu et al. (2008) analysed the relation cost/benefit of using this type of biocontrol, and concluded that only in regions and crops with high AF contamination levels this control measure is advantageous for the producer. Also, care must be taken because *A. flavus* produces other toxins besides AFs. The strategy of using microorganisms native to tree nut orchards as biological control agents has also resulted in the identification of a number of already mentioned organisms with effect against aflatoxigenic fungi (Calistru et al., 1997; Hua et al., 1999; La Penna et al., 2004; Palumbo et al., 2006; Mohadmadipour et al., 2009) but there are no records on the use of such organisms in field trials.

As previously mentioned, different cultivars show different susceptibility to *A. flavus* and AF accumulation. The conversion of orchards into more resistant cultivars is one possible measure of control. Breeding for resistance to AF contamination is underway in almonds (Campbell et al., 2003).

Post-harvest control measures

Post-harvest handling of crops offers additional challenges, but also vital chances to minimise the ultimate AF levels. Nuts may be attacked by fungi in the field which can then rapidly develop and produce mycotoxins during storage when conditions are suitable. Contamination with *A. flavus* and subsequent production of AFs during storage is considered one of the most serious safety problems throughout the world, mainly in hot and humid regions.

To reduce or prevent production of mycotoxins in nuts, drying should take place soon after harvest and as rapidly as feasible. The critical water content for safe storage of nuts (except chestnuts) corresponds to a_W of about 0.7, or relative humidity below 80% (Arrus et al., 2005). Problems in maintaining an adequately low a_W often occur in the tropical and subtropical regions where high ambient humidity makes the control of commodity moisture difficult. Fungal control in stored nuts with high perishability (resulting from high a_W) like chestnuts usually requires a short period of superficial drying followed by anti-fungal chemical treatments and controlled atmospheres in terms of relative humidity, temperature and CO_2 concentration (Mignani & Vercesi, 2003).

The higher value nut and nut product consists of the shelled kernel, whereas in-shell, blanched (seed coat removed), sliced, diced, ground or minced products have reduced market value. Highly processed products are only valuable when integrated in high quality products and delicatessen. Otherwise, they are usually processed from low quality raw material. As an example of this, two surveys on processed Californian almonds (Schade et al., 1975; Schatzki, 1996) showed that AFs were found essentially on diced or ground material, probably as a result of integrating damaged almonds, either by lack of sorting or to hide damages. It has been shown that removing visibly damaged nuts by hand- or mechanical-sorting before processing significantly reduces AF contamination of processed almonds, by reducing the number of potentially contaminated nuts that enter subsequent processing steps (Schade et al., 1975).

AFs are chemically extractable with solvents, but this method, although effective, is not economically practical. Heat treatment, radiation and ammoniation may also reduce, but not eliminate, AFs (reviewed in Klich, 2007).

2.1.6.5 Occurrence of aflatoxigenic fungi and aflatoxins in nuts

Bayman et al. (2002) report the identification of 93% *A. flavus* and 7% *A. tamarii* in field-collected and store-bought Californian almonds. In store-bought almonds from Saudi-Arabia, *A. flavus* constituted 98% of the *Flavi* population, the rest being *A. tamarii* (Abdel-Gawad & Zohri, 1993). In other substrates, Vaamonde et al. (2003) registered the predominance of *A. flavus* in field-collected peanuts (55%) and wheat (75%), and soybeans from local markets (96%) in Argentina. The remaining isolates were *A. parasiticus*.

Similarly, Barros et al. (2003, 2005) analysed peanut-growing soil from Brazil and identified 73% *A. flavus* and 27% *A. parasiticus*. Razzaghi-Abyaneh et al. (2006) detected 88% *A. flavus*, 3% *A. parasiticus* and 9% *A. nomius* from Iranian corn fields. From corn fields and field-collected corn, Wicklow et al. (1998) detected 72% *A. flavus* and 28% *A. parasiticus*. Atehnkeng et al. (2008) analysed corn from Nigeria and detected more than 90% *A. flavus* and only reduced numbers of *A. tamarii* and *A. parasiticus*. In cotton from the USA, Cotty (1997) registered that *A. flavus* and *A. tamarii* accounted for more than 95% of all *Flavi* and that *A. parasiticus* and *A. nomius* occurred at very low frequencies. Also, a comprehensive study on the distribution of *Aspergillus* section *Flavi* from soil and litter (Klich, 2002b) has demonstrated that *A. flavus* was the most commonly isolated species worldwide, and that *A. parasiticus* was reported only rarely for the majority of biomes and latitudes. In the same study, *A. tamarii* was found more frequently than *A. parasiticus* even from cultivated biomes. Other studies have even reported the complete absence of *A. parasiticus*: in Brazil nuts collected from processing plants in Peru (Arrus et al, 2005a) and in stored peanuts (Nakai et al., 2008), processed coffee beans (Batista et al., 2003), and store-bought white sultanas and dried figs (Iamanaka et al., 2007) from Brazil. Sánchez-Hervás et al. (2008) detected an incredibly high percentage of *A. tamarii* isolates (44%), being the rest of the isolates *A. flavus*.

From the studies on the analysis of AFs on almonds, few have reported AF contamination of these nuts. Schade et al. (1975) analysed 74 samples of unsorted, in-shell Californian almonds and found that 10 (14%) were contaminated with 14.8 µg/kg (total weight, kernel plus shell) total AFs, ranging from 1 to 107 µg/kg. Schatzki (1996) reported that 80% of 1547 almonds with different types of processing were contaminated, but at very low levels, averaging 0.67 µg/kg. Abdel-Gawad & Zohri (1993) and Abdulkadar et al. (2000) analysed various nuts marketed in Saudi Arabia and Qatar (no origin reported), respectively, and found that none of the in-shell and shelled almond samples were contaminated. AFB_1 (95 ng/kg) and AFB_2 (15 ng/kg) were found in one sample of almonds from Spain by Jiménez & Mateo (2001). Only traces of AFs were associated with whole almonds from Morocco (Bottalico & Logrieco, 2001).

There are few reports on chestnuts contamination with mycotoxins. Abdel-Gawad & Zohri (1993) analysed a wide range of mycotoxins in chestnuts strongly contaminated with *Fusarium*, *Penicillium* and *Aspergillus*, and detected AFB_1 and AFG_1 in 3 of the 5 samples

analysed, ranging from 20 to 60 µg/kg. On the other hand, Overy et al. (2003) detected significant contamination of Canadian chestnuts with 5 mycotoxins, chaetoglobosins A and C, emodin, OTA and penitrem A, associated with the most prolific penicillia, but no AFs were detected.

AF contamination of other nuts is usually higher than that of almonds. AFs were found in 4 of 11 samples of pistachios from Sicily (up to 45 µg/kg of AFB_2 and AFG_2), in three out of seven samples from Greece (up to 87 µg/kg of AFB_1, AFB_2, AFG_1, and AFG_2) and in three out of six samples from Turkey (up to 102 µg/kg of AFB_1, AFB_2, AFG_1, and AFG_2) (Barbagallo & Russo, 1999). Abdulkadar et al. (2000) detected contamination in 20% of in-shell pistachios, 52% of shelled pistachios and 33% of in-shell peanuts, with total AFs ranging from 0.53 to 289 µg/kg. AFBs were detected in all contaminated samples, whereas only 7% of the pistachio samples were contaminated with low levels of AFGs. In peanuts from Kenya, Mutegi et al. (2009) detected that 36% of the analysed samples were contaminated with AFs, but only a small proportion (7.5%) reached levels > 20 µg/kg. The highest level detected was 7525 µg/kg.

2.1.7 Determination of mycobiota in nuts

2.1.7.1 Detection and enumeration of fungi

The methods for examination of the mycobiota of foods have been originally based on bacteriology or medical mycology. But bacteria and human pathogenic fungi grow optimally on media with high a_W and low carbohydrate content, whereas foodborne fungi, which are typically saprophytic, usually prefer the exact opposite conditions (Samson et al., 2004a). For that reason, in the last decades efforts have been put in the development and standardisation of techniques for food mycological examination (Hocking & Pitt, 1980; Beuchat, 1992a, 1992b; Frändberg & Olsen, 1999; Okuda et al., 2000; Frändberg et al., 2003; Bueno et al., 2004; Samson et al., 2004a).

The dilution plating or colony forming units (CFU) method is one of the commonly used techniques for the examination of foodborne fungi. Homogenising of the samples, preparing 10-fold dilutions and surface spreading on different agar plates are the characteristic steps of this method. After incubation, the resulting colonies are counted and

analysed. But sensitivity and reproduction of this technique may be restricted by the method of homogenisation, by the hydrophobic surface of fungal spores and by the fact that mycelium homogenisation is impossible.

Direct plating is considered the most effective technique for the mycological examination of particulate foods such as nuts (Bueno et al., 2004; Samson et al., 2004a). The results of this analysis are usually expressed as percentage of infected particles. The method does not provide information on the number of propagules infecting each particle, but is gives an accurate idea of the level of contamination.

In the case of cereals and nuts, a surface disinfection with chlorine or ethanol before plating is usually recommended, to allow the enumeration of the fungi effectively invading the food (Pitt & Hocking, 1997; Bueno et al., 2004; Samson et al., 2004a). The differentiation between species which are only present as superficial propagules and those which are effectively contaminating the food (in the case of nuts, the edible part of the fruit) is considered of major importance, since only the latter are taken as associated mycobiota. In general, numerous propagules are present as superficial contaminants, since they are part of the normal environmental mycobiota, but only rare fungi are able to infect the substrate. Numerous studies have compared the mycobiota from surface disinfected and non-disinfected foods, and generally reported a significant decrease of overall fungal contamination after treatment (e.g. Joffe, 1969; Bayman et al., 2002; Batista et al., 2003), showing that contamination is largely superficial.

To our understanding, there are some problems related to surface disinfection of nuts. One of them is that fungi are not equally sensitive to disinfection treatments. Some species of *Aspergillus*, namely *A. flavus* and *A. parasiticus*, appear to be extremely resistant to surface disinfection with chlorine (Sauer & Burroughs, 1986; Andrews, 1996). On the other hand, extreme conditions of treatment with chlorine and 70% ethanol can lead to some inactivation of internal mycobiota (Sauer & Burroughs, 1986). Also, Bayman et al. (2002) reported that, in a few cases, incidence of certain fungi increased in almonds after disinfection. They concluded that superficial mycobiota probably inhibited growth of those fungi on agar in non-treated almonds, and that the removal of surface contaminants allowed them to grow out. In fact, surface disinfection necessarily alters the equilibrium naturally established between fungi, and some fungi that under natural environmental

conditions would not have the competitive ability to grow and produce spoilage may become dominant, and apparently problematic, under laboratory conditions.

Exceptions to generalised surface disinfection are acceptable whenever surface contaminants become part of the downstream mycobiota and potentially influence the quality of the final product. In such cases, Samson et al. (2004a) recommend that foods are investigated with and without surface disinfection. In those cases where the product is going to be subject of long term storage or processing, it is wise to contemplate the overall fungal contamination of foods, even if they are not infecting the foods at the moment, since environmental conditions may evolve to become appropriate for a rare fungus to outgrow others and produce spoilage in downstream stages of production.

Under laboratory conditions, all culture media are selective to some degree, making mycological examination of any food matrix highly dependent on the culture media used for plating. The general medium recommended for fungal isolation from dry foods with $a_W < 0.90$ is Dichloran 18% Glycerol agar (DG18; Hocking & Pitt, 1980), not only because of its low a_W but also because dichloran controls the colony development of fast growing fungi (*Eurotium* species, *Mucorales*) with less inhibition of the development of more slowly growing species. Other media generally used in fungal surveys from foods are Dichloran-Rose Bengal Chloramphenicol (DRBC, King et al., 1979) for less xerophilic fungi (foods with $a_W > 0.90$), and Malt Yeast 50% Glucose agar (MY50G; Pitt & Hocking, 1997), for extremely xerophilic fungi (foods with $a_W < 0.70$).

Malt Salt Agar (MSA; Christensen, 1946) with varying concentration of salt (usually 6% to 10%) has also been used in fungal surveys, mainly when *Aspergillus* and *Penicillium* from low a_W substrates were the central interest of the studies (Joffe, 1969; Phillips et al., 1979; Hocking & Pitt, 1980; Purcell et al., 1980; Ackermann, 1998; Bayman et al., 2002; Samson et al., 2004a; Kaaya & Kyamuhangire, 2006; Medina et al., 2006). Hocking & Pitt (1980) tested DG18 and MSA media on fungal surveys from several dried foods and, in most cases, DG18 produced slightly higher counts than MSA. In fact, the medium used for fungal surveys is determinant for the type and frequencies of fungi detected, and this fact should be taken in consideration when results from different studies are compared. As an example, Abdel-Gawad & Zohri (1993) counted fungal contaminants from 6 different types of nuts using two different media: glucose-Czapek-Dox, with high a_W, and DG18, with low a_W. Besides different counts, these authors also reported that some

species were only detected in one of the media, as was the case of the highly xerophilic *Eurotium* species (which correspond to the teleomorphic state of species from section *Aspergillus*), whose detection was limited to DG18.

2.1.7.2 Fungal isolation and identification

Having detected or made total counts of fungi in food samples, identification to the genus or species level is usually the following step. For that matter, pure cultures must be prepared in the appropriate isolation and identification media. Isolation media should be adapted to the characteristics of the fungus to be isolated, in order to achieve typical growth and sporulation that aid the identification at the group or genus level, but it must also support the growth of various species in order to detect contaminants. The inoculation must be done by streaking, since point-inoculation will not allow contaminants to clearly develop (Samson et al., 2004a). Pure cultures need to be further transferred for specific media, usually more than one, for sub-generic identification. Care should be taken in the preparation and plating of identification media, since variations in media formulae, ingredient quality and poured volume will influence fungal morphology (Okuda et al., 2000). Table 2.2 summarises the media used for isolation and identification of some of the fungal genera usually found in nuts.

Table 2.2 List of media used for isolation and identification of some of the fungal genera usually found in nuts (Samson et al., 2004a; Klich, 2002a; http://www.cbs.knaw.nl/service/foodmedia.aspx, accessed 10.09.2010).

Genus or group	**Isolation media**	**Identification media**
Aspergillus spp.	DRYES, DYSG, DRBC, DG18	CZ, CYA, MEA, CY20S, CREA
Penicillium spp.	DRYES, DYSG, DRBC, DG18	CZ, CYA, MEA, CREA
Fusarium spp.	CZID, TWA	PDA, PSA, SNA
Xerophilic species (except *Eurotium* spp.)	DG18, MY50G	MY50G
Eurotium spp.	CY20S, M40Y	CZ20S, CY20S, MEA20S
Alternaria spp.	MEA	MEA (20 ºC)
Cladosporium spp.	MEA	MEA (20 ºC)

2.1.7.3 Detection and enumeration of aflatoxigenic fungi

Various media have been developed for the rapid screening of aflatoxigenic fungi. In these media, fungi can be directly detected and/or enumerated by dilution plating or direct plating of the sample. Aspergillus flavus and parasiticus Agar (AFPA; Pitt et al., 1983) is the medium most widely used for the purpose, and has been recommended by Samson et al. (2004a). AFPA evolved from the Aspergillus Differential Medium (ADM) developed by Bothast & Fennell (1974, in Samson et al., 2004a), and is used for the detection of the common aflatoxigenic species (*A. flavus*, *A. parasiticus* and some related species), as they are easily differentiated from other species by their bright cadmium-orange reverse. AFs are not produced in these media, but they are indicated by the production of a Ferri chelate of aspergillic acids. Other less common media like RBSAB (Rose Bengal Streptomycin Agar with Botran) (=Dichloran) proposed by Bell & Crawford (1967, in Samson et al., 2004a), M3S1B (Medium with 3% Salt and 1 ppm Botran incubated at 37 ºC) proposed by Giffen & Garren (1974, in Samson et al., 2004a) have also been developed based on the fact that aflatoxigenic species also produce aspergillic acid (Samson et al., 2004a).

Since AFs are not produced by all strains of aflatoxigenic species, various simple and rapid methods have been developed that detect aflatoxigenic strains by the direct visualisation of AFs produced in the culture medium. de Vogel et al. (1965) described a complex medium containing saccharose, various salts and aqueous extract of AF-free peanuts, in which AFs fluoresced bright blue after exposure to short UV radiation (350 to 370 nm). Hara et al. (1974) described a similar but less laborious medium containing corn steep liquor, named AF-producing-ability (APA) medium. Lin & Dianese (1976) described a coconut-based medium (CAM; Coconut Agar Medium), which was later improved by others (Davis et al., 1987; Lemke et al., 1989; Dyer & McCammon, 1994), which was simpler and faster than the previous. In these media, AFs diffused into the medium and fluoresced blue under long UV light. Yabe et al. (1987) reported another simple method based on UV photography. Saito & Machida (1999) also reported a rapid method where colonies from aflatoxigenic strains cultured for two days on AF-inducing media turned pink when exposed to ammonia vapour. Ordaz et al. (2003) described a rapid and reliable method where production of AFs in yeast extract agar medium supplemented with methylated β-cyclodextrin and sodium desoxycholate was detected after three days by a

yellow ring observed under UV light. An innovative analytical methodology based on a similar culture medium was presented by Rojas-Durán et al. (2007), but in this case AFs were detected by using a fibre-optic luminometer that measured the room temperature phosphorescence (RTP) emitted by AFs.

A different approach involves the isolation of the fungi from general isolation media (as described earlier) and their growth under conditions that are known to be inducive of AF production, like carbohydrate-rich or fat-rich media (usually Yeast Extract Saccharose (YES) or CAM). After a period of incubation, AFs are extracted from the medium using appropriate solvents (usually methanol) and are analysed by analytical methods such as thin-layer chromatography (TLC) or high performance liquid chromatography (HPLC) (Samson et al., 2004a). This approach is more expensive and time-consuming than the previous, but is also more accurate.

2.1.8 Determination of aflatoxins in nuts

The fact that most mycotoxins are toxic at very low concentrations requires sensitive and reliable methods for their detection. Furthermore, as regulations become more and more restrictive, requirements for adequate sampling and analytical methods are also imposed. As a consequence, mycotoxin analytical methods need to have low limits of detection, be specific to avoid analytical interferences, be easily applied in routine laboratories, be economical for the laboratory involved and provide a confirmatory test for the analyte of interest (Shephard, 2008). Sampling and analysis are of critical importance since failure to achieve a satisfactory verified analysis can lead to unacceptable consignments being accepted or satisfactory ones being unnecessarily rejected. Due to the varied structures of these compounds, it is not possible to use one standard technique to detect all mycotoxins in any given matrix, as each binomium mycotoxin/matrix will require a different method. Therefore, depending on the physical and chemical properties of both mycotoxin and matrix, procedures have to be developed and optimised around existing analytical techniques.

Mycotoxin analysis in food and feed is generally a multistep process comprised of sampling, sample preparation, toxin extraction from the matrix, extract cleanup and finally detection and quantitative determination. The analytical methods for the determination of

mycotoxins, and particularly AFs, in food have been extensively reviewed (e.g. Jaimez et al., 2000; Krska et al., 2005; Shephard, 2008, 2009; Reiter et al., 2009; Turner et al., 2009). We will briefly address to the various strages of analysis.

2.1.8.1 Sampling

Sampling can be taken as the operations that, applied to a lot of an agricultural product, lead to a laboratory sample of a workable size (some hundreds of grams to some kilograms). This laboratory sample is in turn sub-sampled to a portion that will be assayed. It is, therefore, crucial that the final sample from which the assay portion is sampled is truly representative of the initial lot. In practice, the overall objective of good sampling is to provide samples which represent the true mycotoxin content of an inspected lot.

Sampling plans are particularly relevant in foods or commodities where it is known that the contamination can be heterogeneously distributed. Numerous studies have been directed to the optimisation of sampling plans of nuts, cereals and dried fruits, namely AFs in peanuts (Whitaker & Dickens, 1989; Whitaker et al., 1994a, 1994b, 1995, 1996), almonds (Schade et al., 1975; Schatzki & Ong, 2000, 2001; Whitaker et al., 2010), pistachios (Schatzki, 1995a, 1995b; Schatzki & Pan, 1996; Schatzki & Toyofuku, 2003; MacArthur et al., 2006), and maize (Whitaker & Dickens, 1983; Jewers et al., 1988). These studies conclude that more than 90% of the error associated with mycotoxin assays is attributed to sample collection. The AF distribution among individual kernels is found to be extremely skewed: a very small percentage of the kernels in the lot is contaminated (‘hot spot’), and the concentration on a single kernel can be extremely high (e.g. Schade et al., 1975; Schatzki & Toyofuku, 2003; Ozay et al., 2006). Schade et al. (1975) estimated that only one in 30,000 almonds are contaminated, but that same almond can develop extremely high levels of AF contamination. It is therefore not uncommon to find two samples from the same lot giving extremely different results in terms of contamination levels, even for big samples (Schatzki & Toyofuku, 2003). Because of this extreme mycotoxin distribution among individual kernels in a contaminated lot, it is easy to miss the contaminated kernel with a small sample and underestimate the true concentration in the lot. But if the test sample contains one or more highly contaminated kernels, then the test sample will overestimate the true mycotoxin contamination in the lot. Even with the

use of proper sample selection techniques, the variation among test sample concentrations is inevitable. On the other hand, when almonds get sliced, diced or ground, potentially contaminated nuts can be integrated in this process, and contamination gets dispersed more homogenously in the final processed product.

Because of variability among sample test results, mycotoxin concentrations in lots can never be determined with 100% certainty (van Egmond et al., 2007), which means that lots cannot be classified unambiguously based upon regulatory limits even when samples are taken correctly from a lot. The problem of sampling has been addressed by the development of sampling plans, which are based on statistical evaluations to balance consumer protection (by not accepting contaminated lots) and producer protection (by not rejecting clean lots). Such plans are a compromise between the statistical need for large samples and the practicalities and costs of such samples. Based on scientific studies, EU has produced the Commission Regulation (EC) No. 401/2006, recently amended by the Commission Regulation (EC) No. 178/2010, which establishes sampling plans for nine different groups of food commodities. These EU regulations enforce sampling plans that mandate sample weights that can go up to 30 kg, depending on the lot size. This raises questions on how laboratories should prepare and homogenise such large samples for chemical analysis. For that matter, the sample must first be homogenised and then an aliquot (or subsample) must be taken for analysis. Here the determining factor for variance is the subsample size, as well as the particle size of the grind (Schatzki & Toyofuku, 2003; Spanjer et al., 2006). It is generally accepted that particle size plays a major role in the expected variance: the smaller the particles, the smaller the variance. The subsampling variance may be as much as 10 times that of analysis, depending on the fineness of the grind (Schatzki & Toyofuku, 2003).

2.1.8.2 Aflatoxin extraction from food matrices

Analytical methods require that AFs be extracted from the solid food into a liquid phase. This is done to obtain further isolation of the toxins in sufficient concentration to allow their detection. The assumption inherent in an extraction procedure is that the mycotoxin will be efficiently extracted from the solid phase of the mixture. The extent to

which this assumption is valid will be reflected in the recovery rate of the extraction procedure.

The complexity of food matrices can lead to serious interference during analysis of mycotoxins. Therefore, a large number of procedures for the extraction and purification of selected classes of mycotoxins from a variety of food matrices has been developed. Parameters of importance in mycotoxin extraction are solvent type and composition of a mixture, solvent to sample ratio, type of matrix, extraction method and physical aggregation of the sample.

Mycotoxins are polar compounds, so they are potentially extracted by a range of polar solvents or mixtures of solvents. In the particular case of AFs, traditional methods relied on chloroform extraction, but due to the cost and environmental implications of chlorinated solvents, that solvent has been replaced by aqueous mixtures of methanol, acetonitrile or acetone (Shephard, 2009). Investigations of optimal extraction solvents for AFs from a range of matrices highlighted a series of potential problems which need to be considered. As a start, optimum extraction efficiency requires the analytical sample to be ground to a fine powder (Schatzki & Toyofuku, 2003; Spanjer et al., 2006). Also, the extraction of very dry materials (as is the case of nuts) can lead to a variability associated with water uptake by the dry matrix, an effect that depends on factors such as the matrix, the organic solvent and its ratio in the aqueous extractant and the solvent-to-sample ratio used for the extraction experiment (Shephard, 2009). The most common procedure to address water uptake by the matrix is the addition of sodium chloride to an aqueous methanol extraction.

The physical process of extraction is generally achieved by shaking of the matrix and extractant or by blending with a homogeniser for a short time period. Other methods have been investigated, like pressurised liquid extraction (e.g. Campone et al., 2009; Sheibani & Ghaziaskar, 2009), but the results have not justified the cost of adopting this instrumentation in place of simple shaking.

2.1.8.3 Extract cleanup

The extracts of most matrices are unsuitable for direct analysis due to the large number of co-extracted impurities that mask the analytical signal for the target analyte and

consequently increase the limit of detection. Therefore, for the majority of the analytical methods, the subsequent purification of the extract is also required. For this purpose, several methods have been applied. Traditionally, cleanup of extracts is accomplished by using solid-phase extraction (SPE) columns pre-packed with various stationary phases like silica or Fluorisil (Castro & Varga, 2001; Sobolev, 2007). In some cases, the analyte is retained on the columns while impurities pass through and are washed off. The analyte is then selectively removed by rinsing with the adequate solvent. In other cases, the SPE columns are designed to trap impurities and permit the analyte to pass through.

The development of antibodies raised against individual mycotoxins led to the introduction of immunoaffinity columns (IACs; Trucksess et al., 1991), which is currently the most widely used type of SPE column for extract cleanup (e.g. Stroka et al., 2000; Schatzki & Toyofuku, 2003; Yang et al., 2005; Castegnaro et al., 2006; Kaaya & Kyamuhangire, 2006; Yentür et al., 2006; Muscarella et al., 2009), since IACs have been commercially developed for most of the major mycotoxins. In this method, specific antibodies are immobilised on a gel contained in a small column. The antibodies on the column will recognise and bind the specific mycotoxins(s) and allow impurities to pass through the column. The mycotoxin is eluted with a small amount of methanol, which denatures the antibody and releases the bound analyte.

The advantages of the IACs over other cleanup methods are the effective and specific extract purification provided, the economic use of organic solvents and the improved analytical performance achieved with cleaner samples (Shephard, 2009). The use of these columns is, however, not completely devoid of problems: the complex matrices contain thousands of compounds, some of which may be able to interfere with the antibodies, thus limiting the capacity for the adsorption of the toxin; and the composition of the matrices may interfere with the toxin structure making it not extractable and/or not recognisable by the antibodies (Castegnaro et al., 2006).

2.1.8.4 Separation and detection

AFs are low molecular mass compounds which possess significant UV absorption and fluorescent properties. For this reason, liquid separation techniques coupled with fluorescence detection have predominated in their analysis. The most disseminated ones

include thin layer chromatography (TLC; da Silva et al., 2000; Stroka et al., 2000; Castegnaro et al., 2006; Kaaya & Kyamuhangire, 2006; Nakai et al., 2008; Singh & Shukla, 2008) and reverse-phased high-performance liquid chromatography (HPLC) coupled to fluorescence detector (FLD; e.g. Mahoney & Rodriguez, 1996; Schatzki & Toyofuku, 2003; Yentür et al., 2006; Ardic et al., 2008; Sobolev, 2007; Campone et al., 2009; Muscarella et al., 2009; Shah et al., 2010). Alternative methods based on immunodetection, like enzyme linked immunosorbent assays (ELISA), have also found widespread application in AF analysis (e.g. Leszczynska et al., 2000; Arrus et al., 2005; Mutegi et al., 2009).

TLC and ELISA are perhaps the simpler and most widely used techniques for both qualitative and semi-quantitative monitoring of mycotoxins, and they are useful for screening and routine analysis of large numbers of samples. They do not require expensive equipment or highly trained handling, but they are also less accurate and sensitive techniques. Although ELISA methods are extensively used for rapid qualitative and semi-quantitative screening of AFs, they are not useful in providing a definitive confirmation of the toxins and an accurate quantitative determination (Muscarella et al., 2009), since they have not been validated at sufficiently low levels and are limited in the range of matrices tested (Gilbert & Anklam, 2002). Still, ELISAs are widely used for mycotoxin diagnosis due to the availability of test kits for field use for practically all relevant mycotoxins. On the other hand, TLC has been found to result in more precise and consistent data than ELISA (see Lin et al., 1998, for a review on TLC methods). Yet, the present trend is the use of HPLC for the determination of AFs due to its characteristics of specificity, high sensitivity and simplicity of operation. Additionally, chromatographic determination allows for the quantification of each toxin individually. This is particularly important for food analysis where the determination of AFB_1 is required.

Because AFs are naturally strongly fluorescent, the HPLC detection is most often achieved by application of fluorescent detection. But, in the aqueous mixtures used for reverse-phased chromatography, the fluorescence of AFB_1 and AFG_1 are significantly quenched (Muscarella et al., 2009), and they are required to be converted into more highly fluorescent derivatives, namely their hemiacetals AFB_{2a} and AFG_{2a}. Several derivatisation methods are available, including pre-column derivatisation with trifluoroacetic acid (TFA) (e.g. Trucksess et al., 1994; Doster et al., 1996; Castegnaro et al., 2006; Yentür et al., 2006;

Shah et al., 2010) and post-column derivatisation with iodine (e.g. Yang et al., 2005; Waltking & Wilson, 2006) and bromine (Kobra cell; e.g. Freire et al., 2000; Schatzki & Toyofuku, 2003; Waltking & Wilson, 2006). However, these methods are laborious and present a number of disadvantages, such as handling toxic reagents, instability of the derivatives and low reproducibility (Muscarella et al., 2009). In 1993, a photochemical derivatisation technique was introduced in AF analysis (Joshua, 1993). This method consists of passing the HPLC eluate through a reaction coil wound around a UV light (photochemical reactor, PHRED) at ambient temperature, which causes the hydration of AFB_1 and AFG_1 to their respective hemiacetals. Recently, a collaborative study demonstrated that the PHRED system is equivalent to the iodination and bromination official AOAC procedures (Waltking & Wilson, 2006), with the advantages of being able to obtain reproducible results with simple sample preparation and less chemical waste. Since then, the technique has been preferentially applied (e.g. Sobolev, 2007; Muscarella et al., 2009; Rodrigues et al., 2009; Soares et al., 2010).

In the last years a variety of multi-mycotoxin methods has been reported, showing the special interest for high-throughput multi-mycotoxin routine analysis. These methods are mostly liquid chromatography (LC) coupled to mass spectrometry (MS), and they allow the simultaneous separation and detection of all relevant mycotoxins in a single run, without the need for complex extraction/purification and derivatisation procedures (e.g. Sagawa et al., 2006; Ren et al., 2007; Sulyok et al., 2007, 2010; Spanjer et al., 2008; Santini et al., 2009). The problem with the multi-mycotoxin methods is the incomplete extraction/purification and the high limits of detection of at least some of the analytes, and for that reason these are mostly semi-quantitative methods (Köppen et al., 2010).

The use of matrix-laser assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has also been described for the high throughput of AFs (Catharino et al., 2005), by using a UV-absorbing ionic liquid as matrix.

Table 2.3 lists some of the methods applied to AF analysis in almonds and chestnuts, as well as recovery rates, limit of detection (LOD), limit of quantification (LOQ) and repetibility standard deviation (RSD_r), whenever reported.

Table 2.3 List of the methods applied to AF analysis in almonds and chestnuts, as well as recovery rates, limits of detection/quantification (LOD/LOQ) and repetibility standard deviation (RSDr).

Matrix	Extraction	Cleanup	Separation Mobil phase	Detection	AFs	Spiked levels (ng/g)	LOD (ng/g)	LOQ (ng/g)	Recovery (%)	RSD_r (%)	Reference
Almonds	Chloroform/ Diatomaceous earth	Diatomaceous earth	TLC -	Gold fluorescent light	-	-	-	-	-	-	Schade et al., 1975
Almonds/ /Chestnuts	Chloroform	Chloroform/ silica gel column/ *n*-hexane/ diethyl ether	TLC Chloroform:metanol (97:3)	UV light		-	-	5	80	-	Abdel-Gawad & Zori, 1993
Almonds	Acetonitrile: water (9:1)	Multi-column	HPLC/TFA -	FLD	Total B_1 B_2 G_1 G_2	5-30 3-15 1-3 2-9 1-3	- - - - -	- - - - -	116 91-95 88-92 89-103 98-116	16 9 - 16 -	Trucksess et al., 1994
Almonds	Methanol: 0.1N HCl	SPE (not specified)	HPLC Water:methanol:acetonitrile (74:13:13)	FLD	B_1 B_2 G_1 G_2	10 2.5 10 2.5	- - - -	- - - -	87 95 93 89	6.12 10.93 6.97 8.31	Abdulkadar et al., 2000
Almonds	Methanol: 0.1N HCl/ Methylene chloride	C18 silica gel column	HPLC/TFA	FLD	-	-	-	-	-	-	García-Pascual et al., 2003
Almonds	Dichloromethane	Glass column	TLC Diethyl ether Chloroform:acetone (90:10)	UV Light H_2SO_4	B_1	-	0.5	-	-	-	Gürses, 2006

(continues)

Table 2.3 (continued)

Matrix	Extraction	Cleanup	Separation Mobil phase	Detection	AFs	Spiked levels (ng/g)	LOD (ng/g)	LOQ (ng/g)	Recovery (%)	RSD_r (%)	Reference
Almonds	Acetone 84%	Chloroform; KOH:KCl (1:1)	TLC Chloroform:acetone (1:9)	UV light	-	-	-	-	-	-	Saleemullah et al., 2006
Almonds	Methanol 80%	Florisil column	HPLC/PHRED Water:methanol (63:37)	FLD	B_1 B_2 G_1 G_2	0.5-5 0.5-5 0.5-5 0.5-5	- - - -	- - - -	76.2-84.5 81.7-88.1 77.5-83.2 77.6-93.7	2.1-1.5 3.7-2.1 2.4-2.6 5.7-1.6	Sobolev, 2007
Almonds	Acetonitrile	PLE	HPLC/ PHRED Water:methanol:acetonitrile (60:20:20)	FLD	B_1 B_2 G_1 G_2	0.5-4 0.13-1 0.5-4 0.13-1	0.03 0.01 0.06 0.03	0.1 0.04 0.2 0.1	81-88 83-90 76-91 82-87	2-8 5-8 7-12 3-13	Campone et al., 2009
Almonds	Methanol 80%	IAC	HPLC/ PHRED Water:methanol:acetonitrile (55:15:30)	FLD	B_1 B_2 G_1 G_2	- - - -	0.08 0.02 0.16 0.04	0.22 0.08 0.48 0.10	- - - -	- - - -	Muscarella et al., 2009

2.1.8.5 Validation of methods

In the analysis of contaminants in food commodities, performance criteria are important for obtaining reliable results and laboratories that perform mycotoxin testing must assure that the methods used are both accurate and precise. The precision associated with a mycotoxin test procedure depends on the sampling, sample preparation, and analytical frequency used to estimate the mycotoxin concentration of a bulk lot. Even when using accepted procedures, random variation is associated with each step of the testing procedure.

The term "validation" is usually applied to the evaluation of newly developed methods, or methods that laboratories intend to use as alternatives to reference methods, but laboratories applying reference methods should not be exempted of testing their own ability to implement them correctly by performing "in-house validation". The evaluation of the validation parameters should be based on the intended use of the analytical method. In general different parameters such as specificity, selectivity, precision, accuracy, linearity, range, LOD, LOQ, robustness as well as ruggedness are recommended to be determined. Methods that are used in routine analyses should be tested in appropriate frequency using quality control material or certified reference material to ensure the reliability of analytical results. Furthermore every method has to be revalidated if any parameter in the analysis is changed.

For official control and implementation of mycotoxin regulations, a number of official methods have been validated by interlaboratory collaborative studies conducted under the auspices of international bodies such as AOAC International and the European Standardization Committee (CEN). These organisations are responsible for the development of rapid and accurate analysis techniques, and for the validation and adoption of analytical methods for the enforcement of regulation. The validation process involves testing within-laboratory repeatability, between-laboratory reproducibility, analytical recovery, and limits of detection and quantification. The official methods for AFs detection and quantification in almonds have been reviewed by Gilbert & Anklam (2002).

Several publications give profound insight on the matter of analytical method validation (Thompson, 2000; Gilbert & Anklam, 2002; Chan, 2004; Taverniers et al., 2004; Ermer & Miller, 2005; Nocentini et al., 2008).

2.2 The aflatoxin biosynthesis pathway

2.2.1 Genetic and molecular aspects of aflatoxin biosynthesis

After the discovery that the AF biosynthesis was regulated by a gene cluster (Trail et al., 1995; Yu et al., 1995; Brown et al., 1996), the biosynthetic pathway of AFs has been extensively studied, and most of the enzymes and corresponding genes involved have been identified. Also, most of their functions have been elucidated (e.g. Trail et al., 1994; Yu et al., 1998, 2000, 2004a, 2004b; Ehrlich et al., 2004; Yabe & Nakajima, 2004; Ehrlich et al., 2005; Wen et al., 2005; Cary & Ehrlich, 2006), with possible alternative pathways (Detroy et al., 1973). AF biosynthesis requires at least 25 enzymes and two regulatory proteins encoded by contiguous genes in an 80-kb cluster (reviewed in Yu et al., 2004b). Clustered biosynthetic genes for fungal secondary metabolism are not only regulated by specific transcription factors, as a global epigenetic control mechanism may be conducted by genes, beyond the biosynthetic cluster, which are able to regulate multiple physiological processes and the response to environmental and nutritional factors such as temperature, pH, light, carbon and nitrogen sources (reviewed by Georgianna & Payne, 2009).

The genes involved in the major convertion steps from early precursors to AFs and their funtions are discussed in Yu et al. (2004b). These authors have proposed the use of a three-letter code “afl” to represent AF pathway genes. A capital letter in alphabetical order from “A” to “Y” represents each individual gene confirmed to be (or potentially be) involved in AF biosynthesis, e.g. *aflA* to *aflY* for all of the 25 genes (Figure 2.4). Those genes whose pathway involvement has already been characterised and confirmed are designated *aflA* to *aflQ* from the initial conversion of fatty acids to the final products, AFs. *aflR* and *aflS* (formerly *aflJ*) are named for transcription regulators. Those genes whose pathway involvements are ambiguous or remain unclear are designated *aflT*, *aflU* (= *cypA*), *aflV* (= *cypX*), *aflW* (= *moxY*), *aflX* (= *ordB*), and *aflY* (= *hypA*).

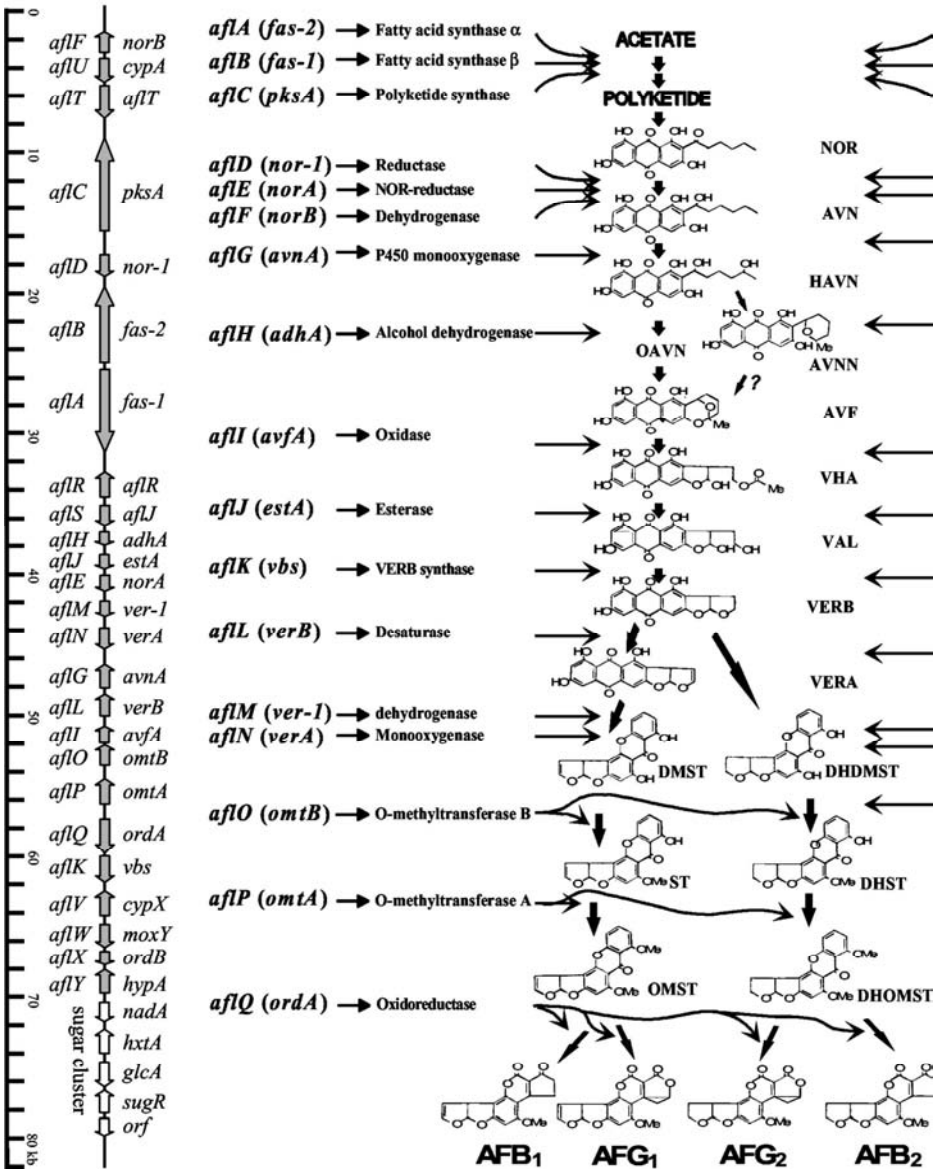

Figure 2.4 Clustered genes and the AF biosynthetic pathway.
The gene names proposed by Yu et al. (2004b) are given on the left of the vertical line and the old gene names are given on the right. Arrows along the vertical line indicate the direction of gene transcription. The ruler at far left indicates the relative sizes of these genes in kilobases. Arrows indicate the connections from the genes to the enzymes they encode, from the enzymes to the bioconversion steps they are involved in, and from the intermediates to the products in the AF bioconversion steps. Abbreviations: NOR, norsolorinic acid; AVN, averantin; HAVN, 5-hydroxyaverantin; OAVN, oxoaverantin; AVNN, averufanin; AVF, averufin; VHA, versiconal hemiacetal acetate; VAL, versiconal; VERB, versicolorin B; VERA, versicolorin A; DMST, demethylsterigmatocystin; DHDMST, dihydrodemethylsterigmatocystin; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMST, O-methylsterigmatocystin; DHOMST, dihydro-O-methylsterigmatocystin (Adapted from Yu et al., 2004b).

Generally, the AF biosynthesis genes of *A. flavus*, *A. parasiticus* and *A. nomius* are highly homologous, the order of the genes within the cluster being the same (Yu et al., 1995; Ehrlich et al., 2005; Chang et al., 2007). Also, AF genes and gene organisation in *A. sojae* are most similar to those of *A. parasiticus* (identity 98-99%). A significant proportion, but not all, of non-aflatoxigenic *A. flavus* isolates have been found to contain various deletions in the AF gene cluster (Prieto et al., 1996; Ehrlich & Cotty, 2004; Ehrlich et al., 2004; Chang et al., 2005, 2006) which are common to some strains of *A. oryzae* (Chang et al., 2005, 2006). Also, additional enzymes are required for AFGs formation in *A. parasiticus*. The loss of the ability to produce AFGs in *A. flavus* seems to result from a deletion in the terminal region of the cluster corresponding to genes *aflF* (= *norB*) and *aflU* (= *cypA*) (Ehrlich et al., 2004). Several studies confirmed that separate pathways lead to the formation of AFBs and AFGs (Henderberg et al., 1988; Bhatnagar et al., 1991; Yabe et al., 1999; Ehrlich et al., 2004).

2.2.2 Molecular differentiation of aflatoxigenic and non-aflatoxigenic strains

Molecular techniques have been widely applied in the attempt to distinguish aflatoxigenic and non-aflatoxigenic strains of *A. flavus* and related species, through the correlation of presence/absence of one or several genes involved in the AF biosynthetic pathway with the ability/inability to produce AFs. Some studies have been able to distinguish these species from other foodborne fungi and, in some cases, they were capable of distinguishing aflatoxigenic from non-aflatoxigenic strains.

The studies by Geisen (1996) and Shapira et al. (1996) can be regarded as the starting point for PCR-based diagnosis of aflatoxigenic and non-aflatoxigenic fungi. Geisen (1996) used multiplex PCR with three sets of primers specific for three structural genes of the AF biosynthetic pathway *aflD*, *aflM* and *aflO*, and was able to differentiate *A. flavus* and *A. parasiticus* from other food-borne fungi, but not aflatoxigenic and non-aflatoxigenic strains of the same species. Shapira et al. (1996) used aflatoxigenic strains and carried out monomeric PCRs with three different sets of primers for *aflR*, *aflO* and *aflM* genes, but they could only discriminate aflatoxigenic strains from other moulds. Färber et al. (1997) detected aflatoxigenic strains of *A. flavus* in contaminated figs by performing a monomeric PCR with the same sets of primer used by Geisen (1996). Other

multiplex PCR with the AF pathway genes *aflR*, *aflD*, *aflM* and *aflO* did not produce a clear pattern that would allow to accurately differenciate aflatoxigenic from non-aflatoxigenic strains (Criseo et al., 2001). Lee et al. (2006) detected the differences in the *aflR* gene of *A. flavus*/*A. oryzae* and *A. parasiticus*/*A. sojae*, but they were not able to clearly differentiate the species. Baird et al. (2006) tested a different methodology based on DNA fingerprinting with two consecutive amplifications with arbitrary primers, with which the majority, but not all, of the aflatoxigenic isolates was differentiated from the non-aflatoxigenic.

AF production ability and aflatoxigenic strains differentiation have also been assessed by monitoring AF genes expression in the *A. flavus* group, using the reverse transcription PCR (RT-PCR) methodology. RT-PCR allows the detection of mRNAs transcribed by specific genes by PCR amplification of cDNA intermediates synthesised by reverse transcription. Such systems have been applied to monitor AF production and AF gene expression based on various regulatory and structural AF pathway genes in *A. parasiticus* and/or *A. flavus* (Sweeney et al., 2000; Mayer et al., 2003a, 2003b; Sherm et al., 2005; Degola et al., 2007), and were found to be very rapid and sensitive. Scherm et al. (2005) studied 13 strains of both species and found consistency of 3 genes (*aflD*, *aflO* and *aflP*) in detecting AF production ability, further indicating them as potential markers.

But, as said, AF biosynthesis is based on a highly complex pathway. It is thus not surprising that genetic protocols that can fully differentiate between AF producers and non-producers have not yet been successfully established. Furthermore, one has to be aware that some genes are not exclusive of the AF biosynthetic pathway, which could create false-positives from sterigmatocystin producing fungi (Paterson, 2006). As an example, *A. nidulans* harbours the complete AF biosynthesis pathway except for the final step that converts sterigmatocystin to AF (Brown et al., 1996).

2.3 *Aspergillus* section *Flavi*

2.3.1 A brief overview of genus *Aspergillus*

Aspergillus is one of the most important genera of microfungi, with many species having great impact on various fields of interest: as human, animal and plant pathogens, as spoilage agents of food, or as producers of toxic metabolites. On the other hand, some of the most important microorganisms used in food fermentations and biotechnology are part of this genus. It is therefore of major importance that we consider the significance of the rigor and stability of its taxonomy, in order to keep the taxonomic system practical and reliable for industrial, economic and regulatory reasons.

Aspergillus is an anamorphic genus belonging to the family Trichocomaceae, order Eurotiales, subclass Eurotiomycetidae, class Eurotiomycetes, phylum Ascomycota. It is characterised by a distinctive round to elongate aspergilla bearing long chains of conidia, which gives the fungus its characteristic morphology. Some members of the genus are known to reproduce sexually, producing teleomorphs, which are invariably cleistothecia bearing inordinately arranged ascospores in dehiscent asci.

As with fungi in general, *Aspergillus* taxonomy is complex and ever evolving. Classic systematics of genus *Aspergillus* and its associated teleomorphs have been based primarily on differences in morphological and cultural characteristics (Raper & Fennell, 1965; Samson, 1979; Klich & Pitt, 1988; Kozakiewicz, 1989). This taxonomic system gives mostly a rough delimitation of the taxa. In several sections of the genus much morphological variation occurs, resulting in complex taxonomic schemes. In the last decades, the taxonomy of the genus has evolved from a simple morphological species concept into a polyphasic approach integrating strong biochemical, ecological, genetic and molecular characters. As methods become more and more sensitive and accurate, species are constantly being added, re-classified or repositioned within the genus (e.g. Peterson, 2000, 2005, 2008; Rigó et al., 2002; Klich et al., 2003; Samson et al., 2004b; Frisvad et al., 2005; Hong et al., 2005, 2008; Serra et al., 2006; Houbraken et al., 2007; Varga et al., 2007a, 2007b; Mares et al., 2008; Peterson et al., 2008; Pildain et al., 2008; Zalar et al.,

2008). Table 2.4 lists the most important publications released on the taxonomy and identification of the genus.

Table 2.4 Important taxonomic treatises and identification manuals for the genus *Aspergillus*.

Year	Reference
1926	Thom C & Church M. The Aspergilli (Williams & Wilkins, Baltimore, USA)
1945	Thom C & Raper KB. A Manual of the Aspergilli (Williams & Wilkins, Baltimore, USA)
1965	Raper KB & Fennell DI. The Genus *Aspergillus* (Williams & Wilkins, Baltimore, USA)
1979	Samson RA. A compilation of the Aspergilli described since 1965 (CBS, Utrecht, The Netherlands).
1985	Samson RA & Pitt JI. Advances in *Penicillium* and *Aspergillus* Systematics (Plenum Press, New York, USA)
1988	Klich MA & Pitt JI. A Laboratory Guide to Common *Aspergillus* Species and Their Teleomorphs (Division of Food Processing, North Ryde, Australia).
1989	Kozakiewicz Z. *Aspergillus* Species on Stored Products (CAB International, Wallingford, UK)
1990	Samson RA & Pitt JI (eds). Modern Concepts in *Penicillium* and *Aspergillus* Classification (Plenum Press, New York, USA)
2000	Samson RA & Pitt JI (eds). Integration of Modern Taxonomic Methods for *Penicillium* and *Aspergillus* classification (Harwood Academic Publications, Amsterdam, The Netherlands)
2002	Klich MA. Identification of Common *Aspergillus* Species (CBS, Utrecht, The Netherlands).
2008	Varga J & Samson RA (eds). *Aspergillus* in the genomic era. (Wageningen Academic Publishers, Wageningen, The Netherlands)

The first complete monograph of *Aspergillus* was written in 1965, by Raper and Fennell, where they recognised 132 species and 18 varieties (Raper & Fennell, 1965). They divided the species into 18 informal groups, which does not constitute a nomenclatural status under the International Code of Botanical Nomenclature (ICBN) scheme. Furthermore, Raper & Fennell (1965) retained the generic name *Aspergillus* for both perfect (teleomorphs) and imperfect (anamorphs) states[1]. In 1979, Samson listed 90 new taxa identified since 1965 and compiled the only 34 accepted ones (following the ICBN requisites) according to the group classification proposed by Raper & Fennell (1965).

In an effort to bring some consistency into the complex taxonomy of the genus, and also in line with the ICBN, the groups were revised and given formal taxonomic status as

[1] Fungi are the only organisms that depart from one of the basic rules of biological nomenclature, i.e. that each taxonomic group can bear only one correct name. Since 1905, the Botanical Code (which governs the naming of plants and fungi) has allowed two different names to be applied to the same organism, depending on whether it is viewed in its sexual or asexual stage. Under this system of taxonomic governance, Article 59 permits dual nomenclature. When a sexual phase is known, the name for this phase takes precedence.

sections, and subgenera were added (Gams et al., 1985). *Aspergillus* names were then typified by Samson & Gams (1986) and Kozakiewicz (1989). In 2000, Pitt and co-workers published a list of accepted species and synonyms in the family Trichocomaceae (Pitt et al., 2000). This list included 204 species accepted in the *Aspergillus* genus (anamorphs) and associated teleomorphs. In the same year, phylogenetic studies using ribosomal RNA from 215 *Aspergillus* led Peterson to propose an alteration to the previous nomenclature, repositioning the species into 3 subgenera and 16 sections (Peterson, 2000).

In a short period of 7 years, Geiser et al. (2007) listed another 40 newly identified species. The latest revision of the genus dates to 2008, when Peterson and co-workers (Peterson et al., 2008), based on phylogenetic analysis of multilocus sequence data, proposed the division of the genus into 8 subgenera and 18 sections, with 12 associated teleomorphic genera (Table 2.5). Since then, numerous other species have been identified (e.g. Hong et al., 2008; Mares et al., 2008; Pildain et al., 2008; Zalar et al., 2008), bringing the total number of *Aspergillus* species to more than 260. Considering that probably fewer than 5% of fungal species are known and identified (Hawksworth, 1991), the number of *Aspergillus* species may ascend to more than 5000. A quick search in the Index Fungorum network site (www.indexfungorum.org) produced, by September 2010, 839 results for the *Aspergillus* query. This number includes not only species, but also subspecies and varieties, as well as numerous synonyms (the names for the teleomorphic states are not herein included); nevertheless, this number reflects the difficulties in creating a stable, workable and reliable taxonomic scheme for the genus.

Table 2.5 Taxonomy of *Aspergillus* at a subgeneric level, as described by various authors.

Raper & Fennell, 1965		**Gams et al., 1985**		**Peterson, 2000**		**Peterson et al., 2008**		
Subgenus	Group	Subgenus	Section	Subgenus	Section	Subgenus	Section	Teleomorphs
Aspergillus	*A. glaucus* *A. restrictus*	*Aspergillus*	*Aspergillus* *Restricti*	*Aspergillus*	*Aspergillus* *Restricti* *Cervini* *Terrei* *Flavipedes* *Wentii* *Flavi* *Nigri* *Circumdati* *Candidi* *Cremei*	*Aspergillus*	*Aspergillus* *Restricti*	*Eurotium* *Eurotium*
Circumdati	*A. wentii* *A. flavus* *A. niger* *A. ochraceus* *A. candidus* *A. cremeus* *A. sarpus*	*Circumdati*	*Wentii* *Flavi* *Nigri* *Circumdati* *Candidi* *Cremei* *Sparsi*	---	---	*Circumdati*	*Circumdati* *Nigri* *Flavi* *Cremei*	*Neopetromyces* *Petromyces* *Chaetosartorya*
Clavati	*A. clavatus*	*Clavati*	*Clavati*	---	---	---	---	
Fumigati	*A. fumigatus* *A. cervinus*	*Fumigati*	*Fumigati* *Cervini*	*Fumigati*	*Fumigati* *Clavati*	*Fumigati*	*Fumigati* *Clavati* *Cervini*	*Neosartorya* *Neocarpenteles,Dichotomomyces*
Ornati	*A. ornatus*	*Ornati*	*Ornati*	---	---	*Ornati*	*Ornati*	*Sclerocleista*
Nidulantes	*A. nidulans* *A. versicolor* *A. ustus* *A. terreus* *A. flavipes*	*Nidulantes*	*Nidulantes* *Versicolores* *Usti* *Terrei* *Flavipedes*	*Nidulantes*	*Nidulantes* *Ornati* *Sparsi*	*Nidulantes*	*Nidulantes* *Sparsi* *Usti*	*Emericella* *Emericella*
---	---	---	---	---	---	*Candidi*	*Candidi*	
---	---	---	---	---	---	*Terrei*	*Terrei* *Flavipedes*	 *Fennelia*
---	---	---	---	---	---	*Warcupi*	*Warcupi* *Zonati*	*Warcupiella* *Penicilliopsis*

Aspergillus species are traditionally identified by morphologic characters. The genus is easily identified by its characteristic conidiophore, but species identification and differentiation is rather complex, for it is traditionally based on a wide range of features. In fact, the defining characteristic of the genus is the aspergillum-like spore-bearing structure. It is the most important microscopic character used in *Aspergillus* taxonomy. During mycelia differentiation certain cells enlarge, develop a heavy cell wall and form 'T' or 'L' shaped 'foot cells' that produce a single conidiophore perpendicular to the long axis of the cell. This erect hyphal branch enlarges at its apex to form a rounded, elliptical or club shaped vesicle. In some species, the fertile area of the vesicle gives rise to a layer of cells called phialides that produce long chains of mitotic spores called conidia or conidiospores. This type is called uniseriate. In other cases, a layer of cells called metullae is produced between the vesicle and the phialides, and the aspergilli are called biseriate.

The size and arrangement of the conidial heads as well as the colour of the spores they bear are important identifying characteristics. Micromorphology characterisation is also dependent on stipe morphology, conidia ornamentation, presence of Hülle cells, and morphology of cleistothecia and ascospores, when present (Kozakiewicz, 1989, Klich, 2002a). Cleistothecia are the sexual reproductive stage that contain the meiotic ascospores borne within asci. Hülle cells are thickened, often globose, cells that are associated with cleistothecia, for which no function is currently known (Z. Kozakiewicz, personal communication, 2008).

The major macromorphological and cultural (physiological) features used in species identification are the colour and diameter of the colony, the production of exudates and soluble pigments, the growth rate, thermotolerance, and the presence of sclerotia and cleistothecia. Sclerotia are rounded masses of mycelium with an outer melanised rind. They are believed to serve as resting structures that allow species to survive adverse growth conditions. Sclerotia morphological features (colour, shape, dimension) also aid in the identification. In addition to these characters, isolates of *Aspergillus* have also been characterised by physiologic responses to various conditions: growth at certain temperatures (Samson et al., 2007), water activities and pH, growth on specific culture media (e.g. creatine-saccharose agar; Varga et al., 2007a; Samson et al., 2007), extracellular enzyme production, profiles of fatty acids (Blomquist et al. 1992; Fraga et al., 2008), etc.

Table 2.6 summarises the major morphological features used in the identification of *Aspergillus* to the section level. The taxonomic scheme presented is that of Gams et al. (1985), since it is the most generally accepted in terms of classic phenotypic identification.

Table 2.6 Morphologic characteristics (based on Klich, 2002a) of the various sections of genus *Aspergillus* (taxonomy after Gams et al., 1985).

Subgenus	Main characteristics	Section	Main characteristics
Aspergillus	Uniseriate, xerophilic, growth on CY20S>CYA25, grey-green conidia,	*Aspergillus*	teleomorph *Eurotium* - yellow cleistothecia with pseudoparenchymatous cells, hyaline ascospores
		Restricti	Strictly anamorphic, slow growth on all media
Clavati	Uniseriate, vesicles predominantly clavate, conidia grey-green,	*Clavati*	The same as subgenus
Circumdati	Uniseriate or biseriate, vesicles spherical to pyriform	*Candidi*	Conidia white or nearly white
		Circumdati	Predominantly biseriate, conidia yellow, buff or ochraceus
		Cremei	Conidia brown, yellow or blue-green
		Flavi	Conidia yellow-green to olive-brown
		Nigri	Stipes smooth-walled, conidia black or near black
		Sparsi	Conidia pale grey to olive-buff
		Wentii	Conidia yellow to brown
Fumigati	Uniseriate, vesicles predominantly pyriform, conidia grey-green, blue-green to orange	*Fumigati*	Conidia grey-green to blue-green
		Cervini	Conidia light-orange to orange-grey
Ornati	Uniseriate, conidia grey-green, yellow-green or olive-brown	*Ornati*	The same as subgenus
Nidulantes		*Flavipedes*	Stipes hyaline to pale brown, conidia white to buff
		Nidulantes	Stipes short often brown, conidia green, Hülle cells often present, most species with *Emericella* teleomorph. Cleistothecia soft-walled, surrounded by Hülle cells, ascospores red to purple
		Terrei	Stipes hyaline, conidia buff to orange-brown
		Usti	Stipes brown, conidia dull red, brown or olive
		Versicolores	Stipes hyaline to brown, conidia green, grey-green or blue-green

2.3.2 The species concept in *Aspergillus*

Many different species concepts have been proposed. The most common ones are the Morphological Species Concept (MSC), the Biological Species Concept (BSC) and the Phylogenetic Species Concept (PSC) (see Taylor et al., 2000 for a review on the various species concepts). A biological species is diagnosed as a group of individuals able to interbreed freely under natural conditions. This species concept is hard to apply to fungi for a number of reasons. Approximately 20% of fungi are not known to reproduce sexually. Other fungi are homothallic and will produce sexual spores without a partner. In addition, some heterothallic fungi cannot be coaxed into mating in cultivation (Taylor et al., 2000). Taking this in consideration, the dominant fungal operational species concept has been, until recently, the MSC, which is founded on the similarity of observable phenotypic (morphological and physiological) characters. Lately, the PSC has been gaining interest among mycologists. A phylogenetic species corresponds to a monophyletic group composed of the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent.

Peterson (2008) considers that the PSC as described by Dettman et al (2003a, 2003b, 2006) is a very attractive option. In this concept, species recognition results from the concordance of independent gene trees; branches are categorised as fully congruent when a single group of isolates always occurs as a terminal group and there is strong statistical support for that grouping. Strong statistical support derived from phylogenetically informative data along with the branch being present in each of the single locus trees is taken as support for the isolate representing a distinct species (Peterson, 2008). PSC can avoid the subjectivity of determining the limits of a species by relying on the concordance of more than one gene genealogy – the genealogical concordance concept. The point where different gene genealogies become concordant is a useful place to assign a species boundary. In practice, this has proven to be a powerful tool in fungi and in the genus *Aspergillus*. Several studies using this genealogical concordance theory resulted in a species recognition system that agreed in part with phenotypic studies and revealed the presence of many undescribed species not resolved by phenotype.

But Rieppel (2007) considers that invoking genetic distance as the basis for distinguishing species is unreasonable because species occur in different sized measures of

time and space. Also, Samson & Varga (2009) consider that, after the introduction of powerful molecular techniques, there has been a tendency to overvalue the contribution of phylogenetic criteria to the description of species. Whatever concept is applied, the question is always put on where to draw the limit of the species. To try to overcome this problem, the integration of various kinds of data and information (phenotypic, genotypic, phylogenetic) into a polyphasic scheme seems to be the most powerfull approach of species identification, as it results in a consensus type of taxonomy (Samson & Varga, 2009).

2.3.3 Schemes of species identification in genus *Aspergillus* – the polyphasic approach

There is no method (morphological, physiological, molecular) that works flawlessly in recognising species. That is why taxonomists are currently sustaining their studies in polyphasic schemes, involving the highest possible number of characters resulting from biological, morphological and phylogenetical approaches. Within these sets of data, features currently used to classify and identify *Aspergillus* isolates are: morphology combined with physiological and ecological features; secondary metabolite profiles and DNA sequences. The more parameters available, the more stability the classification will achieve. In those cases where not all approaches of polyphasic schemes result in a consensus, classification should be a compromise containing a minimum of contradictions (Samson & Varga, 2009).

Morphologic and physiologic characters

As said, morphology and physiology have been extensively used in species recognition. One drawback associated with this type of characters is that they vary greatly within a species. For instance, sclerotia or coloured diffusible metabolites which are characteristic of some species are not always present in all isolates of that species (Rodrigues et al., 2009). Furthermore, most of these characters are dependent on the culture conditions. A variety of subtle effects such as air exchange, light and volume of the medium can affect morphology (Okuda et al., 2000). For this reason, it is of major

importance that species identification is developed with pure cultures grown on known media and under standardised conditions (Klich, 2002a).

Secondary metabolite profiles

Species of *Aspergillus* produce a diverse array of secondary metabolites which can be used in species recognition, since they are believed to have high species specificity (Frisvad 1989; Larsen et al. 2005). Practically all species of *Aspergillus* produce a unique combination of these metabolites. In *Aspergillus*, genes responsible for secondary metabolite biosynthesis are gathered in clusters in the subtelomeric regions of the chromosomes, which are often associated with frequent genome rearrangements and deletions (Yu et al., 2004a; Galagan et al., 2005b; Machida et al., 2005; Nierman et al., 2005; Georgianna et al., 2010).

In various sections of the genus, each species is characterised by a specific profile, and the grouping of species based on the extrolite profile usually correlates well with the groupings obtained by other approaches (e.g. Hong et al., 2005; Houbraken et al., 2007; Samson et al., 2007; Varga et al., 2007a, 2007b). Samson & Varga (2009) recommend that 4 to 8 metabolites should be used in the metabolite profiling of a given species.

Molecular characters

Aspergillus are among the best studied fungi genetically. In fact, the genomes of several *Aspergillus* species are now completed (Nierman et al., 2005; Galagan et al., 2005a; Machida et al., 2005; Payne et al. 2006, 2007; Pel et al., 2007; Fedorova et al., 2008; Rokas et al., 2007) and numerous sequences from several strains are available.

In general, molecular characters provide a big number of variable characters for fungal taxonomy, when compared with other approaches. Furthermore, they can be generated using a widely available technology that makes use of well-developed bioinformatic infrastructures that allow comparison of results, and they produce results that generally correlate well with morphological and physiological characters.

In recent years, molecular tools such as Restriction Fragment Length Polymorphisms (RFLP), Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and DNA sequencing have been applied to taxonomic questions in the genus. Among these, DNA sequence analysis has proven to be a powerful tool in the identification of a large number of *Aspergillus* species. It is a fact that variable

DNA sequence characters provide the best means for inferring relationships among organisms, because it is possible to sample very large numbers of variable characters. But the standard short barcode sequences (*ca.* 600 bp) were found to be unsuitable for inferring accurate phylogenetic relationships among fungi (Min & Hickey, 2007), and they generally lack resolution for species identification among very closely related fungi. Taylor et al. (2000) stated that the phylogenetic analysis of variable nucleic acid characters currently comes closer than the others to recognising species consistent with the Evolutionary Species, but this is considered to be true only if several genes are to be analysed. For both species description and phylogenetic inference, Samson & Varga (2009) recommend that at least 2 gene sequences should be examined, using a Multi Locus Sequence Typing (MLST) approach.

DNA barcoding is a taxonomic method which uses a short genetic marker in an organism's DNA to quickly and easily identify it as belonging to a particular species. A DNA sequence should meet several criteria to be used successfully for species identification. DNA sequences should be orthologous in the examined organisms, and variable enough to allow species identification, with low levels of intraspecific variation (Hebert et al., 2003). A DNA barcode should be easily accessible (universally amplified/sequenced by standardised primers from a wide set of organisms), relatively short (≤ ~ 500–600 bp), simple to sequence and easily alignable.

A variety of loci have been suggested as DNA barcodes for fungi, including coding genes and non-coding spacers in the nuclear genomes, as well as in the mitochondrial DNA. The most widely used DNA target regions for discriminating *Aspergillus* species are the ones in the nuclear ribosomal RNA genes (large subunit, internal transcribed spacers) (e.g. Kanbe et al., 2002; Hinrikson et al., 2005; Serra et al., 2006; Anzai et al., 2008; Peterson et al., 2008; Pildain et al., 2008). Single-copy conserved genes, namely β-tubulin, calmodulin and topoisomerase II, have also been extensively used as targets for taxonomic studies in the genus, when multi-copy segments from the rDNA complex lack variability (e.g. Hong et al., 2005, 2008; Peterson et al., 2008; Pildain et al., 2008). Works on mitochondrial genetics of aspergilli indicated that the genes located on the mtDNA of aspergilla (namely *cox1*) do not meet all criteria needed for a DNA barcode, because of several problems. In fact, several studies indicated that results obtained from mitochondrial and nuclear sequence data are incongruent (Wang et al., 2000; Geiser et al., 2007).

Problems may be averted by selecting genes exhibiting appropriate levels of resolving power, depending on the genetic proximity of the organisms of interest and on the objective of the studies.

Mass Spectra

Matrix-assisted laser desorption/ionisation with time-of flight mass spectrometry (MALDI-TOF MS) is a technique emerged in the late 1980s (Tanaka et al., 1988) that has been successfully applied in the last 15 years in microbial identification. The general principle of MALDI-TOF MS involves the ionisation of large proteins by the rapid photo-volatilisation of a sample embedded in a UV-absorbing matrix followed by time-of-flight mass spectrum analysis (Marvin et al., 2003). The ability of monitoring ions over a broad *m/z* range (mass spectra) forms the basis of taxonomic identification (Marvin et al., 2003). These mass spectra function as "fingerprints" or "spectral signatures", which are unique and representative for individual microorganisms, and unknown sample identification can be performed from comparison with previously constructed databases. Holland et al. (1996) first demonstrated that the identification of whole bacteria was feasible by MALDI-TOF MS. Subsequently, new developments and enhancements of this technology were done in order to characterise a wide spectrum of microbial cells.

The advantages of MALDI-TOF MS over other mass spectrometry methodologies are that it simplified the mass spectral analysis due to gentle ionisation, reducing the number of signals, and the mass range is broader (Kemptner et al., 2009). As a result, very complex samples like whole cells can be investigated. Employing unfractionated cell materials, organism-specific signal patterns in the mass range of 2000 - 20000 Da can be obtained (Kallow et al., 2006). MALDI-TOF MS of intact cells (intact cell mass spectrometry - ICMS) has been shown to produce characteristic mass spectral fingerprints of moieties desorbed from the cell surface (Bright et al., 2002). It is a rapid and reproducible technique, which has been successfully used for the identification and discrimination of various microorganisms, and has shown high potentialities to discriminate very close related taxa.

Welham et al. (2000) presented the first paper describing the use of MALDI-TOF ICMS to characterise different filamentous fungi, using spores of *Penicillium* spp., *Scytalidium dimidiatum* and *Trychophyton rubrum*. Since then, a limited number of reports

have discussed the effectiveness of employing the technique to characterise and identify fungi (Li et al., 2000; Welham et al., 2000; Amiri-Eliasi & Fenselau, 2001; Valentine et al., 2002; Chen & Chen, 2005; Schmidt & Kallow 2005; Kallow et al., 2006; Erhard et al., 2008; Hettick et al., 2008a, 2008b; Qian et al., 2008; Seyfarth et al., 2008; Sulc et al., 2008; Kemptner et al., 2009; Santos et al., 2010). Furthermore, the majority has been devoted to procedure optimisation, where usually a limited number of ex-type strains was tested.

Kallow et al. (2006) report that in filamentous fungi most signals correspond to membrane surface proteins, so their highly characteristic masses can be used for identification and classification. In fact, the composition of fungal cell walls and also of fungal spores exhibits qualitative and quantitative differences within different fungal species, but also between different strains of the same fungal species (Kemptner et al., 2009). Kallow et al. (2006) support that in filamentous fungi most signals correspond to membrane surface proteins, so their highly characteristic masses can be used for identification and classification.

Hettick et al. (2008a, 2008b) reported 100% correct identifications, indicating that MALDI-TOF MS data are a useful diagnostic tool for the objective identification of *Penicillium* and *Aspergillus* species. MALDI–TOF MS was also used to generate highly reproducible mass spectral fingerprints for 12 species of fungi of the genus *Aspergillus* and 5 different strains of *A. flavus* (Hettick et al., 2008b). In this study, the species *A. niger* was not well resolved from *A. chevalieri*, because of poor mass spectra, a situation already reported by Valentine et al. (2002) for *A. niger*. Albeit that problem, the authors concluded that discriminant analysis of the MALDI–TOF MS data was able to correctly classify each *Aspergillus* species with 100% accuracy and was able to correctly classify strains of *A. flavus* with 95 to 100% accuracy. These data indicate that MALDI–TOF MS data may be used for unambiguous objective identification of members of the genus *Aspergillus* at both the species and strain levels. Even though Hettick et al. (2008a, 2008b) report high levels of correct identifications, they also refer to differences observed between laboratories. Hettick et al. (2008a) refer that Chen & Chen (2005) report MALDI-TOF mass spectra of several *Penicillium* species significantly different from their own. The same situation was reported for studies with *Aspergillus* species and strains (Li et al., 2000; Hettick et al., 2008b). The authors attribute the differences in the fingerprint mass spectra

to several factors, namely instrumentation, culture conditions, sample preparation and MALDI matrix. These differences reflect the importance of using standardised methodology for MALDI-TOF.

The same studies also reflected that the obtainable mass spectrum and its reproducibility are essential prerequisites for the successful classification and identification of fungal species. Various parameters are reported to influence mass spectral data, such as the pre-treatment of the fungal sample (growth media, washing procedure), the applied matrix compound and solvent system as well as the MALDI sample preparation technique itself. Furthermore, to correctly identify unknown species and strains, a comprehensive database of fungal fingerprint mass spectra will need to be established (Hettick et al., 2008b). Santos et al. (2010) emphasise that a standard reference library of biological mass spectra needs to be implemented based on inter-laboratories tests.

2.3.4 Taxonomy of section *Flavi*

The diversity of ecological niches occupied by members of *Aspergillus* section *Flavi* and the ability of some species to produce AFs make this group one of the most studied within the genus. Raper & Fennell (1965) originally included nine species and two varieties in the (then called) *A. flavus* group: *A. flavus*, *A. flavus* var. *columnaris*, *A. parasiticus*, *A. oryzae*, *A. oryzae* var. *effesus*, *A. zonatus*, *A. clavato-flavus*, *A. tamarii*, *A. flavofurcatus*, *A. subolivaceus* and *A. avenaceus*. Since then, numerous reports have been adding or repositioning species within section *Flavi* (Kozakiewicz, 1989; Peterson, 2000, 2005, 2008; Rigó et al., 2002; Frisvad et al., 2005; Pildain et al., 2008). The species and varieties that are currently considered to belong to section *Flavi* are summarised in Table 2.7.

Based on phenotypic and/or molecular data, various authors (Kozakiewicz, 1989; Peterson, 2000; Rigó et al., 2002) have suggested that *A. zonatus* and *A. clavatoflavus* should be excluded from section *Flavi*. Peterson (2000) considered *A. flavofurcatus* and *A. tamarii* to be synonyms. Additionaly, *Petromyces alliaceus* and three fungi formerly assigned to section *Wentii*, *A. thomii*, *A. terricola* (synonym of *A. tamarii*) and *A. terricola* var. *americana* have been moved to section *Flavi* (Peterson, 2005). Recent molecular data indicate that *A. flavus* var. *columnaris* and *A. zhaoqingensis* are synonyms of *A. flavus* and

A. nomius, respectively (Pildain et al., 2008). Furthermore, by the time of writing, Index Fungorum considered *A. toxicarius* as a synonym of *A. parasiticus*, and *A. fasciculatus* a synonym of *A. flavus*.

Table 2.7 List of species (by chronological order of description) currently positioned in section *Flavi*.

Species (Current name)	Type culture	Reference
A. flavus Link	CBS 100927	Link (1809)
A. oryzae (Ahlb.) E. Cohn	CBS 100925	Cohn (1883)
A. terricola É.J. Marchal	CBS 579.65	Marchal (1893)
A. parasiticus Speare	CBS 100926	Speare (1912)
A. tamarii Kita	CBS 104.13 = NRRL20818	Kita (1913)
A. terricola var. *americanus* Marchal & É.J. Marchal	CBS 580.65	Thom & Church (1921)
A. avenaceus G. Sm.	CBS 109.46	Smith (1943)
A. thomii G. Sm.	CBS 120.51	Smith (1951)
A. sojae Sakag. & K. Yamada ex Murak.	CBS 100928	Sakaguchi & Yamada (1944) Murakami (1971)
A. alliaceus Thom & Church*	IMI 87209	Thom & Church (1945)
A. flavofurcatus Bat. & H. Maia	CBS 484.65	Batista & Maia (1955)
A. flavus var. *columnaris* Raper & Fennell	CBS 486.65	Raper & Fennell (1965)
A. subolivaceus Raper & Fennell	CBS 501.65	Raper & Fennell (1965)
A. leporis States & M. Chr.	CBS 151.66	States & Christensen (1966)
A. parasiticus var. *globosus* Murak.	CBS 260.67	Murakami et al. (1966)
A. kambarensis Sugiy.	CBS 542.69	Sugiy (1967)
A. lanosus Kamal & Bhargava	CBS 650.74	Kamal & Bhargava (1969)
A. coremiiformis Bartoli & Maggi	CBS 553.77	Bartoli & Maggi (1978)
A. robustus M. Chr. & Raper	CBS 428.77	Christensen & Raper (1978)
P. albertensis J.P. Tewari		Tewari (1985)
A. nomius Kurtzman, B.W. Horn & Hesselt.	CBS 260.88 = NRRL 13137	Kurtzman et al. (1987)
A. caelatus B.W. Horn	CBS 763.97 = NRRL 25528	Horn (1997)
A. beijingensis D.M. Li, Y. Horie, Yu X. Wang & R.Y. Li		Li et al. (1998)
A. qizutongii D.M. Li, Y. Horie, Yu X. Wang & R.Y. Li		Li et al. (1998)
A. bombycis S.W. Peterson, Yoko Ito, B.W. Horn & T. Goto	CBS 117817 = NRRL 26010	Peterson et al. (2001)
A. pseudotamarii Yoko Ito, S.W. Peterson, Wicklow & T. Goto	CBS 766.97 = 93MZ2D = IMI 86979 = NBRC 100702 = NRRL25397 = NRRL25517	Ito et al. (2001)
A. parvisclerotigenus (Mich. Saito & Tsuruta) Frisvad & Samson**	CBS 121.62 = NRRL A-11612 = IBT 3651 = IBT 3851	Frisvad et al. (2005)
A. arachidicola Pildain, Frisvad & Samson	CBS 117610 = IBT 25020	Pildain et al. (2008)
A. minisclerotigenes Vaamonde, Frisvad & Samson	CBS 115635 = IBT 27196	Pildain et al. (2008)

* Teleomorph *P. alliaceus* Malloch & Cain (1973)
** Basionym: *A. flavus* var. *parvisclerotigenus* Saito & Tsuruta (1993)

Considering the most traditional schemes of identification, *Aspergillus* section *Flavi* includes six economically important species that are very closely related morphologically and phylogenetically: *A. flavus*, *A. parasiticus*, *A. nomius*, *A. oryzae*, *A. sojae* and *A. tamarii*. Even though these species share numerous common features, they differ in a major attribute: their ability to produce AFs. In fact, section *Flavi* species are usually divided into two groups. Isolates of the so-called domesticated species, namely *A. oryzae*, *A. sojae* and *A. tamarii*, are widely used in the fermenting process in Asian countries (Kumeda & Asao, 2001). Genetically modified strains of *A. oryzae* are also used as enzyme factories, for industrial production of lactase, pectin-esterase, lipase, protease and xylanase (Pariza & Johnson, 2001). On the other hand, a group of aflatoxigenic species, which include the widely distributed *A. flavus*, *A. parasiticus* and *A. nomius*, is considered a major problem for animal and human health, since those species are able to grow in almost any crop or food.

A. flavus, *A. parasiticus*, *A. oryzae* and *A. sojae* have been shown to possess high degrees of DNA relatedness and similar genome size. Based on DNA complementarity, *A. flavus* and *A. oryzae*, as well as *A. parasiticus* and *A. sojae*, were considered virtually impossible to discriminate, since their DNA similarity was found to be of 100% and 91%, respectively (Kurtzman et al., 1986, 1987). *A. oryzae* and *A. sojae* have been considered non-toxigenic variants of *A. flavus* and *A. parasiticus*, respectively. Phylogenetic studies have indicated that *A. oryzae* may have originated from an ancestral non-aflatoxigenic *A. flavus* (Geiser et al., 1998, 2000; Chang et al., 2006), or that they have lost the ability to produce AFs during the domestication process (Samson et al., 2000). Whole genome comparison of *A. flavus* NRRL3357 and *A. oryzae* RIB40 shows that these two fungi are very similar in genome size and number of predicted genes, although each also has unique genes (Payne et al., 2006). Rokas et al. (2007) report that, at the genome and proteome levels, *A. flavus* and *A. oryzae* share 99.5% and 98% of similarity (respectively), more than the percent of identity found between two strains of *A. niger* (99.3% and 96.7%, respectively). These results support the theory that *A. oryzae* may not be a separate species, but rather a domesticated ecotype of *A. flavus*, as previously advocated by Kurtzman et al. (1986) and Kozakiewicz (1989).

Genome sequence information about *A. sojae* and *A. parasiticus* is not available, but published data showed that genes of *A. parasiticus* and *A. flavus* commonly share 97–99%

nucleotide identity. Whatever the case might be, Geiser et al. (1998) suggest that both *A. oryzae* and *A. sojae* should be retained as separate species for practical reasons, to avoid confusion in food industry. Frisvad et al (2006b) support the idea that, being domesticated forms, *A. oryzae* and *A. sojae* are not expected to be isolated other than from production plants of oriental foods and enzymes.

The close relatedness among those four aspergilla as well as the high intra-specific genetic diversity are the foremost examples of the challenge posed to the accurate classification of *Flavi* species. Misclassification of isolates of these and other species based on morphological characteristics is therefore not uncommon (e.g. Wang et al. 2001; Batista et al., 2008). As molecular methods are being progressively introduced in the identification of *Aspergillus* in the last years, section *Flavi* has suffered extensive developments and adjustments. Also, the analysis of more and more isolates of what is considered to be a given species has shown great variation in AF production, especially within the most common aflatoxigenic species, *A. flavus*. These findings have prompted considerable interest also at the intraspecific level. Many authors have shown evidence that *A. flavus* sensu lato may consist of a paraphyletic group of isolates (Geiser et al., 1998, 2000; Pildain et al., 2008), and high intra-specific genetic diversity has been frequently reported for *A. flavus* populations (Wicklow et al., 1998; Batista et al., 2008; Criseo et al., 2008).

Based on morphological, genetic and physiological criteria, *A. flavus* can be divided into two types of strains (Cotty, 1994). The S-type produces numerous small sclerotia (average diameter <400 μm) and high levels of AFBs, while the L-type produces fewer, larger sclerotia and, on average, less AFBs (Garber & Cotty, 1997). S-type strains producing both AFBs and AFGs, described as *A. flavus* group II by Geiser et al. (2000), together with isolates originating from Argentinian peanuts have been recently described as *A. minisclerotigenes* (Pildain et al., 2008). Also, *A. flavus* var. *parvisclerotigenus*, with morphology and extrolite profile similar to *A. minisclerotigenes*, has been raised to species level (*A. parvisclerotigenus*) by Frisvad et al. (2005). Other species like *A. kambarensis*, *A. fasciculatus*, *A. thomii* and *A. subolivaceus* have shown to be synonymous of *A. flavus* (Pildain et al., 2008).

A. parasiticus and closely related species, although less problematic than *A. flavus*, have also been subject of controversy. Besides *A. sojae*, *A. toxicarius* has not been clearly distinguished from *A. parasiticus* (Pildain et al., 2008; Samson & Varga, 2010). Another

species closely related to *A. parasiticus*, also producing AFBs and AFGs but morphologically resembling *A. flavus* has been recently described as *A. arachidicola* (Pildain et al., 2008).

In a distinct group of species, *A. nomius* and *A. bombycis* are two related species producing both AFBs and AFGs, but not CPA, like *A. parasiticus* (Peterson et al., 2001). *A. bombycis* was isolated from silkworm-rearing houses in Japan and Indonesia, whereas *A. nomius* is more widespread: it was originally isolated from mouldy wheat in the USA, and later from various substrates in India, Japan and Thailand. Recently, *A. nomius* has been reported as an important contaminant and AF producer in Brazil nuts (Johnsson et al., 2008; Olsen et al., 2008).

In the *A. tamarii* group of species, isolates grow in a shade of olive to bronze brown. *A. tamarii* is traditionally used in oriental food industry, since it is non-aflatoxigenic. Goto et al. (1996) reported AF production by one isolate defined as *A. tamarii;* however, Ito et al. (2001) later described this isolate as a new closely related species, *A. pseudotamarii*.

P. alliaceus and *P. albertensis* are the only two sexually reproducing species (teleomorphs) classified in section *Flavi* (Tamura et al., 2000; Frisvad et al., 2005; Peterson, 2008). Data on sexual recombination between vegetative compatibility groups (VCGs) in members of section *Flavi*, namely *A. flavus* and *A. parasiticus*, have been reported (Horn et al., 2009a, 2009b), but sexual stages have not yet been found in nature.

2.3.5 Identification of species in *Aspergillus* section *Flavi*

Within the genus, the identification of species belonging to section *Flavi* has been strongly based on morphological and biochemical characterisation. Conidial wall ornamentation and colony colour on CYA have been regarded as the primary morphological diagnostic characters for separation of isolates into groups of species. Conidia of *A. flavus*-like species have relatively thin walls which are finely to moderately rough. Their shape can vary from spherical to elliptical. Conidia of *A. parasiticus*-like species are more spherical and noticeably echinulate or spinulose. When grown on Czapek-Dox (CZ), colonies of *A. flavus* are yellow-green and those of *A. parasiticus* have a distinctly darker green (Klich, 2002a; Samson et al., 2004a). *A. nomius* is morphologically similar to *A. flavus* in colour, but conidia are more roughened (Kurztman

et al., 1987). Species of the *A. tamarii* group are very distinctive from the others. They have olive to bronze-brown colonies on CYA and their conidia are thick and echinulate.

The mycotoxigenic profile regarding AFBs, AFGs and CPA of these strains has also been routinely used for identification purposes. *A. parasiticus* strains are usually strongly aflatoxigenic, producing both AFBs and AFGs, but not CPA. Non-aflatoxigenic strains have rarely been reported (Horn et al., 1996; Razzaghi-Abyaneh et al., 2006; Tran-Dinh et al., 1999; Vaamonde et al., 2003). On the other hand, *A. flavus* populations have been found to be extremely diverse in terms of morphology and toxigenicity, and have thus been divided into groups, depending on their toxigenic profile (Vaamonte et al., 2003; Razzaghi-Abyaneh et al., 2006; Giorni et al., 2007). Five groups have been proposed (Vaamonde et al., 2003): (i) chemotype I for AFBs and CPA producers; (ii) chemotype II for AFBs, AFGs and CPA producers; (iii) chemotype III for AFBs producers; (iv) chemotype IV for CPA producers; and (v) chemotype V for non-producers. Other extrolites like aspergillic acid, kojic acid, parasiticolides, chrysogine and aflatrems have also been found useful for species characterisation, but have been used less frequently (Samson et al., 2004a; Pildain et al., 2008).

Table 2.8 compiles the most significant morphological and physiological characteristics of species from section *Flavi*.

Table 2.8 Compilation of the major morphological and biochemical characters used in the distinction of species of *Aspergillus* section *Flavi* (Tewari, 1985; Kurtzman et al., 1987; Horn, 1997; Li et al., 1998; Ito et al., 2001; Peterson et al., 2001; Klich, 2002a; Samson et al., 2004, 2006; Frisvad et al., 2005; Hedayati et al., 2007; Pildain et al., 2008).

Species	Seriation[a,b]	Conidia texture	Conidia size (µm)	Sclerotia[b]	Colony colour	Reverse on AFPA[b]	Colony diameter (cm) on CZ42[b]	AFBs [c]	AFGs [c]	CPA [c]
A. arachidicola	b or u	echinulate	4.5-5	no	olive to olive-brown	Cream	good	+	+	-
A. avenaceus	b	smooth	4-5 x 3.2-4	elongate	yellow to olive	n.d.	n.d.	-	-	-
A. beijingensis	u	verrucose	3.5-6.5	n.d.	olive-yellow	n.d.	n.d.	n.d.	n.d.	n.d.
A. bombycis	b	rough	4-7	n.d.	green to bronze	n.d.	n.d.	+	+	-
A. caelatus	u or b	coarse	5-6	elongate	olive to brown	Brown	no growth	-	-	-
A. coremiiformis	b	encrusted	6.9-9	n.d.	cream to brown	n.d.	n.d.	n.d.	n.d.	n.d.
A. flavus	b or b/u	smooth	3.5-4.5	globose	yellow-green	Orange	2.4-3.6	+	-	+
A. lanosus	b	smooth	2.2-2.8	n.d.	yellow	n.d.	n.d.	-	-	-
A. leporis	b	smooth	3-3.5	elongate	olive	n.d.	n.d.	-	-	-
A. minisclerotigenes	b	smooth	3-4	small	grayish-green	n.d.	n.d.	+	+	+
A. nomius	u or u/b	echinulate	4.5-6.5	elongate	yellow-green to olive-green	Orange	0-1.5	+	+	-
A. oryzae	variable	smooth	4.5-8.0	no	brown	Cream	1.8-3.2	-	-	+
A. parasiticus	u or u/b	rough	3.5-5.5	elongate	dark-green	Orange	1.8-3.3	+	+	-
A. parvisclerotigenus	n.d.					n.d.	n.d.	+	+	+
A. pseudotamarii	b or u	rough	6.1-7.8	globose	bronze to brown	Brown	n.d.	+	-	+
A. qizutongii	u	smooth	5-6.5	n.d.	olive-yellow	n.d.	n.d.	n.d.	n.d.	n.d.
A. robustus	b	echinulate	3.5-4.5 x 2.8-3.4	irregular	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
A. sojae	u	rough	5-6	n.d.	brown-green	Orange	1.5-2.7	-	-	-

(continues)

Table 2.8 (continued)

Species	Seriation[a]	Conidia texture	Conidia size (µm)	Sclerotia[b]	Colony colour	Reverse on AFPA[b]	Colony diameter (cm) on CZ42[b]	AFBs [c]	AFGs [c]	CPA [c]
A. subolivaceus	b	smooth	4 x 3	globose to elongate	olive	n.d.	n.d.	n.d.	n.d.	n.d.
A. tamarii	u or b	echinulate	5-8	no	dark-brown	Brown	0.2-1.0	-	-	+
A. terricola	b or u	echinulate	4.5-9	n.d.	olive to brown	n.d.	n.d.	n.d.	n.d.	n.d.
A. thomii	b/u	rough	3-5.5	n.d.	ochraceous	n.d.	n.d.	n.d.	n.d.	n.d.
P. albertensis	b	smooth	2.3-3.5	elongate	olive	n.d.	n.d.	-	-	-
P. alliaceus	b/u	smooth	2.5-4	ovoid	brown	n.d.	n.d.	-	-	-

[a] u: uniseriate; b: biseriate; u/b: predominantly uniseriate; b/u: predominantly biseriate;
[b] n.d.: no data found
[c] +: producer; -: non-producer.

Because species identification based on morphological and biochemical characters is time-consuming and not always straight-forward, several molecular genetic techniques have been tested to differentiate and identify species and strains within section *Flavi*, as well as for establishing phylogenetic relationships between species. Since the 1990's, several methods have been applied with aim of producing genetic markers capable of distinguishing the closely related species *A. flavus*, *A. oryzae*, *A. parasiticus*, *A. sojae* and *A. nomius*. Fingerprinting techniques such as AFLP, RFLP, RAPD and inter-simple sequence repeats (ISSR) have been applied (Klich & Mullaney, 1987; Moody & Tyler, 1990a, 1990b; Yuan et al., 1995; Montiel et al., 2003; Baptista et al., 2008; Godet & Munaut, 2010). Other methods based on the analysis of PCR amplified DNA fragments have also been used: single-strand conformation polymorphism (PCR-SSCP), PCR-RFLP, heteroduplex panel analysis (PCR-HPA), single nucleotide polymorphism (SNP) (Chang et al., 1995; Kumeda & Asao, 1996, 2001; Somashekar et al., 2004). DNA sequence analysis from both nuclear and mitochondrial DNA has also been applied (Wang et al., 2001). Other non-genetic, innovative methods based on mass spectrometry and spectroscopy have also been used for species differentiation and identification (Li et al., 2000; Garon et al., 2010).

Although these studies provided important information about the phylogenetic relationships between species, none of them used singly was able to solve problems of identification. High similarity between species of section *Flavi*, as well as a high degree of intraspecific variability, has resulted in the inability to produce a unique biological marker capable of consistently differentiating the various species.

Table 2.9 lists some of the studies related to taxonomic issues within section *Flavi* as well as the characteristics and results obtained.

Table 2.9 Methods and characters used by various authors in species identification and description within *Aspergillus* section *Flavi*, with reference to the major achievments.

Reference	Method				Achievements
	Morphological	**Physiological**	**Molecular**	**Other**	
Kurtzman et al., 1986			DNA complementarity		High genetic relatedness between species *A. flavus*, *A. oryzae*, *A. parasiticus* and *A. sojae*
Klich & Mullaney, 1987			RFLP		Differentiated some *A. oryzae* isolates from *A. flavus*
Moody & Tyler, 1990a			RFLP of mtDNA		Differentiated *A. flavus*, *A. parasiticus* and *A. nomius*
Moody & Tyler, 1990b			RFLP of nuclear DNA		Differentiated *A. flavus*, *A. parasiticus* and *A. nomius*
Chang et al., 1995			SNP of *aflR* gene		Differentiated *A. parasiticus* from *A. sojae*, and *A. flavus* from *A. oryzae*
Yuan et al., 1995			RAPD		Differentiated *A. sojae* from *A. parasiticus*, and the *A. sojae* strains were further separated into two groups
Kumeda & Asao, 1996			PCR-SSCP and PCR-RFLP of ITS region		Differentiated *A. flavus*/*A.oryzae*, *A. parasiticus*/*A. sojae*, *A. nomius* and *A. tamarii*
Nikkuni et al., 1996, 1998			DNA sequence analysis of ITS region		Differentiated *A. flavus*/*A.oryzae*, *A. parasiticus*/*A. sojae*, *A. nomius* and *A. tamarii*
Li et al., 2000				MALDI-TOF ICMS of spores	Differentiated *A. parasiticus* and *A. flavus*
Kumeda & Asao, 2001			PCR-HPA of ITS region		Differentiated *A. flavus*/*A.oryzae*, *A. parasiticus*/*A. sojae*, *A. nomius* and *A. tamarii*

(continues)

Table 2.9 (continued)

Reference	Method				Achievements
	Morphological	**Physiological**	**Molecular**	**Other**	
Peterson, 2001	Macro-and micromorphology	Growth on various media; extrolites	DNA sequence analysis MLST		Phylogenetic relationships. Description of *A. bombycis*
Wang et al., 2001			DNA sequence analysis of *cox1* (mtDNA)		Differentiated *A. parasiticus* from *A. flavus*
Quirk & Kupinski, 2002			RFLP of mtDNA		Differentiated *A. flavus*/*A. oryzae*, *A. parasiticus*/*A. sojae*, *A. nomius* and *A. tamarii*
Rigó et al., 2002	Colony colour, sclerotia	Ubiquinone system	DNA sequence analysis of ITS region		Differentiated *A. parasiticus*/*A. sojae* and *A. flavus*/*A. oryzae*
Ehrlich et al., 2003	Sclerotia size	AF production	DNA sequence analysis of *aflJ* and *aflR* genes		Phylogenetic relationships. Differentiated S_{BG} *A. flavus* isolates from other *A. flavus*
Montiel et al., 2003			AFLP		Differentiated *A. parasiticus*/*A. sojae* and *A. flavus*/*A. oryzae*
Lee et al., 2004	Macro-and micromorphology		AFLP		Differentiated *A. flavus* from *A.oryzae*
Somashekar et al., 2004			RFLP (*PvuII*) of the *aflR* gene		Differentiated *A. flavus* from *A. parasiticus*
Frisvad et al., 2005	Macro-and micromorphology	Growth on various media; extrolites	DNA sequence analysis of β-tubulin gene		Phylogenetic relationships. Description of *A. parvisclerotigenus*
Baptista et al., 2008			RAPD/ ISSR		Demonstrated high inter- and intra-specific genetic diversity within section *Flavi* species that allowed species identification
Peterson, 2008			DNA sequence analysis MLST		Phylogenetic relationships

(continues)

Table 2.9 (continued)

Reference	Method				Achievements
	Morphological	**Physiological**	**Molecular**	**Other**	
Pildain et al., 2008	Macro-and micromorphology	Growth on various media; extrolites	DNA sequence analysis of ITS region and calmodulin/ β-tubulin genes		Phylogenetic relationships. Description of *A. arachidicola* and *A. minisclerotigenes*
Garon et al., 2010		AFs production		FT-IR Spectroscopy	Differentiated *A. flavus* from *A. parasiticus*
Godet & Munaut, 2010			real-time PCR/ RAPD/RFLP		Six-step strategy to set up a decision-making tree for identification

3 MATERIALS AND METHODS

3.1 Mycobiota and mycotoxins of almonds and chestnuts

3.1.1 Almond and chestnut sampling

3.1.1.1 Study area

Samples were collected in the Portuguese northeast District of Bragança, in the former Province of Trás-os-Montes e Alto Douro, now called North region, divided in several sub-regions. Chestnut samples (which were all from the field) originated from Santa Comba de Rossas, council of Bragança.

Almond samples were collected in the parish of Larinho, council of Torre de Moncorvo (Moncorvo; field and storage samples), and in the council of Alfândega da Fé (Alfândega; processor samples). Almonds from field and storage samples originated from Moncorvo, and almonds from samples collected at the processor originated from Moncorvo and Faro. Bragança, Moncorvo and Faro will herein be characterised geographically as well as climatically, because of the significance of these characteristics to the fungal contamination of chestnuts and almonds. Even though we did not collect Faro samples directly at the producer (samples were collected at the processor), we considered it important to characterise Faro as well. Alfândega will not be characterised in terms of climate, since only processor samples were collected at this study area, which does not significantly influence, in terms of environmental conditions, the fungal contamination of the almonds.

3.1.1.2 Geographic characterisation

Chestnut sampling was conducted in Santa Comba de Rossas, council of Bragança, which lies in the utmost north of Bragança District, sub-region Alto Trás-os-Montes, just a few kilometres from the border with Spain. It is located at a latitude of 41º49'N, longitude of 06º45'W and an altitude of approximately 720 m. This area lies in Terra Fria de Planalto, a regional denomination attributed by the prominent local climatologist Prof.

Dionísio Gonçalves (Gonçalves, 1991), after profound studies on the orographic and climatic conditions of the region.

Almonds from field and storage, as well as part of those collected at the processor, were produced in Moncorvo, which lies in the south of Bragança District, sub-region Douro, at a latitude of 41º04'N, longitude of 07º01'W and an altitude of approximately 410 m. This study area is located in Terra Quente (Gonçalves, 1991), and is naturally bordered by the Douro River. Just north from Moncorvo is Alfândega, where the processor plant is located.

Part of the almond samples collected at the processor was produced in Faro. The council of Faro is integrated in the District of Faro, the southernmost district of Portugal, which coincides with the new region of Faro (former Province of Algarve), positioned at a latitude of 37º02'N, longitude of 07º56'W and an altitude of approximately 10 m. It is bathed by the Atlantic Ocean, but suffers a strong influence of the Mediterranean Sea. Figure 3.1 illustrates the Portuguese country, and marks the areas under study.

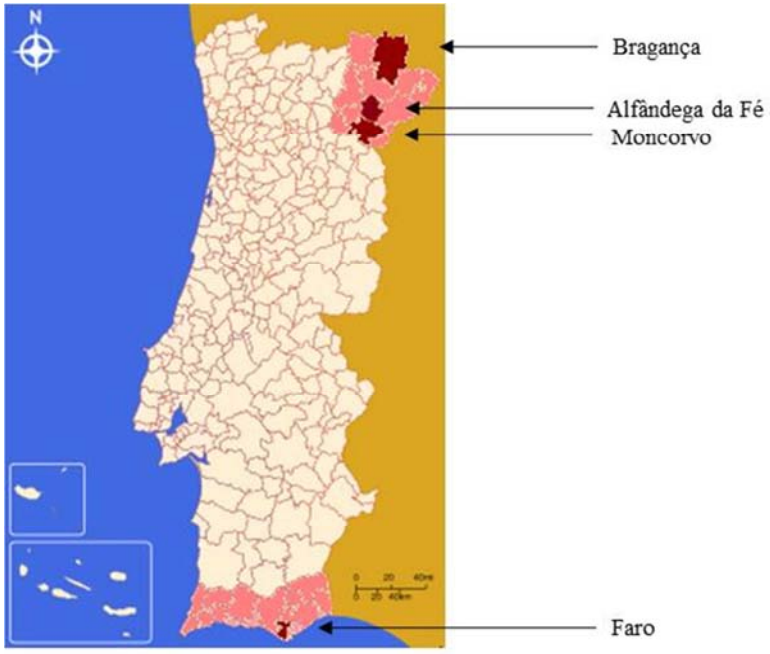

Figure 3.1 Map of Portugal. Study areas are marked in red. Districts where the study areas are integrated are marked in pink.

3.1.1.3 Climatic characterisation

Study areas were characterised in terms of bioclimate using the bioclimatic models of Rivas-Martinez (2004). Data used for climatic characterisation of the study areas were obtained from the reference values given by the closest weather station of Instituto de Meteorologia (INMG, 1991a, 1991b). Table 3.1 lists and characterises the weather stations used in this study. Table 3.2 indicates the bioclimatic diagnostic obtained for each study area.

Table 3.1 Weather stations used for climate characterisation of the study areas.

	Nearest weather station				
Study area	**Local**	**Lat (N)**	**Long (W)**	**Alt (m)**	**Period**
Bragança	Bragança	41º48’	6º 44’	690	1951-1980
Moncorvo	Mirandela	41º31’	7º12’	250	1958-1980
Faro	Faro	37º01’	7º58’	8	1964-1980

Table 3.2 Bioclimatic classification of the study areas, based on Rivas-Martinez (2004).

Study area	**Bioclimatic classification**			
	Bioclimate	**Continentality**	**Thermotype**	**Ombrotype**
Bragança	Mediterranean pluviseasonal oceanic	Euoceanic	Lower Supramediterranean	Upper subhumid
Moncorvo	Mediterranean pluviseasonal oceanic	Semi-continental	Upper Mesomediterranean	Upper dry
Faro	Mediterranean pluviseasonal oceanic	Semi-hyperoceanic	Lower Thermomediterranean	Lower dry

Bragança is characterised by a humid climate (yearly rainfall of 741.1 mm/year), with moderate water deficit during summer, and great excess in winter, with 86% of the rainfall occurring from October to May. The summer is warm, with mean temperatures around 21 ºC, and the winter is cold, with temperatures frequently dropping below 0 ºC.

Moncorvo is characterised by hot summers, with mean temperatures around 24 ºC, but 40 ºC being registered with some frequency during July and August. Moncorvo registers mean temperatures of 6 ºC in the cold months of December and January, and a yearly rainfall of 520.1 mm with 83% concentrated in the period from October to May.

Faro has a typical Mediterranean climate, with hot and dry summers and mild winters. Summer temperatures follow those of Moncorvo, but winter mean temperatures are around 12 ºC. Rainfall assumes big amplitudes throughout the year: 96% of the 513.6 mm fall from October to May.

3.1.1.4 Sampling plans

The sampling plans proposed for this study included the collection of samples of both chestnuts and almonds at different stages of production (field, storage and processing) for two consecutive crops (2007 and 2008). However, for a number of reasons, which will be exposed whenever found necessary, the sampling plans had to be adjusted along the course of the work.

The most significant almond and chestnut producers and processing industries of the region were identified, and, from those, one for each matrix was selected. The selected processors represent key industries in the northeast region, and are usually responsible for processing the majority of the local production. Among the farmers, the one contributing the most for the processing industry was selected.

It is worth noting that the present study is not limited to one single variety. Local orchards are a mix of varieties. Furthermore, in the processing industries, almonds and chestnuts from local producers are generally processed as mixtures. In fact, they are not separated by variety but by size, given that they are similar in quality. This study intended to be a survey of fungal and mycotoxin contamination of local almonds and chestnuts. It was not our intention to study the vulnerability of the different varieties to fungal growth and mycotoxin accumulation. The only discrimination in terms of varieties set in this study was for almond samples (field, storage and processor samples), which consisted only of a mixture of soft shell varieties, since these are more likely to be damaged by insects and fungi.

Because almond and chestnut productions vary in their characteristics, a general workflow from production to commercialisation will be described for each matrix, and sampling plans will be described separately.

3.1.1.4.1 Chestnuts

General workflow

Chestnuts are usually ready to harvest from the beginning of October until the end of November. Fruits are collected from the ground, at first by hand into 50 kg bags, on a weekly basis. When all fruits have fallen from the trees, collection is made mechanically with a suction machine, cleaned from soil, leaves and other dirt, and sacked. Chestnuts are then transported to the warehouse, spread on the floor and left to dry for 3 to 4 days. They are then put in 50 kg net bags and stored in a controlled atmosphere chamber (controlled temperature, relative humidity and CO_2) until being processed.

In the 2007 crop, chestnuts were ready to harvest at the beginning of November, later than usual, because of excessive rainfall in the normal harvesting period. The producer started to harvest at 01/11/2007 and finished three to four weeks later. Because this year's crop produced very low yields, chestnuts were processed and expedited immediately after harvest. Also, in the mycological analysis of field samples a very limited number of *Aspergillus* isolates was detected and none of them belonged to section *Flavi*, which was the main interest of this study. For these reasons, we considered that this matrix was of limited interest to our objectives, and, contrary to the proposed sampling plan, samples were not taken at storage and processing stages. Furthermore, field samples were only collected and analysed for the 2007 crop.

Field samples

Three chestnut orchards, approximately 500 m apart from each other, where selected for field sampling, and were coded *Px* (where *x* refers to the number of the orchard). Five actively producing trees per orchard (one in each corner and one in the center of the orchard) were selected as sampling points, and were coded *Cy* (where *y* refers to the number of the chestnut tree). In total, we took 15 sampling spots, coded *PxCy*.

Three samples were taken from each sampling spot. The first sampling time-point coincided with the beginning of the cropping (01/11/2007), when the farmer began to crop fallen nuts from the ground. At this time-point, we collected one sample of nuts from the ground (coded *PxCy/Ch1/07*, where *Ch1/07* means the first sampling from the ground taken in the 2007 crop), and one sample from the tree (coded *PxCy/Arv1/07*, where *Arv1*

means the first sampling from the tree) (Figure 3.2). The second sampling time-point occurred at 20/11/2007, and corresponded to the period when all nuts had fallen from the trees and the farmer began to crop them mechanically. From the first to the second sampling time-point, the farmer collects the fallen chestnuts erratically, so sampled chestnuts from 20/11/2007 had been on the ground between one to 20 days. At this time-point, only one sample of chestnuts was taken from the ground, and was coded *PxCy/Ch2/07* (second sampling from the ground).

Figure 3.2 Chestnut orchard P1 (A), chestnut tree (B) and chestnuts on the ground (C), at the time of sample collection.

Samples were composed of 50 nuts, randomly collected. Nuts were collected by hand, freed from the spiny exocarp and put in a C4 craft paper envelope (229 x 324 mm). The envelope was immediately sealed and stored in a portable refrigerator. Hands were disinfected with 70% ethanol between each sampling spot. Samples arrived to the laboratory no more than 3 hours later.

Climatic data for the year of 2007 were collected by an agro-climatic station of the Polytechnic Institute of Bragança, localised in the same farm as the orchards (Lat. 41º49'N, Long. 06º45'W, Alt. 720 m). Climatic data were registered daily, and included Temperature, Precipitation and Relative Humidity. Information regarding irrigation and plant disease treatments was collected from the producer.

3.1.1.4.2 Almonds

General workflow

The almond is not a true nut, but a drupe, which consists of an outer dehiscent hull (exocarp) and a hard shell (endocarp) with the edible seed (kernel) inside, involved by a brown seed coat. Shelling almonds refers to removing the shell to reveal the kernel. Almonds are commonly sold shelled, i.e. after the shells have been removed, or in-shell, i.e. with the shells still attached. Blanched almonds (or nutmeat) are shelled almonds that have been treated with hot water to soften the seed coat, which is then removed to reveal the white embryo.

Almonds are generally ready to harvest from the beginning of September onwards. A few almonds naturally fall from the tree and are collected from the ground. However, they are predominantly harvested by shaking the tree branches and making nuts fall into a net placed on the ground, underneath the tree. Nuts are collected into 50 kg bags and transported to the producer's warehouse. Once in the warehouse, almonds are spread on the floor and left to dry naturally. When sufficiently dry (usually 3 to 4 weeks later), they are again bagged and piled in the warehouse until expedited to the processor. The product is rarely sold immediately, and it is common for it to stay in the warehouse for a large number of months, depending on market conditions.

Field samples

Almond field sampling spots, sample coding and nut collection were identical to those previously described for chestnut samples. In sample codes, *Cy* was replaced by *Ay* to indicate almonds.

Two samples were taken from each sampling spot, regarding two consecutive crops: 2007 and 2008. The sampling time-points (06/09/2007 and 12/09/2008) corresponded to the day before the beginning of harvesting. Samples were coded as *PxAy/07* and *PxAy/08*, respectively. Samples were composed of 50 nuts collected randomly from the tree canopy (Figure 3.3).

Climatic data for the years of 2007 and 2008 were collected from a local data logger (agro-climatic station) of the Direcção Regional de Agricultura e Pescas do Norte (DRAPN), localised at Quinta de Água D'Alta, Moncorvo (Lat 41º18'N, Long 7º07'W, Alt

274 m). Climatic data were registered daily, and included Temperature, Precipitation and Relative Humidity. Information regarding irrigation and plant disease treatments was collected from the producer.

Figure 3.3 Almond orchard P3 (A) and almond tree (B), at the time of sample collection.

Storage samples

Sampling during storage took place for the 2008 crop only. From 13/09/2008 onwards, almonds were continuously collected by the producer, spread in the warehouse and left to dry. On 24/10/2008, almonds began to be put in 50 kg bags (by order of arrival) and piled (Figure 3.4). At this time-point (coded *Storage 1*), two bags from the pile were selected, one from the top (Bag A) and one from inside the pile (Bag B), and marked. One data logger was put inside each bag. One sample of each bag was collected. Samples were collected every 3 months, until the almonds were expedited. So, after the first sampling time-point (24/10/2008), two other samples were taken, at days 16/01/2009 (*Storage 2*) and 20/03/2009 (*Storage 3*) from the same bags. The day after *Storage 3*, almonds were expedited, not to our selected industry (contrary to what had happened the previous years), but to Spain, so we lost track of them.

Figure 3.4 Producer´s warehouse (A) and detail of stored almonds piled in 50 kg bags (B).

Approximately 2 kg of in-shell almonds per sample were collected from various parts of each selected bag. Samples from the bag at the top of the pile were coded *A1*, *A2* and *A3*, and samples from the bag inside the pile were coded *B1*, *B2* and *B3*, depending on the time-point of collection. Samples were treated as previously described.

Temperature and Relative Humidity of the warehouse were registered every 3 hours with two data loggers Hygrochron coupled to the software Eclo ExpressThermo 2007, one placed in the middle of the warehouse and the other near an inexistent wall (open to the outside), where almonds began to be distributed and piled.

Processor samples

The following general categories of almonds were sampled from the processor: i) unsorted in-shell nuts, representing incoming almonds as received by the processor; ii) "in-process" nuts, representing nuts in different processing stages; and iii) processed nutmeats, representing a finished product ready to be sold for food consumption. Temperature and Relative Humidity of the warehouse were registered as previously mentioned by one data logger. Sample details are summarised in Table 3.3.

Since a significant group of processor samples was relative to almonds originating from Faro, climatic data of Faro for the year of 2008 were also collected. Data were retrieved from the official site of DRAPALG (URL: http://www.drapalg.min-agricultura.pt/; accessed 20.06.2010). They were registered by an agro-climatic station of the Direcção Regional de Agricultura e Pescas do Algarve (DRAPALG), localised at Patacão, Faro (Lat. 37º02'N, Long. 07º56'W, Alt. 13 m), and included Temperature,

Precipitation and Relative Humidity. It was not possible to collect information regarding irrigation and plant disease treatments for Faro almonds, since the samples were taken from the processor and the farmer was not identified.

Table 3.3 List of almond samples and description of conditions on which samples were collected.

Production stage	Code	Collection date	Local of collection	Almonds Origin	Characterisation	Size
2007 crop						
Field	P1/A1	06.09.2007	Moncorvo	Moncorvo	Each sample corresponds to one tree.	50 fruits
	P1/A2					50 fruits
	P1/A3					50 fruits
	P1/A4					50 fruits
	P1/A5					50 fruits
	P2/A1					50 fruits
	P2/A2					50 fruits
	P2/A3					50 fruits
	P2/A4					50 fruits
	P2/A5					50 fruits
	P3/A1					50 fruits
	P3/A2					50 fruits
	P3/A3					50 fruits
	P3/A4					50 fruits
	P3/A5					50 fruits
Processor	A	29.02.2008	Alfândega da Fé	Moncorvo	In-shell nuts. Received at the processor 10 days before sample collection. Stored in 50 kg bags, in the warehouse.	2 kg
	B	29.02.2008	Alfândega da Fé	Moncorvo	Shelled nuts. The same as A, but shelled the day before sample collection: wet in cold water →dried at 40 ºC for 5 hours →mechanically shelled → stored in the warehouse in 50 kg bags.	700 g
	C1	20.03.2009	Alfândega da Fé	Moncorvo	Shelled nuts. Nuts from the 2007 crop. Received and shelled by the processor in October 2008. Stored in 50 kg bags.	700 g
	C2	22.05.2009				700 g

(continues)

Table 3.3 (continued)

Production stage	Code	Collection date	Local of collection	Almonds Origin	Characterisation	Size
2008 crop						
Field	P1/A1	12.09.2008	Moncorvo	Moncorvo	Each sample corresponds to one tree.	50 fruits
	P1/A2					50 fruits
	P1/A3					50 fruits
	P1/A4					50 fruits
	P1/A5					50 fruits
	P2/A1					50 fruits
	P2/A2					50 fruits
	P2/A3					50 fruits
	P2/A4					50 fruits
	P2/A5					50 fruits
	P3/A1					50 fruits
	P3/A2					50 fruits
	P3/A3					50 fruits
	P3/A4					50 fruits
	P3/A5					50 fruits
Storage	A1	24.10.2008	Moncorvo	Moncorvo	In-shell nuts. Harvested at 15.09.2008. Spread in the warehouse to dry, bagged and piled. Sample from the top of the pile. (Climatic data logger No 39)	2 kg
	A2	16.01.2009				2 kg
	A3	20.03.2009				2 kg
	B1	24.10.2008	Moncorvo	Moncorvo	In-shell nuts. Harvested at 15.09.2008. Spread in the warehouse to dry, bagged and piled. Sample from inside the pile. (Climatic data logger No 61)	2 kg
	B2	16.01.2009				2 kg
	B3	20.03.2009				2 kg
Processor	D1	20.03.2009	Alfândega da Fé	Moncorvo	Shelled nuts. Shelled in January and stored in the warehouse in 50 kg bags.	700 g
	D2	22.05.2009				700 g
	F1	20.03.2009	Alfândega da Fé	Faro	In-shell nuts. Just received and stored in the silo.	2 kg
	F2/kernel	22.05.2009			Shelled nuts (nutmeat with seed coat). Nuts from the silo being shelled at the moment of sample collection.	700 g
	F2/shell	22.05.2009			Shell corresponding to sample F2/M.	
	F3/nutmeat	26.05.2009			Nutmeat from nuts shelled at 22.05.2009. Seed coat was being removed at the moment of sample collection.	700 g
	F3/seed coat	26.05.2009			Seed coat corresponding to sample F3/M	100 g

Determination of water activity

Water activity was measured for storage and processor samples. As soon as the samples arrived to the laboratory, they were left at room temperature for 2 hours and water activity was measured at approximately 22 ºC, in triplicate, using Rotronic Hygropalm AW1 equipment.

3.1.2 Mycological analysis

3.1.2.1 Implementation of the procedures for fruit plating

Some tests were made to determine the best procedure for fruit plating. For this matter, we tested the necessity and efficacy of fruit superficial disinfection, as well as the selectivity of various culture media towards *Aspergillus* fungi.

3.1.2.1.1 Superficial disinfection

The following superficial disinfection methods were tested:

Method 1

Fruits were submerged in 0.5% sodium hypochloride for 2 minutes, washed twice in sterile tap water and plated.

Method 2

Fruits were submerged in 95% ethanol for 30 seconds, washed twice in sterile tap water and plated.

Method 3

Fruits were submerged in 95% ethanol for 30 seconds, flamed to eliminate the ethanol and plated.

Method 4

Fruits were briefly flamed (3 to 4 seconds) and plated.

For each disinfection method, 5 fruits were plated with the shell (in-shell), and 5 other fruits were plated without the shell (shelled). The same number of fruits was used as control (no treatment). Each fruit was plated in a 9 cm Petri dish containing 15 to 20 mL of Dichloran Glycerol 18% (DG18; Oxoid). Given the size of in-shell fruits (for both almonds and chestnuts) and of shelled chestnuts, they had to be cut in order for them to fit the Petri

dish. In-shell chestnuts were cut alongside with a sterile kitchen knife and the two halves were directly plated; for shelled chestnuts, the shell was cut off, the fruit was cut alongside and the two halves were plated (Figure 3.5). Almonds were put in a sterile plastic bag and shells were broken with a nutcraker. For in-shell fruit analysis, the kernel and the broken shell were plated, and for shelled fruits shell was discarded and only the kernel was plated (Figure 3.6).

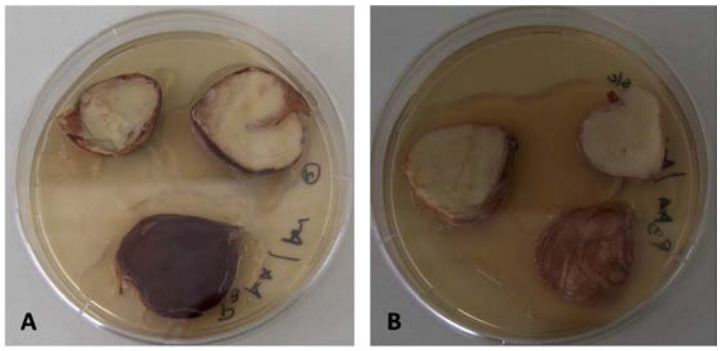

Figure 3.5 Chestnuts plated in-shell (A) and shelled (B).

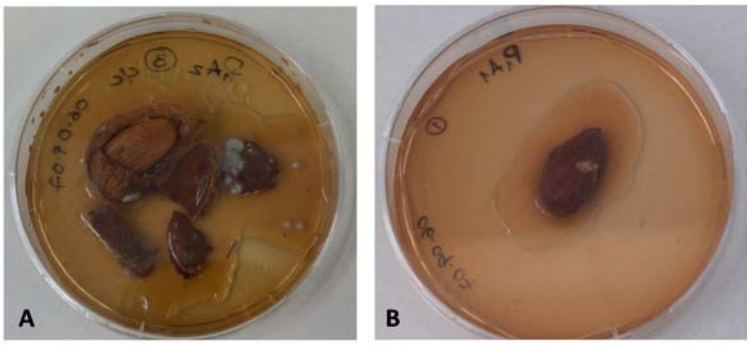

Figure 3.6 Almonds plated in-shell (A) and de-shelled (B).

Results were analysed by a qualitative appreciation of the fruits, for both number and type of fungi. Superficial disinfection by methods 1 to 3 was considered inadequate for almonds, because, given the porosity of the shell, the fruit would get completely soaked and rotten. For chestnuts, the 4 methods were considered similar. In terms of fungal growth, we did not detect differences between methods. In-shell treated fruits showed little or no fungal growth, whereas untreated fruits showed a high level of shell contamination. Considering shelled fruits, both treated and untreated fruits showed little fungal growth.

Superficial disinfection almost completely eliminated the shell contaminants. Bearing in mind that our goal was to detect and quantify fungi from *Aspergillus* genus and

particularly potentially aflatoxigenic fungi (*Aspergillus* section *Flavi*), we considered that it would be of interest to detect not only those fungi effectively colonising the edible part of the fruit at that moment, but also those that were present on the shell as environmental contaminants, and that could colonise the fruits at a posterior stage, if inductive environmental conditions were met.

Taking this into account, we chose to plate both in-shell and shelled fruits (for almonds and chestnuts). In-shell fruits were not submitted to any superficial disinfection treatment. Shelled fruits were superficially disinfected by method 4 (for both almonds and chestnuts) before being shelled, in order to eliminate the possibility of contamination of the kernel by the fungi present on the shell.

3.1.2.1.2 Culture medium

There is a wide variety of culture media available for fungi isolation from food commodities. As previously mentioned in the Literature Review, the medium recommended for low water activity foodstuffs is DG18 (Hocking & Pitt, 1980; ISO 21527-2:2008), but Dichloran Red Bengal Chloramphenicol (DRBC; King et al., 1979) is also available, even though it is used preferably for high water activity foodstuffs (ISO 21527-1:2008). Malt Salt Agar (MSA; Malt Extract Agar (MEA) supplemented with 6 to 10% of NaCl) has also been used for this purpose, especially when the goal was to reduce the variety of fungi to some genera of interest, namely *Aspergillus* and *Penicillium* (Joffe, 1969; Phillips et al., 1979; Hocking & Pitt, 1980; Purcell et al., 1980; Ackermann, 1998; Bayman et al., 2002; Samson et al., 2004a; Kaaya & Kyamuhangire, 2006; Medina et al., 2006).

In order to determine the most appropriate culture media for our study, three media were tested: DRBC (Oxoid), DG18 (Oxoid) and MEA (Malt Extract 20 g/L, Glucose 20 g/L, Peptone 1 g/L, Agar 20 g/L) supplemented with 10% NaCl (MSA10) - by direct plating of 3 in-shell almonds and 3 in-shell chestnuts on each media. The fruits were plated individually on 9 cm Petri dishes containing 15 mL of culture media and incubated in the dark for 7 days at 25-28 ºC. After the first test, we observed that the parts of the fruits that were not in direct contact with the medium would get very dry, and no fungi would grow on that area. So, we further tested the effect of covering the fruit with a thin layer of the

same medium, in order to allow medium selectivity and fungal growth to occur all over the fruit. For this test, we plated the fruits as previously described, and covered them with a thin layer of liquefied medium (near 45 ºC) with the aid of a pipette.

Figure 3.7 shows the results obtained for almonds plated on, and covered with, the three media, after 7 days of incubation. Contrary to the expectations, fruits plated on DRBC and on DG18 were completely invaded by rapid growing fungi (*Mucorales*). On the other hand, on MSA10 the growth of these fungi was restrained and various other fungi were able to grow and be detected. We also observed that covering the fruits with a medium layer resulted in a larger variety of fungi present at the upper part of the fruit (Figures 3.8 and 3.9).

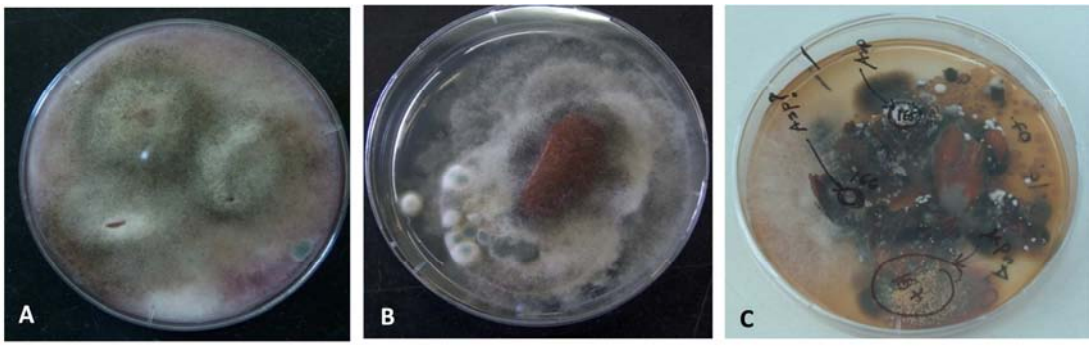

Figure 3.7 In-shell almonds plated on DRBC (A), DG18 (B) and MSA10 (C), covered with a thin layer of the same medium. Growth corresponds to 7 days of incubation.

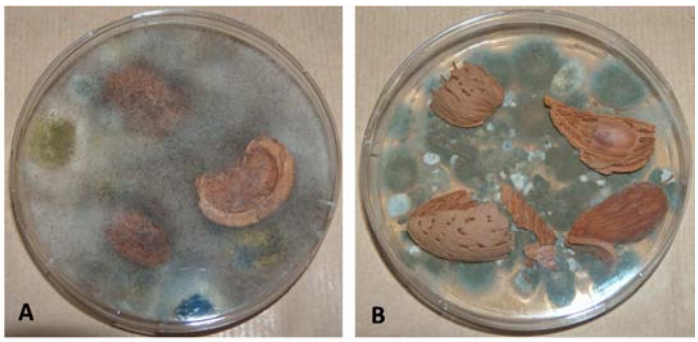

Figure 3.8 In-shell almonds plated on DG18 (A) and MSA10 (B), not covered with a thin layer of the same medium. Growth corresponds to 7 days of incubation.

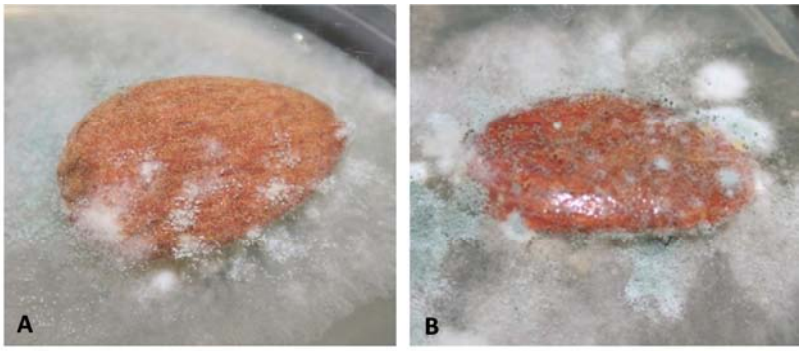

Figure 3.9 Detail of almond not covered (A) and covered (B) with MSA10 after 7 days of incubation. Only *Eurotium* grows on not-covered fruit. On covered fruit, we can detect the presence of various colonies of *Aspergillus* sections *Nigri* and *Flavi*.

3.1.2.2 Fungal isolation from samples

From each sample, fruits were taken randomly from the bags using a sterile forceps. For field samples, 3 in-shell fruits and 3 shelled fruits per sample were plated, in a total of 45 in-shell fruits and 45 shelled fruits for each sampling time-point. For storage samples and processor in-shell samples, 10 in-shell fruits and 10 shelled fruits per sample were plated. For shelled processor samples, 20 shelled fruits per sample were plated. For sample F3 (see Table 3.3), 20 blanched nuts (nutmeat) and seed coats corresponding to 20 nuts were plated.

Fungal isolation followed the method of direct plating on MSA10 without surface disinfection and with medium covering that was previously established and described. Chestnuts and in-shell almonds were plated individually. For shelled almonds, 4 kernels were directly plated in each 9 cm Petri dish containing. Petri dishes were incubated in the dark, at 25-28 ºC, for 7 to 10 days.

All plates were inspected after 3, 5 and 7 days of incubation, using a stereomicroscope (Nikon SMZ-U), to accompany fungal growth. After 7 days of incubation, all fungi belonging to genus *Aspergillus* and some fungi representative of other genera were transferred into 9 cm Petri dishes containing 15 mL of MEA with an inoculation needle previously wet in a sterile solution of 0.1% Tween80. The inoculum was selected with the aid of a stereomicroscope, to avoid contaminations from adjacent colonies. The inoculation was preferentially made by 3 point inoculation but, whenever

considered necessary (i.e., in cases where there were colony juxtapositions), the inoculum was distributed on the Petri dish by a continuous streak. Cultures were incubated at room temperature (approximately 25 ºC) for 7 days and sub-cultured in 6 cm plates by single point inoculation the number of times necessary to obtain pure cultures.

All isolates were attributed a code *yyXZn*, where *yy* means the year of isolation (e.g. *07* for 2007), *X* refers to the commodity (*A* for almond; *C* for chestnut), *Z* refers to the first 3 letters of the genus (*Asp* for *Aspergillus*, *Pen* for *Penicillium*, etc) and, *n* to the isolate number.

3.1.2.3 Conservation of fungal isolates

All isolates were stored at -20 ºC immediately after being isolated as pure cultures. After identification, all isolates considered of interest were also stored at -80 ºC. Conservation procedures were as follows:

Conservation at -20 ºC

Cultures were prepared in 6 cm diameter Petri dishes containing 10 mL of MEA and incubated at 27 ºC until being well sporulated. The purity of the culture was confirmed with the aid of a stereomicroscope. 1.5 mL of 20% glycerol [v/v] were dropped on the colony and the spores were suspended by reflux with a plastic Pasteur pipette. This suspension was transferred to a 2.0 mL criovial and left for 1 hour at room temperature and then at 4 ºC over-night to allow glycerol to diffuse into the cells. The criovials were then stored at -20 ºC.

Conservation at -80 ºC

Cultures were prepared in 6 cm diameter Petri dishes containing 10 mL of MEA and incubated at 27 ºC until being well sporulated. The purity of the culture was confirmed with the aid of a stereomicroscope. 1.5 mL of 20% glycerol [v/v] were dropped on the colony and the spores were suspended by reflux with a plastic Pasteur pipette. This suspension was transferred to a 2.0 mL criovial and left for 1 hour at room temperature to allow glycerol to diffuse into the cells. The criovials were then put in a *CryoFreezer Container* containing isopropanol and stored at -80 ºC. This way, the temperature would decrease at a controlled rate of 1 ºC/min. After 4 hours, the criovials were then removed from the *CryoFreezer Container* and stored at -80 ºC.

Isolates ahead mentioned by the code MUM were further cultured in duplicate on 15 mL tubes containing 3 mL of MEA (slants) for 7 days at 25 ºC and deposited in the fungal culture collection Micoteca da Universidade do Minho (MUM), Braga, Portugal (www.micoteca.deb.uminho.pt), following the internally established procedures.

3.1.2.4 Identification of filamentous fungi from genus *Aspergillus*

All fungi belonging to genus *Aspergillus* were identified morphologically, based on various macro and micro morphological characters.

3.1.2.4.1 Culture conditions

Before identification, isolates were grown on MEA in the dark for 7 days at 25 ºC. From this culture, a loop full of spores was suspended in 500 µL of 0.2% agar with 0.05% Tween 80, and this suspension was used for three-point inoculations on 9 cm diameter Petri dishes containing MEA, Czapek Yeast Autolysate (CYA: Saccharose 30 g/L, Powder Yeast Extract 5 g/L, K_2HPO_4 1 g/L, $NaNO_3$ 2 g/L, KCl 0.5 g/L, $MgSO_4.7H_2O$ 0.5 g/L, $FeSO_4.7H_2O$ 0.01 g/L, $ZnSO_4.7H_2O$ 0.01 g/L, $CuSO_4.5H_2O$ 0.005 g/L, Agar 20 g/L) and CY20S (CYA but with 200 g/L saccharose). Cultures were incubated in the dark at 25 ºC.

Some isolates were also cultured on Czapek-Dox Agar (CZ: the same as CYA, but without the Yeast Extract) at 25 ºC and CYA at 37 ºC (CYA37). Culturing on CZ helps in the differentiation of colony colour for similar species of section *Flavi* and culturing on CYA37 is essential when identifying some species of section *Nigri*. The volume of plated media was kept constant (approximately 20 mL per plate), since media depth or head space differences can lead to morphological changes (Okuda et al., 2000). Also, chemicals used were brand-fixed and of analytical grade, to maintain media consistence (Klich, 2002a). All media were sterilized by autoclaving at 121 ºC for 15 minutes.

3.1.2.4.2 Monosporic culture

Whenever necessary for accurate fungal identification, the purity of the fungal cultures was confirmed by a monosporic culture. From a sporulating culture, a spore suspension was prepared in a 0.05% Tween 80 solution. This suspension was thoroughly homogenised by vortexing for a few minutes and diluted by successive 10-fold dilutions. The diluted suspensions were also vortexed and the spore dispersion and dilution was confirmed on the microscope with the aid of a Neubauer counting chamber. The suspension showing a reduced number of spores per microliter and, most importantly,

fewer spore clusters, was used to inoculate 3 Petri dishes (6 cm diameter) containing a very thin layer (approximately 3 mL) of MEA with 2% agar. These dishes were inoculated with 15 µL of spore suspension on 5 equidistant points. The cultures were incubated at 28 ºC over night, just the time necessary to allow spore germination. After incubation, germination was checked at the stereomicroscope. Four germinated well isolated spores were collected with the aid of an inoculation needle and individually transferred to a new Petri dish with 10 mL of MEA, in order to obtain 4 monosporic cultures. Each of these cultures would garanty genetic purity of the culture, necessary to obtain reliable results in further studies.

3.1.2.4.3 Macroscopic analysis

Cultures from all media were analysed after 7 days of incubation. Analysed characters are listed in the Identification Sheet represented in Figure 3.10. Cultures on CYA were kept for two more weeks to confirm colony colour and, when teleomorphs were identified, to obtain mature ascospores. Whenever necessary, the stereomicroscope was used to aid macroscopic examination. Colony colour and surface texture was compared with those presented in the taxonomic guides. Sclerotia were measured at the stereomicroscope, using the Measuring command of a previously calibrated Nikon Control Unit DS-LI/DS Camera. Photographs were taken with the same system. The mean of 50 sclerotia (when present) was used to score them as > 400 nm (L-type) or ≤ 400 nm (S-type).

3.1.2.4.4 Microscopic analysis

Microscopic examination of spores and reproductive structures was made on actively sporulating material from cultures grown on CYA and MEA. Slides were prepared as wet mounts on distilled water (for pigmented fungi) or cotton blue (for hyaline fungi). Strongly sporulated material was previously washed with a drop of 96% ethanol and gently teased with the needle, to remove excessive conidia. Ethanol was removed with absorbent paper and only then a drop of distilled water or stain was added. This procedure was essential for a good microscopic examination, since we were dealing with *Aspergillus* spp., which have

numerous hydrophobic conidia. Slides were examined with a compound light microscope (Leitz Labourlux 12) under bright field and Nomarski contrast. Stipes, vesicles, conidia and, when present, ascospores and cleistothecia were measured with the system previously mentioned. Stipe, conidia and ascospore surface textures as well as head seriation were observed with 1000x magnitude amplification.

3.1.2.4.5 Section/Species Identification

The various macro and micro morphological characters of each isolate were registered on an Identification Sheet (Figure 3.10) adapted from Klich (2002a). Identification followed the taxonomic keys and guides available for genus *Aspergillus* (Klich, 2002a; Samson et al., 2004a). Some isolates were identified to the species level, while others were identified only to the section or aggregate levels. The taxonomic scheme proposed by Gams et al. (1985) was followed, since it is the one generally followed in the reference guides herein used for identification.

IDENTIFICATION SHEET

Isolate Code: _____ ID: _____

Incubation time: _____

Macromorphology (colony characteristics):

	MEA25	CYA25	CY20S25	CZ25	CYA37
Diameter (7 days)					
Color					
Conidia color					
Mycelium					
Exudate					
Reverse color					
Soluble pigment					
Cleistothecia					
Sclerotia					

Micromorphology:

Stipe: Length _____
Width _____
Surface Texture _____

Vesicle: Diameter _____
Shape _____

Seriation: Uniseriate / Biseriate

Conidia: Length _____
Shape _____
Color _____
Surface Texture _____

Sclerotia: Length _____
Shape _____
Color _____

Cleistothecia Length _____
Shape _____
Color _____
Surface cells: hyphae / parenchyma

Ascospores: Days to maturation _____
Length _____
Width _____
Surface texture _____
Furrows /Flanges _____

Observations:

Figure 3.10 Identification Sheet used in the identification of *Aspergillus* isolates.

3.1.3 Identification of aflatoxigenic fungi

3.1.3.1 Morphological identification

Besides the morphological characterisation described in Section 3.1.2.4 all isolates of *Aspergillus* identified as belonging to section *Flavi* were cultured on Aspergillus flavus and parasiticus Agar (AFPA; Oxoid, Basingstoke, United Kingdom) for 3 to 5 days at 25 ºC, in the dark, to confirm identification at the section level by colony reverse colour.

3.1.3.2 Aflatoxigenic profile

Two approaches were followed to verify AFs production: fluorescence on Coconut Agar Medium (CAM) and HPLC analysis.

3.1.3.2.1 Fluorescence on Coconut Agar Medium

Isolates were tested for AFs production ability by culturing on CAM. This medium was chosen because it is inductive of AFs production (Dyer & McCammon, 1994), and, because of the reaction with coconut fats, producer isolates can be identified by fluorescence in the reverse side of the culture (Lin & Dianese, 1976; Davis et al., 1987).

CAM was prepared as described by Davies et al. (1987): 100 g of shredded coconut were thoroughly mixed with 300 mL of hot water for 5 minutes, filtered through cheese cloth and 20 g/L of agar were added; the medium was autoclaved for 15 minutes at 121 ºC. Strains were inoculated at a central point on a 6 cm diameter Petri dish containing 10 mL of CAM and incubated for 7 days in the dark at 25 ºC. Cultures were observed for fluorescence under long-wave UV light (365 nm) after 3, 5 and 7 days. Isolates were scored by presence/absence of fluorescence and by fluorescence colour.

3.1.3.2.2 HPLC analysis

All isolates were tested for AFs production in AF-inducing Yeast Extract Saccharose medium (YES: Yeast Extract 20 g/L, Saccharose 150 g/L, Agar 15 g/L). Some isolates

(MUM92.01, MUM92.02, 07AAsp05, MUM 10.204 and 08AAsp68) were also tested for AFs production in the non-inducing Yeast Extract Peptone medium (YEP: Yeast Extract 20 g/L, Peptone 200 g/L, Agar 15 g/L). Strains were inoculated on 6 cm diameter Petri dishes and incubated at 25-27 °C for 7 days, in the dark. Then the methodology of Bragulat et al. (2001) was employed: 3 agar plugs were removed from one colony, placed into a 4 mL ambar vial, and 1 mL of methanol was added. After 60 minutes, the extract was filtered by 0.45 μm syringe filters, diluted 20 times in mobile phase and analysed by HPLC.

Chromatographic separations were performed on an HPLC system equipped with: an autosampler (Spark Basic Marathon); a pump (Varian 9002); a reverse phase C18 column (Waters Spherisorb ODS2, 4.6 mm x 250 mm, 5 μm), fitted with a precolumn with the same stationary phase; and a fluorescence detector (Jasco FP-920). On-line photochemical derivatisation was performed using a photochemical post-column derivatisation reactor (PHRED unit - Aura Industries, USA) placed between the separation column and the fluorescence detector, which consisted of a 254-nm low-pressure mercury lamp and a knitted reaction coil fitted around the UV lamp.

The mobile phase consisted of an isocratic programme of water:acetonitrile:methanol (3:1:1, v/v/v) and was pumped at 1.0 mL/min for a total run time of 28 minutes. The injection volume was 100 μL. The fluorescence detection was carried out at excitation and emission wavelengths of 365 nm and 435 nm, respectively. The gain was set to 1000 and the attenuation to 16.

Instrumentation control, data acquisition and processing were computed via chromatographic software Varian 850-MIB Data System Interface. Samples were taken as positive for each of the toxins when yielding a peak at a retention time similar to each standard, with a height five times higher than the baseline noise. A standard solution of AFB_1, AFB_2, AFG_1 and AFG_2 (Biopure, Austria) was used.

3.1.4 Aflatoxins detection in almonds

3.1.4.1 Chemicals and materials

The standard solution of AFB_1, AFB_2, AFG_1 and AFG_2 used (Biopure, Austria) had a total concentration of 5 mg/mL (in acetonitrile) corresponding to a concentration of 2.0 mg/mL each for AFB_1 and AFG_1 and of 0.5 mg/mL each for AFB_2 and AFG_2. From this (standard stock solution), standard working solutions were prepared by 100-fold dilution in methanol, corresponding to 20 ng/mL of AFB_1 and AFG_1, and 5 ng/mL of AFB_2 and AFG_2, and stored in ambar flasks at -20 ºC when not in use.

HPLC grade solvents (methanol and acetonitrile) were used in the preparation of AF standards, in sample extraction, and in the preparation of mobile phase. For extracts purification, AflaTest WB immunoaffinity columns (IACs) were obtained from VICAM (Watertown, MA, USA). Phosphate buffer saline (PBS) 0.1M pH 7.0 was prepared by adding 500 mL of 0.1M NaH_2PO_4 and 500 mL of 0.1M $Na_2HPO_4.12H_2O$ and pH was corrected with NaOH.

3.1.4.2 Safety considerations

Due to the toxicity of AFs, all the necessary safety considerations were taken into account when handling this substance, as recommended by Castegnaro et al. (1980). Solutions were handled with protective gear; all disposable materials were decontaminated by autoclaving before being disposed; reusable materials were decontaminated by immersion in 10% bleach over-night, immersion in 5% acetone for one hour and washed with distilled water several times.

3.1.4.3 In-house method validation

AFs from almond samples were extracted and purified by IACs, and were detected and quantified by HPLC. Before sample analysis, the extraction and quantification methods were submitted to in-house validation. Method validation is based on various

parameters, namely *linearity*, *accuracy* and *precision* (Chan, 2004). These parameters were determined for our method and are herein described.

In consequence of the EU legal limits for AFs in almonds (2 µg/kg of AFB_1 and 4 µg/kg for total AFs, by the time of these analyses; Commission Regulation No 1881/2006), different sets of standard solutions and of spiked samples (one time and three times the legal limits) were used for the validation of the AFs extraction method.

3.1.4.3.1 Linearity

The *linearity* of an analytical procedure can be defined as the ability (within a given range) to obtain test results of variable data which are directly proportional to the concentration of the analyte in the sample. The calibration parameters evaluated for each AF were LOD and LOQ, which reflect the linearity of the equipment. LOD can be defined as the lowest amount of an analyte in a sample which can be detected but not necessarily quantified as an exact value; LOQ corresponds to the analyte concentration which is measurable within a certain level of confidence (Taverniers et al., 2004). Below LOD and LOQ, determination and quantification are possible, but become unreliable as the uncertainty associated with it at these lower levels is higher than the measurement value itself (Taverniers et al., 2004).

The linearity of the method was determined by two series of analyses (on two different days), by injecting four standard solutions of AFB_1 and AFG_1 each at concentrations of 0.2, 0.4, 1.0 and 2.0 ng/mL, and AFB_2 and AFG_2 each at concentrations of 0.05, 0.1, 0.25 and 0.5 ng/mL. These analyses were used to obtain a calibration curve and to determine LOD and LOQ of the method. The data variable used for quantitation of the analyte was the peak area.

3.1.4.3.2 Accuracy (recovery) and precision

The *accuracy*, or *recovery*, of an analytical procedure is the closeness of agreement between the values that are accepted either as conventional true values or an accepted reference value and the value found (Chan, 2004). Accuracy is usually reported as percent

recovery by assay, using the proposed analytical procedure, of known amount of analyte added to the matrix (spiking).

The *precision* of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple samples of the same homogeneous sample under prescribed conditions. Precision is usually investigated at three levels: repeatability, intermediate precision, and reproducibility (Chan, 2004).

Repeatability is a measure of the precision under the same operating conditions over a short interval of time. *Intermediate precision* is defined as the variation within the same laboratory (e.g., day-to-day variation, analyst variation, equipment variation). *Reproducibility* measures the precision between laboratories as in collaborative studies. This parameter is not applied to our study. The standard deviation (SD) and relative standard deviation (RSD) are usually reported for the referred parameters (Chan, 2004).

Spiking

Precision and recovery tests were made by sample fortification (spiking) with known amounts of the 4 AFs. For this matter, 2 kg of blanched ground almond were bought from a local industry. This sample was thoroughly homogenised and stored at -20 ºC until use. Tests were performed on three sets of almond blank samples, six replicates each, tested in two different days (three replicates each day). Two of these sets were spiked with different concentrations of AFs (corresponding to 3 times and 1 time the legal limits allowed for AFB_1) and the third set was left unspiked, to serve as blank.

A working standard solution with a concentration of 2 ng/mL of AFB_1 and AFG_1, and 0.5 ng/mL of AFB_2 and AFG_2 was used for spiking purposes. In the first day, 9 sample units of 25 g were weighted into 250 mL conical flasks. Three were spiked with 7.5 mL of the working solution (corresponding to spiking concentrations of 6 µg/kg of AFB_1 and AFG_1 and 1.5 µg/kg of AFB_2 and AFG_2); three were spiked with 7.5 mL of a three-fold dilution of the working solution (corresponding to spiking concentrations of 2 µg/kg of AFB_1 and AFG_1 and 0.5 µg/kg of AFB_2 and AFG_2), and three other units with 7.5 mL of methanol (blank). These sample units were coded as *AM_Sx_n*, where *AM* sets for matrix; *S* for spiked; *x* corresponds to the spiking concentration of AFB_1; and *n* is the number of the replica. The flasks were involved (but left uncovered) with aluminum foil and left over

night, for the methanol to evaporate, before being submitted to the extraction protocol. This procedure was repeated the following day.

Aflatoxins extraction

The extraction method described by VICAM for this matrix was applied, with some modifications, on sample preparation and extraction.

Five grams of NaCl and 125 mL of methanol:water (60:40) were added to the spiked samples (25 g). The flask was covered and the mixture was stirred in a magnetic plate for 30 min. The extract was then poured into fluted filter paper and 20 mL were collected in a clean vessel. This filtrate was diluted with 20 ml of 0.1M PBS pH 7.0 and further filtered through a glass microfibre filter. The extract was then purified with an AflaTest WB immunoaffinity column (IAC) containing immobilised antibodies against the four AFs. The IAC was adapted to a 10 mL syringe set on a hose clamp. Ten mL of the extract were poured into the syringe and passed through the IAC by gravity, at a rate of about 1-2 drops/sec. As soon as air came through the column, the column was washed twice with 10 mL of purified water, at a rate of about 2 drops/sec. The AFs were then eluted from the affinity column by passing 2.0 mL of HPLC grade methanol through the column at a rate of 1-2 drops/sec and the sample eluate was collected into an amber vial. By eluting the AFs in 2.0 mL of methanol, the concentration of the eluate corresponded to a 2-fold dilution of the level of spiking (e.g., the spiking level of 6 ng of AFB_1 per gram of almond sample corresponded to a concentration of 3 ng/mL of methanolic eluate).

The stability of purified extracts was high enough to allow autosampler overnight injections. No significant change in concentration or purity of AFs within 12 hours of analysis was observed.

Aflatoxins detection and quantification

AF detection and quantification were performed by HPLC as described in Section 3.1.3.2.2.

3.1.4.4 Aflatoxins extraction and quantification in almond samples

3.1.4.4.1 Sample preparation

Samples from the 5 trees (*A1* to *A5*) of each orchard (*P1* to *P3*) collected in 2007 and 2008 were gathered in a single sample, resulting in samples *P1/2007* to *P3/2007*, and *P1/2008* to *P3/2008*. The fruits left over after mycological analysis were shelled by hand with a hammer and blanched by rapid immersion in hot water. Kernels were dried on a drying oven, ground with a coffee mill, homogenised and stored at -20 ºC until use.

3.1.4.4.2 Aflatoxins extraction

AFs were extracted from almond samples following the same procedure as that described for validation tests (cf. Section 3.1.4.3.2).

3.1.4.4.3 Aflatoxins detection and quantification

AF detection and quantification were performed by HPLC as described in Section 3.1.3.2.2.

3.1.5 Data analysis

3.1.5.1 Mycological data

Samples were compared for overall distributions of fungi. Contingency tables were produced with fungal frequencies of infection, which were then compared by two-tailed *Fisher's exact test*. *Fisher's exact test* and *Qui-square test* are the tests most commonly used to compare proportions between independent samples when testing dichotomous variables (Maroco, 2003). In our specific case, Chi-square test could not be used because the premises for its application were not always met (in many cases, more than 20% of the

cross-tabulation cells showed count values of observed frequencies ≤ 5) (Maroco, 2003). In all cases, the mean differences were significant at $P < 0.05$.

For the comparison of means of quantitative variables, samples were first tested for normal distribution by *Shapiro-Wilk test* (for $n < 30$) or *Kolmogorov-Smirnov test* (for $n \geq 30$), and for homogeneity of variances by *Levene's test*. Whenever samples followed these criteria, variances were analysed by *one-way ANOVA*, and Multiple Comparisons between samples pairs were made by *Bonferroni's test* (for $n < 30$) or *Tukey's test* (for $n \geq 30$). For samples failing the premise of homogeneity of variances, *Tamhane's T2 test* was applied. Whenever samples failed the two premises, normality and homogeneity of variances, samples were analysed pairwise by the non-parametric *Mann-Whitney test* (Maroco, 2003; Pestana & Gageiro, 2005). In all cases, the mean differences were significant at $P < 0.05$.

In order to determine the fungal diversity in our samples, the indices *Richness* and *Simpson Diversity Index* (SDI) were calculated based on Zak & Willig (2004). *Richness* corresponds to the number of species identified in each sample. Because in our study identification only reached the section level, this index was adapted to the number of *Aspergillus* sections present in each sample. *SDI* takes into consideration the number of species (sections) present in the sample, as well as the abundance of each species (section). This index was calculated as the reciprocal form of *Simpson's Index* (1/*D*), as follows:

$$SDI = 1 / \sum(P_i/P_n)$$

where:

$\sum(Pi/Pn)$ corresponds to D (Simpson's Index)

P_i is number of individuals of a given section

P_n is the total number of individuals

SDI is preferably used because it is more easily interpreted than D, for two major reasons: *i)* the reciprocal form of Simpson's Index ensure that the index increases with increasing diversity; *ii)* the resulting value can be taken as the number of species (section) which effectively contribute to diversity.

Associations among fungi were tested pairwise by comparing observed values (the number of nuts infected by both fungi) with expected values (the product of the

frequencies of each fungus alone), and by using *Phi coefficient* as measure of nominal association. *Phi* is used to assess association between two dichotomous variables (2 x 2 table). Associations between fungal contamination and nominal non-dichotomous variables (origin, type of collection, stage of processing; 2 x *n* table) were tested by using *Cramer's V* as measure of nominal association (Maroco, 2003). In both tests, association is calculated by first calculating chi-square. Chi-square determines if there is a significant relationship between variables, but it does not measure the level of this association. *Phi coefficient* and *Cramer's V* measures of association are post-tests that give this additional information.

Both *Phi coefficient* and *Cramer's V* vary between 0 and 1. Close to 0 there is little association between variables. Close to 1, it indicates a strong association. Association values were interpreted as follows: very association $0.00 \leq$ Phi (or *Cramer's V*) < 0.30; low $0.30 \leq$ Phi (or *Cramer's V*) < 0.50; moderate $0.50 \leq$ Phi (or *Cramer's V*) < 0.70; high $0.70 \leq$ Phi (or *Cramer's V*) < 0.90; and very high $0.90 \leq$ Phi (or *Cramer's V*) ≤ 1.00 (adapted from low http://www.westgard.com/lesson42.htm, accessed 20.09.2010). The Statistical Package for Social Science SPSS Statistics version 17.0 was used for all statistical analyses.

3.1.5.2 Aflatoxins extraction and quantification

Method validation was carried out taking into account the harmonised guidelines for in-house method validation presented in the Commission Regulation (EC) No 401/2006. Linearity, limits of detection (LOD), limits of quantification (LOQ), accuracy (recovery) and precision were the parameters used to test the performance of the HPLC procedure for AFB_1, AFB_2, AFG_1 and AFG_2 quantification.

LOD and LOQ were calculated according to the following equations (Taverniers et al., 2004): $LOD=3s_a/b$ and $LOQ=10\ s_a/b$, where s_a is the standard deviation of the intercept of the regression line obtained from the calibration curve and b is the slope of the line. The calibration curve used for quantification was calculated by the least-squares method in Excel 2007.

The recovery rates of each AF were calculated for the 6 replicates of the two spiking levels, by the ratio of recovered AF concentration relative to the known spiked

concentration. Precision was calculated in terms of intra-day repeatability (n = 3) and intermediate precision (inter-day within-laboratory reproducibility; 2 different days) for each AF at the two contamination levels in spiked almond samples. Intra-day repeatability (RSD_r) is a measurement of the variation obtained within the replicates tested each day, and is given by the corresponding relative standard deviation. Intermediate precision (RSD_{int}) is a measurement of the variation obtained between days, and is given by the corresponding relative standard deviation.

Definitions for the performance criteria are as follows (Commission Regulation (EC) No 401/2006; Taverniers et al., 2004):

> SD_r = Standard deviation, calculated from results generated under repeatability conditions.
>
> RSD_r (%) = Relative standard deviation, calculated from results generated under repeatability conditions [(SD_r/mean) × 100].
>
> SD_{int} = Standard deviation, calculated from results under intermediate precision conditions.
>
> RSD_{int} (%) = Relative standard deviation calculated from results generated under intermediate precision conditions [(SD_{int} /mean) × 100].

Commission Regulation (EC) No. 401/2006 and the Codex Committee on Contaminants in Foods (CCCF, 2008), based on the equations determined by Thompson (2000) and Horwitz & Albert (2006), issued that the recommended values of experimental RSD_r for each concentration level must be lower than, or equal to, two-thirds of the value derived by Horwitz equation (which determines the value for reproducibility RSD, RSD_R). The theoretical RSD% is calculated on the basis of the analyte concentration, independently of the matrix and analytical method used. The Horwitz equation is as follows:

$$RSD_R = 2^{(1-0.5\log C)}$$

where:

C corresponds to the analyte concentration rate (e.g. 6×10^{-9} for a spiked concentration of 6 µg/kg).

In the absence of reference values for intermediate precision, our experimental data for intermediate precision were compared with the value recommended for reproducibility. In our case, because there are only two values for mean recovery to calculate Intermediate Precision, Mean Deviation (MD) and Relative Mean Deviation (RMD) substitute the commonly used Standard Deviation (SD) and Relative Standard Deviation (RSD).

Peak identification was achieved with the retention times obtained after injection of an AF standard solution under the same conditions. AFs quantification was determined by following a linear equation, as suggested by Miller & Miller (1993):

$$y = bx + a$$

where:
y is the peak area
b is the curve slope
x is AF concentration in the sample
a is the intercept on the y-axis

Since extracted AFs were eluted in 2 mL of methanol instead of 1 mL, the AF concentrations measured from the calibration graph (in ng/mL) were multiplied by 2 (in ng/g).

3.2 Molecular differentiation of aflatoxigenic and non-aflatoxigenic isolates

For the differentiation of aflatoxigenic and non-aflatoxigenic isolates, two molecular approaches were followed. In both cases, two genes involved in the AF production pathway, *aflD* and *aflQ*, were subject of analysis. One approach consisted of detecting the presence of the two genes by PCR amplification of genomic DNA; the other consisted of analysing their expression, under AF inductive conditions, by RT-PCR of total RNA. The nomenclature of genes adopted in this study followed that proposed by Yu et al. (2004b). Gene *aflD* is synonymous to *nor1*, and gene *aflQ* is synonymous to *ordA* and *ord1*.

3.2.1 Detection of aflatoxin biosynthetic pathway genes

3.2.1.1 Materials and Reagents

All reagents, solutions and disposable material were previously autoclaved at 121 ºC for 15 minutes, except for chloroform, isoamil alcohol, isopropanol and ethanol.

3.2.1.2 Selection of genomic DNA extraction protocol

The development of a sensitive extraction protocol for nucleic acids from pure cultures was one important basic demand for the application of PCR in this work. Simple handling and the use of non-toxic reagents were desired for this extraction method. Furthermore, optimisation of the mechanical disruption process for fungal mycelia was additionally necessary. The disruption of fungal cell walls is a requirement and a major challenge during nucleic acid extraction. Problems during the extraction of nucleic acids from filamentous fungi are basically caused by the compact cell wall structures from chitin, cellulose, s-1-3-glucan, chitosan, and mannan.

Usually, grinding with mortar and pestle in liquid nitrogen (N_2) is the most efficient method for disruption of the rigid fungal cell walls of hyphae and conidia. Unfortunately, this handling was not suitable for the present study, since N_2 is not always available in our

laboratory. In the DNA purification steps, the use of phenol/chloroform generally improves the purity of the extracted nucleic acids, but it is toxic and not suitable in routine handling. So, we aimed at implementing a protocol that would be user friendly (fast and non-toxic), economic and non-dependable on irregular supplies, but producing genomic DNA of good quality for PCR purposes. For this matter, we tested two types of biological material (mycelium and conidia) and two protocols for DNA extraction (*Sodium Dodecyl Sulfate* [SDS] protocol and *Cetyl Trimethyl Ammonium Bromide* [CTAB] protocol), using glassbeads for mechanical disruption of cell walls. In total, four protocols were tested: *Mycelium/SDS*, *Mycelium/CTAB*, *Conidia/SDS* and *Conidia/CTAB*. These tests were run on 5 isolates of section *Flavi*.

3.2.1.2.1 Biological material

DNA extraction was tested using two types of biological material: mycelium and conidia. Both types of material have disadvantages for this purpose: conidia are heavily pigmented and have hard walls, which can result on low DNA purity and concentration; on the other hand, young mycelium walls are not as hard, but they can form a mesh around glass beads, turning cell disruption difficult to accomplish. On this matter, the aim was not to obtain exclusively conidia and mycelium, but rather obtain predominantly one or the other. The biological materials tested for DNA extraction were prepared as follows:

Mycelium

Ten mL of Malt Extract Broth (MEB: Malt Extract 20 g/L, Glucose 20 g/L, Peptone 1 g/L) (in a 50 mL Falcon tube) were inoculated with a loop full of spores and incubated for 72 h at 25 ºC, in the dark, with agitation. Mycelium was collected by centrifugation at 14,000 x g for 10 min, washed twice with 10 mL of 0.85% NaCl and centrifuged at 14,000 x g for 10 min. The mycelium was collected and used for DNA extraction.

Conidia

A 6 cm Petri dish containing 10 mL of MEA was inoculated with a small number of spores and incubated for 7 days at 25 ºC in the dark. Conidia were used directly for DNA extraction.

3.2.1.2.2 DNA extraction

The two methods tested for genomic DNA extraction were as follows:

SDS (Adapted from Santos, 2004)

The biological material (mycelium or loop full of spores) was transferred to a 15 mL tube containing 1.5 mL of Lysis Buffer SDS (200 mM Tris-HCl pH 8.5; 250 mM NaCl; 25 mM EDTA; 0.5% [w/v] SDS) and approximately 1 g of sterile 0.4- to 0.6-mm diameter glass beads (Sigma, St. Louis, MO, USA), previously washed with nitric acid. The mixture was vortexed for 5 min at maximum speed. Polysaccharides and proteins were precipitated by adding 750 µL of cold 3 M sodium acetate, pH 5.5. This was gently mixed by inversion, placed at -20 ºC for 10 min and centrifuged twice at 14,000 x g for 10 min (4 ºC). Clean supernatant was then transferred to a new tube and precipitated with one volume of cold isopropanol (-20 ºC). This solution was gently mixed by inversion for a few minutes, incubated at -20 ºC for one hour and centrifuged at 14,000 x g for 10 min (4 ºC). DNA pellet was washed twice with 1.0 mL of cold 70% ethanol, centrifuged at 6,000 x g for 7 min (4 ºC) and air dried. DNA was dissolved in 50 to 100 µL of ultra-pure water, depending on the yield, and stored at -20 ºC.

CTAB (Adapted from http://www.*aspergillus*.org/protocols/, accessed 13.03.2007)

The biological material (mycelium or loop full of spores) was transferred to a 15 mL tube containing 1.5 mL of Lysis Buffer CTAB (100 mM Tris-HCl pH 8.0; 1.4 M NaCl; 20 mM EDTA pH 8.0; 2% CTAB [p/v]; 0.4% polyvinylpyrrolidone (PVP) [p/v]; 0.05% β-mercaptoethanol [v/v]) and 1 g of 0.4- to 0.6-mm-diameter glass beads (Sigma, St. Louis, MO, USA), previously washed with nitric acid, vortexed for 5 min at maximum speed and incubated at 65 ºC for 15 min. Vortexing and incubation were repeated and 1.5 mL of 24:1 chloroform:isoamil alcohol were added. The mixture was thoroughly homogenized by agitation and centrifuged for 10 min at 14,000 x g. 1.2 mL of the aqueous phase were transferred into a tube containing 0.7 mL of isopropanol and 0.1 mL of 7.5 M NH_4OAc. The mixture thoroughly homogenized by agitation and centrifuged for 10 min at 14,000 x g. The supernatant was discarded and the pellet (DNA) was washed with 1.5 mL of cold 70% ethanol (-20 ºC), followed by a centrifugation for 10 min at 14,000 x g. The supernatant was discarded and the pellet was air-dried until all the ethanol had evaporated. DNA was dissolved in 50 to 100 µL of ultra-pure water and stored at -20 ºC.

Quality and concentration of genomic DNAs obtained from the different protocols were determined by horizontal gel electrophoresis and by spectrophotometry. Electrophoretic analysis was done on 1% agarose gels with Tris-Acetate-EDTA buffer (TAE: 40 mM Tris-HCl; 40 mM acetic acid; 1.0 mM EDTA, pH 8.0) stained with GelRed (VWR). Runs were made in TAE buffer, at constant voltage of 5 V/cm for approximately one hour. Five µL of genomic DNA and one µL of Orange Blue Loading Buffer (Promega) were loaded on the gel. DNA was visualised under UV light and images were obtained by the image analysis system Eagle Eye II (Stratagene). For the spectrophotometric analysis,

absorbance readings were made at 230 nm (A230), 260 nm (A260) and 280 nm (A280) in quartz cuvettes. DNA concentration was calculated as follows:

$$[DNA]\ \mu g/mL = A260 \times Dilution\ Factor \times 50$$

DNA purity relative to protein contamination is given by the ratio A_{260}/A_{280}, which should render between 1.8 and 2.1. Lower values denote high protein contamination, and DNA should be further treated with proteinase K for protein digestion. The ratio A_{260}/A_{230} indicates salt and amino acid contamination. If outside the range 1.8-2.1, DNA should be further washed with ethanol. Figures 3.11 and 3.12 represent the results for the spectrophotometric analysis of the genomic DNA obtained by the 4 extraction protocols. Gel electrophoresis results are given in Figure 3.13.

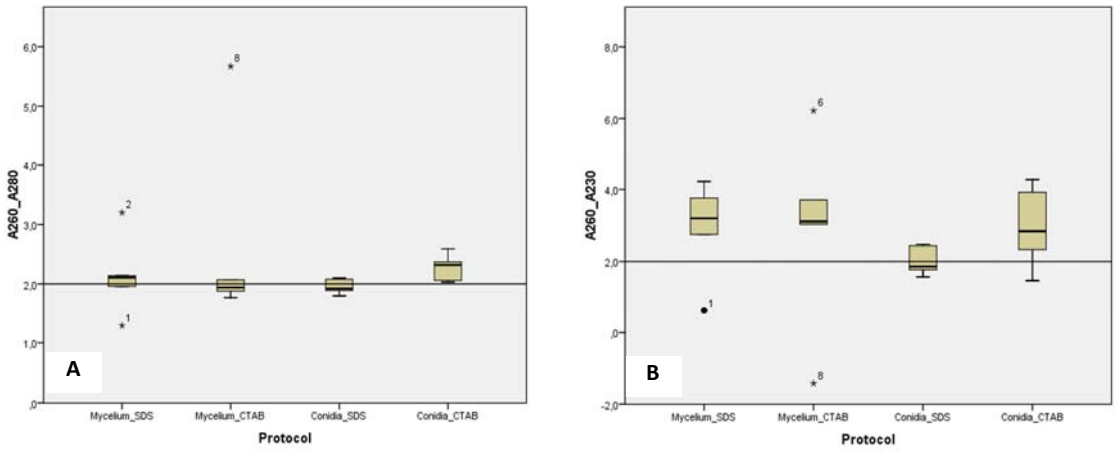

Figure 3.11 Spectrophotometric results of the levels of purity for the genomic DNA obtained by the 4 extraction protocols (n=5): A) A260/A280; B) A260/A230 ratios. Horizontal bar indicates reference value.

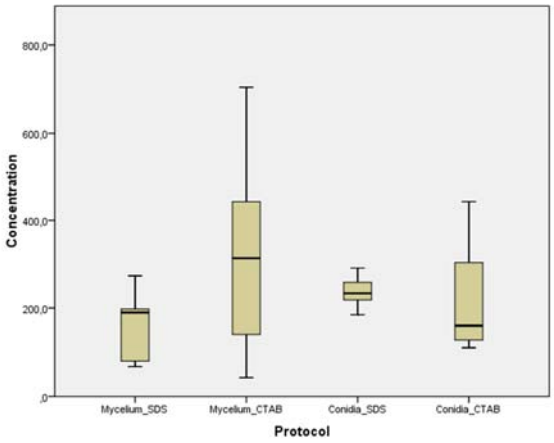

Figure 3.12 DNA concentration obtained by spectrophotometry (n=5). Vertical bars indicate maximum and minimum values.

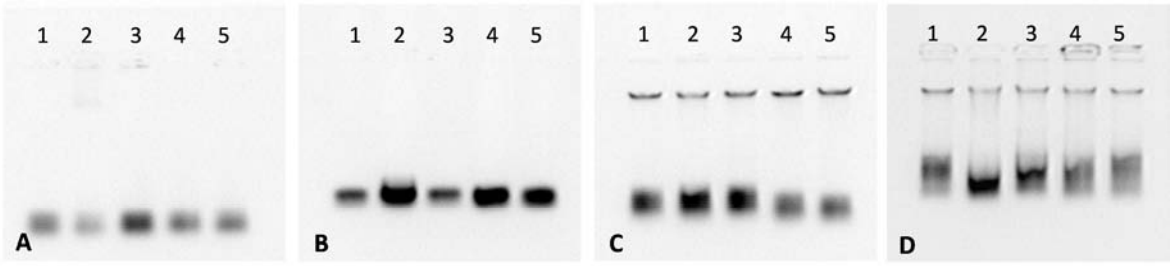

Figure 3.13 Electrophoresis results for the genomic DNAs obtained by the four protocols tested. A: *Mycelium/SDS*; B: *Mycelium/CTAB*; C: *Conidia/SDS*; D: *Conidia/CTAB*. Lanes 1 to 5 in each gel correspond to the 5 isolates tested.

Considering the results of spectrophotometric and electrophoretic analyses, DNAs extracted from mycelium, independently of the extraction protocol, were considered unsuitable for genomic DNA extraction of *Aspergillus* section *Flavi*. These results denote that mycelium is not suitable for mechanical disruption with glass beads. On the other hand, DNAs obtained from protocols using conidia seemed to have good quality and were selected for further testing.

Genomic DNAs obtained by the selected methods *Conidia/SDS* and *Conidia/CTAB* were further tested for purity and concentration by PCR. The universal primers ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990; Gardes & Bruns, 1993), which amplify a 600 bp segment of the ITS1/5.8S/ITS2 region of the rRNA gene, were used for this purpose. PCRs were run on 25 µL reaction mixtures in a BioRad Mycycler thermalcycler. Table 3.4 summarises the PCR mix and programme used.

Table 3.4 PCR conditions used for the amplification of the ITS region.

Reaction Mix (25 µL)	**ITS (ITS1-F/ITS4)**	
GoTaq Flexi Colourless Buffer without $MgCl_2$	1x	
$MgCl_2$	1.5 mM	
GoTaq Flexi DNA Polymerase (Promega, #M8305)	1.25 U	
dNTPs (dNTP Mix, Promega, #U1511)	0.2 mM	
Primer Forward	0.2 µM	
Primer Reverse	0.2 µM	
Genomic DNA	50 ng	
Amplification Programme		
Initial denaturation	94 ºC, 3 min	
Denaturation	94 ºC, 30 sec	35x
Annealing	55 ºC, 30 sec	
Extension	72 ºC, 2 min	
Final extension	72 ºC, 10 min	

PCR products were separated on a 1.5% agarose/TAE gel, stained with GelRed and compared to the DNA size marker 100 bp DNA Ladder (Promega, #G2101). Electrophoretic runs and image acquisition were as previously described.

Figure 3.14 illustrates the results of the ITS region amplification. As can be observed, genomic DNAs obtained by both methods were found to be equally suitable for PCR analysis. Since protocol *conidia/SDS* is more economic and user-friendly (faster and non-toxic), it was elected as the best method and was therefore adopted for further DNA extractions.

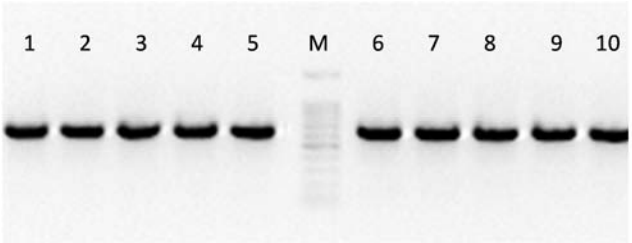

Figure 3.14 Electrophoretic results of the ITS region amplification. Lanes: 1 to 5 – Samples obtained by the protocol *Conidia/SDS*; 6 to 10 – Samples obtained by the protocol *Conidia/CTAB*; M – 100 bp DNA ladder.

3.2.1.3 Detection of *aflD* and *aflQ* genes

3.2.1.3.1 Isolates tested

Thirty-five isolates previously identified and characterised for their aflatoxigenic ability were tested for the presence of *aflD* and *aflQ*. The list of isolates is given in Table 4.27 (Chapter 4).

3.2.1.3.2 Genomic DNA extraction

Genomic DNA for the detection of *aflD* and *aflQ* genes was extracted with the protocol *conidia/SDS*, as described in Section 3.2.1.2.

3.2.1.3.3 Multiplex-PCR amplification

Genes *aflD* and *aflQ* were amplified simultaneously in a single PCR amplification (multiplex-PCR), using the primer pairs nor1-F/nor1-R and ord1-gF/ord1-gR, respectively. *aflD* primers were specifically designed in this study; *aflQ* primers were selected from previous studies. Table 3.5 shows a list of primers and details. Table 3.6 summarises the multiplex-PCR conditions.

The housekeeping gene *tub1* coding for β-tubulin (primer pair tub1-F/tub1-R) was used as internal amplification control. PCR products were visualised by electrophoresis in agarose gel as previously described.

Table 3.5 Details of the target genes, primer sequences and expected product length in base pairs (bp) for PCR and RT-PCR.

Primer pair	Gene	Primer sequence (5´→3')	PCR product size (bp)	RT-PCR product size (bp)	Reference
Tub1-F		GCT TTC TGG CAA ACC ATC TC			Scherm et al., 2005
Tub1-R	*tub1*	GGT CGT TCA TGT TGC TCT CA	1406	1198	Scherm et al., 2005
Nor1-F	*aflD*	ACC GCT ACG CCG GCA CTC TCG GCA C			This study
Nor1-R		GTT GGC CGC CAG CTT CGA CAC TCC G	400	400	This study
Ord1-gF		TTA AGG CAG CGG AAT ACA AG			Sweeney et al., 2000
Ord1-gR		GAC GCC CAA AGC CGA ACA CAA A	719	599	Sweeney et al., 2000
Ord1-cR	*aflQ*	GAATATCTGGACGTTTACCC	----	487	Degola et al., 2007

Table 3.6 PCR conditions used for the multiplex amplification of genes *tub1*, *aflD* and *aflQ*.

Reaction Mix		
GoTaq Flexi Colourless Buffer without $MgCl_2$	1x	
$MgCl_2$	1.5 mM	
GoTaq Flexi DNA Polymerase (Promega, #M8305)	1.25 U	
dNTPs (dNTP Mix, Promega, #U1511)	0.2 mM	
Each primer Forward	0.2 µM	
Each primer Reverse	0.2 µM	
Genomic DNA	25 ng	
Amplification Programme		
Initial denaturation	94 ºC, 3 min	
Denaturation	94 ºC, 1 min	30x
Annealing	55 ºC, 1 min	
Extension	72 ºC, 1 min	
Final extensión	72 ºC, 10 min	

3.2.2 Analysis of aflatoxin gene expression

3.2.2.1 Materials and reagents

All non-plastic materials used for RNA extraction (spatulae, filter paper, mortar and pestle) were sterilised in a sterilisation oven at 160 ºC, over-night, and refrigerated (-20 ºC) before use. Plastic material (eppendorf tubes, PCR tubes) was sterilised by autoclaving at 121 ºC for 1 hour. RNase-free filter pipette tips were used. Water was treated with 0.1% diethyl pyrocarbonate (DEPC), left over night and autoclaved at 121 ºC for 1 hour to eliminate DEPC. All the solutions were prepared with DEPC-treated water. The electrophoresis equipment (tank, trays and combs) was used exclusively for RNA analysis, and was regularly washed with 10% SDS and DEPC-treated water.

3.2.2.2 Biological material preparation

Biological material for total RNA extraction was prepared by growing the isolates under both AF inducive and non-inducive conditions. Twenty five mL of YES (AF inducive) and YEP (non-inducive) broths (in a 50 mL Falcon tubes) were inoculated with a loop full of spores. This culture was incubated horizontally for 4 days at 25-28 ºC, in the dark, with slight agitation. The mycelia were collected with a sterile spatula, dried in sterile

absorbent paper and rapidly divided into aliquots of 100 mg. Mycelia were immediately used for RNA extraction. All RNA extractions were performed on freshly produced material.

3.2.2.3 Selection of total RNA extraction protocol

As for DNA extraction, we aimed at establishing a protocol for total RNA extraction that would be economic, fast, user-friendly and not dependable on irregular supplies (namely liquid nitrogen). For that matter, we tested 3 protocols of biological material maceration and 2 protocols of total RNA extraction.

3.2.2.3.1 Maceration of biological material

The maceration of the biological material used for RNA extraction is probably the most important step of the procedure when it comes to keeping RNA integrity. Maceration with liquid N_2 is the most feasible method for fungal material, but requires extra cares and skills to avoid RNA contamination and degradation, as is dependent on regular supply. In this study, we attempted to implement a maceration protocol that would substitute this procedure. The protocols tested, maceration with N_2, glass beads and the mechanical homogeniser *TissueRuptor* (Qiagen), are herein described.

Liquid Nitrogen

One hundred mg of mycelium were ground to a fine powder with N_2 in a cold mortar and pestle. The powder was transferred with a residual amount of N_2 into a 2.0 mL eppendorf tube previously refrigerated in N_2. Immediately after the N_2 had completely evaporated, the extraction buffer (dependent on the extraction protocol) was added. The mixture was homogenised by inversion and used for RNA extraction.

Glass beads

One hundred mg of mycelium were placed in a 2.0 mL eppendorf tube containing the extraction buffer and 0.5 g of glass beads (previously washed with nitric acid and DEPC-treated water). The mixture was vortexed for 5 min and directly used for RNA extraction.

TissueRuptor

One hundred mg of mycelium were placed in a 2.0 mL eppendorf tube containing the extraction buffer. The mixture was homogenised for 5 min with the TissueRuptor and directly used for RNA extraction.

3.2.2.3.2 Total RNA Extraction

After mycelium maceration, two RNA extraction protocols were tested. The *RNeasy Plant MiniKit* (Qiagen) is one of the most cited methods of RNA extraction, and gives good results for filamentous fungi. The disadvantage is that it is very expensive, and therefore not suitable for a large number of samples. So, we tried to establish an alternative protocol with the *TRIzol* reagent (Invirogen). The procedures followed the protocols described by the manufacturers, with minor adaptations.

RNeasy Plant Mini Kit (Qiagen)

Mycelium was homogenised in 450 µL of RLT or RLC buffer containing 10 µL/mL of β-mercaptoethanol as described ahead and vortexed vigorously. The lysate was transferred to a QIAshredder spin column (lilac) placed in a 2 ml collection tube, and centrifuged at 12000 g for 2 min. The supernatant of the flow-through was carefully transferred to a new microcentrifuge tube without disturbing the cell-debris pellet in the collection tube. 0.5 volumes of 96% ethanol were added to the cleared lysate, and mixed immediately by pipetting. The sample was transferred to an RNeasy spin column (pink) placed in a 2 mL collection tube and centrifuged for 15 sec at 8,000 x g. The flow-through was discarded and 700 µL of Buffer RW1 were added to the RNeasy spin column. The lid was gently closed and the sample was centrifuged for 15 sec at 8,000 x g. The flow-through was discarded and 80 µL of DNase I mix (Qiagen) were placed directly on the membrane. The sample was incubated for 15 min at room temperature and 350 µL of Buffer RW1 were added. The lid was gently closed and the sample was centrifuged for 15 sec at 10,000 x g. The flow-through was discarded and 500 µL of Buffer RPE were added to the RNeasy spin column to wash the column membrane. The sample was centrifuged for 15 sec at 8,000 x g and the flow-through was discarded. The washing procedure was repeated. The sample was centrifuged for 1 min at 12,000 x g. The RNeasy spin column was placed in a new 1.5 mL collection tube and 40 µL of RNase-free water was added directly to the spin column membrane. The RNA was eluted by centrifugation for 1 min at 8000 g, and stored at -80 ºC.

TRIzol (Invitrogen)

Mycelium was homogenised in 1 mL of TRIzol as described ahead and then incubated for 5 min at room temperature. 200 µL of chloroform were added and the tube was securely capped. The tube was vigorously shaken by hand for 15 sec and incubated at 15 to 30 °C for 2 to 3 min. Samples were centrifuged at 12,000 x g for 15 min at 4 °C. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. The aqueous phase was transferred into a fresh tube and 500 µL of isopropanol were added to precipitate the RNA. Samples were incubated for 10 min at room temperature and centrifuged at 12,000 x g for 10 min at 4 °C. Supernantant was removed and the pellet (RNA) was washed once with 1 mL of cold 75% ethanol. The sample was mixed by vortexing and centrifuged at 7500 g for 5 min at 4 °C. The RNA pellet was air-dried for 5 to 10 min, redissolved in 40 µL of RNase-free water and stored at -80 °C.

Total RNA samples were visualised by horizontal electrophoresis in 1.5% agarose gels, under the conditions described for DNA analysis. Considering the electrophoresis

results shown in Figure 3.15, we rejected maceration with glass beads and extraction with TRIzol. The next step was to determine the suitability of the samples for reverse-transcription PCR (RT-PCR), in terms of RNA quality and DNA contamination. RT-PCR was tested on samples obtained by N_2 and TissueRuptor maceration and extraction with RNeasy Plant MiniKit.

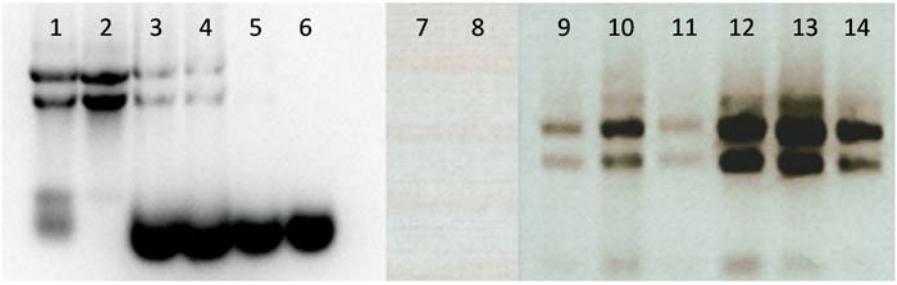

Figure 3.15 Electrophoresis of total RNA samples obtained by different protocols. 1 - N_2, RNeasy Plant Mini Kit (RLT buffer); 2 - N_2, RNeasy Plant Mini Kit (RLC buffer); 3 and 4 - N_2, Trizol; 5 and 6 - Glass Beads, Trizol; 7 and 8 - Glass Beads, RNeasy Plant Mini Kit (RLC buffer); 9 to 11 - TissueRuptor, RNeasy Plant Mini Kit (RLC buffer); 12 to 14 - N_2, RNeasy Plant Mini Kit (RLC buffer).

RT-PCR was performed in 20 µL reaction One-Step RT-PCR Pre-Mix kit (INTRON Biotechnology, Gyeonggi-do, South-Korea). RT-PCR details are described in Table 3.7. The PCR amplification parameters followed those reported by Degola et al. (2007).

Table 3.7 RT-PCR conditions used for the multiplex amplification of genes *tub1*, *aflD* and *aflQ*.

Reaction Mix (20 µL)		
One-Step RT-PCR Pre-Mix	8 µL	
Each primer Forward	0.2 µM	
Each primer Reverse	0.2 µM	
Total RNA	1 µg	
Amplification Programme		
Reverse Transcription	45 ºC, 30 min	
Initial denaturation	94 ºC, 4 min	
Denaturation	94 ºC, 1 min	5x
Annealing	60 ºC, 1 min	
Extension	72 ºC, 1 min	
Denaturation	94 ºC, 1 min	30x
Annealing	55 ºC, 1 min	
Extension	72 ºC, 1 min	
Final extension	72 ºC, 6 min	

To check for the presence of genomic DNA contamination in the RNA samples, PCR was carried out as described for the amplification step of RT-PCR, using the same sets of primers and 1 µg of total RNA as template. The primers used for gene expression detection included those previously mentioned (see Table 3.5). The housekeeping gene *tub1* was used as IAC in both PCR and RT-PCR amplifications. The amplification results are shown in Figure 3.16.

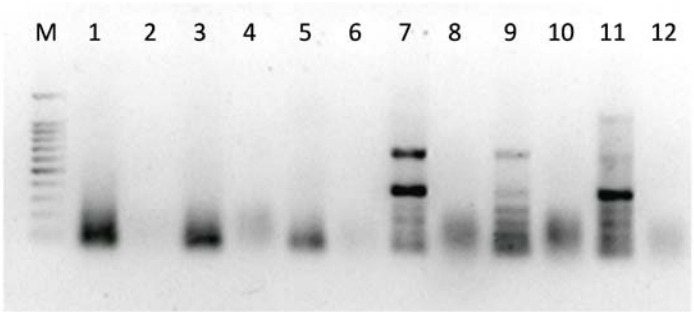

Figure 3.16 RT-PCR and PCR amplifications of total RNA for genes *tub1*, *aflD* and *aflQ*. M - 100 bp DNA ladder (Promega); 1, 3 and 5 – RT-PCR for TissueRuptor macerated samples; 2, 4 and 6 – PCR for TissueRuptor macerated samples; 7, 9 and 11 - RT-PCR for N_2 macerated samples; 8, 10 and 12 - PCR for N_2 macerated samples.

As can be observed in Figure 3.16, RNA obtained with TissueRuptor maceration was not suitable for RT-PCR under the tested conditions. On the other hand, N_2 maceration produced total RNA with quality and concentration adequate to RT-PCR, and no genomic DNA contamination was detected. Given these results, we found that, for RNA extraction, we could not eliminate the use of N_2 for biological material maceration. Furthermore, the TRIzol extraction protocol was found to be inadequate for our samples. Consequently, RNA samples used in subsequent analysis were obtained by N_2 mycelium maceration followed by extraction with the Qiagen RNeasy Plant MiniKit.

3.2.2.4 Detection of *aflD* and *aflQ* gene expression

3.2.2.4.1 Isolates tested

All isolates were produced under AF inductive conditions (YES broth). In order to confirm that the AF genes were not expressed under non-inductive conditions, 5 of them were also grown on YEP broth. Even though all these isolates had already been characterised for their aflatoxigenic ability, after mycelium collection both YEP and YES broths were analysed by HPLC, to confirm the correlation between gene expression and AF production. This test is important because AF production is extremely dependent on growth conditions, so it was important to determine aflatoxigenic ability under the current test conditions.

3.2.2.4.2 Total RNA extraction

Total RNA was extracted with the protocol described in Section 3.2.2.3.

3.2.2.4.3 Reverse-Transcription PCR (RT-PCR)

RT-PCR was performed as described in Section 3.2.2.3.2. (Table 3.7). RT-PCR products were visualised by electrophoresis in agarose gels as previously described.

3.3 *Aspergillus* section *Flavi*

3.3.1 Identification and characterisation of strains from *Aspergillus* section *Flavi*

After general morphological identification, isolates identified as belonging to section *Flavi* were subject of further analysis. This Section of the thesis describes the various methods - phenotypic, genetic and spectral – that were applied to the isolates for taxonomic purposes.

Classic phenotypic analysis included several macro- and micromorphological features and the extrolite profile relative to AFs and CPA. A group of selected isolates was identified based on DNA sequence analysis of two genomic DNA regions – the ITS region and the calmodulin gene. Spectral analysis was based on protein mass spectra by MALDI-TOF ICMS.

3.3.1.1 Phenotypic analysis

3.3.1.1.1 Morphological characterisation

Besides the morphological characterisation described in Section 3.1.2.4 and Section 3.1.3, isolates were also cultured on CZ at 42 ºC, and colony diameter was measured after 7 days of incubation (Kurtzman et al., 1987).

3.3.1.1.2 Mycotoxigenic profile

Production and analysis of aflatoxins

All isolates were tested for aflatoxigenic ability by fluorescence on CAM and HPLC analysis, as described in Section 3.1.3.2.

Production and analysis of cyclopiazonic acid

The strains were tested for cyclopiazonic acid in CYA. All strains were inoculated on 6 cm diameter Petri dishes and incubated at 25 °C for 14 days, in the dark (Gqaleni et al., 1997). Then the methodology of Bragulat et al. (2001) was employed, as already described for AF analysis (Section 3.1.3.2.2).

Samples were analysed using a HPLC equiped with the same autosampler and pump previously mentioned, and with a Varian 2050 UV detector set to 285 nm. Chromatographic separations were performed on a EuroSpher 100 NH_2 column (Knauer, 4.6 mm x 250 mm, 5 μm), fitted with a precolumn with the same stationary phase. The mobile phase consisted of an isocratic program of acetonitrile:50 mM ammonium acetate (3:1, v/v), pH 5, and was pumped at 1.0 mL/min. The injection volume was 100 μL.

Cyclopiazonic acid standard was supplied by Sigma (St. Louis, MO, USA). Samples were taken as positive when yielding a peak at a retention time similar to the CPA standard, with a height five times higher than the baseline noise.

3.3.1.1.3 Data analysis

Twenty-four isolates spanning the various phenotypes were selected for phenotypic cluster analysis. From these isolates, four of them were used as laboratory reference strains (MUM 92.01 and MUM 10.220 for *A. flavus*, MUM 92.02 for *A. parasiticus* and MUM 09.03 for *A. tamarii*). The remaining 20 isolates were field isolates obtained in this study and were divided in two groups: one group of eight isolates with consistent phenotypic characterisation that were easily assigned to one of the previously mentioned species (MUM 10.200, MUM 10.202, MUM 10.207, MUM 10.209 and MUM 10.218 identified as *A. flavus*; MUM 10.201 and MUM 10.216 identified as *A. parasiticus*; and MUM 10.217 identified as *A. tamarii*), and another group of 12 isolates with problematic phenotypic characterisation that could not be assigned to any of the main species (MUM 10.203, MUM 10.204 MUM 10.205, MUM 10.206, MUM 10.208, MUM 10.210, MUM 10.211, MUM 10.212, MUM 10.213, MUM 10.214, MUM 10.215 and MUM 10.219).

A cluster analysis was made to create homogeneous clusters of strains based on 8 phenotypic characters: colony colour on CYA; conidia surface; reverse on AFPA; AFBs production; AFGs production; CPA production; chromatographic pattern of AFs; and

chromatographic pattern of CPA. Twenty-four isolates spanning the various phenotypes were selected for phenotypic cluster analysis. From these isolates, four of them were used as laboratory reference strains (MUM 92.01 and MUM 10.220 for *A. flavus*, MUM 92.02 for *A. parasiticus* and MUM 09.03 for *A. tamarii*). The remaining 20 isolates were field isolates obtained in this study and were divided in two groups: one group of eight isolates with consistent phenotypic characterisation that were easily assigned to one of the previously mentioned species (MUM 10.200, MUM 10.202, MUM 10.207, MUM 10.209 and MUM 10.218 identified as *A. flavus*; MUM 10.201 and MUM 10.216 identified as *A. parasiticus*; and MUM 10.217 identified as *A. tamarii*), and another group of 12 isolates with problematic phenotypic characterisation that could not be assigned to any of the main species (MUM 10.203, MUM 10.204 MUM 10.205, MUM 10.206, MUM 10.208, MUM 10.210, MUM 10.211, MUM 10.212, MUM 10.213, MUM 10.214, MUM 10.215 and MUM 10.219).

The cluster analysis and the dendrogram of relatedness were performed with the statistical package JMP 8.0.2 (SAS Institute Inc). The Complete Linkage method with Euclidean distance was used as the distance index, after variable normalisation as *z*-scores, to yield equal metrics and equal weighting.

3.3.1.2 Genetic analysis

3.3.1.2.1 Isolates tested

The isolates submitted to genetic analysis were the same as those used for phenotypic cluster analysis (Section 3.3.1.1.3).

3.3.1.2.2 DNA extraction

DNA extraction for sequence analysis followed the protocol *Conidia/SDS* previously described (Section 3.2.1.2).

3.3.1.2.3 PCR amplification

Genotypic analysis was done for two DNA segments generally used for taxonomic purposes of fungi: a portion of the rRNA gene (spanning part of the 18S ribosomal RNA gene, the internal transcribed spacer 1, the 5.8S ribosomal RNA gene, the internal transcribed spacer 2, and part of the 28S ribosomal RNA gene), and a portion of the calmodulin gene (*cmd*; comprising part of exon 2, exons 3 to 5, part of exon 6 and introns 2 to 5). Primer pairs used for this analysis were V9D-LS266 and CL1-CL2A, respectively. Primer details are listed in Table 3.8. PCR amplifications were run on 50 µL reaction mixtures in a thermal cycler BioRad Mycycler. PCR amplification details are presented in Table 3.9.

Table 3.8 Primers used in this study, target gene, sequence and expected PCR product size.

Primers	**Gene**	**Primer sequence (5´→3')**	**PCR product size (bp)**	**Reference**
V9D	ITS	TTAAGTCCCTGCCCTTTGTA	950	Gerrits van den Ende
LS266	region	GTAGTCATATGCTTGTCTC		& de Hoog, 1999
CL1	*cmd*	GARTWCAAGGAGGCCTTCTC	730	O'Donnell et al.,
CL2A		TTTTGCATCATGAGTTGGAC		2000

Table 3.9 PCR conditions used for the amplification of the ITS region and partial calmodulin gene.

Reaction Mix (50 µL)	**ITS**		**Calmodulin**	
GoTaq Flexi Colourless Buffer without $MgCl_2$	1x		1x	
$MgCl_2$	1.5 mM		1.5 mM	
GoTaq Flexi DNA Polymerase (Promega, #M8305)	1.25 U		1.25 U	
dNTPs (dNTP Mix, Promega, #U1511)	0.2 mM		0.2 mM	
Primer Forward	0.4 µM		0.4 µM	
Primer Reverse	0.4 µM		0.4 µM	
Genomic DNA	50 ng		100 ng	
Amplification Programme				
Initial denaturation	95 ºC, 5 min		95 ºC, 10 min	
Denaturation	95 ºC, 30 sec		95 ºC, 50 sec	
Annealing	62 ºC, 1 min	30x	55 ºC, 50 sec	35x
Extension	72 ºC, 2 min		72 ºC, 1 min	
Final extension	72 ºC, 5 min		72 ºC, 7 min	
Reference	Gerrits van den Ende & de Hoog, 1999		O'Donnell et al., 2000	

PCR products were visualised as previously described. Concentration was compared to that of the nearest band of the 100 bp DNA ladder. When PCR product concentration was lower than 10 ng/µL, the PCR reaction was repeated, in order to obtain sufficient amount for sequencing.

3.3.1.2.4 PCR product purification

Before sequencing, the PCR products previously obtained were purified from excessive dNTPs and primers with the commercial kit PCR Product Purification Genomed JetQuick, according to the instructions of the manufacturer. The protocol is herein described. After the purification step, purified PCR product concentration was confirmed by electrophoresis as previously described and sent for sequencing.

Protocol for PCR Product Purification (Genomed JETQUICK)

Four hundred µL of H1 solution were added to the PCR product and thoroughly mixed. This mixture was loaded on a JETQUICK spin column placed in a 2 mL receiver tube and centrifuged at 12,000 x g for 1 min. The flowthrough was discarded. The column was again loaded with 500 µL of solution H2 and centrifuged at 12,000 x g for 1 min. The flowthrough was discarded and centrifugation was repeated. The JETQUICK spin column was transferred to a 1.5 mL eppendorf, loaded with 50 µL of sterile ultra-pure water and centrifuged at 12,000 x g for 2 min. The column was discarded and the collected DNA was stored at -20 ºC.

3.3.1.2.5 DNA sequencing

Sequence analyses were performed on an ABI 3730xl DNA Analyser (Applied Biosystems), by outsourcing. PCR products were sequenced in both directions.

3.3.1.2.6 Sequence analysis

Sequence analysis was done on the 24 sequences obtained in the present study plus 22 sequences retrieved from GenBank. The GenBank sequences corresponded to the reference strains for the most important species currently identified in section *Flavi*. All strains and the corresponding accession numbers are listed in Table 3.10.

Table 3.10 *Aspergillus* section *Flavi* included in this study (^{T} designates a culture ex-type).

Strain number	Source	GenBank accession number Calmodulin	GenBank accession number ITS	Species name
MUM 10.238^T (CBS 117610)	*Arachis glabrata* leaf; Argentina	EF202049		*A. arachidicola*
CBS 117187^T	Frass in a silkworm rearing house; Japan	EF202029		*A. bombycis*
CBS 763.97^T	Soil; USA	EF202035		*A. caelatus*
MUM 10.234^T (CBS 110.55)	Air contaminant; Brazil	EF202056		*A. fasciculatus*
CBS 484.65^T	Air contaminant; Brazil	EF202032		*A. flavofurcatus*
MUM 10.237^T (CBS 100927)	Cellophane; South pacific Islands	EF202063		*A. flavus*
CBS 485.65^T	Butter, Japan	EF202053		*A. flavus* var. *columnaris*
MUM 10.236^T (CBS 542.69)	Stratigraphic core sample; Japan	EF202069		*A. kambarensis*
MUM 10.239^T (CBS 151.66)	Dung of *Lepus townsendii*; USA	EF202078		*A. leporis*
MUM 10.240^T (CBS 117635)	*Arachis hypogea* seed; Argentina	EF202072		*A. minisclerotigenes*
MUM 09.02^T (NRRL 13137)	Wheat; USA	EF202028		*A. nomius*
MUM 10.242^T (CBS 100925)	Unknown source; Japan	EF202055		*A. oryzae*
CBS 100926^T	*Pseudococcus calceolariae*, sugar cane mealy bug; Hawaii, USA	EF202043		*A. parasiticus*
CBS 260.67^T	Unknown source; Japan	EF202042		*A. parasiticus* var. *globosus*
MUM 10.235^T (CBS 121.62)	*Arachis hypogea*; Nigeria	EF202077		*A. parvisclerotigenus*
CBS 766.97^T		EF202030		*A. pseudotamarii*
MUM 10.241^T (CBS 100928)	Soy sauce; Japan	EF202041		*A. sojae*
CBS 501.65^T	Cotton lintafelt; UK	EF202064		*A. subolivaceus*
CBS 104.13^T	Activated carbon;	EF202034		*A. tamarii*
CBS 580.65^T	Soil; USA	EF202047		*A. terricola* var. *americanus*
CBS 120.51^T	Culture contaminant; UK	EF202070		*A. thomii*
CBS 822.72^T	*Arachis hypogea*; Uganda	EF202046		*A. toxicarius*
MUM 92.01 (NRRL 6412)		HQ340098	HQ340109	*A. flavus*
MUM 92.02 (NRRL 3386)		HQ340099	HQ340110	*A. parasiticus*
MUM 09.03 (NRRL 427)		HQ340100	HQ340111	*A. tamarii*
MUM 10.200 (07AAsp37)	*Prunus dulcis* nut; Portugal	HQ340078	HQ340101	*A. flavus*
MUM 10.201 (07AAsp43)	*Prunus dulcis* nut; Portugal	HQ340079	HQ340102	*A. parasiticus*
MUM 10.202 (08AAsp35)	*Prunus dulcis* nut; Portugal	HQ340080	HQ340103	*A. flavus*
MUM 10.203 (08AAsp37)	*Prunus dulcis* nut; Portugal	HQ340081		*A. flavus*
MUM 10.204 (08AAsp42)	*Prunus dulcis* nut; Portugal	HQ340082	HQ340104	*A. flavus*
MUM 10.205 (08AAsp67)	*Prunus dulcis* nut; Portugal	HQ340083	HQ340105	*Aspergillus* sp.
MUM 10.206 (08AAsp116)	*Prunus dulcis* nut; Portugal	HQ340084	HQ340106	*A. flavus*
MUM 10.207 (08AAsp179)	*Prunus dulcis* nut; Portugal	HQ340085		*A. flavus*
MUM 10.208 (08AAsp183)	*Prunus dulcis* nut; Portugal	HQ340086		*Aspergillus* sp.
MUM 10.209 (08AAsp225)	*Prunus dulcis* nut; Portugal	HQ340087	HQ340107	*A. flavus*
MUM 10.210 (08AAsp252)	*Prunus dulcis* nut; Portugal	HQ340088		*A. parasiticus*
MUM 10.211 (09AAsp146)	*Prunus dulcis* nut; Portugal	HQ340089		*Aspergillus* sp.
MUM 10.212 (09AAsp187)	*Prunus dulcis* nut; Portugal	HQ340090		*A. parasiticus*
MUM 10.213 (09AAsp240)	*Prunus dulcis* nut; Portugal	HQ340091		*A. parasiticus*
MUM 10.214 (09AAsp260)	*Prunus dulcis* nut; Portugal	HQ340092		*Aspergillus* sp.
MUM 10.215 (09AAsp266)	*Prunus dulcis* nut; Portugal	HQ340093		*A. parasiticus*
MUM 10.216 (09AAsp304)	*Prunus dulcis* nut; Portugal	HQ340094		*A. parasiticus*
MUM 10.217 (09AAsp392)	*Prunus dulcis* nut; Portugal	HQ340095		*A. tamarii*
MUM 10.218 (09AAsp478)	*Prunus dulcis* nut; Portugal	HQ340096		*A. flavus*
MUM 10.219 (09AAsp494)	*Prunus dulcis* nut; Portugal	HQ340097		*Aspergillus* sp.
MUM 10.220 (05UasBr01)	Grapes; Brazil	HQ340077	HQ340108	*A. flavus*

3.3.1.2.7 Sequence editing and alignment

For the sequences obtained in this study, a consensus sequence was created from the assembly of the forward and backward sequences using the package Sequencher 4.9 (Gene Codes, Ann Arbor Michigan). The consensus sequences were manually adjusted by chromatogram comparison.

Sequence alignments were made with CLUSTAL W (Thompson et al., 1994). Distance matrices were produced and analysed with the package MEGA version 4 (Tamura et al., 2007).

3.3.1.2.8 Phylogenetic analysis

The phylogenetic analysis was used for taxonomic purposes. Our data were analysed by four different methods of phylogenetic inference: *Distances* (*Neighbour-Joining*, NJ), *Maximum Parsimony* (MP), *Maximum Likelihood* (ML) and *Bayesian Inference* (BI). Each of these methods is herein briefly explained, based on Hall (2008). NJ is a distance method. It converts aligned sequences into a distance matrix of pairwise differences (distances) between the sequences, and uses the matrix as the data for phylogenetic tree construction. MP, ML and BI are character-based methods, and use multiple alignment directly by comparing characters within each column (site) in the alignment. MP looks for the tree or trees with the minimum number of changes. It can happen that there are several trees only slightly different with the same number of changes, and that are therefore equally parsimonious. ML looks for the tree that, under some model of evolution, maximises the likelihood of observing the data. It almost always recovers a single tree. One advantage of ML is that the likelihood of the resulting tree is known. The confidence in the structure of the trees obtained with these three methods needs to be assessed by bootstrapping for a number of replicates. BI is a variant of ML. Instead of seeking the tree that maximises the likelihood of observing the data, it seeks those trees with the greatest likelihoods given the data. Instead of producing a single tree, Bayesian analysis produces a set of trees with roughly equal likelihoods. The frequency of a given clade in any set of trees is virtually identical to the probability of that clade. In this case, no bootstrapping is necessary.

Trees are generally rooted by using an outgroup representative of a different species or section. For our study, *A. leporis* was used as outgroup. This species belongs to the same section as test strains, but is somewhat distant from the other species of the section, so we considered that it could be successfully used as outgroup on phylogenetic analysis of our isolates, which are very closely related. Monophyly was imposed for all taxa. Phylogenetic trees were edited using the program TreeView (Page, 1996).

Nucleotide Substitution Model

For NJ, ML and BI analysis, the sequence alignments representing raw data need to be corrected by a Nucleotide Substitution (Evolutionary) Model. The appropriate evolutionary model is dependent on the type of data under analysis, and has to be determined by specific software. The optimal evolutionary correction model was found with the jModeltest 0.1.1 package (Posada, 2008), using the Akaike Information Criterion (AIC) (Akaike, 1973). The GTR+G (general time-reversible; Tavaré, 1986) model with gamma-distribution was selected to correct raw data.

Neighbour-Joining

Corrected distance matrices were used to construct the NJ tree using MEGA 4. Gaps were treated as 5^{th} character and all sites were included. To determine the support of each clade, a bootstrap analysis was performed with 1000 replications.

Maximum Parsimony

The MP analysis was done with PAUP* (Swofford, 2003), using raw data. All sites were included in the analysis, and were unordered and of equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option with 100 random taxa additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all equally parsimonious trees were saved. A consensus tree was generated. The robustness of the trees obtained was evaluated by 1000 bootstrap replications.

Maximum Likelihood

ML trees were created with PAUP* using corrected data. Gaps were treated as 5th character and all sites were included. To determine the support of each clade, a bootstrap analysis was performed with 1000 replications.

Bayesian Inference

The phylogenetic inference using a Bayesian approach was tested with the program MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2003). Commands obtained with jModeltest for distance correction were adapted to MrBayes. The Monte Carlo Markov Chain (MCMC) analysis was run for a number of generations enough to reach convergence (standard deviation of split frequencies < 0.01), usually 5 x 10^5. Every 100 generations, a tree was sampled, and the first 25% trees were discarded as burn-in. Branches whose support was < 50% were collapsed into a polytomy (Cut-off Value for Consensus Tree = 50%). The consensus tree with the posterior probability of each internal node was calculated from 75% of the obtained trees.

3.3.1.3 Spectral analysis by MALDI-TOF ICMS

3.3.1.3.1 Isolates tested

One-hundred and nineteen isolates were included in this analysis. Ten of these isolates correspond to type-strains or reference strains for the most significant species: *A. flavus* (MUM 92.01 and MUM 00.06), *A. parasiticus* (MUM 92.02), *A. tamarii* (MUM 09.03 and MUM 09.04), *A. arachidicola* (MUM 10.238), *A. minisclerotigenes* (MUM 10.240), *A. oryzae* (MUM 10.242), *A. parvisclerotigenus* (MUM 10.235) and *A. sojae* (MUM 10.242). The remaining isolates were field isolates selected on a way that they would be representative of all phenotypes identified, with major incidence on those phenotypes that could not be assigned to any known species. From each phenotype, typical and atypical isolates were submitted to analysis. Atypical isolates were those bearing one or more features found atypical for that phenotype, e.g. cream, instead of orange, reverse on AFPA; green, instead of blue, fluorescence on CAM; different shade of green. *A. leporis* (MUM 10.239) was used as outgroup.

3.3.1.3.2 Growth conditions

For the analysis of the *Aspergillus* isolates, a small number of spores was transferred from 7 day old cultures to 6 cm Petri dishes containing 10 mL of MEA. Cultures were incubated in the dark for 5 days at 28 ºC. *Escherichia coli* strain DH5α, used as control (external calibration), was obtained from the Micoteca da Universidade do Minho. *E. coli* cells were grown on Luria-Bertani medium agar (LB; 10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl) at 37 °C for 20 hours.

3.3.1.3.3 Flex target plate preparation

Approximately 50 µg of spores and young mycelium of each microorganism were transferred directly from the culture plate to the 48-well MALDI-TOF plate. Immediately, 0.5 µL of matrix solution (75 mg/mL 2,5-dihydroxybenzoic acid in ethanol/water/acetonitrile [1:1:1] with 0.03% trifluoroacetic acid) were added to fungi and mixed gently. To the calibrant *E. coli*, the same procedure was used. The sample mixtures were air dried at room temperature. Each sample was spotted in duplicate to test reproducibility. During the analyses all solutions were prepared and stored at 5 °C.

3.3.1.3.4 Data acquisition

The analyses were performed in the Laboratory of Mycology and Molecular Biology of MUM on an Axima LNR system (Kratos Analytical, Shimadzu, Manchester, UK) equipped with a nitrogen laser (337 nm), where the laser intensity was set just above the threshold for ion production. *E. coli* DH5α strain with known mass values of ribosomal proteins was used as external calibration. The mass range from m/z = 2,000 to 20,000 Da was recorded using the linear mode. The mass spectrometer was used in the linear mode with a delay of 104 ns and using an acceleration voltage of +20 kV. Final spectra were generated by summing 20 laser shots accumulated per profile and 50 profiles produced per sample, leading to a total of up to 10,800 laser shots per summed spectrum. The resulting peak lists were exported to the SARAMIS™ software package (Spectral Archiving and

Microbial Identification System, AnagnosTec, Postdam-Golm, Germany, www.anagnostec.eu).

In SARAMIS™, peak lists of individual samples were compared with the SuperSpectra database generating a ranked list of matching spectra. SARAMIS™ uses a point system based on peak list with mass signals weighted according to their specificity. The weighting is based on empirical data from multiple samples of the reference strains. SuperSpectra are consensus spectra containing a pattern of mass signals which are specific for each species or other taxonomic units. These SuperSpectra are characteristic for individual microbial taxa and allow the identification of specimens as well as cluster analyses of spectra of multiple samples. The similarity between individual spectra is expressed as the relative or absolute number of matching mass signals after subjecting the data to a single link agglomerative clustering algorithm. Dendrograms of spectral proximity between isolates were created.

4 RESULTS

4.1 Mycobiota and aflatoxigenic profile of almonds and chestnuts

4.1.1 Environmental conditions

Field

For the year 2007, chestnuts' maturation occurred from August to October, and harvest took place during November. For almonds, maturation occurred from June to September, and they were harvested in the middle of September, in both 2007 and 2008. As such, these were probably the periods that mostly influenced the final characteristics of the nuts. Table 4.1 lists the climatic conditions of Bragança for the period August-November of 2007, as registered by a local agro-climatic station. Table 4.2 lists the climatic conditions of Moncorvo locally registered for the periods June-September of 2007 and 2008, and of Faro for the period June-September 2008. Anomalies to Normal values are also described.

Table 4.1 Climatic conditions of Bragança for the months before and during chestnut harvest for the 2007 crop, and anomalies relative to Normal values.

	Total Rainfall (mm)		Mean Temperature (ºC)		Relative Humidity (%)	
	Registered[a]	Anomaly[b]	Registered[a]	Anomaly[c]	Registered[a]	Anomaly[c]
Aug	45.0	312.5%	18.0	-2.4	60.8	+8.1
Sept	48.2	147.5%	16.5	-1.2	60.6	+0.3
Oct	38.2	58.2%	11.9	-0.9	73.6	+0.9
Nov	56.2	64.5%	6.8	-1.0	64.9	-5.1
Total	187.6	93.8%	13.3	-1.4	65.0	-1.2

[a] values registered by a local agro-climatic station.
[b] Percentage of registered values relative to Normal values for the period 1951-1980 (INMG, 1991a).
[c] Difference between registered values and Normal values for the period 1951-1980 (INMG, 1991a).

Table 4.2 Climatic conditions of Moncorvo and Faro for the months before and during almond harvest, and anomalies relative to Normal values.

	Total Rainfall (mm)		Mean Temperature (ºC)		Relative Humidity (%)	
	Registered[a]	Anomaly[b]	Registered[a]	Anomaly[c]	Registered[a]	Anomaly[c]
Moncorvo 2007						
June	26.8	73.2%	19.8	-0.6	63.2	-22.3
July	1.8	18.9%	23.1	-0.5	52.7	-28.3
Aug	10.6	101.0%	23.0	+0.1	51.2	-22.3
Sept	0.0	0.0%	22.8	+2.8	47.3	-19.2
Total	39.2	46.0%	22.2	+0.5	53.6	-23.0
Moncorvo 2008						
June	1.6	4.4%	22.0	+1.6	56.1	-29.4
July	0.0	0.0%	24.0	+0.4	46.3	-34.7
Aug	4.0	38.1%	24.0	+1.1	49.1	-24.4
Sept	5.8	20.2%	20.3	+0.3	61.9	-4.6
Total	11.4	13.4%	22.6	+0.9	53.4	-23.2
Faro 2008						
June	0.01	0.5%	23.3	+2.8	55	-10.0
July	0.01	3.0%	24.6	+1.4	53	-7.0
Aug	0.4	8.3%	24.2	+1.0	56	-3.0
Sept	101.4	709%	21.1	+0.2	69	+4.3
Total	101.8	323%	23.3	+1.3	58.3	

[a] values registered by local agro-climatic stations.
[b] Percentage of registered values relative to Normal values for the period 1951-1980 (INMG, 1991a, 1991b).
[c] Difference between registered values and Normal values for the period 1951-1980 (INMG, 1991a, 1991b).

Moncorvo and Faro are similar in climate, even though Faro is warmer, but Bragança represents a totally different climatic reality, with more rain and milder temperatures in late summer.

In Bragança, for the referred period, the year of 2007 was slightly colder and drier than usual. Even though total rainfall in October was only 44% of the Normal values, the preceding months had been extremely wet and it rained strongly for a few days in middle October, which led to the postponing of harvest from October to November.

In Moncorvo, 2007 had an extremely dry and hot summer, with September registering no rain and temperatures almost 3 ºC higher than normal. These conditions allowed the almond harvest to occur early in September. September 2008 was not as dry and hot as the previous year. Faro was, in general, slightly warmer than usual. September was extremely wet, with 709% more rainfall than normal, but this rainfall was concentrated in the last days of the month, after almond harvest.

Storage (producer's warehouse)

The environmental conditions (temperature and relative humidity) inside the producer´s warehouse were registered by two data loggers (A and B; placed in different parts of the warehouse) from the first day of almond harvest (13.09.2008) until the almonds were expedited (20.03.2009). When almonds started to be bagged (24.10.2008), two other data loggers (C and D) registered the environmental conditions inside the bags. Table 4.3 shows the mean values of temperature (T) and relative humidity (RH) for each data logger. Figure 4.1 represents the evolution of those conditions, taken as the average of the two data loggers.

Table 4.3 Mean Temperature (T) and Relative Humidity (RH) registered at the warehouse (environment) and inside the bags, relative to the months of almond storage.

	Environment						Bags					
	T (ºC)			RH (%)			T (ºC)			RH (%)		
	A[a]	B[b]	Mean	A	B	Mean	C[c]	D[d]	Mean	C	D[e]	Mean
Sep'08	18.7	17.9	18.3	57.2	58.3	57.8	-	-	-	-	-	-
Oct	14.2	13.7	14.0	67.0	67.8	67.4	10.6	11.5	11.1	72.9	-	72.9
Nov	7.7	7.1	7.4	81.8	83.5	82.7	7.9	8.5	8.2	78.7	-	78.7
Dec	5.9	5.2	5.6	83.9	91.5	87.7	6.0	6.5	6.3	83,0	-	83.0
Jan'09	6.1	5.3	5.7	85.6	90.7	88.2	6.0	6.4	6.2	85.6	-	85.6
Feb	7.8	7.2	7.5	72.9	75.5	74.2	6.5	7.4	7.0	84.9	-	84.9
Mar	13.0	12.3	12.7	55.5	57.3	56.4	12.3	13.5	12.9	67.8	-	67.8

[a] Logger *A* – back of the warehouse, near an open wall
[b] Logger *B* – middle of the warehouse
[c] Logger *C* – top of the pile
[d] Logger *D* – inside the pile
[e] A problem with the logger occurred, RH was not logged.

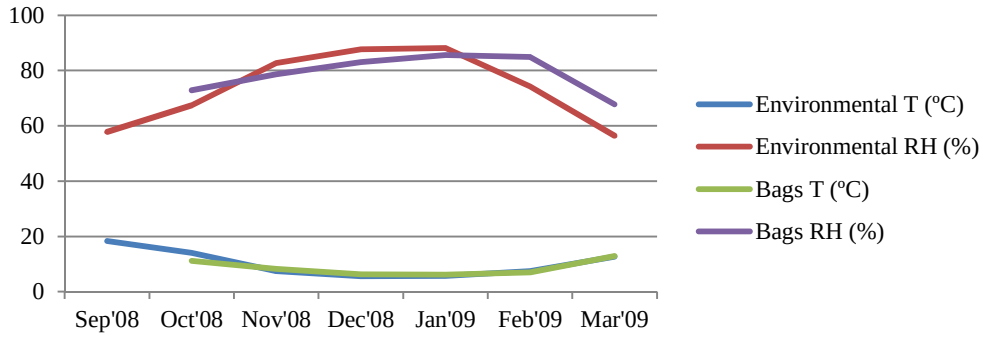

Figure 4.1 Mean Temperature (T) and Relative Humidity (RH) of the warehouse environment and inside the bags, relative to the months of almond storage (mean of two data loggers for each condition except Bags RH).

4.1.2 Water activity

Storage samples

Water activity (a_W) of storage samples is presented in Table 4.4. The significance of differences (P-value) between samples *A* (top of the pile) and *B* (inside the pile) for each time of collection is also shown.

Table 4.4 Water activity of storage samples throughout the storage period (n=3, mean ± standard deviation).

	Storage 1				Storage 2				Storage 3			
	A1[a]	**B1[a]**	**Mean[b]**	**P [c]**	**A2[a]**	**B2[a]**	**Mean[b]**	**P [c]**	**A3[a]**	**B3[a]**	**Mean[b]**	**P [c]**
In-shell	0.672 ± 0.003	0.589 ± 0.006	0.630 ± 0.046	0.000	0.717 ± 0.012	0.726 ± 0.019	0.721 ± 0.015	1.000	0.416 ± 0.009	0.396 ± 0.010	0.406 ± 0.014	0.661
Shelled	0.696 ± 0.012	0.645 ± 0.007	0.671 ± 0.029	0.092	0.710 ± 0.005	0.720 ± 0.003	0.715 ± 0.006	0.491	0.452 ± 0.020	0.399 ± 0.014	0.426 ± 0.033	0.300

[a] mean ± standard deviation, n=3.
[b] mean ± standard deviation, n=6.
[c] difference significance, as determined by *Tamhane's T2* test for $P < 0.05$.

Samples *A* and *B* were collected from different bags, to test if there were significant differences between nuts stored inside the pile or on the top of the pile of bags. In terms of a_W, no significant differences were detected between samples *A* and *B* throughout the storage period, except for in-shell almonds on the first time-point of collection. For this reason, we considered it reasonable to treat samples *A* and *B* as one sample only in the following analyses. From this point onwards, whenever the analyses result from the conjugation of data of samples *A* and *B*, samples will be referred to as *Storage 1* (*A1 + B1*), *Storage 2* (*A2 + B2*) and *Storage 3* (*A3 + B3*). Figure 4.2 represents the evolution of a_W in storage samples.

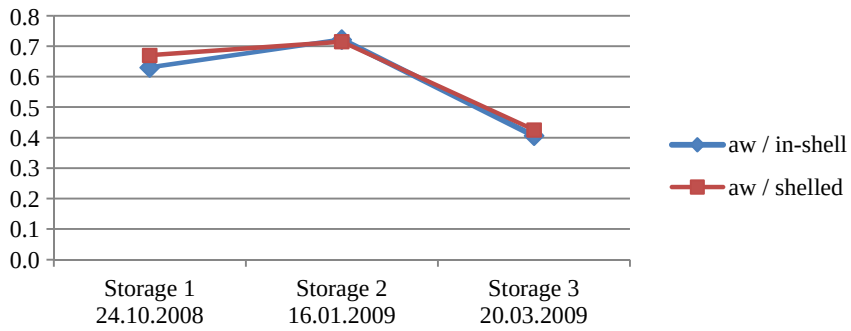

Figure 4.2 Water activity of storage samples (mean of samples *A* and *B*) throughout the storage period.

Between *Storage 1* and *Storage 2*, a_W increased, but not significantly, for both in-shell and shelled almonds (P = 0.537 and P = 0.950, respectively; Table AI.1, Appendix I), and then significantly decreased between *Storage 2* and *Storage 3* (P < 0.001 in both cases). This trend correlated directly with Relative Humidity and inversely with Mean Temperature registered inside the warehouse.

Processor samples

Water activity of processor samples is presented in Table 4.5. The significance of differences (P-value) between samples, as determined by the *Tamhane's T2* test, is shown in Table AI.1 of Appendix I.

Table 4.5 Water activity registered for the processor samples (n=3, mean ± standard deviation).

	Processor samples						
	C		D		F		
	C1	C2	D1	D2	F1	F2	F3
In-shell	-	-	-	-	0.428 ± 0.010	-	-
Shelled	-	-	-	-	0.461 ± 0.027	-	-
Shell	-	-	-	-	-	0.561 ± 0.012	-
Kernel	0.425 ± 0.006	0.534 ± 0.009	0.521 ± 0.039	0.520 ± 0.002	-	0.502 ± 0.004	-
Seed coat	-	-	-	-	-	-	0.877 ± 0.008
Nutmeat	-		-	-	-	-	0.370 ± 0.009

Regarding processor samples, sample *C1* had lower a_W than samples *C2*, *D1* and *D2*, but the difference was only significant between *C1* and *C2* (P = 0.045). In fact, almonds from lot C had been harvested in 2007, a year before arriving to the processor, and that storage period as well as the extremely dry and hot conditions in September 2007 may be the reasons for the extremely low a_W of sample *C1*. Throughout the storage period in the processor's warehouse, sample *C1* levelled with the other samples.

Sample *F1* had an extremely low a_W, similar to sample *C1* (P = 1.000). We did not follow Faro's warehouse climatic conditions during storage, but February and March 2009 were very dry, possibly allowing almonds to get well dry before being expedited to the processor. The a_W of Faro's samples significantly increased from *F1* to *F2* (P = 0.030). By

22.05.2009, when samples *C2*, *D2* and *F2* were collected, differences between samples' a_W had become insignificant ($P > 0.753$). This evolution in almonds a_W towards a common level could mean that the environmental conditions inside the processor's warehouse allow the a_W to increase in extremely dry samples, but apparently to levels that still refrain the development of fungi.

Samples *F3/nutmeat* and *F3/seed coat* had extremely low and high values of a_W, respectively. These values resulted from the processing they were submitted to: *F2/kernel* was wet to promote the peeling, the seed coat was peeled off and discarded still wet, and the nutmeat was oven-dried before being packed.

4.1.3 Fungal contamination of almonds and chestnuts

This section describes the results regarding the monitoring of mycobiota in field, storage and processor samples of almonds and chestnuts. In all cases, total fungal incidence was assessed, but contaminating fungi other than those belonging to the genera *Aspergillus* and *Penicillium* were analysed only qualitatively. The frequency of contamination with *Penicillium* species was determined only at the genus level. The isolates of *Aspergillus* genus, which were the main interest of the study, were identified to the section level, for both frequency of infection and number of individuals. Diversity was also assessed for the *Aspergillus* sections, through the *Richness* index, which in this case corresponds to the number of sections identified, and the *Simpson Diversity Index* (*SDI*), which reflects how many of those sections have significant impact on the diversity level of the samples.

Even though the majority of the isolates was identified to the species level, this identification was based solely on morphological and physiological characters (except for section *Flavi*, which will be discussed later), which we considered limited for an accurate identification in a number of cases. For this reason, we opted to analyse the results at the section and not at the species level, in order to minimise identification errors.

4.1.3.1 Fungal incidence and diversity

4.1.3.1.1 General overview

Samples under study represent different types of nuts sampled under different conditions. As much as we understand that they cannot be analysed in bulk, a general overview of the fungal contamination observed in the different samples can be of significance in order to better understand the more refined analyses that follow. Figures 4.3 and 4.4 show, respectively, the percentage of nuts infected by the different fungi and the percentage of *Aspergillus* propagules isolated (by section).

More than eighty percent of the nuts analysed in this study were contaminated with some kind of fungi. The most common fungi were identified as belonging to the genera *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium*, *Fusarium* and *Penicillium*. From these, only *Aspergillus* and *Penicillium* incidences were quantified. As to genus *Aspergillus*, only 48% of the nuts were contaminated, from which 1959 propagules were isolated and identified. They were grouped into 9 different sections: *Aspergillus* (represented by the highly xerophylic species belonging to the teleomorphic genus *Eurotium*) was the most disseminated section, with the highest number of isolates (998 isolates), followed by *Flavi* (352 isolates), *Nigri* (228 isolates), *Wentii* (174 isolates) and *Versicolores* (165 isolates). Four of the nine sections were only rarely isolated: *Circumdati* (27 isolates), *Flavipedes* (12 isolates), *Fumigati* (2 isolates) and *Nidulantes* (1 isolate). The maximum number of propagules and sections isolated from an individual nut was obtained for in-shell almonds from Faro, with as much as 24 isolates from 6 different sections.

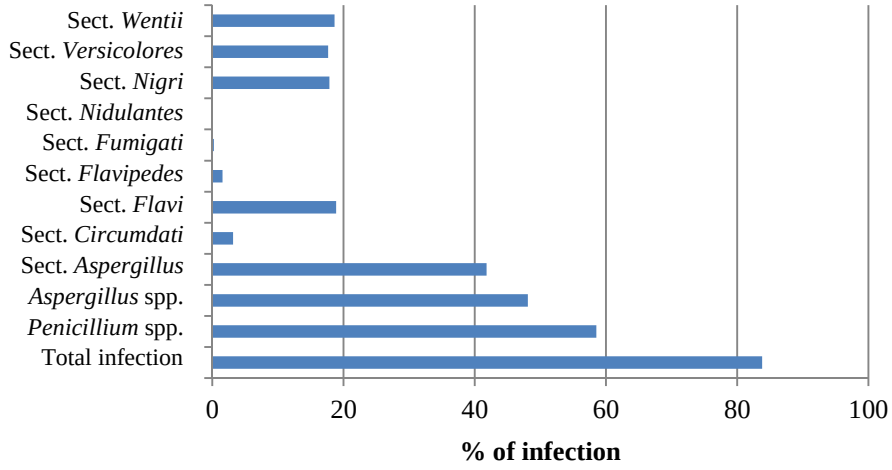

Figure 4.3 Percentage of nuts infected by the different fungi.

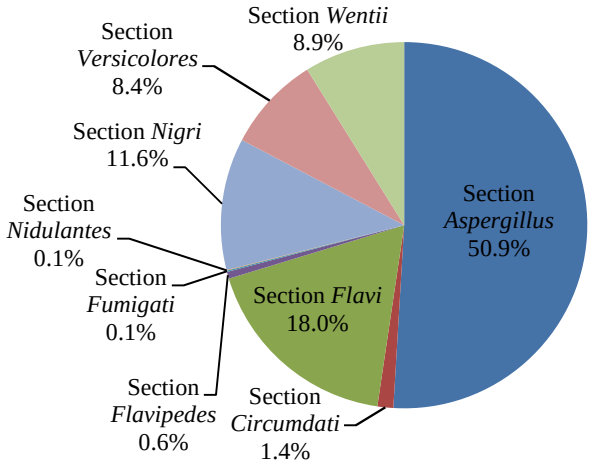

Figure 4.4 Percentage of *Aspergillus* propagules isolated, by section.

In both chestnut and almond field samples, in-shell nuts were highly contaminated with a wide variety of fungi. These mostly included *Alternaria*, *Botrytis*, *Cladosporium* and *Fusarium*, with numerous propagules developing in each nut. Chestnut samples were also rich in unidentified yeasts. *Penicillium* and *Aspergillus* were also present, but usually with few propagules per nut. When analysing storage and processor samples, fungal incidence was at the same level as that of field samples. If we were to analyse incidence levels on their own, we could conclude that samples were similar in terms of fungal contamination. Yet, contaminating fungi differed greatly between field and

storage/processor samples. In fact, the latter were contaminated almost exclusively by the genera *Aspergillus* and *Penicillium*, whereas those fungi reported as major contaminants in field samples were extremely rare.

From the total number of *Aspergillus* isolates, 1944 (99.2%) were isolated from almonds, and only 15 (0.8%) from chestnuts. Even though these figures correspond to different sized samples (total of 270 chestnuts and 500 almonds) with different characteristics (field, storage and different processing stages), we can state that chestnuts have contributed with a very limited number of isolates to the present study.

4.1.3.1.2 Field samples

Three chestnut field samples were taken on the year 2007, one from the tree (*Arv/07*), and two from the ground, at two different time points (*Ch1/07* and *Ch2/07*). Almonds were sampled from the tree in two consecutive years, 2007 and 2008 (*Arv/07* and *Arv/08*, respectively). In all cases, an equal number of nuts was plated with and without the shell (in-shell and shelled, respectively), without surface disinfection.

Table 4.6 lists the fungal incidence observed in chestnut and almond field samples, for both in-shell and shelled nuts, by genus and, in case of *Aspergillus*, by section. The number of *Aspergillus* isolated from these samples and the diversity indices *Richness* and *Simpson Diversity Index* (SDI) (relative to sections) are given in Table 4.7 and Figure 4.5.

Table 4.6 Percentage of mold-infected nuts for chestnut and almond field samples.

	Chestnut								Almond					
	Arv/07		Ch1/07		Ch2/07		Mean		Arv/07		Arv/08		Mean	
	In-shell	Shelled	In-shell	Shelled	In-shell	Shelled	In-shell	Shelled	In-shell	Shelled	In-shell	Shelled	In-shell	Shelled
# nuts	45	45	45	45	45	45	135	135	45	45	45	45	90	90
Genus														
Total	100	71	100	42	100	89	100	67	100	64	100	69	100	67
Penicillium	40	9	38	11	56	13	45	11	73	7	100	18	87	13
Aspergillus	11	9	11	2	-	-	7	4	53	-	80	-	67	-
Section														
Aspergillus	-	2	7	-	-	-	2	1	24	-	38	-	31	-
Circumdati	-	-	-	-	-	-	-	-	2	-	7	-	5	-
Flavi	-	-	-	-	-	-	-	-	7	-	27	-	17	-
Flavipedes	-	-	-	-	-	-	-	-	4	-	4	-	4	-
Nidulantes	-	-	-	-	-	-	-	-	-	-	2	-	1	-
Nigri	11	7	-	2	-	-	4	3	13	-	44	-	29	-
Versicolores	-	-	-	-	-	-	-	-	18	-	7	-	13	-
Wentii	-	-	4	-	-	-	1	-	7	-	20	-	14	-

Table 4.7 Number of *Aspergillus* isolates and diversity indices in chestnut and almond field samples.

	Chestnut								Almond					
	Arv/07		*Ch1/07*		*Ch2/07*		*Total*		*Arv/07*		*Arv/08*		*Total*	
	In-shell	Shelled	In-shell	Shelled	In-shell	Shelled	In-shell	Shelled	In-shell	Shelled	In-shell	Shelled	In-shell	Shelled
# nuts	45	45	45	45	45	45	135	135	45	45	45	45	90	90
Section														
Aspergillus	-	1	3	-	-	-	3	1	19	-	21	-	40	-
Circumdati	-	-	-	-	-	-	-	-	1	-	3	-	4	-
Flavi	-	-	-	-	-	-	-	-	3	-	14	-	17	-
Flavipedes	-	-	-	-	-	-	-	-	2	-	2	-	4	-
Nidulantes	-	-	-	-	-	-	-	-	-	-	1	-	1	-
Nigri	5	3	-	1	-	-	5	4	11	-	25	-	36	-
Versicolores	-	-	-	-	-	-	-	-	8	-	3	-	11	-
Wentii	-	-	2	-	-	-	2	-	3	-	9	-	12	-
Total	5	4	5	1	-	-	10	5	47	-	78	-	125	-
Mean/nut	0.11	0.09	0.11	0.02	-	-	0.07	0.04	1.04	-	1.73	-	1.39	-
Diversity Indices														
Richness[a]	1	2	2	1	-	-	3	2	7	-	8	-	8	-
SDI[b]	1	1.6	1.92	1	-	-	2.63	1.47	3.88	-	4.45	-	4.49	-

[a] Corresponds to number of sections

[b] Simpson Diversity Index relative to sections (*SDI = 1/Σ(Pi/Pn*), where *Pi* is the number of individuals of a given section and *Pn* is the total number of individuals).

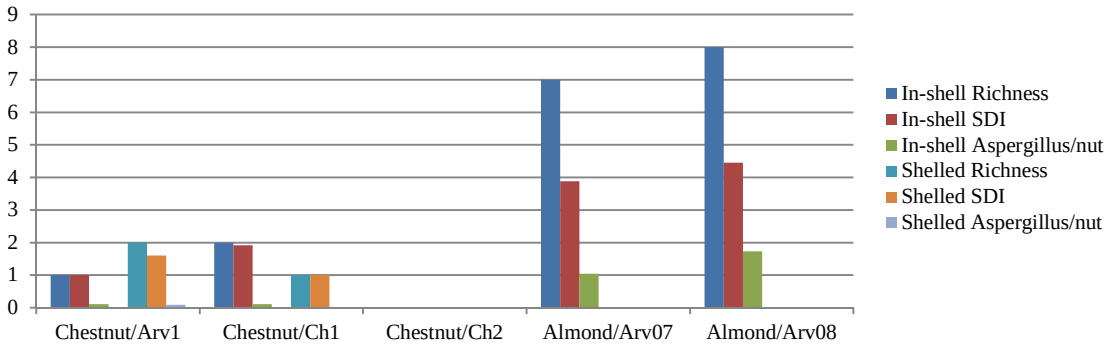

Figure 4.5 Diversity indices (*Richness* and *SDI*) and average number of *Aspergillus* per nut, for in-shell and shelled nuts of field samples.

Chestnut samples

In chestnut samples, a high level of overall contamination was observed, especially for in-shell nuts, which showed 100% contamination. *Alternaria*, *Botrytis*, *Cladosporium*, *Fusarium* and unidentified yeasts were the most commonly found fungi (data not quantified). *Penicillium* and *Aspergillus* were present at lower levels, the first being generally more frequent than the latter (Table 4.5). In fact, genus *Aspergillus* was very

weakly represented in chestnuts, in terms of frequency of contamination as well as in number and variety of fungi (Tables 4.6 and 4.7; Figure 4.5). Chestnuts contamination with *Aspergillus* included only 3 sections: *Nigri* (60% of the isolates), *Aspergillus* (26.6%) and *Wentii* (13.3%). Chestnuts showed a mean number of *Aspergillus* per nut (taken as the average of the three samples) of 0.07 and 0.04 for in-shell and shell nuts, respectively. *Richness* and *SDI*, which varied from 0 to 2, reflect the extremely low diversity of these samples relative to genus *Aspergillus*.

Chestnut samples *Arv1/07*, *Ch1/07* and *Ch2/07* were pairwise compared in order to determine the influence of the time and method of chestnut collection on fungal contamination. In general, chestnuts that had been on the ground for up to 3 weeks (sample *Ch2/07*) showed higher levels of contamination with overall fungi and *Penicillium* than the other samples, but were less contaminated with *Aspergillus* spp.

In-shell nuts were 100% contaminated, regardless of the sample. Contamination with *Penicillium* spp. was higher, but not significantly, in sample *Ch2/07* than in samples *Arv1/07* and *Ch1/07* ($P = 0.205$ and $P = 0.139$, respectively; Table AI.2). As opposed to that, *Aspergillus* spp. were less frequent in sample *Ch2/07* (0% incidence). In this particular case, *Penicillium* and *Aspergillus* incidences seem to have an opposite trend of evolution. Considering *Aspergillus* sections, contamination was very low, and no significant differences were registered between samples.

In shelled nuts, sample *Ch1/07* showed significantly less overall contamination than samples *Arv1/07* and *Ch2/07* ($P < 0.001$), but no other significant differences were registered ($P > 0.117$). When comparing in-shell with shelled nuts, shelled nuts had significantly less contamination with general fungi and *Penicillium* than in-shell nuts, for all samples ($P < 0.026$, Table AI.3). On the other hand, contamination with *Aspergillus* was not significantly different between in-shell and shelled nuts ($P > 0.361$).

Almond samples

The fungal genera predominantly found in almonds' field samples were generally the same as those found in chestnuts, namely *Alternaria*, *Botrytis*, *Cladosporium* and *Fusarium*. But in almonds the genera *Penicillium* and *Aspergillus* were also important contaminants (Tables 4.6 and 4.7; Figure 4.5). As for *Aspergillus* genus, the most common sections found in almonds were *Aspergillus* (32% of the isolates), *Nigri* (28.8%), *Flavi*

(13.6%), *Wentii* (9.6%) and *Versicolores* (8.8%). Sections *Circumdati*, *Flavipedes* and *Nidulantes* were rarely found.

All in-shell nuts showed contamination with overall fungi. Contamination levels with *Penicillium* and *Aspergillus* were of 87% and 67%, respectively, with almonds having a mean of 1.39 *Aspergillus* per nut. Maximum values of *Richness* and *SDI* were 8 and 4.49, respectively. Contrary to the high in-shell contamination levels, shelled nuts showed significantly lower levels of contamination with overall fungi and *Penicillium* spp. ($P < 0.001$), with rare colonies developing on infected fruits. None of the shelled nuts was found to be contaminated with *Aspergillus*.

By comparing in-shell samples from 2007 and 2008 crops, no differences were detected in total contamination (100% contamination in both samples), but *Penicillium* and *Aspergillus* were significantly more frequent in 2008 than in 2007 samples ($P < 0.001$ and $P = 0.013$, respectively; Table AI.4). When considering *Aspergillus* sections, 2008 nuts had higher contamination levels for all sections except for section *Versicolores*, but only sections *Flavi* and *Nigri* showed significant differences between years ($P = 0.011$ and $P = 0.002$, respectively).

4.1.3.1.3 Storage samples

Storage samples were taken from the warehouse of the almond producer, at 3 time-points along the storage period: one month of storage (*Storage 1*, 24.10.2008), four months (*Storage 2*, 16.01.2009) and six months (*Storage 3*, 20.03.2009). Table 4.8 lists the fungal incidence observed in storage samples, for both in-shell and shelled nuts, by genus and, in the case of *Aspergillus*, by section. The number of *Aspergillus* isolated from these samples and the diversity indices *Richness* and *SDI* are given in Table 4.9.

Table 4.8 Frequency (%) of mold-infected nuts in almond storage samples.

	Storage 1				Storage 2				Storage 3			
	A1		*B1*		*A2*		*B2*		*A3*		*B3*	
	In-shell	Shelled	In-shell	Shelled	In-shell	Shelled	In-shell	Shelled	In-shell	Shelled	In-shell	Shelled
# nuts	10	10	10	10	10	10	10	10	10	10	10	10
Genus												
Total	100	50	100	60	100	50	100	20	100	40	100	50
Penicillium	100	20	100	30	100	40	100	20	100	20	100	30
Aspergillus	100	30	100	30	100	40	100	10	100	30	100	40
Section												
Aspergillus	100	10	100	10	100	30	100	10	100	20	100	40
Circumdati	-	-	20	-	10	-	20	-	-	-	-	-
Flavi	20	-	10	-	20	10	70	-	30	-	10	-
Flavipedes	10	-	10	-	-	-	10	-	-	-	-	-
Fumigati	-	10	-	-	-	-	-	-	-	-	-	-
Nigri	-	-	-	-	40	-	30	-	-	-	10	-
Versicolores	90	10	90	-	90	-	60	-	70	10	30	-
Wentii	50	-	60	20	70	-	80	-	70	-	60	-

Table 4.9 Number of *Aspergillus* isolates and diversity indices in almond storage samples.

	Storage 1				Storage 2				Storage 3			
	A1		*B1*		*A2*		*B2*		*A3*		*B3*	
	In-shell	Shelled	In-shell	Shelled	In-shell	Shelled	In-shell	Shelled	In-shell	Shelled	In-shell	Shelled
# nuts	10	10	10	10	10	10	10	10	10	10	10	10
Section												
Aspergillus	17	1	20	1	26	3	29	1	23	3	37	5
Circumdati	-	-	2	-	1	-	3	-	-	-	-	-
Flavi	2	-	1	-	2	1	9	-	4	-	1	-
Flavipedes	1	-	1	-	-	-	1	-	-	-	-	-
Fumigati	-	1	-	-	-	-	-	-	-	-	-	-
Nigri	-	-	-	-	4	-	6	-	-	-	1	-
Versicolores	24	2	22	-	19	-	11	-	9	1	3	-
Wentii	8	-	9	2	13	-	13	-	9	-	6	-
Total	52	4	55	3	65	4	72	1	45	4	48	5
Mean/Nut	5.2	0.4	5.5	0.3	6.5	0.4	7.2	0.1	4.5	0.4	4.8	0.5
Diversity Index												
Richness[a]	5	3	6	2	6	2	7	1	4	2	5	1
SDI[b]	2.9	2.7	3.1	1.8	3.44	1.6	4.12	1	2.86	1.6	1.63	1

[a] Corresponds to number of Sections
[b] Simpson Diversity Index

Samples A and B were collected from different bags, to test if there were significant differences between nuts stored inside the pile or on the top of the pile of bags. No significant differences in fungal incidence were detected between samples A and B along the storage period for any of the taxa considered ($P > 0.070$ for all cases; Table AI.6). For this reason, we considered it reasonable to treat samples A and B as one sample only in further analysis.

In the next Figures of this section, data refer to the mean incidences of samples *A* and *B* for each storage sampling time-point. Since storage samples relate to each other chronologically, results of fungal incidences and diversity are represented as trend lines.

Figure 4.6 shows the frequencies of in-shell almonds contamination with the various taxa identified throughout the storage period. Incidences of total fungi and *Penicillium* spp. showed to be homogeneous between samples (100% in all samples). Infection with Sections *Flavi* and *Nigri* increased throughout the first stage of storage (*Field* to *Storage 1*), and decreased again in the following period, but only section *Nigri* varied significantly ($P < 0.001$ and $P = 0.008$, respectively; Table AI.7). In January, a new period of climatic changes occurred. From January to March, environmental conditions began to invert relative to the last three months: temperature began to rise and RH decreased. Under these conditions, samples got substantially drier. The trend at this stage was for general reduction of *Aspergillus* incidence, but only section *Nigri* suffered a significant reduction ($P = 0.044$). *Aspergillus* diversity in in-shell almonds followed the trend of a_W and environmental RH: during periods of higher RH, *Aspergillus Richness* and *SDI* increased, and vice-versa (Figure 4.8).

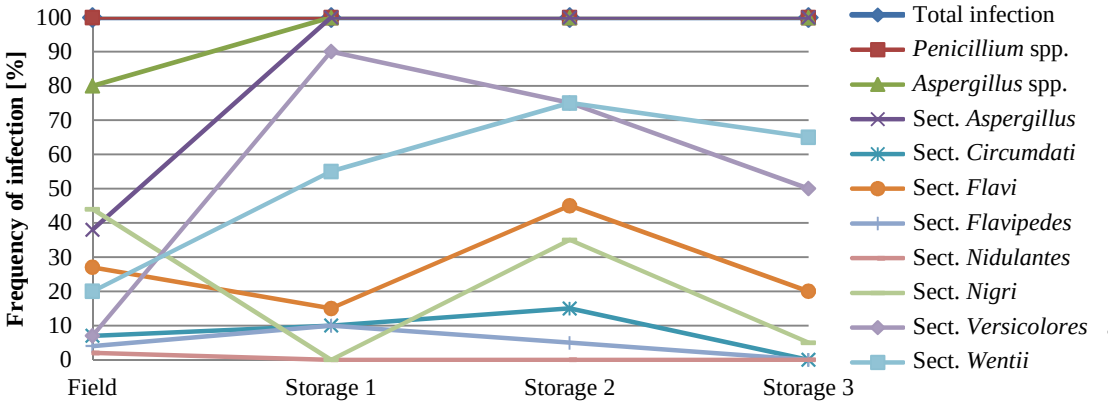

Figure 4.6 Evolution of in-shell almonds infection for the various taxa under study, from the field until the end of storage. Frequency of infection corresponds to the number of nuts infected by fungi from a given taxon.

Shelled almonds generally followed a different trend from in-shell nuts in terms of fungal infection (Figure 4.7). Total infection decreased from *Field* to *Storage 2*, but infection with *Penicillium* increased over that period. *Aspergillus* spp. significantly

increased during the first period of storage ($P < 0.001$), but then slightly decreased from *Storage 1* to *Storage 2*. No significant differences were observed for each section alone in the overall period of storage, probably due to the extremely low levels of incidence, but they generally slightly increased from *Field* to *Storage 1*, decreased from *Storage 1* to *Storage 2* and increased again from *Storage 2* to *Storage 3*. The only exception to this trend was section *Flavi*, whose incidence was higher in *Storage 2* than in the other samples. In fact, this was the only storage sample where section *Flavi* was detected as a kernel contaminant. Shelled nuts' *Richness* and *SDI* slightly decreased during storage (Figure 4.8). In spite of the analyses that have just been done for shelled nuts, we have to consider that the extremely low level of *Aspergillus* isolated from shelled almonds (21 from a total of 150 almonds analysed) restricts the conclusions that can be drawn.

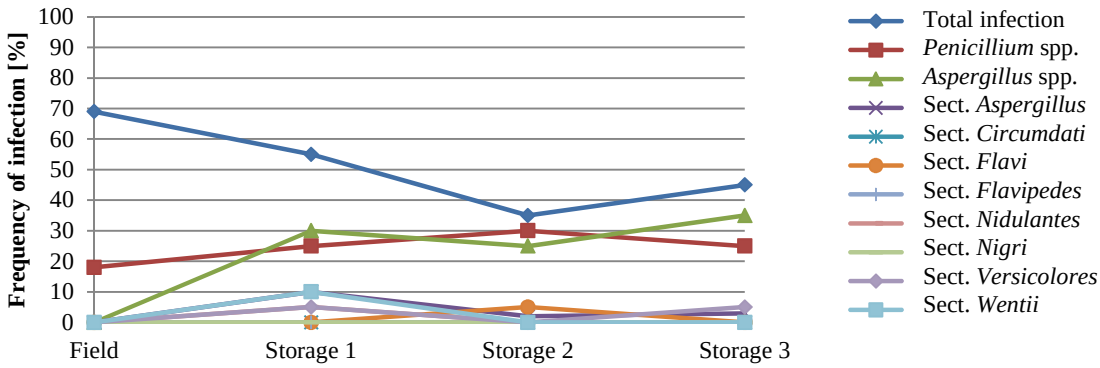

Figure 4.7 Evolution of shelled almonds infection for the various taxa under study, from the field until the end of storage. Frequency of infection relates to the number of nuts infected by fungi from a given taxon.

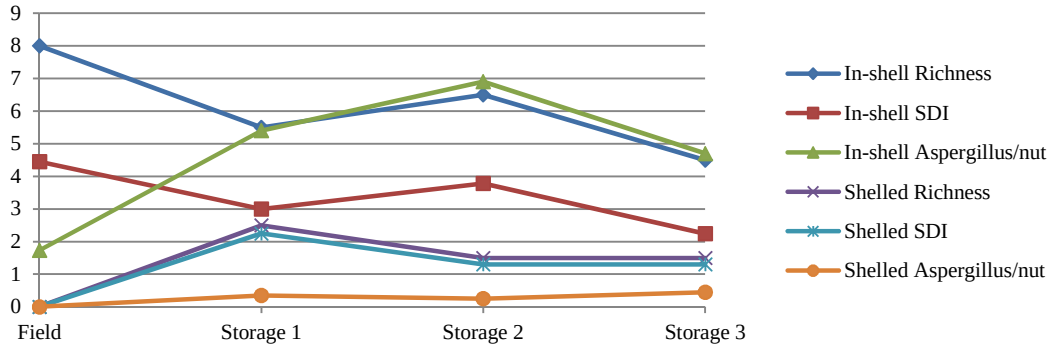

Figure 4.8 Diversity indices (*Richness* and *SDI*) and average number of *Aspergillus* per nut for in-shell and shelled almonds from the field until the end of storage.

4.1.3.1.4 Processor samples

Frequencies of fungal infection of processor samples are shown in Table 4.10. Number of *Aspergillus* and diversity indices are presented in Table 4.11 and Figure 4.9.

Table 4.10 Frequency (%) of mold-infected nuts of almond processor samples.

Sample	A			C		D		F					
	A		B	C1	C2	D1	D2	F1		F2		F3	
	In-shell	Shelled	Kernel	Kernel	Kernel	Kernel	Kernel	In-shell	Shelled	Shell	Kernel	Nutmeat	Seed coat
# nuts	10	10	10	20	20	20	20	10	10	10	20	20	20
Genus													
Total	100	100	100	100	100	100	100	100	100	100	100	100	100
Penicillium	100	100	100	100	100	100	100	100	100	100	100	100	100
Aspergillus	100	100	100	100	100	100	100	100	100	100	100	90	100
Section													
Aspergillus	100	100	100	100	100	100	100	100	100	100	100	90	100
Circumdati	30	-	10	-	-	-	-	15	-	30	25	-	-
Flavi	50	30	40	70	50	85	85	100	40	100	95	-	15
Flavipedes	-	-	-	5	-	-	-	30	-	-	5	-	-
Fumigati	-	10	-	-	-	-	-	-	-	-	-	-	-
Nigri	30	10	-	65	65	90	100	100	10	100	85	5	5
Versicolores	20	20	100	30	-	25	20	40	10	70	55	5	15
Wentii	80	20	30	45	10	80	15	90	10	80	40	-	5

Table 4.11 Number of *Aspergillus* isolates and diversity indices in almond processor samples.

Sample	A		B	C1	C2	D1	D2	F1		F2		F3	
	In-shell	Shelled	Kernel	Kernel	Kernel	Kernel	Kernel	In-shell	Shelled	Shell	Kernel	Nutmeat	Seed coat
# nuts	10	10	10	20	20	20	20	10	10	10	20	20	20
Section													
Aspergillus	50	10	50	100	100	100	100	53	13	50	100	20	32
Circumdati	3	-	1	-	-	-	-	5	-	3	5	-	-
Flavi	19	6	7	17	15	36	50	63	4	45	50	-	3
*Flavi*pedes	-	-	-	1	-	-	-	3	-	-	1	-	-
Fumigati	-	1	-	-	-	-	-	-	-	-	-	-	-
Nigri	4	1	-	15	19	27	35	17	1	25	26	1	1
Versicolores	4	2	13	6	-	5	4	4	1	8	12	1	3
Wentii	12	2	3	10	2	25	3	18	1	14	9	-	1
Total	92	22	74	149	136	193	192	163	20	145	203	22	40
Mean	9.2	2.2	7.4	7.5	6.8	9.7	9.6	16.3	2.0	14.5	10.2	1.1	2.0
Diversity Index													
Richness	6	6	5	6	4	5	5	7	4	6	7	3	5
SDI[a]	2.6	3.3	2.0	2.1	1.7	2.9	2.7	3.6	2.0	3.7	1.5	1.2	1.5

[a] Corresponds to number of Sections
[b] Simpson Diversity Index

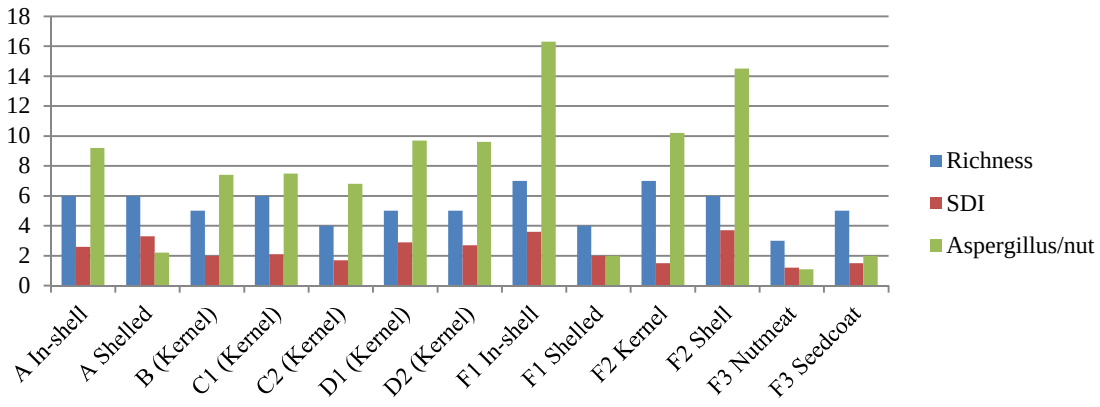

Figure 4.9 Diversity indices (*Richness* and *SDI*) and average number of *Aspergillus* per nut for processor samples.

Samples A and B

Samples *A* and *B* originated from the same lot of almonds, from the 2007 crop. These almonds were stored at the farmer's warehouse for 5 months before being delivered to the processor and shelled. Samples were collected simultaneously: sample *A* was still in-shell, whereas sample *B* had been shelled by the processor the day before sample collection. These samples were taken with the aim of determining the effect of shelling at the processor on the fungal infection of kernels. Samples *A* and *B* were strongly contaminated with both genera *Penicillium* and *Aspergillus*, whereas fungi from other genera were almost absent (data not quantified). In-shell nuts of sample *A* were also highly contaminated with section *Wentii*, but those fungi significantly decreased after shelling (P = 0.023; Table AI.8). Besides section *Aspergillus*, section *Flavi* was the most significant section contaminating shelled nuts of sample *A*. This section decreased after shelling, but not in a significant way (P = 0.370).

Fungal incidence of sample *B* kernels was, in general, similar to sample *A* (for both in-shell and shelled nuts), differing from sample *A* only for section *Versicolores*, which was significantly higher (P = 0.001 for both in-shell and shelled nuts).

Samples C and D

Samples *C* and *D* were collected simultaneously from different lots of almonds. Samples *C* originated from a lot of almonds cropped in 2007, whereas samples *D*

originated from almonds cropped in 2008. Lot *C* was stored at the producer for one year and delivered to the processor in October 2008. Lot *D* was stored at the producer for 4 months and delivered to the processor in January 2009. They had both been shelled by the processor a few months before the first sample collection.

The genera *Penicillium* and *Aspergillus* were present in all almonds. Section *Aspergillus* was the most representative of the genus, but sections *Flavi* and *Nigri* were also key contaminants. Samples *D* were generally more contaminated than samples *C*, but differences were only significant for sections *Flavi*, *Nigri* and *Wentii* (Tables 4.12 and AI.9). Throughout the 2 months that separated the 2 time-points of sample collection - 20.03.2009 (samples *C1 + D1*) and 22.05.2009 (samples *C2 + D2*) -, section *Wentii* was the only one that significantly decreased.

Table 4.12 Differences in mold-incidence between samples *C* and *D*.

	Genus			Section				
	Total	*Penicillium*	*Aspergillus*	*Aspergillus*	*Flavi*	*Nigri*	*Versicolores*	*Wentii*
C1 vs D1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-
C2 vs D2	n.s.	n.s.	n.s.	n.s.	-	-	n.s.	n.s.
C vs D	n.s.	n.s.	n.s.	n.s.	-	--	n.s.	n.s.
C1 vs C2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	+	+
D1 vs D2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	++
1 vs 2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	++

n.s., differences not significant at the P = 0.05 level (as determined by two-tailed Fisher's exact test)
+, the fungus was significantly more frequent on the first than on the latter, $0.001 < P < 0.05$
++, $P < 0.001$
-, the fungus was significantly less frequent on the first than on the latter, $0.001 < P < 0.05$
--, $P < 0.001$

Samples F

Samples *F* were collected from a lot of almonds originated from Faro. They were harvested in September 2008 and stored at the producer until March 2009. Lot *F* had just arrived to the processor at the time of sample *F1* collection, and was stored in the silo until processing. Samples *F2* (kernel and shell) were collected on the first stage of processing, as nuts were being shelled, after 2 months of storage in the silo. Samples *F3* (nutmeat and seed coat) were collected 4 days later, when shelled kernels were further processed to

bleached (pealed) nutmeat. At this processing stage, kernels were soaked in hot water, the seed coat was removed and discarded, and the nutmeat was oven-dried.

All samples *F* were highly contaminated with *Penicillium* spp. and *Aspergillus* spp., as well as with *Aspergillus* section *Aspergillus*, even those samples that suffered processing. Contamination with fungi from other genera was found only rarely. In-shell *F1* nuts were also strongly contaminated with sections *Flavi*, *Nigri* and *Versicolores*, but that contamination was reduced in a significant way for *Flavi* and *Nigri* when nuts were plated shelled (*F1 shelled*), meaning that only a small part of the contaminating fungi were infecting the kernel. But in the case where almonds were shelled by the processor, the resulting kernel (*F2 kernel*) was nearly as contaminated as in-shell almonds (*F1 in-shell*).

By comparing the evolution of kernel contamination along the stages of processing (Figure 4.10), it is evident the increase of fungal incidence after shelling at the processor (*F1 in-shell* vs. *F2 shelled*), which reflects that, during the shelling process, fungi are transferred from the shell (and probably the environment) to the kernel. At this stage, the increase of sections *Flavi* and *Nigri* incidence was highly significant (Tables 4.13 and AI.10). For nuts that were further processed, i.e, bleached, contamination was almost exclusively reduced to *Penicillium* spp. and *Aspergillus* section *Aspergillus*, since the contamination with other sections of *Aspergillus* was significantly reduced to a small proportion of the F3 nutmeats.

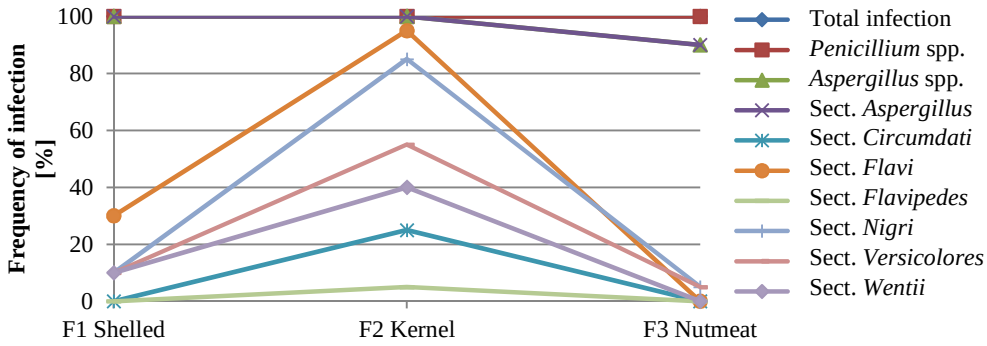

Figure 4.10 Frequency (%) of mold-infected kernels on processor samples *F* before and after shelling.

Table 4.13 Differences in fungal contamination between samples *F*.

	Genus			Section						
	Total	*Penicillium*	*Aspergillus*	*Aspergillus*	*Circumdati*	*Flavi*	*Flavipedes*	*Nigri*	*Versicolores*	*Wentii*
In-shell F1 vs shelled F1	n.s.	n.s.	n.s.	n.s.	n.s.	+	n.s.	++	n.s.	++
In-shell F1 vs shell F2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
In-shell F1 vs kernel F2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	+
Shelled F1 vs kernel F2	n.s.	n.s.	n.s.	n.s.	n.s.	- -	n.s.	- -	-	n.s.
Kernel F2 vs shell F2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-
Kernel F2 vs nutmeat F3	n.s.	n.s.	n.s.	n.s.	+	++	n.s.	++	++	+

n.s., differences not significant at the P = 0.05 level (as determined by two-tailed Fisher's exact test)
+, the fungus was significantly more frequent on the first than on the latter, 0.001 < P < 0.05
++, P < 0.001
-, the fungus was significantly less frequent on the first than on the latter, 0.001 < P < 0.05
- -, P < 0.001

4.1.3.1.5 Differences between in-shell and shelled nuts

Incidence of fungi was compared between in-shell and shelled nuts to determine if fungal inoculum was superficial or internal. Table 4.14 lists the differences found between in-shell and shelled nuts contamination. More detailed data can be found in Table AI.3.

In chestnuts, in-shell nuts had significantly more contamination with total fungi and *Penicillium* spp. than shelled nuts, independently of chestnuts being collected from the tree of from the ground, or even from being on the ground for 3 weeks, meaning that contamination was mostly superficial. Contamination with *Aspergillus* spp. was very low or absent in both in-shell and shelled fruits, so differences were not significant for any of the sections.

Table 4.14 Differences in frequency of fungi between in-shell and shelled nuts.

	Genus			Section						
Sample	Total	*Penicillium*	*Aspergillus*	*Aspergillus*	*Circumdati*	*Flavi*	*Flavipedes*	*Nigri*	*Versicolores*	*Wentii*
Chestnut, field										
Arv/07	++	+	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Ch1/07	++	+	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Ch2/07	+	++	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
All	++	++	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Almond, field										
Arv/07	++	++	++	++	n.s.	n.s.	n.s.	+	+	n.s.
Arv/08	++	++	++	++	n.s.	++	n.s.	++	n.s.	+
All	++	++	++	++	n.s.	++	n.s.	++	++	++
Almond, storage										
Storage 1	++	++	++	++	n.s.	n.s.	n.s.	n.s.	++	+
Storage 2	++	++	++	++	n.s.	+	n.s.	+	++	++
Storage 3	++	++	++	++	n.s.	n.s.	n.s.	n.s.	+	++
All	++	++	++	++	+	++	+	++	++	++
Almond, processor										
A	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	+
F	n.s.	n.s.	n.s.	n.s.	n.s.	+	n.s.	++	n.s.	++
All	n.s.	n.s.	n.s.	n.s.	+	+	n.s.	++	n.s.	++

n.s., differences not significant at the P = 0.05 level (as determined by two-tailed Fisher's exact test)
+, the fungus was significantly more frequent on in-shell than on shelled nuts, $0.001 < P < 0.05$
++, $P < 0.001$
-, the fungus was significantly less frequent on in-shell than on shelled nuts, $0.001 < P < 0.05$
--, $P < 0.001$

In almonds, we considered only data of samples collected in-shell, i.e, all field and storage samples, and processor samples *A* and *F*. We found significantly more contamination with total fungi, *Penicillium* spp., *Aspergillus* spp. and section *Aspergillus* on in-shell nuts ($P < 0.001$) in all field and storage samples, but the differences were eliminated in processor samples, where shelling the fruits had no significant effect on removing these fungi. We can perceive a tendency for the aforementioned taxa to transpose the shell barrier and progressively infect the fruit as storage time goes by. For the remaining *Aspergillus* sections analysed, shelling generally resulted in a significant reduction of inocula for sections *Flavi*, *Nigri*, *Versicolores* and *Wentii*.

4.1.3.1.6 Differences between nuts

To compare fungal contamination of almonds and chestnuts, we analysed only data from tree samples from 2007 crop, because these were the only samples with common characteristics.

While total contamination of in-shell chestnuts and almonds reached 100%, contamination levels with *Penicillium* and *Aspergillus* were always higher in almonds than in chestnuts, with significant differences observed for genera *Penicillium* ($P = 0.001$; Table AI.5) and *Aspergillus* ($P < 0.001$), and for sections *Aspergillus* ($P < 0.001$) and *Versicolores* ($P = 0.006$). Almonds showed a mean of 1.04 *Aspergillus* per nut, while chestnuts had a mean contamination level of 0.07 per nut. From the 56 *Aspergillus* isolated in 2007 from in-shell field samples, 84% originated from almonds, while only 9 (16%) originated from chestnuts. If we consider all samples, the difference is even more acute, with 92.6% of the 135 isolates resulting from almonds.

For shelled nuts, there were no significant differences between chestnuts and almonds ($P > 0.117$). In both nuts, fungal contamination with *Penicillium* spp. and *Aspergillus* spp. was extremely low or null. In fact, when considering field samples, shelled chestnuts showed higher contamination with *Aspergillus* spp. than shelled almonds.

4.1.3.1.7 Differences among almond origin

Almonds analysed in the present study had 2 different origins: Moncorvo and Faro. In order to analyse differences in fungal incidence between Faro and Moncorvo almonds, we only compared sample *Storage 3* from Moncorvo with sample *F1* from Faro, since they corresponded to almonds collected in September 2008, and both samples were collected at the same time-point (20.03.2009), after approximately 6 months of storage at the producer.

Figures 4.11 and 4.12 make a comparative analysis of fungal incidence of both samples for in-shell and shelled nuts, respectively.

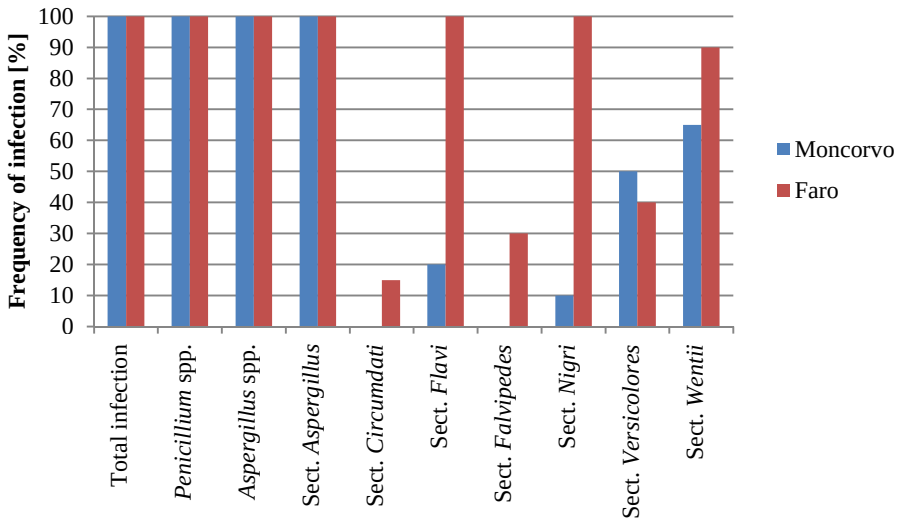

Figure 4.11 Frequency (%) of mold-infected in-shell almonds for Moncorvo and Faro samples.

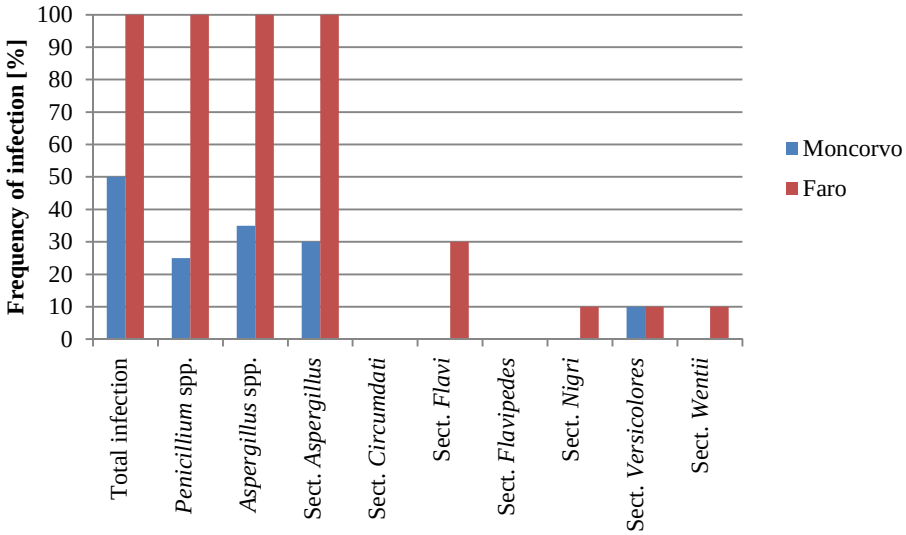

Figure 4.12 Frequency (%) of mold-infected shelled almonds for Moncorvo and Faro samples.

Samples from Faro were generally more contaminated than those from Moncorvo. While in-shell almonds from both origins showed no differences in total fungi, *Penicillium* spp., *Aspergillus* spp. and section *Aspergillus* incidences, Faro samples were significantly more contaminated with sections *Circumdati*, *Flavi*, *Flavipedes* and *Nigri* (Tables 4.15 and AI.11). On the other hand, internal contamination (shelled almonds) was significantly higher in Faro's samples, since the majority of the fungi of Moncorvo´s almonds was eliminated by removing the shell.

Table 4.15 Differences in frequency of fungi of almonds relative to origin.

	Genus			Section						
Faro vs Moncorvo	Total	*Penicillium*	*Aspergillus*	*Aspergillus*	*Circumdati*	*Flavi*	*Flavipedes*	*Nigri*	*Versicolores*	*Wentii*
In-shell	n.s.	n.s.	n.s.	n.s.	+	++	+	++	n.s.	n.s.
Shelled	+	++	++	++	n.s.	+	n.s.	n.s.	n.s.	n.s.
All	+	++	+	+	+	++	+	++	n.s.	n.s.

n.s., differences not significant at the P = 0.05 level (as determined by two-tailed Fisher's exact test)
+, the fungus was significantly more frequent on Faro than on Moncorvo nuts, 0.001 < P < 0.05
++, P < 0.001
-, the fungus was significantly less frequent on Faro than on Moncorvo nuts, 0.001 < P < 0.05
--, P < 0.001

4.1.3.1.8 Differences between field, storage and processor samples

In order to study the evolution of fungal contamination from production until processing, we compared samples with the same background. For this matter, only samples from Moncorvo and from crop 2008 were used (*Arv/08*, *Storage 1*, *Storage 2*, *Storage 3* and *D1*). Because sample *D1* had already been shelled by the processor, we compared that sample with both in-shell and shelled nuts from field and storage. By comparing processed almonds (almonds shelled by the processor) with in-shell field and storage samples (Figure 4.13), we can determine the evolution of environmental contamination, but comparing processed almonds with shelled field and storage samples (Figure 4.14), allows us to determine how many of those propagules really contact with the edible part of the fruit on each stage of production. The significances of fungal differences are presented in Table AI.12.

As can be concluded by the analysis of Figures 4.13 and 4.14, fungal contamination of almonds was mainly superficial, since kernels only suffered a big increase in contamination after being shelled at the processor (sample *D1*). On both in-shell and shelled nuts, contamination generally tended to increase significantly from field to processing, but that effect on shelled nuts is almost restricted to the final stage of shelling.

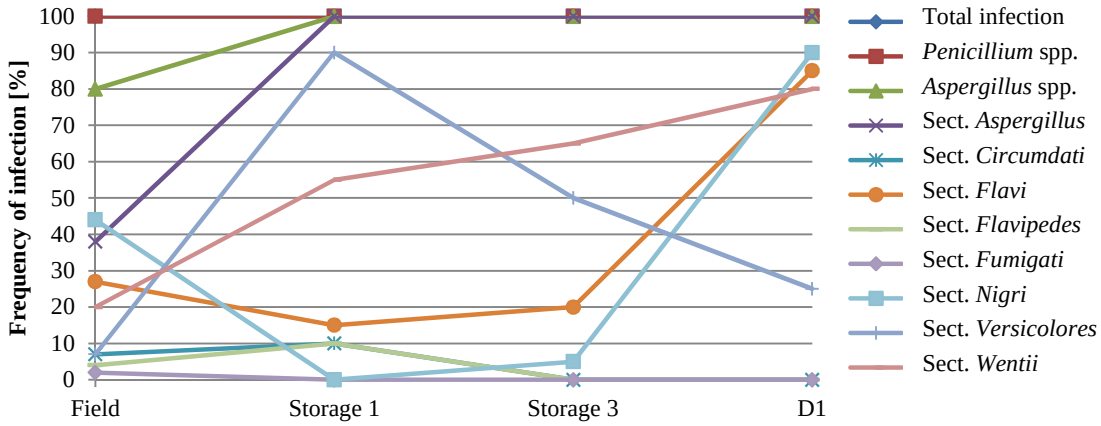

Figure 4.13 Evolution of fungal incidence from field to processing (in-shell Field and Storage samples).

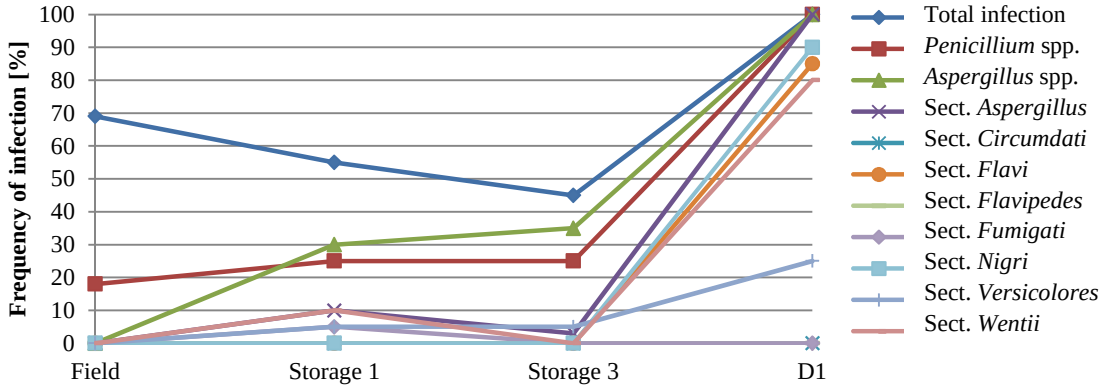

Figure 4.14 Evolution of fungal incidence from field to processing (shelled Field and Storage samples).

4.1.3.1.9 Associations among fungi

Associations among fungi were tested pairwise by comparing observed values (the number of nuts infected by both fungi) with expected values (the product of the frequencies of each fungus alone) using Phi correlation as measure of nominal association.

Because samples were so heterogeneous in their characteristics, which reflected strongly in fungal incidence, we opted to analyse fungal associations by dividing samples in two groups: i) "unprocessed" samples, which included those samples without any processing (*in-shell* samples) but also those that, after being processed, maintained high levels of contamination (*kernel* and *shell* samples); ii) "processed" samples, which

included those resulting from shelling at the lab (*shelled* samples) and samples for which processing greatly altered contamination level (*nutmeat* and *seed coat* samples). This division was necessary because, if samples were analysed in bulk, levels of association between fungi would be biased by the fact that almost all "unprocessed" nuts were contaminated by a given group of fungi and almost none of the "processed" were contaminated with that same fungi. This would result in levels of association higher than those obtained if samples were analysed in groups with more homogeneous characteristics.

Tables 4.16 and 4.17 list the association between fungi (*Phi coefficient*), and the corresponding significance (P-value), for "processed" and "unprocessed" samples. In the crosstabulations where at least one of the fungi contaminated 100% of the nuts, measures of association were not computed. On the other hand, associations between fungi with low or null incidence, namely those involving chestnut samples and sections *Circumdati*, *Flavipedes*, *Fumigati* and *Nidulantes* of almond samples, all resulted in extremely low *Phi* values and generally high P-values, meaning that no association could be established between those fungi. For that reason, those results are not shown. Also, association between a pair of intertwined variables is not valid, i.e., association cannot be established if one of the variables (e.g. *Penicillium* spp. or *Aspergillus* spp. contamination) is a component of the other variable (Total contamination). Consequently, associations between the referred taxa were not determined.

Table 4.16 Association between fungi in chestnuts and almonds on "unprocessed" samples (*in-shell*, *kernel*), as determined by *Qui-square test* and *Phi coefficient*, based on differences between observed and predicted co-infection frequencies.

Fungi	Observed	Predicted	Phi	P
Almonds				
Penicillium spp. & *Aspergillus* spp.	253	250.1	0.170	0.004
Penicillium spp. & Sec. *Aspergillus*	225	218.4	0.289	0.000
Penicillium spp. & Sec. *Flavi*	139	133.7	0.191	0.001
Penicillium spp. & Sec. *Nigri*	137	132.8	0.153	0.009
Penicillium spp. & Sec. *Versicolores*	100	99.1	0.034	0.560
Penicillium spp. & Sec. *Wentii*	117	112.6	0.163	0.005
Sec. *Flavi* & Sec. *Aspergillus*	130	108.8	0.355	0.000
Sec. *Flavi* & Sec. *Nigri*	97	66.1	0.426	0.000
Sec. *Flavi* & Sec. *Versicolores*	56	49.4	0.096	0.103
Sec. *Flavi* & Sec. *Wentii*	75	56.1	0.266	0.000
Sec. *Nigri* & Sec. *Aspergillus*	122	108	0.234	0.000
Sec. *Nigri* & Sec. *Versicolores*	44	49.0	-0.072	0.218
Sec. *Nigri* & Sec. *Wentii*	62	55.7	0.089	0.130
Sec. *Versicolores* & Sec. *Aspergillus*	95	80.6	0.251	0.000
Sec. *Versicolores* & Sec. *Wentii*	54	41.6	0.183	0.002
Sec. *Wentii* & Sec. *Aspergillus*	110	91.6	0.314	0.000

Table 4.17 Association between fungi in chestnuts and almonds on "processed" samples (all samples except *in-shell* and *kernel*), as determined by *Qui-square test* and *Phi coefficient*, based on differences between observed and predicted co-infection frequencies.

Fungi	Observed	Predicted	Phi	P
Almonds				
Penicillium spp. & *Aspergillus* spp.	69	31.8	0.746	0.000
Penicillium spp. & Sec. *Aspergillus*	64	29.3	0.710	0.000
Penicillium spp. & Sec. *Flavi*	12	5.0	0.290	0.000
Penicillium spp. & Sec. *Nigri*	4	1.7	0.164	0.017
Penicillium spp. & Sec. *Versicolores*	12	5.0	0.201	0.004
Penicillium spp. & Sec. *Wentii*	6	2.5	0.202	0.003
Sec. *Flavi* & Sec. *Aspergillus*	11	4.0	0.304	0.000
Sec. *Flavi* & Sec. *Nigri*	1	0.2	0.116	0.093
Sec. *Flavi* & Sec. *Versicolores*	3	0.5	0.252	0.000
Sec. *Flavi* & Sec. *Wentii*	2	0.3	0.204	0.003
Sec. *Nigri* & Sec. *Aspergillus*	4	1.3	0.197	0.004
Sec. *Nigri* & Sec. *Versicolores*	0	0.2	-0.029	0.669
Sec. *Nigri* & Sec. *Wentii*	0	0.1	-0.024	0.729
Sec. *Versicolores* & Sec. *Aspergillus*	7	3.0	0.200	0.004
Sec. *Versicolores* & Sec. *Wentii*	1	0.3	0.105	0.129
Sec. *Wentii* & Sec. *Aspergillus*	4	2.0	0.121	0.079

4.1.3.2 Aflatoxigenic fungi

Even though five species outside section *Flavi* have been identified as AF producers (AF^+), only fungi belonging to *Aspergillus* section *Flavi* have been previously implicated in the production of AFs in food and food commodities. Therefore only isolates of section Flavi were considered for this Section. The identification of these isolates was based on various phenotypic characters, involving morphological and physiological features, as well as the extrolite profile. For the purpose of this analysis, isolates were grouped in 3 morphotypes – *Aspergillus flavus*, *A. parasiticus* and *A. tamarii* – depending on their morphological resemblance with these 3 species, and characterised by their AF pattern. Results on isolate identification to the species level will be discussed in Section 4.3.

Three-hundred and fifty two isolates were identified as belonging to section *Flavi*: 128 (36.4%) were grouped in the *A. flavus* morphotype, 195 (55.4%) as *A. parasiticus* morphotype and 29 (8.2%) as *A. tamarii* morphotype. Table 4.18 shows a detailed list of the number of *Flavi* isolates by sample.

The highest number of isolates originated from processor samples (315 isolates, 89.5%), which also had the highest average number of *Flavi* per nut (Figure 4.15A). Only 17 (4.8%) and 20 (5.7%) isolates originated from field and storage samples, respectively. Field, storage and processor samples were more strongly contaminated with *A. parasiticus* than with *A. flavus*, and *A. tamarii* was only rarely isolated (Figure 4.15B). *A. flavus* and *A. tamarii* were isolated more often in processor samples. These samples differed significantly from field and storage samples for all morphotypes ($P < 0.001$; Table AI.13), whereas that difference was not significant between field and storage samples ($P > 0.060$).

Table 4.18 Number of *Aspergillus* section *Flavi* isolates, grouped by morphotype, isolated from the various samples, percentage of each morphotype relative to total *Flavi*, and average number of *Flavi* per nut.

		Morphotypes						Total	
		A. flavus		*A. parasiticus*		*A. tamarii*			
Sample		#	% *Flavi*	#	% *Flavi*	#	% *Flavi*	#	*Flavi*/nut
Field									
Arv/07	in-shell	1	33.3	2	66.7	0		3	0.07
	shelled	0		0		0		0	
Arv/08	in-shell	2	14.3	11	78.6	1	7.1	14	0.31
	shelled	0		0		0		0	
Storage									
Storage 1	in-shell	0	0.0	3	100.0	0		3	0.15
	shelled	0		0		0		0	
Storage 2	in-shell	3	27.3	8	72.7	0		11	0.20
	shelled	0	0.0	1	100.0	0		1	0.10
Storage 3	in-shell	1	20.0	4	80.0	0		5	0.25
	shelled	0		0		0		0	
Processor									
A	in-shell	8	42.1	11	57.9	0		19	0.95
	shelled	2	33.3	3	50.0	1	16.7	6	0.30
B	kernel	5	71.4	2	28.6	0		7	0.70
C1	kernel	7	41.2	9	52.9	1	5.9	17	0.85
C2	kernel	10	66.7	3	20.0	2	13.3	15	0.75
D1	kernel	11	30.6	20	55.6	5	13.9	36	1.80
D2	kernel	27	54.0	16	32.0	7	14.0	50	2.50
F1	in-shell	17	27.0	43	68.3	3	4.8	63	6.30
	shelled	0	0.0	4	100.0	0		4	0.40
F2	shell	16	35.6	22	48.9	7	15.6	45	4.50
	kernel	17	34.0	32	64.0	1	2.0	50	2.50
F3	nutmeat	0		0		0		0	0.00
	seedcoat	1	33.3	1	33.3	1	33.3	3	0.15
Total		128	36.4	195	55.4	29	8.2	352	0.68

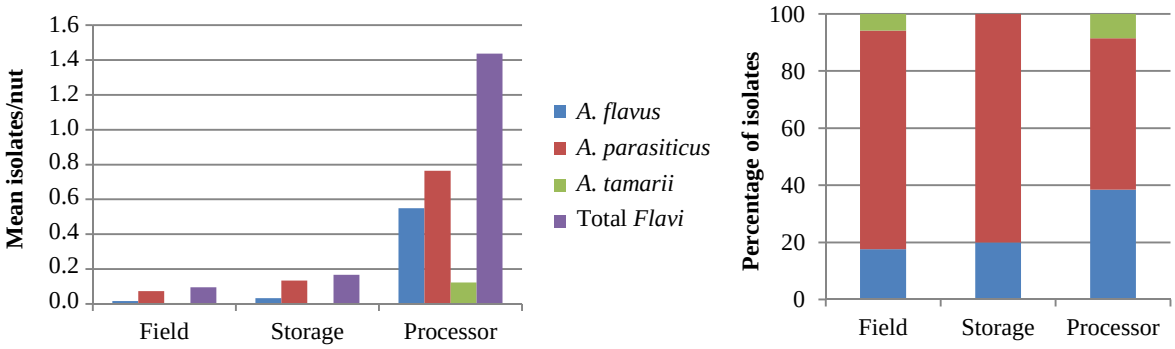

Figure 4.15 Average number of isolates (A) and percentage of isolates of each morphotype (B) by stage of production.

In order to study the evolution of average *Aspergillus* section *Flavi* per nut from production to processing, we compared samples with the same background. For this matter, only samples from Moncorvo and from crop 2008 were used (*Arv/08*, *Storage 1*, *Storage 2*, *Storage 3*, *D1* and *D2*). Figures 4.16 and 4.17 illustrate the fungal evolution throughout the stages of production, for in-shell and shelled nuts respectively. The comparison of these data allows us to determine how many of the contaminating fungi are effectively in contact with the edible part of the fruit on each stage of production.

As can be observed, both superficial (in-shell) and internal (shelled) contamination were extremely low or null during field production and storage. As the almonds got shelled by the processor, the number of section *Flavi* isolates significantly increased ($P < 0.001$; Table AI.13).

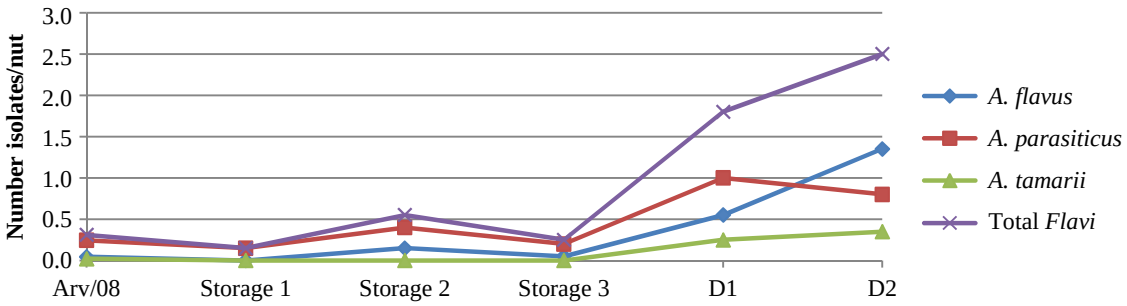

Figure 4.16 Average number of isolates of each morphotype throughout production, for Moncorvo 2008 samples. Field and storage samples refer to in-shell almonds only.

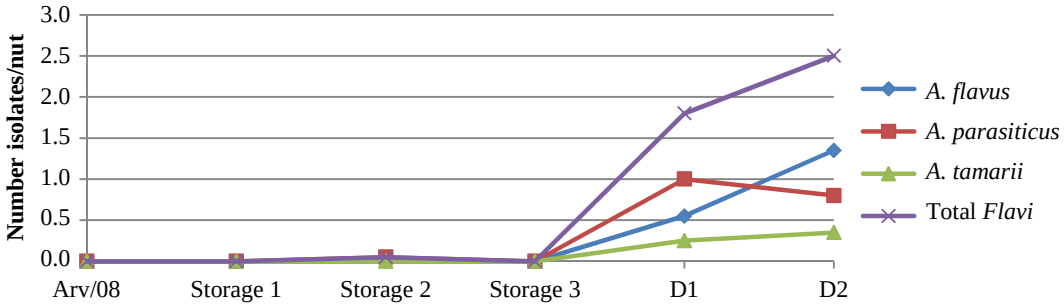

Figure 4.17 Average number of isolates of each morphotype throughout production, for Moncorvo 2008 samples. Field and storage samples refer to shelled almonds only.

Relative to almond's origin, we compared samples *Storage 3* (Moncorvo In-shell and shelled) and *D* (Moncorvo kernel) from Moncorvo with samples *F1* (Faro in-shell and shelled) and *F2* (Faro kernel) from Faro, because of their common characteristics. Figures 4.18 and 4.19 show the difference between Moncorvo and Faro samples, in terms of percentage of isolates of each morphotype and average number of *Flavi* isolates per nut, respectively. In terms of morphotype distribution, *A. parasiticus* morphotype is more frequent than the other morphotypes in samples from both Moncorvo and Faro, except for kernels originating from Moncorvo. It is worthnoting that *A. tamarii* is almost exclusive of samples collected at the processor; it is absent from storage samples and only one isolate was detected from field samples (not included in this analysis). This fact may indicate that this is a rare morphotype in almonds from Moncorvo, and it has probably been disseminated in the processor's plant by almonds originationg from Faro or from California, United States of America, resulting in cross-contamination of Moncorvo's almonds.

Samples from Faro were significantly more contaminated than those from Moncorvo ($P < 0.001$, Table AI.13). In fact, storage samples from Moncorvo were almost free of *Aspergillus* section *Flavi*, but almonds shelled at the processor were significantly more contaminated ($P < 0.001$). Once again, this may have to do with cross-contamination from the environment.

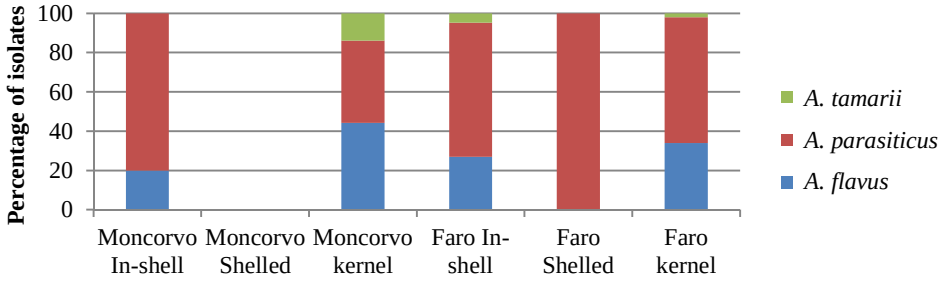

Figure 4.18 Percentage of isolates of each morphotype by almond's origin.

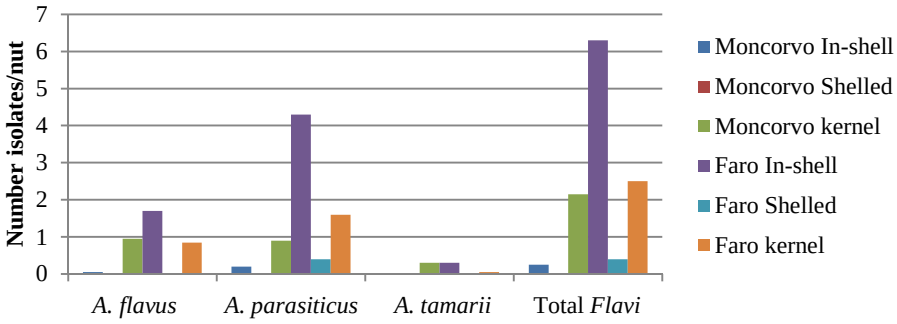

Figure 4.19 Average number of isolates by almonds' origin.

Figures 4.20 and 4.21 illustrate the percentage of each morphotype and the average number of isolates per nut in Faro samples for the various stages of processing. For almonds that are originally strongly contaminated by *Aspergillus Flavi*, processing can have an important role on the final level of contamination. By shelling at the processor, a significant contamination of the kernel occurred (shelled vs. kernel; $P < 0.001$; Table AI.13), i.e, propagules present at the shell or at the environmet entered in direct contact with the kernel. But, by submitting those kernels to oven-drying, those fungi were eliminated, rendering this final product a high quality in terms of fungal contamination.

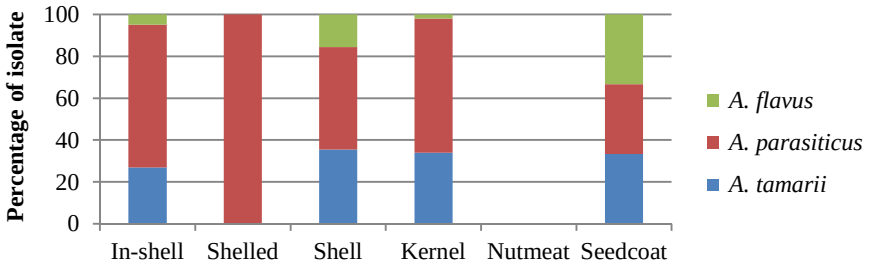

Figure 4.20 Percentage of isolates of each morphotype by type of processing, for Faro samples.

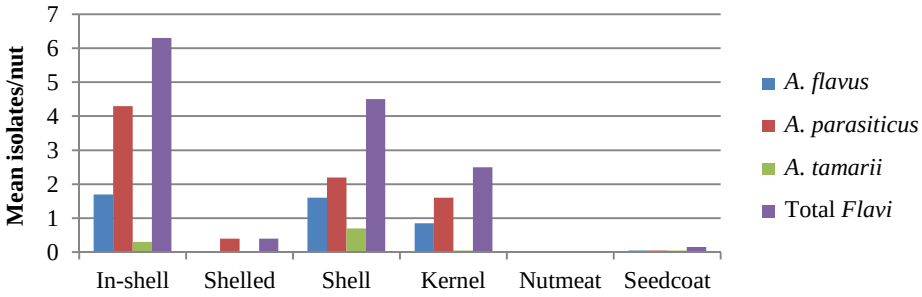

Figure 4.21 Average number of isolates of each morphotype by type of processing, for Faro samples.

4.1.3.2.1 Frequency of aflatoxigenic and non-aflatoxigenic strains

Table 4.19 lists the number and percentage of aflatoxigenic (AF^+) isolates by sample.

Table 4.19 Number of isolates and percentage of AF producers of each morphotype, by sample.

		Morphotypes											
		A. flavus			*A. parasiticus*			*A. tamarii*			Total		
Sample		#	AF^+	% AF^+	#	AF^+	% AF^+	#	AF^+	% AF^+	#	AF^+	% AF^+
Field													
Arv/07	in-shell	1	1	100.0	2	2	100.0	0			3	3	100.0
	shelled	0						0			0		
Arv/08	in-shell	2	2	100.0	11	11	100.0	1	0	0.0	14	13	92.9
	shelled	0						0			0		
Storage													
A1	in-shell	0			2	2	100.0	0			2	2	100.0
	shelled	0						0			0		
A2	in-shell	0			2	2	100.0	0			2	2	100.0
	shelled	0			1	1	100.0	0			1	1	100.0
A3	in-shell	0			4	4	100.0	0			4	4	100.0
	shelled	0						0			0		
B1	in-shell	0			1	1	100.0	0			1	1	100.0
	shelled	0						0			0		
B2	in-shell	3	1	33.3	6	6	100.0	0			9	7	77.8
	shelled	0						0			0		
B3	in-shell	1	0	0.0				0			1	0	0.0
	shelled	0						0			0		
Processor													
A	in-shell	8	0	0.0	11	11	100.0	0			19	11	57.9
	shelled	2	0	0.0	3	3	100.0	1	0	0.0	6	3	50.0
B	kernel	5	5	100.0	2	2	100.0	0			7	7	100.0
C1	kernel	7	1	14.3	9	9	100.0	1	0	0.0	17	10	58.8
C2	kernel	10	3	30.0	3	3	100.0	2	0	0.0	15	6	40.0
D1	kernel	11	3	27.3	20	20	100.0	5	0	0.0	36	23	63.9
D2	kernel	27	4	18.5	16	16	100.0	7	0	0.0	50	21	42.0
F1	in-shell	17	2	11.8	43	43	100.0	3	0	0.0	63	45	71.4
	shelled	0			4	4	100.0	0			4	4	100.0
F2	shell	16	6	37.5	22	22	100.0	7	0	0.0	45	28	62.2
	kernel	17	8	41.2	32	32	100.0	1	0	0.0	50	39	78.0
F3	nutmeat	0			0			0			0		
	seed coat	1	0	0.0	1	1	100.0	1	0	0.0	3	1	33.3
Total		128	36	28.1	195	195	100.0	29	0	0.0	352	231	65.6

AF production was detected in 65.6% (231 of the 352) of the isolates. The majority of the AF producing isolates belonged to the A. parasiticus morphotype, for which 100% of the isolates tested positive for aflatoxigenic ability. For the *A. flavus* morphotype, only 28.1% of the isolates (10.2% of total A. *Flavi*) were considered AF^+, whereas none of the isolates of the *A. tamarii* morphotype was identified as AF^+.

Relative to almonds' origin, the isolates were predominantly AF^+ in both Faro and Moncorvo (Table 4.20) samples, but there were significantly more AF^+ isolates in Faro samples ($P < 0.001$; Table AI.13).

Table 4.20 Number of isolates and percentage of AF producers of each morphotype, grouped by origin, stage of production and type of processing.

	Morphotypes									Total			
	A. flavus			*A. parasiticus*			*A. tamarii*						
	#	AF^+	% AF^+	#	AF+	% AF^+	#	AF^+	% AF^+	#	AF^+	% AF^+	AF^+/nut
Origin													
Moncorvo	77	20	26.0	93	93	100.0	17	0	0.0	187	113	60.4	0.27
Faro	51	16	31.4	102	102	100.0	11	0	0.0	165	118	71.5	1.31
Stage of production													
Field	3	3	100.0	13	13	100.0	1	0	0.0	17	16	94.1	0.09
Storage	4	1	25.0	16	16	100.0	0	-	-	20	17	85.0	0.14
Processor	121	32	26.4	166	166	100.0	28	0	0.0	315	198	62.9	0.90
Type of processing													
In-shell	32	6	18.8	82	82	100.0	4	0	0.0	118	88	74.6	0.49
Shelled	2	0	0.0	8	8	100.0	1	0	0.0	11	8	72.7	0.04
Shell	16	6	37.5	22	22	100.0	7	0	0.0	45	28	62.2	2.90
Kernel	77	24	31.2	82	82	100.0	16	0	0.0	175	106	60.6	0.96
Nutmeat	0	-	-	0	-		0	-	-	0	-	-	0.00
Seed coat	1	0	0.0	1	1	100.0	1	0	0.0	3	1	33.3	0.05
Total	128	36	28.1	195	195	100.0	29	0	0.0	352	231	65.6	0.45

Field and Storage samples were contaminated by a small number of *Aspergillus* section *Flavi*, which were mainly AF^+. On the other hand, isolates from processor samples were significantly more numerous ($P < 0.001$), but a smaller percentage of them was AF^+. The population of *A. flavus* from Field samples was 100% AF^+, but we have to consider the small number of isolates (3).

When considering samples by type of processing, in-shell and shelled almonds, which corresponded mainly to Field and Storage stages of production, were the ones with the highest percentage of AF^+ isolates, but they were weakly contaminated. The sample with the highest number of *Flavi* isolates per nut was the shell of Faro almonds (after being shelled by the processor), but the kernels resulting from this processing also had high levels of contamination. These were also the samples where the percentage of AF^+ *A. flavus* isolates was higher, but the difference relative to in-shell almonds was not significant ($P > 0.266$).

4.1.3.2.2 Associations among fungi

Associations between *Aspergillus* section *Flavi* contaminating almond samples is presented inTable 4.21. The association among the 3 morphotypes is low. Also, contamination with aflatoxigenic *A. flavus* does not seem to be associated to contamination with *A. parasiticus* and *A. tamarii* morphotypes.

Table 4.21 Associations between fungi, as determined by *Phi coefficient*.

Fungi	Observed	Predicted	Phi	P
A. flavus AF+ vs *A. parasiticus*	14	6.0	0.165	0.001
A. flavus AF+ vs *A. tamarii*	5	1.5	0.136	0.011
A. flavus vs *A. parasiticus*	44	17	0.352	0.000
A. flavus vs *A. tamarii*	16	4.1	0.287	0.000
A. parasiticus vs *A. tamarii*	17	5.8	0.236	0.000

The results presented in Table 4.21 correspond to the analysis of all samples in bulk. When considering the analysis using uniform groups of samples (by origin, by stage of production and by type of processing), *Phi coefficients* and P-values showed to be within the same magnitude (data not shown). The major exception was that the association between *A. flavus* and *A. parasiticus* in Faro samples was higher than for general analysis (*Phi* = 0.614, $P < 0.001$).

4.1.3.2.3 Associations between fungi and other variables

Tables 4.22 and 4.23 list the associations of *Aspergillus* section *Flavi* isolates (in terms of morphotypes and aflatoxigenicity) with the origin of the almonds and with the stage of production, respectively. All groups of isolates considered were more common on Faro samples than expected (and less common in Moncorvo), but the levels of association of the almonds' origin with the aflatoxigenicity of the isolates, or even with any of the morphotypes, were low to very low. On the other hand, the associations with the stage of production ranged between low to moderate (always determined with extremely high statistical certainty). In all cases, almonds collected at the processor were more

contaminated than expected, and the opposite was verified for field and storage samples. Isolates from section *Flavi* and aflatoxigenic isolates were only moderately associated with the stage of production.

Table 4.22 Associations between *Aspergillus* section *Flavi* and almond's origin, as determined by *Cramer's V*.

	Moncorvo		Faro			
Fungi	**Observed**	**Predicted**	**Observed**	**Predicted**	***Cramer's V***	**P**
AF^+	82	104.2	44	21.8	0.263	0.000
AF^+ *A. flavus*	15	23.2	13	4.8	0.184	0.000
Total *Flavi*	108	127.3	46	26.7	0.215	0.000
A. flavus	49	65.3	30	13.7	0.231	0.000
A. parasiticus	70	92.6	42	19.4	0.280	0.000
A. tamarii	16	22.3	11	4.7	0.145	0.001

Table 4.23 Associations between *Aspergillus* section *Flavi* and almond's stage of production, as determined by *Cramer's V*.

	Field		Storage		Processing			
Fungi	**Observed**	**Predicted**	**Observed**	**Predicted**	**Observed**	**Predicted**	***Cramer's V***	**P**
AF^+	14	43.6	15	29.1	97	53.3	0.399	0.000
AF^+ *A. flavus*	3	9.7	1	6.5	24	11.8	0.210	0.000
Total *Flavi*	15	53.3	17	35.5	122	65.2	0.487	0.000
A. flavus	3	27.3	3	18.3	73	33.4	0.429	0.000
A. parasiticus	11	38.8	15	25.8	86	47.4	0.370	0.000
A. tamarii	1	9.3	0	6.2	26	11.4	0.256	0.000

4.1.4 Aflatoxin contamination of almond samples

4.1.4.1 Method validation

Instrumental precision was assessed by analysing 4 standard solutions over 2 consecutive days. The HPLC conditions allowed the determination of the 4 AFs with retention times of approximately 15.5, 18, 20.5 and 24.5 minutes for AFG_2, AFG_1, AFB_2 and AFB_1, respectively (Figure 4.22).

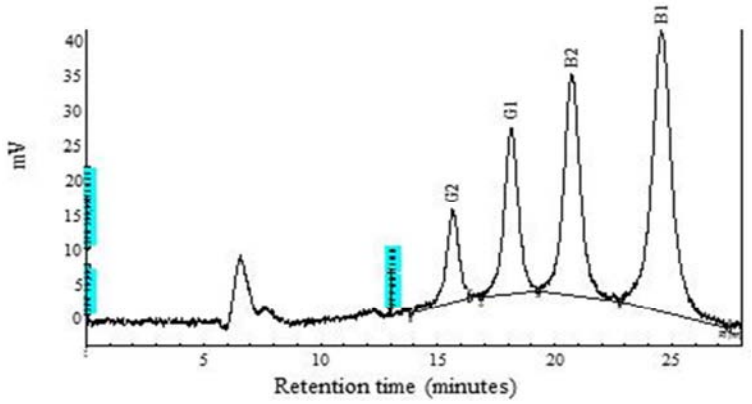

Figure 4.22 HPLC chromatogram for a standard solution of mixed AFs (3 µg/mL of AFB_1 and AFG_1, and 0.75 µg/mL of AFB_2 and AFG_2).

Calibration parameters are presented in Table 4.24. To evaluate the precision and recovery of the extraction method, blank samples were spiked at two different AFs concentrations; AFs were extracted and analysed in triplicate over 2 consecutive days. Clean chromatograms, with well resolved peaks corresponding to the 4 AFs, were obtained with spiked almonds (Figure 4.23). Chromatograms of AF-free almond samples showed no background interference from other substances. Results for Recovery, Relative Standard Deviation (RSD_r and RSD_{int}), LOD and LOQ are expressed in Table 4.25.

Table 4.24 Calibration parameters of instrumentation.

AF	Calibration curve	r^2	LOD (ng/mL)	LOQ (ng/mL)
B_1	y = 6E-07x + 3E-05	0.998	0.133	0.384
B_2	y = 2E-07x + 5E-06	0.998	0.028	0.083
G_1	y = 1E-06x + 2E-05	0.991	0.230	0.725
G_2	y = 7E-07x + 1E-05	0.993	0.059	0.175

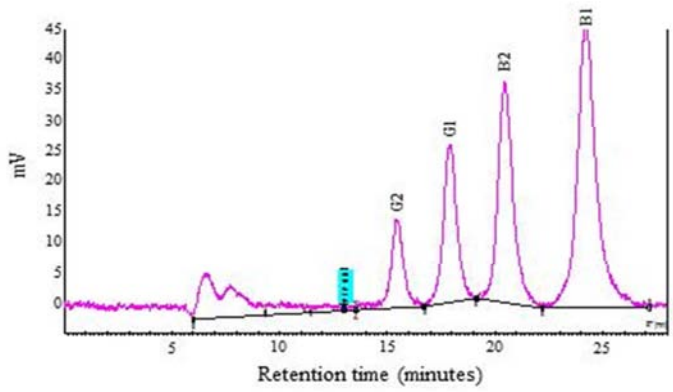

Figure 4.23 Chromatogram of AFs extracted from almonds spiked with 6 µg/kg of AFB_1 and AFG_1, and 1.5 µg/kg of AFB_2 and AFG_2.

Table 4.25 Performance and precision of AFs extraction method, for each AF.

	B_1		**B_2**		**G_1**		**G_2**	
	6 µg/kg	**2 µg/kg**	**1.5 µg/kg**	**0.5 µg/kg**	**6 µg/kg**	**2 µg/kg**	**1.5 µg/kg**	**0.5 µg/kg**
Day 1								
Recovery (%)								
Replicate 1	96.0	92.2	98.7	101.8	88.4	106.9	97.8	105.9
Replicate 2	90.6	91.4	95.2	112.7	80.4	108.5	94.8	112.8
Replicate 3	85.3	92.7	90.3	93.7	77.7	96.5	94.3	95.7
Mean	90.6	92.1	94.7	102.7	82.2	104.0	95.6	104.8
SD	5.35	0.66	4.22	9.5	5.58	6.53	1.91	8.57
RSD_r (%)	5.9	0.7	4.5	9.3	6.8	6.3	2.0	8.2
Day 2								
Recovery (%)								
Replicate 1	91.1	114.7	98.0	96.5	88.6	102.0	89.5	103.9
Replicate 2	98.7	96.0	100.2	91.3	91.3	104.7	95.2	103.2
Replicate 3	100.4	93.7	95.9	85.6	89.9	99.0	85.7	112.0
Mean	96.7	101.5	98.0	91.1	89.9	101.9	90.1	106.4
SD	5.0	11.5	2.1	5.5	1.4	2.9	4.7	4.9
RSD_r (%)	5.1	11.3	2.2	6.0	1.5	2.8	5.3	4.6
Mean Recovery (%)	93.7	96.8	96.4	96.9	86.0	103.0	92.9	105.6
MD_{int}*	4.3	6.6	2.3	8.2	5.4	1.5	3.9	1.1
RMD_{int}* (%)	4.6	6.9	2.4	8.5	6.3	1.4	4.2	1.1
LOD (µg/kg)	0.266		0.057		0.461		0.119	
LOQ (µg/kg)	0.768		0.166		1.451		0.350	
Recommended range								
Recovery (%)	70-110		50-120		70-110		50-120	
RSD_r (%)	22	27	28	33	22	27	28	33
RSD_R(%)	34	41	42	47	34	41	42	47

* Because there are only two values for mean recovery to calculate Intermediate Precision, mean deviation (MD) and relative mean deviation (RMD) substitute the commonly used standard deviation (SD) and relative standard deviation (RSD).

** As recommended by the Codex Committee on Contaminants in Foods (CCCF, 2008), based on the equations determined by Thompson (2000) and Horwitz & Albert (2006), and adopted by the European Regulation No 178/2010.

4.1.4.2 Sample analysis

Results of AFs detection and quantification on Portuguese almonds, which were undertaken on nutmeat only, are listed in Table 4.26. Figure 4.24 shows AFs chromatograms of naturally contaminated samples. AFs were only detected on sample A1 from storage. The level of contamination with AFB_1 and total AFs did not reach the current maximum levels legally set either for almonds to be sorted and processed or for ready to eat almonds.

Table 4.26 Aflatoxin contamination of almond samples.

	AFs (µg/kg)				
Sample	B_1	B_2	G_1	G_2	Total
Field					
P1/2007	< LOD	< LOD	< LOD	< LOD	< LOD
P2/2007	< LOD	< LOD	< LOD	< LOD	< LOD
P3/2007	< LOD	< LOD	< LOD	< LOD	< LOD
P1/2008	< LOD	< LOD	< LOD	< LOD	< LOD
P2/2008	< LOD	< LOD	< LOD	< LOD	< LOD
P3/2008	< LOD	< LOD	< LOD	< LOD	< LOD
Storage					
A1	4.8	0.17	< LOD	< LOD	4.97
A2	< LOD	< LOD	< LOD	< LOD	< LOD
A3	< LOD	< LOD	< LOD	< LOD	< LOD
B1	< LOD	< LOD	< LOD	< LOD	< LOD
B2	< LOD	< LOD	< LOD	< LOD	< LOD
B3	< LOD	< LOD	< LOD	< LOD	< LOD
Processor					
A	< LOD	< LOD	< LOD	< LOD	< LOD
B	< LOD	< LOD	< LOD	< LOD	< LOD
C1	< LOD	< LOD	< LOD	< LOD	< LOD
C2	< LOD	< LOD	< LOD	< LOD	< LOD
D1	< LOD	< LOD	< LOD	< LOD	< LOD
D2	< LOD	< LOD	< LOD	< LOD	< LOD
F1	< LOD	< LOD	< LOD	< LOD	< LOD
F2	< LOD	< LOD	< LOD	< LOD	< LOD
F3	< LOD	< LOD	< LOD	< LOD	< LOD
Maximum levels*					
To be processed	12	-	-	-	15
Ready to eat	8	-	-	-	10

* Commission Regulation (EU) No 165/2010 of 26 February 2010, setting maximum levels for certain contaminants in foodstuffs as regards AFs

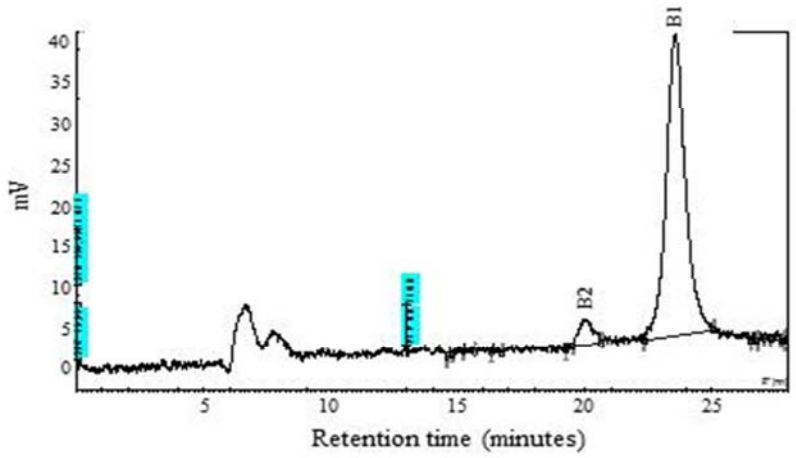

Figure 4.24 Chromatogram of AFs extracted from almonds naturally contaminated with AFB_1 and AFB_2 (storage sample *A1*).

4.2 Molecular differentiation of aflatoxigenic and non-aflatoxigenic isolates

Thirty-five isolates were analysed for the presence of genes *aflD* and *aflQ* (PCR), as well as for their expression (RT-PCR) under conditions that where inducive and non-inducive of AFs production. Multiplex PCR was developed on genomic DNA with 3 primer pairs: tub1-F/tub1-R for the *tub1* gene (internal amplification control); ord1-gF/ord1-gR for *aflQ*; and nor1-F/nor1-R for *aflD*. PCR on cDNA (RT-PCR) involved an extra pair of primers for *aflQ*, ord1-cF/ord1-cR. Figure 4.25 illustrates the expected results for each primer pair amplification, for RT-PCR (uniplex amplification on 2 isolates) and for PCR (multiplex amplification on one isolate).

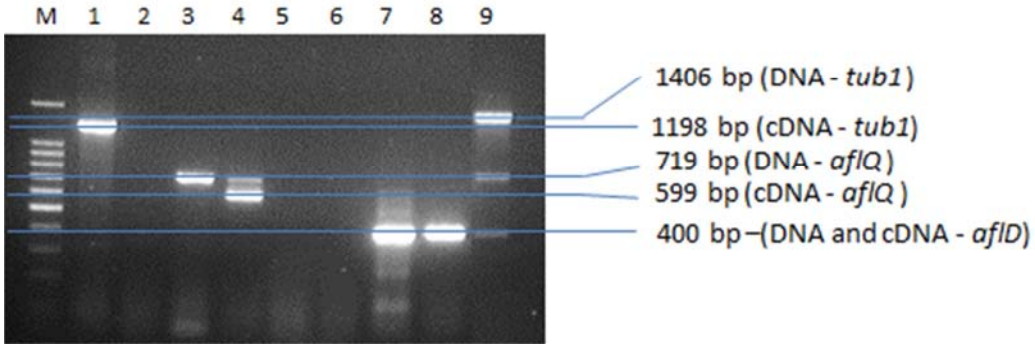

Figure 4.25 Band sizes expected from PCR and RT-PCR reactions. M - 100 bp DNA ladder (Promega); 1 and 2 - RT-PCR with tub1-F/tub1-R (MUM 10.202 [AF^-] and MUM 10.225 [AF^+], respectively); 3 and 4 - RT-PCR with ord1-gF/ord1-gR; 5 and 6 - RT-PCR with ord1-cF/ord1-cR; 7 and 8 - RT-PCR with nor1-F/nor1-R; 9 - PCR with tub1-F/tub1-R, ord1-gF/ord1-gR and nor1-F/nor1-R (MUM 10.225 [AF^+]).

When using the primer pair ord1-gF/ord1-gR proposed by Sherm et al. (2005) for the amplification from RNA, we detected a band corresponding to the size of the expected amplification from DNA (719 bp) in all isolates and another band corresponding to the expected amplification from RNA (599 bp) in the AF^+ isolates. To exclude the possibility of DNA contamination of the RNA, we ran a PCR for various RNA samples and no amplification occured (data not shown), confirming the efficacy of the DNase treatment. Furthermore, if any DNA contamination was to be present, two bands for the *tub1* gene would appear at sizes 1406 bp (DNA) and 1198 bp (RNA). Only the smaller band was present, further confirming the purity of the RNA samples. The primer pair

ord1-gF/ord1-cR proposed by Degola et al. (2007) did not produce any amplicon, even when tested in a wider number of isolates. We later came to the knowledge that these primers's sequences were wrongly published, thus justifying the inexistence of amplification.

Figures 4.26 and 4.27 illustrate the multiplex PCR and RT-PCR results for various AF^+ and AF^- isolates, indicating the expected band sizes. PCR and RT-PCR results for all isolates tested are presented in Table 4.27.

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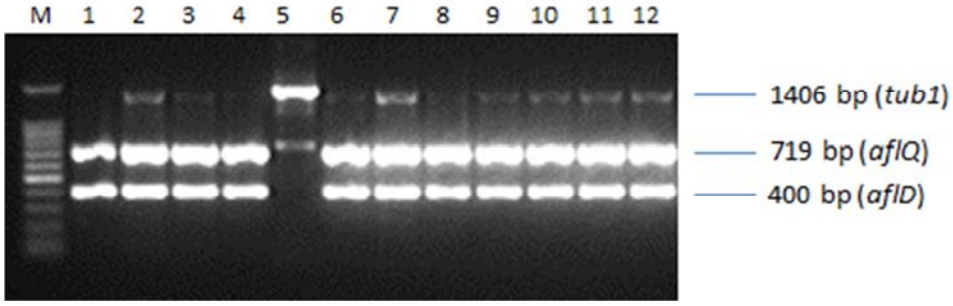

Figure 4.26 Agarose gel electrophoretic pattern of PCR products. M - 100 bp DNA ladder (Promega); 1 - 07AAsp05 (AF^+); 2 - MUM 10.200 (AF^+); 3 - MUM 10.201 (AF^+); 4 - 08AAsp34 (AF^+); 5 - MUM 10.202 (AF^-); 6 - MUM 10.225 (AF^+); 7 - MUM 10.203 (AF^+); 8 - 08AAsp38 (AF^+); 9 - 08AAsp39 (AF^+); 10 - 08AAsp72 (AF^+); 11 - 08AAsp76 (AF^-); 12 - 08AAsp77 (AF^-); 13 - 08AAsp83 (AF^+); 14 - MUM 10.220 (AF^-).

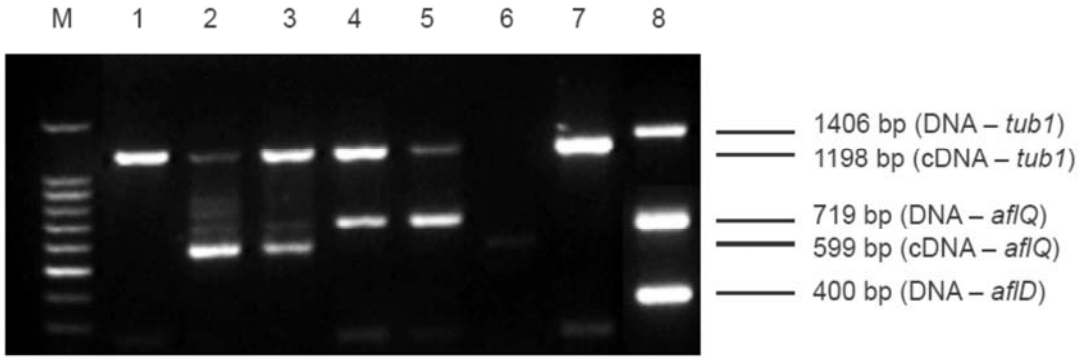

Figure 4.27 Agarose gel electrophoretic pattern of RT-PCR products. M - 100 bp DNA ladder (Promega); 1 - MUM 10.202 (AF^-); 2 - MUM 10.225 (AF^+);3 - 08AAsp72 (AF^+); 4 - 08AAsp76 (AF^-); 5 - 08AAsp77 (AF^-); 6 - 08AAsp83 (AF^+); 7 - MUM 10.220 (AF^-); 8 - DNA-PCR control.

Table 4.27 Presence of genes *aflD* and *aflQ* (PCR) and their expression (RT-PCR) in *Aspergillus* section *Flavi* isolates.

			Gene presence (PCR)		Gene expression (RT-PCR)	
Isolate code	Classification	AFB_1	*aflD*	*aflQ*	*aflD*	*aflQ*
07AAsp05	*A. parasiticus*	+	+	+	+	+
MUM 10.200	*A. flavus*	+	+	+	+	+/-
MUM 10.201	*A. parasiticus*	+	+	+	+	+
08AAsp34	*A. parasiticus*	++	+	+	+	+
MUM 10.202	*A. flavus*	-	-	+	-	-
MUM 10.225	*A. parasiticus*	++	+	+	n.d.	+
MUM 10.203	*A. flavus*	+/-	+	+	+	-
08AAsp38	*A. parasiticus*	++	+	+	+	+
08AAsp39	*A. parasiticus*	++	+	+	+	+
MUM 10.204	*A. flavus*	+/-	+	+	+	-
08AAsp43	*A. flavus*	-	+	+	+	-
08AAsp66	*A. parasiticus*	++	+	+	+	+
MUM 10.205	*A. parasiticus*	+	+	+	n.d.	+/-
08AAsp68	*A. parasiticus*	++	+	+	n.d.	+
08AAsp72	*A. parasiticus*	++	+	+	n.d.	+
08AAsp76	*A. flavus*	-	+	+	n.d.	-
08AAsp77	*A. flavus*	-	+	+	n.d.	-
08AAsp83	*A. parasiticus*	++	+	+	n.d.	+
08AAsp101	*A. parasiticus*	++	+	+	n.d.	+
08AAsp103	*A. parasiticus*	++	+	+	n.d.	+
08AAsp105	*A. flavus*	-	+	+	n.d.	-
08AAsp108	*A. parasiticus*	++	+	+	n.d.	+
08AAsp109	*A. flavus*	-	+	+	n.d.	-
08AAsp110	*A. parasiticus*	++	+	+	n.d.	+
08AAsp111	*A. parasiticus*	++	+	+	n.d.	+
08AAsp112	*A. flavus*	-	+	+	n.d.	-
08AAsp113	*A. flavus*	-	+	+	n.d.	-
08AAsp115	*A. flavus*	-	+	+	n.d.	-
MUM 10.206	*A. flavus*	+/-	+	+	n.d.	-
08AAsp117	*A. parasiticus*	++	+	+	n.d.	+
08AAsp158	*A. parasiticus*	+++	+	+	n.d.	+
Controls						
MUM 92.01	*A. flavus*	+/-	+	+	+	-
MUM 92.02	*A. parasiticus*	++	+	+	+	+
MUM 10.220	*A. flavus*	-	-	-	-	-
01UAs55	*A. flavus*	-	-	-	-	-

n.d. not determined
+: strong signal; +/-: weak signal; -: no signal detected

From Portuguese almond isolates (field isolates), and considering the presence of both genes under study (*aflD* and *aflQ*), only MUM 10.202 (AF^-) was negative for the *aflD* amplicon, whereas 01UAs55 (AF^-) and MUM 10.220 (AF^-), herein used as negative controls (lab strains), showed no amplification for both *aflD* and *aflQ*.

RT-PCR for *aflQ* showed a confusing, but consistent, band pattern. All isolates tested for *aflD* expression gave a positive result, even for AF^- isolates. The only exceptions were, as expected, the isolates negative for *aflD* presence (MUM 10.202, 01UAs55 and MUM 10.220). *aflQ* expression was tested for all isolates. All strong AFB_1 producers showed an amplicon near 600 bp, corresponding to the expected *aflQ* mRNA. This fragment was not detected in AF^- isolates. Among the weak producers of AFB_1, isolates MUM 10.200 and MUM 10.205 showed a weak expression signal, and isolates MUM 10.203, MUM 10.204, MUM 10.206 and MUM92.01 showed no signal for *aflQ* expression.

4.3 *Aspergillus* section *Flavi*

4.3.1 Isolates characterisation and identification

Since species identification within section *Flavi* is very difficult to attain, our isolates were characterised using a polyphasic approach. This approach involved a preliminary morphological identification, which resulted in the separation of the isolates into morphotypes, followed by the characterisation of their extrolite profile in respect to AFs (B_1, B_2, G_1 and G_2) and CPA production (chemotypes). The combination of morphological features and extrolite profile resulted in the phenotypic identification of the isolates. A restricted group of isolates, selected from the different phenotypes identified, were further characterised by genetic sequence analysis and protein mass spectral analysis.

Isolates were characterised by comparison to ex-type and authentic strains of species belonging to the section.

4.3.1.1 Phenotypic analysis

In our survey, we isolated 352 fungi belonging to section *Flavi*. All isolates showing colony colour on CYA in a shade of green or bronze/brown were tested on AFPA. Those isolates simultaneously showing green colony on CYA and cadmium orange (and, in rare cases, cream) on AFPA, or brown colony on CYA and brown reverse on AFPA were confirmed as *Aspergillus* section *Flavi*. Isolates were subjected to a batch of morphological and physiological analysis for species identification: colony colour and diameter on CYA, MEA, CY20S and CYA at 42 ºC; conidia morphology and ornamentation; aspergilli head seriation; sclerotia morphology and size, fluorescence on CAM; and production of CPA and AFs. Results for all isolates are shown in detail in Appendix AII.

As a result of preliminary morphological characterisation, isolates were divided into three morphotypes, based on colony colour on CYA, conidia ornamentation and colony reverse colour on AFPA. Figure 4.28 shows the characteristics typical of each of the morphotypes. One group of 29 isolates (8.2%) was very distinctive from the others, and

was characterised by bronze to dark-brown velvety colonies on CYA with conspicuously roughened thick walls, and colonies with a dark-brown reverse on AFPA (Figure 4.28A). Because they were very similar to *A. tamarii*, they were grouped as '*A. tamarii* morphotype'. The remaining isolates were less distinctive between each other. They had yellow–green to dark-green colonies on CYA and smooth to rough conidia. They also had a cadmium orange- or, less frequently, cream-coloured reverse on AFPA. These isolates were further divided into 2 morphotypes: 127 isolates (36.1%) resembled *A. flavus*, with yellow-green colonies and smooth conidia, and were included in the '*A. flavus* morphotype' (Figure 4.28B). All these isolates had orange reverse on AFPA. Isolates similar to *A. parasiticus*, with olive-green to dark-green colonies and rough conidia were included in the '*A. parasiticus* morphotype' (196 isolates, 55.7%; Figure 4.28C). Almost all isolates showed the typical orange reverse on AFPA, but six isolates showed cream reverse, a characteristic that has been associated with *A. oryzae* and *A. arachidicola*, but not to *A. parasiticus*.

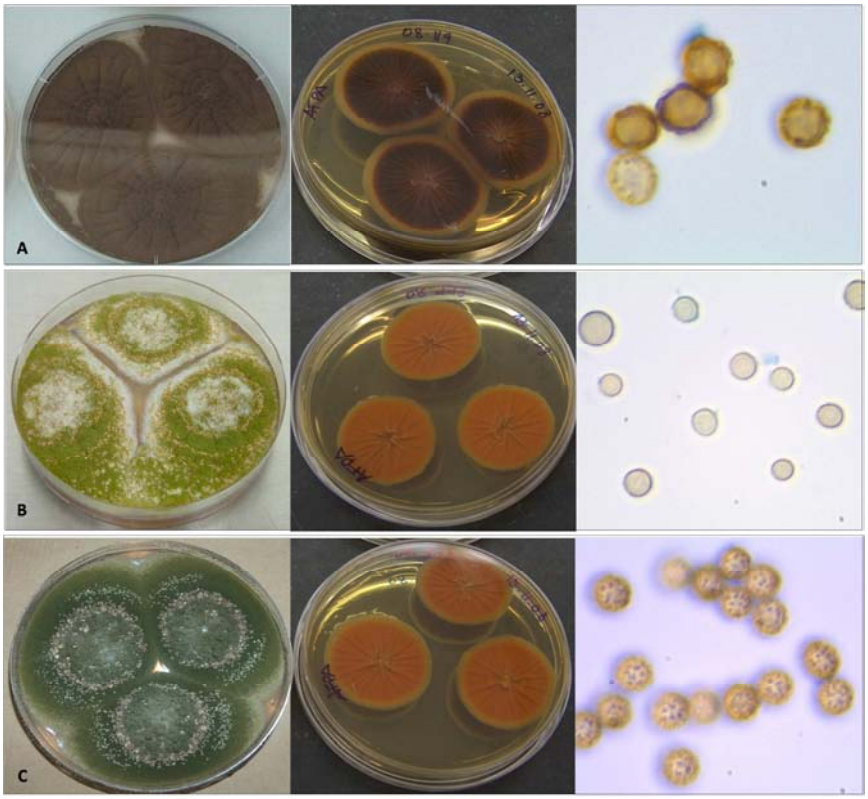

Figure 4.28 Morphological characteristics used for morphotype differentiation. A) morphotype *A. tamarii*; B) morphotype *A. flavus*; and C) morphotype *A. parasitius*. From left to right: colony colour on CYA; colony reverse on AFPA; conidia ornamentation.

Albeit this separation, isolates within morphotypes showed some morphological variance when cultured under the same conditions. In both *A. flavus* and *A. parasiticus* morphotypes we could observe velvety and floccose colonies, as well as sclerotia producers and non-producers. Also, colony colour on CYA showed various shades of green within *A. flavus* and *A. parasiticus* morphotypes (Figure 4.29), as well as various degrees of brown in the *A. tamarii* morphotype. Parallel to that, each isolate also showed high plasticity depending on culture conditions (Figure 4.30).

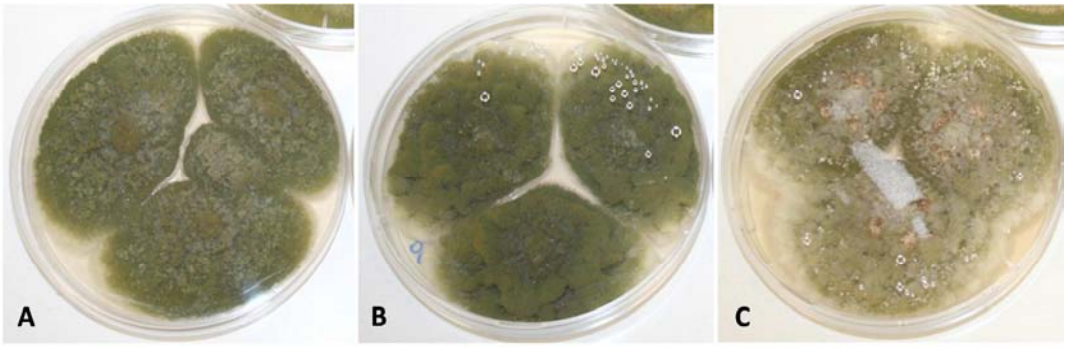

Figure 4.29 Isolates 09AAsp01 (A), 09AAsp04 (B) and 09AAsp06 (C) identified as *A. parasiticus* morphotype, showing different textures and different shades of dark-green.

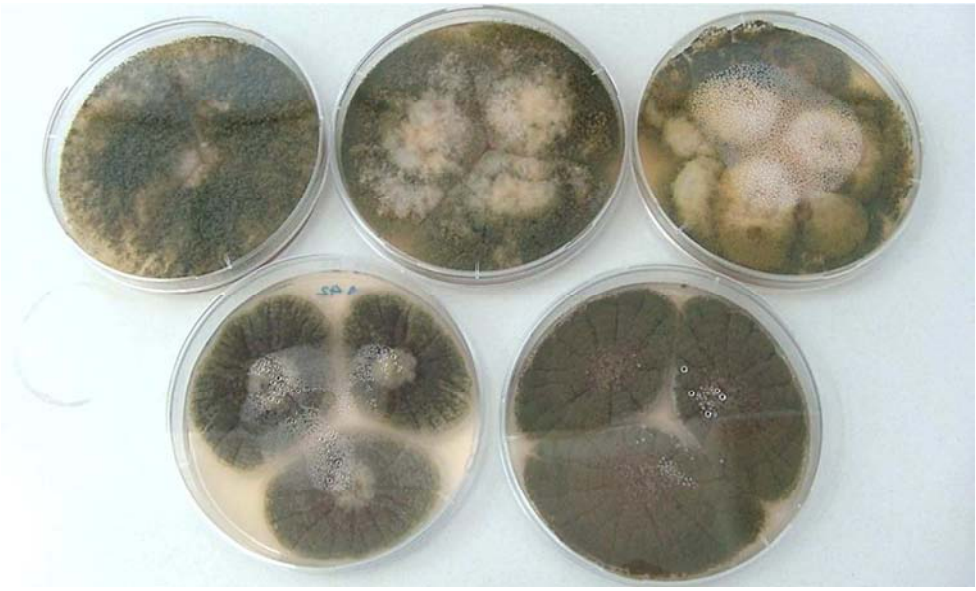

Figure 4.30 Different morphologies shown by the isolate MUM 10.209 (*A. flavus* morphotype), when grown under different culture conditions. From top left to bottom right: growth on CY20S, CYA, MEA, CYA42, CYA37.

All isolates showed good growth on the various media at 25 ºC, consistently reaching diameters > 7 cm after 7 days of incubation. Sclerotia were all of the L-type (> 400 µm), round to elongate, and were present in 80.7% of the *A. flavus* isolates and in 52.6% of the *A. parasiticus* isolates. Thirty-five per cent of the *A. tamarii* isolates showed rare white elongate sclerotia. As to conidial head seriation, 63% of the *A. flavus* morphotype isolates were predominantly biseriate, whereas 94.1% of the *A. parasiticus* morphotype were predominantly uniseriate.

Isolates were characterised on the basis of mycotoxigenic profile, namely AFBs, AFGs and CPA production ability. By associating morphotypes to chemotypes, isolates were grouped into phenotypes (Table 4.28). Interestingly, we observed that each phenotype was associated to a characteristic chromatographic pattern for both AFs and CPA, and that the chromatograms were helpful in phenotype determination. So, chromatographic patterns for AFs and CPA were included in the analysis as extra features. Figures 4.31 and 4.32 show, respectively, examples of CPA and AFs chromatograms characteristic of the different chemotypes.

Table 4.28 Morphotypes, chemotypes and phenotypes (resulting from the association between morphotype and chemotype) of the *Aspergillus Flavi* isolates.

Morphotype	Chemotype			Phenotype	# isolates
	CPA	AFBs	AFGs		
A. flavus	-	-	-	*A. flavus* I	23
	+	-	-	*A. flavus* II	69
	+	+	-	*A. flavus* III	32
	+	+/-	+/-	*A. flavus* IV	2
A. parasiticus	-	++	+	*A. parasiticus* I	169
	(?)	+	++	*A. parasiticus* II	14
	+	++	+	*A. parasiticus* III	5
	-	+	-	*A. parasiticus* IV	8
A. tamarii	+	-	-	*A. tamarii*	29

-: not detected
+: detected
++: detected in high levels
+/-: detected in very low levels
(?): unable to determine

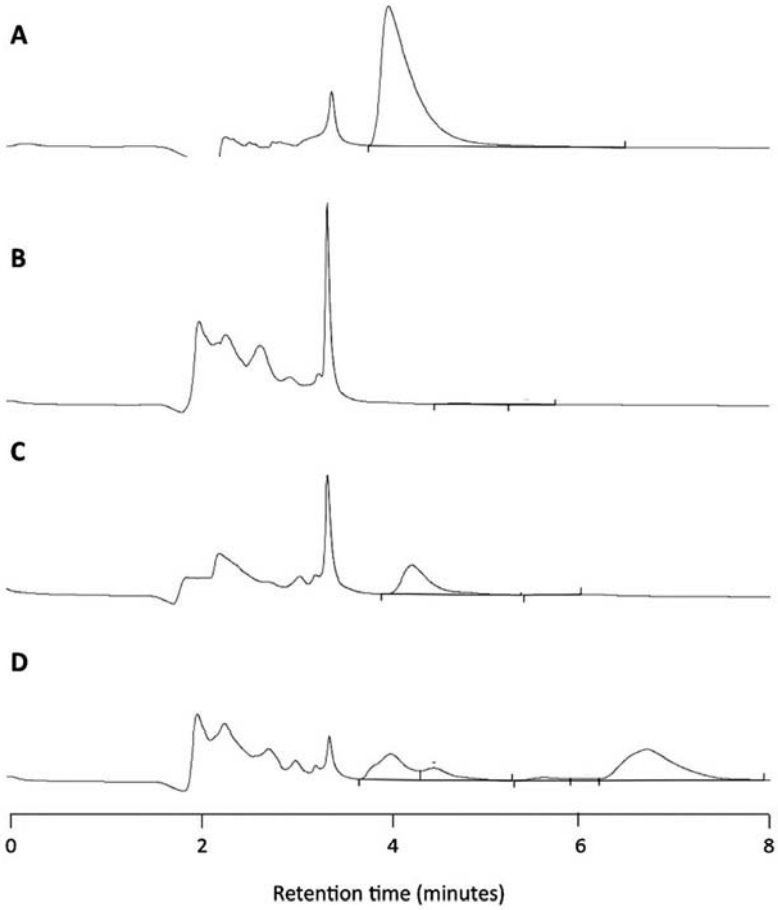

Figure 4.31 HPLC chromatograms of CPA production analysis. A) CPA standard; B) *A. flavus* I and *A. parasiticus* I phenotypes (CPA^-); C) *A. flavus* II, *A. flavus* III, *A. flavus* IV and *A. parasiticus* III phenotypes (CPA^+); D) *A. parasiticus* II phenotype (CPA^-).

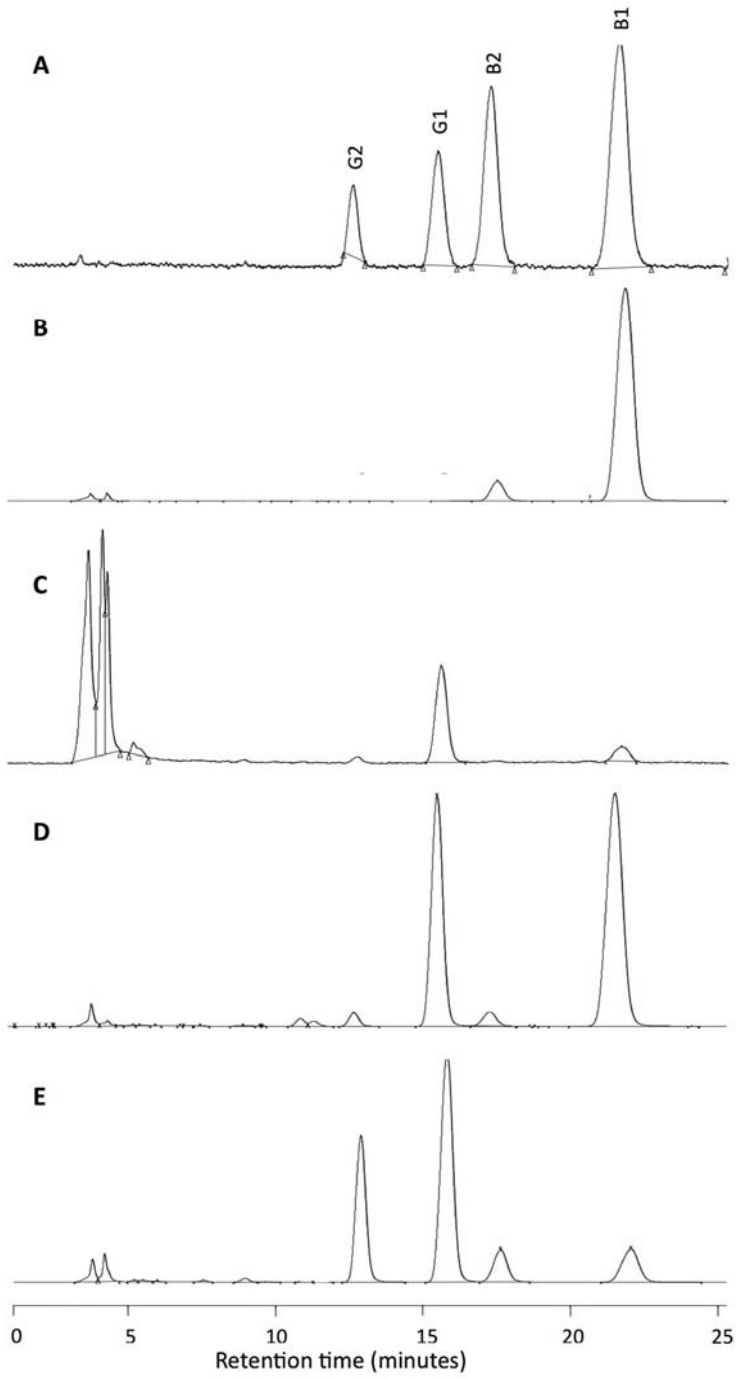

Figure 4.32 HPLC chromatograms of AFs production analysis. A) Standard solution of mixed AFG_2, AFG_1, AFB_2 and AFB_1; B) *A. flavus* III and *A. parasiticus* IV phenotypes (AFB^+ and AFG^-); C) *A. flavus* IV phenotype ($AFB^{+/-}$ and $AFG^{+/-}$); D) *A. parasiticus* I and *A. parasiticus* III phenotypes (AFB^+ and AFG^+); E) *A. parasiticus* II phenotype (AFB^+ and AFG^{++}).

The *A. tamarii* morphotype showed a highly consistent profile, with all isolates producing CPA but not AFs and exhibiting similar HPLC chromatograms. On the other hand, the *A. flavus* morphotype was more variable and four chemotypes were identified: 18% of the isolates were found to be non-toxigenic for both AFs and CPA under the tested conditions, 54% produced CPA only and 26% produced AFBs and CPA. Two isolates had the unusual characteristic of producing CPA and small amounts of AFBs and AFGs.

The *A. parasiticus* morphotype also varied in terms of extrolite profile, with four chemotypes identified. The majority of the isolates (86%) had the typical profile of *A. parasiticus*, i.e, were strong producers of AFBs and AFGs and did not produce CPA. Unexpectedly, 8 isolates (4%) were found to be AFGs negative, showing AFs chromatograms similar to those from aflatoxigenic *A. flavus*. Another group of isolates (7%) showed the atypical characteristic of producing more AFGs than AFBs. These isolates also showed a particularly different CPA chromatogram where the determination of CPA production was dubious due to various peaks near the CPA retention time (Figure 4.31D).

AFs production was also tested on CAM, for which fluorescence production and colour were recorded. Presence of fluorescence was correlated with AFs production at a very high level (*Cramer's V* = 0.988, P = 0.000), since only two isolates producing AFs did not show fluorescence on CAM. These two isolates, MUM 10.203 and 08AAsp41, showed to be weak AFs producers, as determined by HPLC. Whenever present, fluorescence assumed different colours: blue, violet or green. Isolates producing AFBs only (AFB^+) also produced a violet fluorescence on CAM; isolates AFB^+ and AFG^+ generally produced a blue fluorescence, the exception being those isolates producing higher levels of AFGs than AFBs, which in some cases produced a green fluorescence.

Twenty-four of the 352 isolates characterised phenotypically (4 lab strains and 20 field isolates) were selected to construct a dendrogram based on phenotypic similarity. These isolates were representative of the various phenotypes and, in some cases, even if belonging to the same phenotype, they were selected because of a special characteristic that differentiated them from the reference strain (e.g. different shade of green or floccose texture of colony on CYA). Only colony colour on CYA, conidia ornamentation, colony reverse on AFPA and mycotoxigenic profile (including chromatographic patterns) were considered for the phenotypic cluster analysis. Table 4.29 lists the 24 isolates used in this

analysis and the corresponding phenotypic features. The phenotypic dendrogram is presented in Figure 4.33.

Table 4.29 List of strains used for analysis of phenotypic similarity, with details on the features used for the analysis.

Code	Conidia on CYA	Colony colour on CYA	Reverse on AFPA	AFGs on YES	AFBs on YES	CPA on CYA	Phenotypic ID
Control strains							
MUM 92.01	smooth	yellow-green	orange	-	+/-	+	*A. flavus* III
MUM 92.02	rough	dark-green	orange	+	+	-	*A. parasiticus* I
MUM 09.03	rough	brown	brown	-	-	+	*A. tamarii*
MUM 10.220	smooth	yellow-green	orange	-	-	-	*A. flavus* I
Field isolates							
MUM 10.200	smooth	yellow-green	orange	-	++	+	*A. flavus* III
MUM 10.201	rough	dark-green	orange	+	+	-	*A. parasiticus* I
MUM 10.202	smooth	yellow-green	orange	-	-	-	*A. flavus* I
MUM 10.203	smooth	yellow-green	orange	-/+	+/-	+	*A. flavus* IV
MUM 10.204	smooth	yellow-green	orange	-	+/-	+	*A. flavus* III
MUM 10.205	rough	dark-green	orange	++	+	(?)	*A. parasiticus* II
MUM 10.206	smooth	yellow-green	orange	-	+	+	*A. flavus* III
MUM 10.207	rough	yellow-green	orange	-	++	+	*A. flavus* III
MUM 10.208	rough	dark-green	orange	++	++	+	*A. parasiticus* III
MUM 10.209	smooth	yellow-green	orange	-	++	+	*A. flavus* III
MUM 10.210	rough	dark-green	cream	++	++	-	*A. parasiticus* I
MUM 10.211	rough	dark-green	cream	++	+	(?)	*A. parasiticus* II
MUM 10.212	rough	dark-green	orange	-	++	-	*A. parasiticus* IV
MUM 10.213	rough	dark-green	cream	++	++	-	*A. parasiticus* I
MUM 10.214	rough	dark-green	orange	++	+	(?)	*A. parasiticus* II
MUM 10.215	rough	dark-green	cream	+	++	-	*A. parasiticus* I
MUM 10.216	rough	dark-green	orange	+	++	-	*A. parasiticus* I
MUM 10.217	rough	brown	brown	-	-	+	*A. tamarii*
MUM 10.218	smooth	yellow-green	orange	-	+	+	*A. flavus* III
MUM 10.219	rough	dark-green	orange	++	++	+	*A. parasiticus* III

-: not detected
+: detected
++: detected in high levels
+/-: detected in very low levels
(?): unable to determine

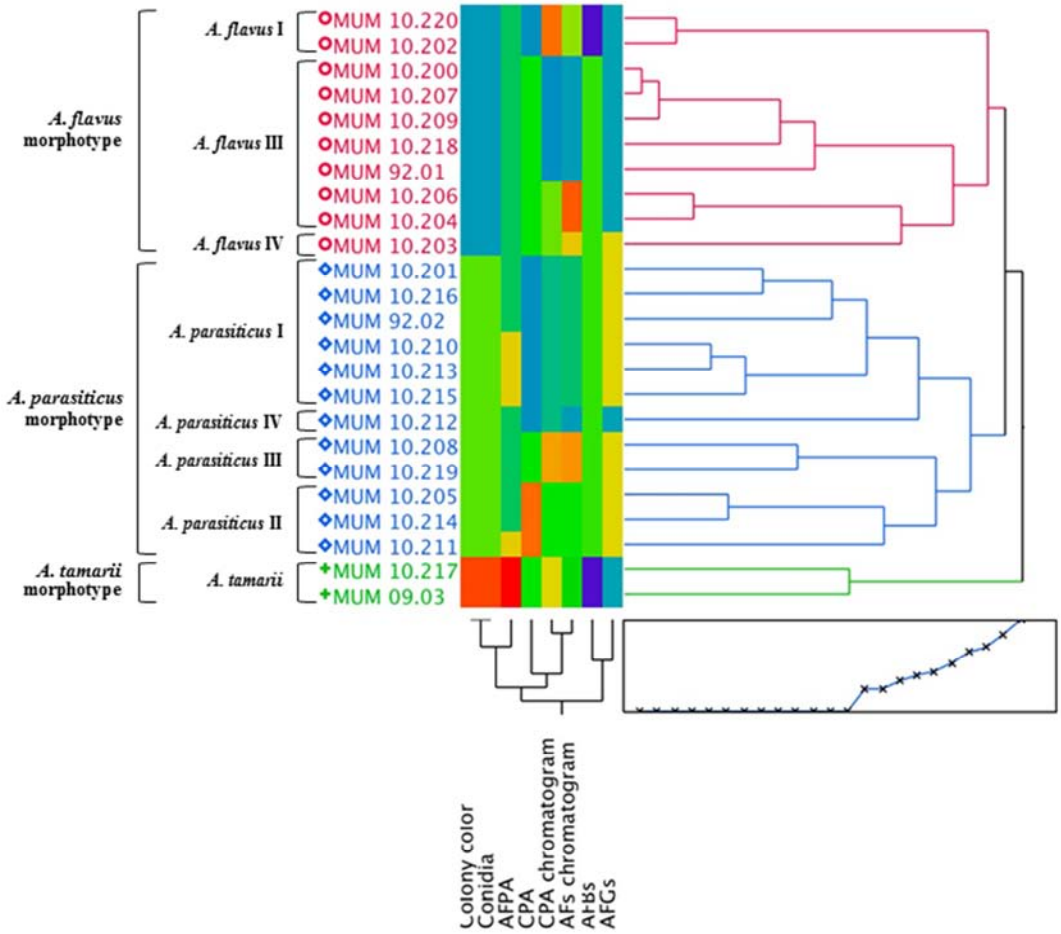

Figure 4.33 Dendrogram of relatedness between section *Flavi* isolates based on the analysis of 8 phenotypic characters: colony colour on CYA; conidia surface; reverse on AFPA; AFB production; AFG production; CPA production; chromatographic pattern of AFs; and chromatographic pattern of CPA. The colour map indicates the differences between isolates for each of the features.

As expected, three major clusters corresponding to the three morphotypes were created, with *A. tamarii* being more distantly related to *A. flavus* and *A. parasiticus*. Within each major cluster, sub-clusters are easily related to the various phenotypes. It is noteworthy that isolates MUM 10.204 and MUM 10.206, although included in the *A. flavus* III phenotype, grouped closer to MUM 10.203 than to the other *A. flavus* III isolates, mostly because they share common CPA chromatograms.

4.3.1.2 Molecular analysis

For the molecular analysis, two regions of the genome were analysed, namely part of the rRNA gene and part of the calmodulin gene, in order to determine which was more informative for the phylogenetic analysis. For this matter, 11 of the previously referred 24 isolates were submitted to sequence analysis of both regions. These 11 isolates included individuals from *A. flavus* I, *A. flavus* III, *A. flavus* IV, *A. parasiticus* I, *A. parasiticus* II and *A. tamarii* phenotypes.

The phylogenetic relationship among our isolates was analysed by various inference methods: Neighbour-Joining (NJ), Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI). For the different methods, various analytical parameters were tested. The analyses resulted in trees with similar topologies and similar confidence levels (bootstrap values for NJ, MP and ML, and posterior probabilities for BI). Hall (2005) and Ogden & Rosenberg (2006) have stated that BI is slightly more accurate than ML, that MP is next, and that NJ is the least accurate approach. For that reason, results presented in this section are those obtained by Bayesian Inference. Trees obtained by the other methods are presented in Appendix III.

Figures 4.34 and 4.35 represent the consensus trees obtained by Bayesian Inference for the ITS region and the calmodulin gene, respectively. The values on the branches correspond to the posterior probabilities of each internal node, which reflect the level of confidence for each clade. No outgroup was defined in these analyses.

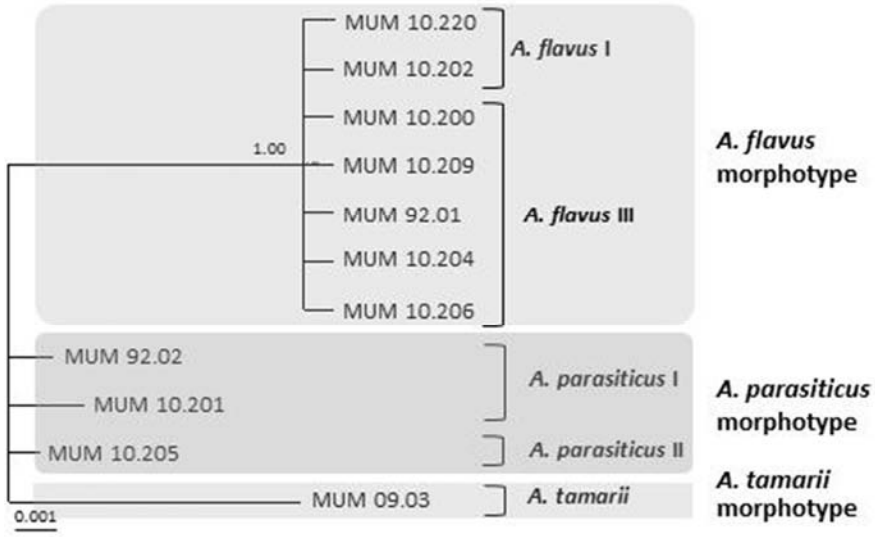

Figure 4.34 Consensus tree for the ITS region obtained by Bayesian Inference. Node labels represent the posterior probabilities of each internal node.

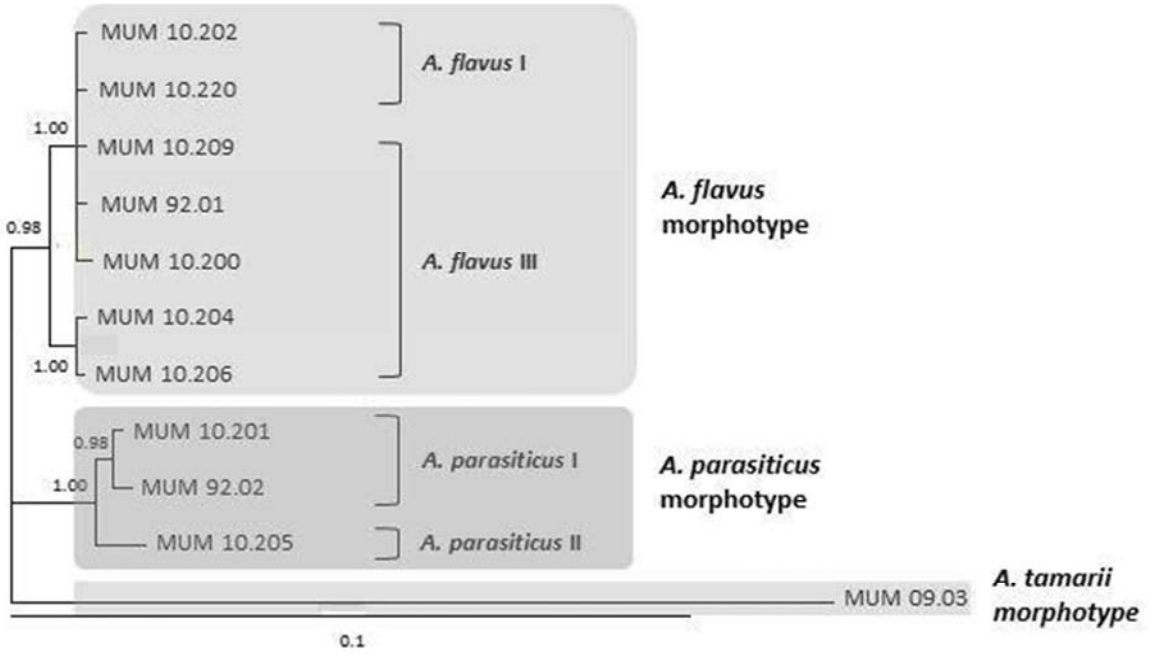

Figure 4.35 Consensus tree for the calmodulin gene obtained by Bayesian Inference. Node labels represent the posterior probabilities of each internal node.

For the analysis of the calmodulin gene, 730 sites were analysed, of which 16 (2.2%) were phylogenetically informative. The ITS dataset included 908 sites, with 6 (0.66%) sites being considered informative. Because of the limited number of isolates tested and the significant proximity between them, the observed extremely low number of informative

sites was expectable. The ITS region allowed to discriminate only to a level matching that of the morphotypes, and created 3 clades corresponding to the *A. flavus*, *A. parasiticus* and *A. tamarii* morphotypes. On the other hand, the calmodulin gene showed higher level of resolution, and resulted in clades matching the phenotypes previously identified in the phenotypic dendrogram.

Considering these results, we concluded that the ITS region did not have a sufficient level of resolution for the identification of our field isolates. On the other hand, the calmodulin gene showed to be a good genetic marker for the identification of our isolates. Taking this into consideration, the genetic analysis of the remaining 13 field isolates was developed with the calmodulin gene only. The phylogenetic relationship between the 24 isolates under study is presented inFigure 4.36. In order to clarify the identification of these isolates, we compared the calmodulin sequences of our 24 isolates with the sequences of 22 type strains of section *Flavi* available in GenBank. In this analysis, the sequences corresponding to the Portuguese isolates included 730 sites, whereas GenBank sequences were shorter, and included around 550 sites. The approximately 180 sites in excess in the Portuguese isolates were maintained in the analysis in order to sustain a higher level of resolution. In these analyses, 82 sites were considered informative for the phylogenetic inference.

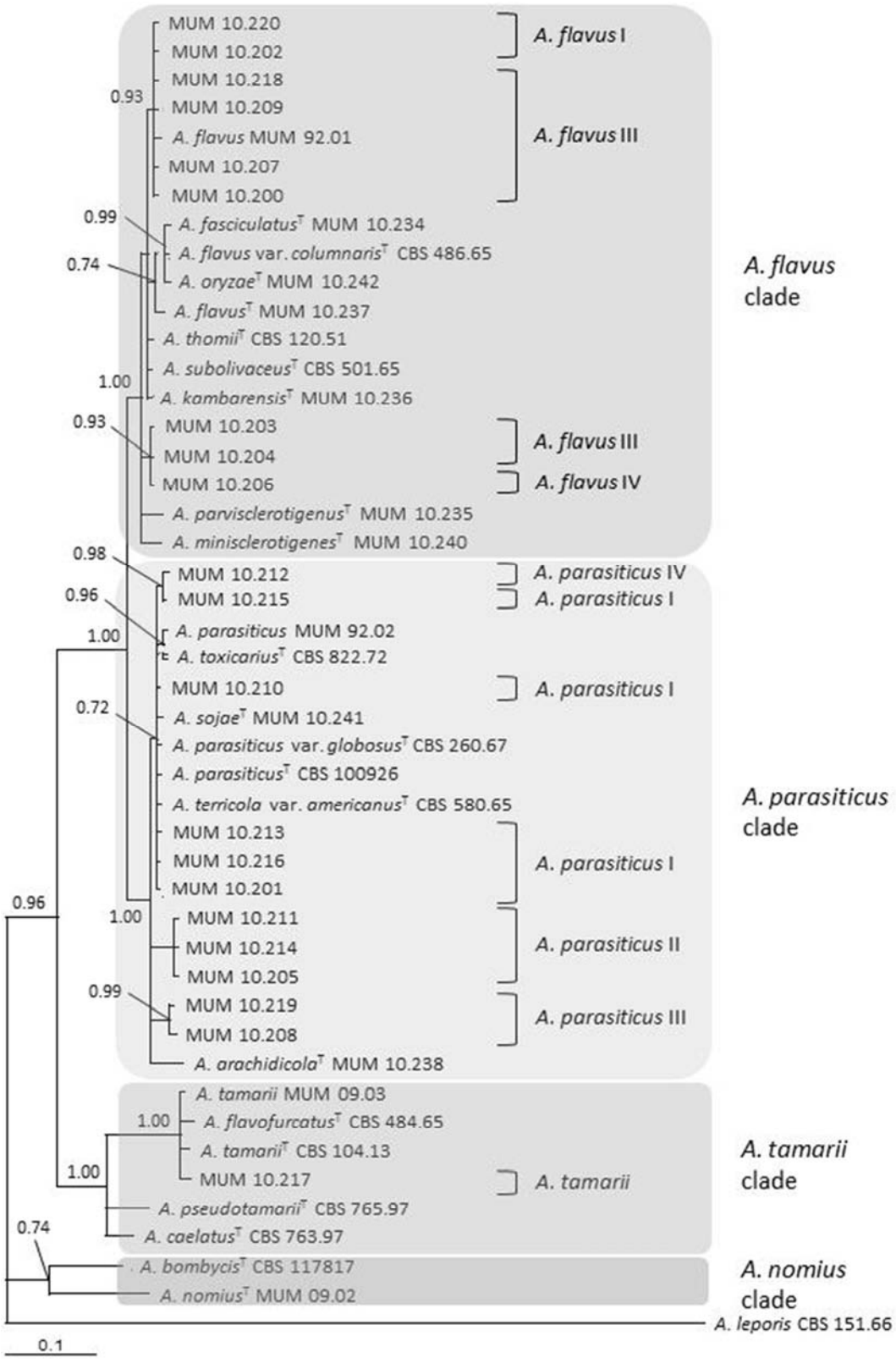

Figure 4.36 Consensus tree for the calmodulin gene obtained by Bayesian Inference. Node labels represent the posterior probabilities of each internal node.

4.3.1.3 MALDI-TOF ICMS spectral analysis

Concomitantly to the phenotypic and genetic analyses, section *Flavi* isolates were also analysed based on their protein mass spectra by MALDI-TOF ICMS. Figure 4.37 shows a dendrogram of relatedness between 69 isolates out of the 119 isolates tested (58%). Besides the 11 type-strains, the 58 field isolates included in the dendrogram herein presented were selected as representative of all the clusters obtained in the complete analysis, and were distributed as follows: 4 *A. flavus* I (18% of all *A. flavus* I isolates), 9 *A. flavus* II (13%), 8 *A. flavus* III (24%), 2 *A. flavus* IV (100%), 11 *A. parasiticus* I (7%), 11 *A. parasiticus* II (79%), 3 *A. parasiticus* III (60%), 8 *A. parasiticus* IV (89%) and 2 *A. tamarii* (7%). *A. leporis* type strain was used as out-group.

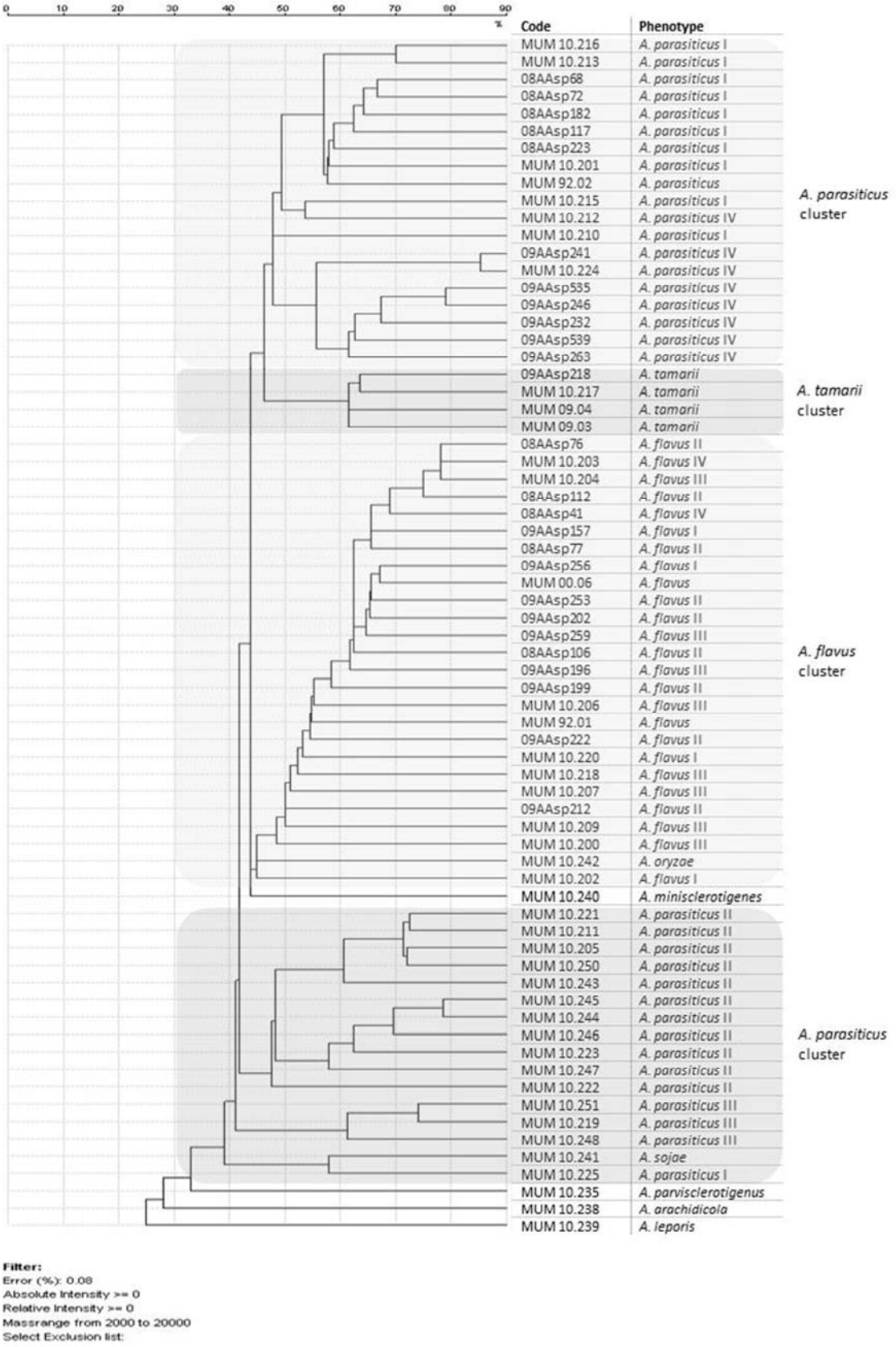

Figure 4.37 Dendrogram of relatedness between isolates of section *Flavi* based on MALDI-TOF ICMS analysis.

4.3.1.4 Identification of key taxonomic characters for species identification

The association between phenotypic features and species identification was also determined. For this matter, species identification was based on the conjugation of phenotypic, molecular and spectral analysis. The four phenotypes of *A. parasiticus* were considered as being different species, whereas all *A. flavus* phenotypes were identified as *A. flavus*. Results are shown inTable 4.30.

Table 4.30 Association between the various morphological and chemical features and the isolate classification in terms of phenotypic identification and species identification based on the consensus of the three data sets, as determined by *Qui-square* and *Cramer's V*.

	Phenotypic ID		Species	
Characteristic	*Cramer's V*	P	*Cramer's V*	P
Reverse on AFPA	0.726	< 0.001	0.726	< 0.001
Colour on CYA	0.994	< 0.001	0.994	< 0.001
Sclerotia production	0.368	< 0.001	0.336	< 0.001
Conidia ornamentation	1.000	< 0.001	1.000	< 0.001
Seriation	0.691	< 0.001	0.679	< 0.001
Fluorescence on CAM	0.860	< 0.001	0.689	< 0.001
CPA	1.000	< 0.001	0.943	< 0.001
AFBs	1.000	< 0.001	0.821	< 0.001
AFGs	0.994	< 0.001	0.983	< 0.001

5 DISCUSSION

5.1 Mycobiota of almonds and chestnuts

5.1.1 Fungal contamination of samples

In a general overview, more than eighty percent of the nuts analysed in this study were contaminated with some kind of fungi. The most common fungi were identified as belonging to the genera *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium*, *Fusarium* and *Penicillium*. Filtenborg et al. (2004) refer that normally less than 10 species are present in a given food commodity, and only 1 to 3 dominate and are responsible for spoilage. Our study was not intended to be an extensive survey on all contaminating fungi, and results are not presented at the species level, but they somewhat reflect that assumption. Even though mycobiota varied in terms of nuts and stage of production, we observed that, from among all fungi identified, few had significant impact on nuts contamination under each variable situation. For instance, whenever *Alternaria*, *Botrytis*, *Cladosporium* and *Fusarium* were widely distributed, *Penicillium* and *Aspergillus* were less significant, and vice-versa. And even in those samples where *Aspergillus* became the major contaminant, less than 5 sections (SDI) from the entire *Aspergillus* population (maximum Richness of 8) were of significance for the overall mycobiota.

5.1.1.1 Chestnuts

Field-collected chestnuts showed to be highly contaminated by unidentified yeasts and by filamentous fungi mainly belonging to the genera *Alternaria*, *Botrytis*, *Cladosporium* and *Fusarium*. *Penicillium* represented an incidence of 45% in in-shelled nuts, whereas aspergilla were identified in only 7% of the analysed nuts. From the *Aspergillus* genus, sections *Nigri*, *Aspergillus* and *Wentii* were the only ones present. The low representativeness of *Aspergillus* when compared to total fungal contamination reveals that probably this matrix and the environmental conditions in which chestnuts are produced are not suitable for *Aspergillus* establishment in the presence of other fungi. Chestnuts have high starch and moisture contents (Wareing et al., 2000; Barreira et al., 2009), which give them the potential to support the growth of a large spectrum of fungi. On the other

hand, during the period of chestnut maturation and harvest of 2007, Bragança registered mean temperatures that dropped from 18 ºC to 6.8 ºC from August to November, and the RH varied from 60.8% to 64.9%. During this period of 2007, to which these data refer, the temperature was only slightly lower than usual, but the rain was more abundant. Because we only have data for one crop year, we cannot clearly establish a correlation between mycological contamination and environmental conditions, but we can withdraw some conclusions on the basis of the physiological characteristics of the different fungi contaminating chestnuts from Trás-os-Montes. As previously mentioned, field fungi such as *Alternaria, Cladosporium* and *Fusarium* have ecophysiological conditions clearly different from those of *Aspergillus* and *Penicillium*. The first group of fungi is adapted to colder and more humid conditions and the latter have a more xerophilic and thermophilic nature (Rosso & Robinson, 2001; Filtenborg et al., 2004; Magan, 2006). Under the environment of chestnut production and the matrix characteristics, field fungi are notably more competitive in the presence of other fungi.

In our samples, aspergilla belonging to section *Flavi* were completely absence. Besides the inadequate environmental conditions, there is also the possibility that yeasts, which were present abundantly, exerted some kind of antagonism over these aspergilla (Wicklow et al., 1980; La Pena et al., 2004).

Few studies have been devoted to determining fungal contamination of chestnuts, and none has analysed chestnuts originating from the major producing countries. In fact, some of those studies are relative to marketed chestnuts with unknown origin. In a survey on commercial chestnuts collected from Canadian markets, Overy et al. (2003) detected twelve species, predominantly from genus *Penicillium*, and only two species of *Aspergillus*, *A. ochraceus* (section *Circumdati*) and *A. japonicus* (section *Nigri*), were isolated at very low frequencies. Sieber et al. (2007) found that chestnuts from Switzerland orchards were colonised predominantly by *Penicillium* spp. and *Mucor* spp., while *Aspergillus* spp. had little or no significance. On the other hand, Wells & Payne (1975) analysed freshly collected chestnuts from Georgia, USA, and reported strong contamination with *Penicillium*, *Rhizopus*, *Alternaria*, *Fusarium* and *Aspergillus* spp., mainly those from sections *Wentii*, *Flavi* and, to a lesser extent, *Nigri*. Abdel-Gawad & Zohri (1993) reported 100% contamination with *Eurotium*, *Aspergillus* and *Penicillium* in a survey on chestnuts from Saudi Arabia markets (province of Ar'Ar). In this case, sections

Flavi and *Nigri* were present in all chestnuts. Even if none of these studies refer to the origin of the analysed chestnuts, we can consider the hypothesis that they had been stored under different environmental conditions, since Georgia, USA, and Ar'Ar, Saudi Arabia, are hot and dry whereas Canada and Switzerland are cold and wet (URL: http://worldweather.wmo.int/, accessed 01.09.2010). These studies confirm that *Aspergillus* spp. are significantly more relevant in surveys from hot and dry places than from cold and wet places. Our environmental conditions as well as our results compare mostly with the latter.

Chestnuts from Trás-os-Montes are always collected from the ground. They are allowed to drop from trees and they usually rest for several days or weeks on wet ground until gathered. Sieber et al. (2007) analysed nuts collected from the ground 2 and 7 days after falling and nuts collected from nets fixed below the tree canopy and they did not register significant differences in fungal contamination between harvest methods. In our study, 3 harvest methods were tested: from the tree, from the ground at the beginning of harvest, and from the ground 3 weeks after the beginning of harvest. Results showed that harvest method had no significant influence on superficial contamination, but internal total contamination varied significantly. Chestnuts collected after up to 3 weeks on the ground were significantly more contaminated internally with overall fungi than the samples that were collected from the tree or from the ground at the beginning of the harvest period. This result can be explained by the fact that the contact of nuts with the wet soil leads to an increased contact with fungal propagules and, under high humidity, fungi can more easily grow and reach the kernel. On the other hand, the fact that aspergilla were found less frequently in nuts collected from the ground reflects the lower competitiveness of these fungi under high humidity, when in the presence of other fungi.

5.1.1.2 Almonds

5.1.1.2.1 Field-collected samples

Field-collected almonds showed strong contamination with the same fungi identified in chestnuts, mainly *Cladosporium*, but, besides those, *Aspergillus* species were also found to be important superficial contaminants of almonds. Eight different *Aspergillus* sections

were identified, with the most common being sections *Aspergillus*, *Nigri* and *Flavi*. Nevertheless, no internal contamination was associated with aspergilla.

Similar results had been reported for field-collected Californian almonds (Phillips et al., 1979; Purcell et al., 1980; Teviotdale & Hendricks, 1994; Bayman et al., 2002). In these studies, *Cladosporium* and/or *Alternaria* were also considered major contaminants, and *Mucor* spp. or *Rhizopus* spp. were also reported. In our case, these two genera were also found but not at significant frequencies. That might be due to different culture conditions, namely the salt concentration on the culture medium being higher in our study (10% against 6 to 7.5% in the other studies). Phillips et al. (1979), Purcell et al. (1980) and Bayman et al. (2002) had also referred to *Aspergillus* spp. at significant frequencies, and reported section *Nigri* as the major *Aspergillus* contaminant, whereas other sections like *Circumdati*, *Flavi*, *Fumigati* and *Nidulantes* were only rarely isolated. A negligible internal contamination at this stage of almond production has also been reported by these authors.

Contrary to our data, Phillips et al. (1979) and Purcell et al. (1980) mentioned the detection of section *Aspergillus* at much lesser frequencies than section *Nigri*. Teviotdale & Hendricks (1994) and Bayman et al. (2002) do not even refer to any *Eurotium* species (or any of their anamorphic counterparts) in their surveys. This was, to some extent, expected in the case of Teviotdale & Hendricks (1994), where culture medium used for fungal isolation (Potato Dextrose Agar) was not adequate for xerophilic fungi, but not in the others, where mycobiota was analysed in a culture medium very similar to ours (6% or 7.5% salt agar). It is possible that the higher salt concentration in our culture medium (10%) gave selective advantage to *Eurotium* species. Other possible explanation, even if less probable, is that the difference might be the result of different environmental conditions and almonds' a_W during maturation and harvest. In neither case the almonds' a_W was determined, but other almonds from Trás-os-Montes produced under similar conditions showed a_W near 0.7.

Considering climatic data, almonds from both Trás-os-Montes and California are produced under similar stressful conditions. During maturation and harvest, mean temperatures in Moncorvo are around 20-24 ºC, but maximum temperatures are around 31 ºC, frequently reaching 40 ºC. Relative humidity varies between 40 and 50%. California temperatures vary from 23 to 27 ºC, with even less rainfall than Moncorvo (http://worldweather.wmo.int/, accessed 01.09.2010). It is not likely, but it is possible, that

Californian almonds analysed in those studies were produced under more humid conditions than usual. Whatever the case might be, our results are supported by King et al. (1983), which analysed field-collected almonds with different water activity and observed that *A. glaucus* (anamorph for *E. herbariorum*) was the major contaminant of almonds with low a_W (0.75-0.80), whereas *A. niger* was the most frequent species isolated from almonds with $a_W > 0.9$.

Penicillium and *Aspergillus*, and sections *Nigri* and *Flavi* specifically, were significantly more frequent in 2008 than in 2007. And, even though Richness and Diversity were not considerably different, the average number of *Aspergillus* isolates per nut increased. In fact, the climatic conditions of Moncorvo during the period of June to September of those two years were very different. During the maturation period of 2007 (June to August), temperatures rounded 23 ºC, and rainfall was normally distributed, but the harvest period (early September) was extremely hot and dry. These conditions probably allowed less xerophilic fungi to preferably establish in almonds during maturation, with their development being further hampered by extreme dryness at the end of the harvest period. On the other hand, 2008 was very dry and hot during almond maturation period. The rainfall was almost inexistent, and mean temperatures reached 24 ºC. This period was probably more suitable for the establishment of more xerophilic *Aspergillus* spp. and *Penicillium* spp. during maturation, and less adequate for the usually predominant field fungi. Almonds were then harvested under more humid conditions, which may have favoured the growth of the already established fungi.

5.1.1.2.2 Storage-collected samples

When considering storage-collected almonds, *Penicillium* and *Aspergillus* were the predominant contaminants, both externally (in-shell) and internally (shelled). In fact, other genera like *Cladosporium* and *Fusarium* were still present, but were considerably less frequent than in field-collected nuts. Even though total contamination was not significantly different from field samples (100% in most cases), *Aspergillus* became significantly more important as a contaminant. This was evidenced by the average number of *Aspergillus* per nut in almonds harvested in 2008. External contamination with *Aspergillus* increased from 1.7 in the field to 5.6 in storage, and internal contamination increased from 0 to 0.4.

On the other hand, Richness suffered a reduction from 8 to 5.5 (with only 3 sections being representative) on in-shell nuts, but increased from 0 to 2 on shelled nuts. This seems to be a reflex of the effect of extreme environmental conditions on fungal distribution. At the end of this period, almonds registered a_W of approximately 0.63, which is too low for the majority of species to grow or even survive. Under these conditions, the most xerophilic sections *Aspergillus*, *Versicolores* and *Wentii* (Filtenborg et al., 2004; Hoekstra et al., 2004) were responsible for almost all the contamination. Without the competitiveness of other fungi, a few propagules of these sections were able transpose the shell barrier and contaminate the kernel.

Similar results in terms of mycobiota evolution throughout storage periods had been observed by others. The mycobiota of sorghum grains (da Silva et al., 2000) and peanuts (Nakai et al., 2008) from Brazil, and kolanuts from Nigeria (Adebajo & Popoola, 2003) was analysed throughout storage periods of up to 12 months and, in all cases, progressive increase of *Aspergillus* and *Penicillium* incidences was detected along with the decrease of other genera like *Cladosporium* and *Fusarium*.

In our survey, sections *Aspergillus*, *Versicolores* and *Wentii* became strong contaminants in storage almonds, and accounted for the majority of fungal contamination. Section *Flavi* also became significant, whereas other sections like *Circumdati*, *Flavi*pedes and *Nigri* were only rarely isolated. It is noteworthy that section *Nigri*, which was an important *Aspergillus* contaminant in the field, almost disappeared during the storage period. This was not expected, since almost all reports on mycobiota of almonds and other similar substrates during storage periods refer to this section as one of the major contaminants under storage conditions, alongside with section *Flavi*, with evident increases being registered throughout the storage period (e.g. Purcell et al., 1980; Adebajo & Popoola, 2003; Kaaya & Kyamuhangire, 2006). But a few studies (da Silva et al., 2000; Nakai et al., 2008) have reported results more close to ours, where section *Nigri* was isolated only rarely from long-term storage samples. Studies on unprocessed almonds marketed in California, USA (Joffe, 1969; King & Schade, 1986; Bayman et al., 2002), Spain (Jiménez et al., 1991) and Saudi Arabia (Abdel-Gawad & Zohri, 1993) have also identified remarkably high contamination with both sections *Flavi* and *Nigri*.

Section *Aspergillus* was, by far, the predominant contaminant in our study. Other studies have reported the presence of these fungi, but never at incidence levels as high as

ours (Joffe, 1969; Purcell et al., 1980; Jiménez et al., 1991; Abdel-Gawad & Zohri, 1993). As previously referred for field-collected almonds, King et al. (1983) observed that *Eurotium* spp. were more associated with low a_W almonds, whereas section *Nigri* was more frequently isolated from almonds with higher a_W. If we transpose these results to stored almonds, we can hypothesise that the higher incidence of section *Nigri* and absence of section *Aspergillus* reported in the previously mentioned studies may be due to storage under higher RH than in our case or, as previously mentioned, to different culture conditions during fungal isolation. Storage conditions of almonds from Trás-os-Montes seem to be adequate and effective in the control of the problematic sections *Nigri* and *Flavi*.

Our results also showed that section *Nigri* was only present during the storage stage where RH was higher, and decreased greatly in drier periods. These results point to a correlation between storage environmental conditions and mycobiota, since fungal contaminants varied throughout the 6 months of storage. The storage period to which our samples were subjected could be divided into 3 different stages. In the first stage (mid September to late October) mean temperatures and RH were still mild and therefore adequate for almond drying. At the end of this period in-shell almonds registered a_W of 0.63, and the mycobiota evolved in a way that accompanied the increased dryness of the substrate. Contamination with *Aspergillus* spp. significantly increased, due mainly to sections *Aspergillus*, *Versicolores* and *Wentii*, which are recognised as the most xerophilic of the genus (Filtenborg et al., 2004; Hoekstra et al., 2004). On the contrary, sections *Flavi* and *Nigri*, slightly less xerophilic (Filtenborg et al., 2004), decreased.

During the second period of storage (late October to late January), mean temperatures dropped drastically to 5.7 ºC and RH increased to almost 90%. As a result, almonds' a_W increased to 0.72, and sections *Flavi* and *Nigri* also increased, despite the extremely low temperatures. This was also the stage with the highest Richness index, with seven sections identified, even if only four were of significance to the overall population. With the third stage (late February to late March) came a new increase of temperature and decrease of RH, and almonds' a_W was strongly reduced to 0.43. Once again, the less xerophilic sections reduced their incidence. These results are in accordance with the principle that relative humidity and matrix a_W are more important in governing fungal growth than temperature (Samapundo et al., 2007b).

5.1.1.2.3 Processor-collected samples

The majority of the Portuguese almonds is submitted to further processing after a period of storage at the producer. In that case, they are received in-shell by the industrial processor and are shelled by mechanical cracking after being water-soaked. Kernels can at that point be directly expedited as final product or further processed to obtain a blanched nutmeat. This nutmeat can also be expedited as final product, or it can be sliced or minced to be sold as food ingredient. Following the fate of a single batch of nuts from field to the end of processing is difficult, because it strongly depends on producer's offer and market conditions. In our study, processor samples originated from both Moncorvo and Faro, as well as from 2007 and 2008 crops. None originated from the selected producer, and so we could not directly compare field and storage samples with processor samples, but other conclusions could be withdrawn.

In a general overview, all processor-collected nuts (including those not yet processed) were strongly contaminated by *Aspergillus* and *Penicillium*, only rarely showing any other kind of fungi. Almonds suffered strong contamination with section *Aspergillus*, but sections *Flavi*, *Nigri*, *Versicolores* and *Wentii* were also present at high frequencies and with numerous propagules. In fact, with the exception of the ubiquitous section *Aspergillus*, sections *Flavi* and *Nigri* were the predominant fungi on these samples, while sections *Circumdati*, *Flavipedes* and *Fumigati* remained, as in previous samples, minor contaminants. In those samples where internal and superficial contaminations were independently determined, we could generally register that internal contamination was significantly lower that superficial contamination. Internal contaminants were mainly those from sections *Aspergillus* and *Flavi*.

In-shell almonds from both Moncorvo and Faro gave some information on the effect of industrial shelling on kernel's mycobiota. The average number of *Aspergillus* per nut contacting the kernel before industrial shelling, as determined by aseptically removing the shell in the laboratory, was limited to 2, whereas that number increased to 10 in industrially shelled kernels. These data suggest that mechanical shelling under industrial conditions leads to the contact of the kernel with a high number of fungi that would otherwise be limited to the shell. In fact, besides those fungi that were already highly

frequent superficially, other less frequent fungi like sections *Circumdati* and *Versicolores* became strong contaminants of the kernel after shelling.

In a survey on Brazil nuts, Arrus et al. (2005) also found that whole in-shell nuts were not contaminated with section *Flavi*, but that contamination was present in processed nuts, both in-shell and shelled. These results might be due to various factors. Fungi in particulate foods have the characteristic of being heterogeneously distributed, i.e. a given fungus can strongly contaminate some nuts but not the others, so processing can result in its dissemination into a wider number of nuts. This dissemination is aggravated by the fact that fungal contamination is usually not evident in foods such as nuts, and those almonds that are visibly damaged are usually discarded by hand sorting only after shelling, so strongly contaminated in-shell nuts might be incorporated in the shelling processing stage and contaminate equipments and environment. Also, fungi easily disseminate in industrial environments through a number of different propagules, namely conidia, ascospores, hyphae and sclerotia, so cross-contamination from other samples via industrial environment constitutes a real problem.

Some processor samples in our study had already been shelled a few months before sample collection. These almonds, which originated from Moncorvo, showed superficial contamination levels with sections *Flavi* and *Nigri* that were significantly higher than in-shell storage- and processor-collected almonds with the same origin. Despite the fact that in this case superficial and internal contaminants were not differentiated, the high incidence of these sections in short-term stored kernels suggests that these are the fungi which are best fitted to the substrate and will probably cause injury in the edible part of the almond during storage, given appropriate conditions.

These data suggest that, in the cases where no further processing exists, these fungi will remain as strong contaminants until being consumed. Sections *Flavi* and *Nigri*, although not necessarily producing evident deterioration of almonds, have been implicated in the adulteration of chemical features of various nuts (almonds, walnuts, cashewnuts, coconuts, peanuts; Bilgrami et al., 1983; Saleemullah et al., 2006; Singh & Shukla, 2008), and, more than that, are responsible for the production of mycotoxins such as AFs, CPA and OTA. So, they constitute real problems in terms of nuts' safety and nutritional quality.

Almonds from Faro were followed from the moment they arrived at the processor as in-shell nuts until the moment they were packed as blanched nutmeat (final product).

Processing involved the storage in silo for 2 months, mechanical cracking, blanching after being water-soaked and oven-drying. In-shell samples were highly contaminated mainly with sections *Aspergillus*, *Flavi* and *Nigri*, reaching 16 propagules per in-shell nut. After shelling, the contamination spread to the kernel, but processing after shelling eliminated the majority of the fungi. The number of *Aspergillus* contaminating the blanched nutmeat was reduced to 1 per nut, and belonged mostly to section *Aspergillus*. This means that peeling and drying the kernel to a_W below 0.4 before packing was an appropriate processing that resulted in good quality product (in terms of fungal contamination). Generally, a variety of fungal survival structures (conidia, sclerotia, chlamydospores and ascospores) exhibit significant heat resistance at temperatures between 55 and 95 ºC. Conidia from *A. niger*, *A. flavus*, *A. parasiticus* and mostly *E. rubrum*, have been reported to be extremely resistant to temperatures around 55 ºC (see Scholte et al., 2004 for revision), but these fungi were almost completely eliminated from our samples after being submitted to oven-drying.

5.1.1.3 Differences among nuts

In-shell almonds and chestnuts were both strongly contaminated by fungi commonly present in the environment, but almonds were significantly more contaminated with *Aspergillus* than chestnuts. The main difference between the two nuts in terms of *Aspergillus* contamination was the presence of sections *Aspergillus*, *Flavi* and *Nigri* in almonds, which were rare or even absent in chestnuts. One of the possible explanations for the higher contamination of almonds with *Aspergillus* species is the differences in environmental conditions under which each type of nut is produced. Almonds are produced under considerably dryer and warmer conditions than chestnuts, and these conditions probably make *Aspergillus* more competitive when facing other fungi.

The differences in intrinsic biological and chemical characteristics of both matrices may also account for *Aspergillus* advantage in almonds when compared to chestnuts, in the presence of competing fungi. As previously referred, almonds are extremely rich in protein and fat, whereas chestnuts are mainly composed of water and carbohydrates. Sections *Flavi* and *Nigri* seem to be well adapted to almonds. This is probably related to their ability

to produce a wide range of hydrolytic proteins such as lipases that confer them advantage in fat-rich substrates (Mellon et al., 2000; Yu et al., 2003; Mellon et al., 2007).

Even though *Aspergillus* were more abundant in the outer shell of almonds, internal contamination was higher in chestnuts. This suggests that, even though almonds are exposed to higher levels of *Aspergillus* contamination, spore germination and colonisation of internal tissues is probably easier in chestnuts. This fact is potentially due to the fact that the shell is thinner and more prone to damage in chestnuts (Wells & Payne, 1975). Furthermore, they are somewhat more perishable (Mignani & Vercesi, 2003) and are easily spoiled by insects (Wells & Payne, 1975; Jermini et al., 2006).

5.1.1.4 Differences among origin

Almonds from Faro were significantly more contaminated with sections *Circumdati*, *Flavi*, *Nigri* and *Flavipedes* than those from Moncorvo. Internal contamination with *Penicillium* spp, section *Aspergillus* and section *Flavi* was also significantly higher in Faro's almonds. The higher levels of contamination in these samples probably have to do with two main factors. Whereas in Trás-os-Montes the almond culture continues to be a strong source of local financial income, the almond production in the region of Algarve has been in great decline for the last decade (INE, 2005), and producers do not develop an intensive production of this culture. This means that almonds from Algarve are not harvested every year or, when they are, they can be maintained in storage by the producer for years, depending on the market conditions. The mix of old nuts with new ones and the storage for long periods can be the source of high levels of contamination with the most common storage fungi, like *Penicillium* spp. and *Aspergillus* spp.

On the other hand, the environmental conditions under which Faro's almonds were produced and harvested were more prone to *Aspergillus* development than those from Moncorvo. In fact, almonds from Faro (2008 crop) were produced under high drought and temperature stress, with 4% the usual rainfall and mean temperature 1.7 ºC higher, but harvest was done under high humidity, with September having 700% more rain than usual. Even though we did not follow those almonds at the producer and we do not know the conditions to which the monitored almonds were submitted during storage, we can suppose that probably the maturation period selected for more xerophilic fungi, but then the drying

period after harvest was longer than usual, allowing for some of those fungi, namely from sections *Flavi* and *Nigri*, to more easily establish and develop. Similar situation, even if not so marked, had been observed between the two monitored years for Moncorvo's almonds.

5.1.1.5 Associations among fungi

Associations among fungi were almost always positive and generally highly significant, but high levels of association between fungi were rare. In "unprocessed" samples, section *Flavi* correlated mostly (but only moderately) with section *Nigri*. This means that, in general, nuts superficially contaminated with representatives of section *Flavi* are more likely to be co-contaminated with section *Nigri* than with any other fungus. On the other hand, in "processed" almond samples, i.e. those samples which suffered some kind of processing that altered the type and level of superficial contamination, *Penicillium* spp. contamination highly and significantly correlated with *Aspergillus* spp. and section *Aspergillus*, but sections *Flavi* and *Nigri* associated very poorly with each other.

Numerous studies have been devoted to fungal surveys on nuts, but few studies have tested for associations among fungi on individual seeds or crop parts. Doster et al. (1996) and Bayman et al. (2002) reported a highly significant association between *A. flavus* and *A. niger* for Californian figs and for Californian nuts (pistachios, almonds, walnuts and Brazil nuts), respectively. Also, and even though the association is not clearly determined, the majority of studies on *Aspergillus* surveys refer to similar numbers or incidences of both sections *Flavi* and *Nigri*, being that on nuts (King & Schade, 1986; Abdel-Gawad & Zohri, 1993; Freire et al., 2000; Singh & Shukla, 2008) or on other foods (e.g. Freire et al., 2000; Sanchéz-Hervás et al., 2008), which suggests that they are, in fact, associated to some degree. This association is probably due to the fact that sections *Flavi* and *Nigri* share common habitats and ecophysiological characteristics (Rosso & Robinson, 2001; Magan, 2006; Klich, 2007), so conditions that favour one of these fungi probably favour the other. An alternative explanation given by Bayman et al. (2002) is that infection by one *Aspergillus* species makes a fruit more susceptible to other aspergilli. Results obtained by Phillips et al. (1979) did not reflect the same type of correlation, as they reported that section *Flavi* was negatively correlated with sections *Nigri* and *Aspergillus*.

Doster et al. (1996) and Bayman et al. (2002) reported a negative association between *Aspergillus* spp. and *Penicillium* spp. Bayman et al. (2002) postulated that there is probably some degree of antagonism or competitive exclusion between *Aspergillus* spp. and *Penicillium* spp. Our results do not support this theory. Also, a study on the antagonistic effect of various fungi against *Aspergillus* spp., especially section *Flavi*, in Californian almonds detected that growth of aspergilli in the presence of penicilli was not significantly deterred (Phillips et al., 1979). Joffe (1969) observed that fungi with higher a_W requirements had stronger antagonistic effect over *A. flavus* than those fungi usually associated with dry foods. It is not completely understood if competition is due to antagonistic effects or to the physical and chemical environment. Whatever the case might be, our results corroborate that perception, since samples with high incidences of field fungi were less contaminated with *Aspergillus* spp. and vice-versa.

5.1.2 Aflatoxigenic fungi

In this section, isolates will be referred to as *A. flavus*, *A. parasiticus* and *A. tamarii* lato sensu, based only on their identification to the phenotypic level in terms of morphology and aflatoxigenic profile. Identification to the species level will be considered later. In our survey, only almonds were contaminated with *Aspergillus* section *Flavi*, so chestnuts will not herein be considered.

In our survey, *A. parasiticus* was found to be the predominant species, corresponding to 55.4% of all isolates, followed by *A. flavus* (36.4%) and *A. tamarii* (8.2%). Our results go against the majority of the reports, being that on almonds (Abdel-Gawad & Zohri, 1993; Bayman et al., 2002) or on other foods (Cotty, 1997; Wicklow et al., 1998; Klich, 2002b; Barros et al., 2003, 2005; Batista et al., 2003; Vaamonde et al., 2003; Arrus et al, 2005a; Razzaghi-Abyaneh et al.; 2006; Iamanaka et al., 2007; Atehnkeng et al., 2008; Nakai et al., 2008; Sánchez-Hervás et al., 2008), where *A. flavus* is usually found to be the dominant species, and *A. parasiticus*, *A. nomius* and *A. tamarii* are found only rarely.

In terms of aflatoxigenicity, 65.6% of our isolates were found to produce at least one type of AFs. *A. parasiticus* were found to be all aflatoxigenic, whereas only 28.1% of the *A. flavus* isolates were detected to produce AFs. None of the *A. tamarii* isolates produced

detectable amounts of AFs. *A. parasiticus* and *A. tamarii* toxigenic profiles were as expected, as they are very consistent throughout regions and substrates.

The factors responsible for the toxigenicity profile of *A. flavus* populations in a region or substrate are not fully understood. The fact that low levels of aflatoxigenic *A. flavus* were found in almonds, a rich-carbon substrate, may be related to the theory proposed by Bilgrami et al. (1988) and Horn & Dorner (2001), which suggests that AF production ability and other wild-type characters in *A. flavus* are lost in nutritionally rich environments.

Another interesting observation from our study was that *A. parasiticus* was more significant in field and storage samples (nearly 80%) than *A. flavus* and that, as samples were submitted to storage, at both producer and processor, *A. flavus* became progressively more significant. In processor samples, the first samples taken (late March) had an incidence of 27 to 42% of *A. flavus*, and two months later that incidence ranged from 35 to 71%. This fact may in part be the result of *A. flavus* being more adapted to the environmental conditions at the processor's warehouse and the almonds' a_W than *A. parasiticus*. Water activities from processor samples were always very low (below 0.56 in all samples), but were slightly higher at the end of the storage period for most of the samples (increased from 0.43 to 0.53, in average). The warehouse environmental conditions were not fully and correctly registered, because of problems with the data logger installed at the warehouse, but temperatures during the monitored period (March to May) were higher than normal, reaching almost 30 ºC, and relative humidity was below 70%. Gonçalez et al. (2008) reported that *A. parasiticus* was more frequent in dry pods of Brazilian peanuts whereas *A. flavus* was found more frequently in less mature pods.

5.1.3 Aflatoxin extraction and detection

5.1.3.1 Method performance

In the present work, an analytical procedure was tested and in-house validated for the determination of AFB_1, AFB_2, AFG_1 and AFG_2 in almonds, based on immunoaffinity column sample cleanup and HPLC coupled with photochemical derivatisation and fluorescence detection. The calibration parameters (linearity) that determine the precision

of the equipment were satisfactory. Linearity is considered to be achieved when the coefficient of determination (r^2) is ≥ 0.997 (Chan, 2004). In our case, r^2 was slightly below that level for AFG_1 and AFG_2 (0.991 and 0.993, respectively). LOQ values were 0.77, 0.17, 1.45 and 0.35 µg/kg for AFB_1, AFB_2, AFG_1 and AFG_2, respectively. LOQ values from other reports using methodologies similar to ours vary widely. Campone et al. (2009) and Muscarella et al. (2009) reported LOQ levels in the range of 0.1-0.22, 0.04, 0.2-0.5 and 0.1 µg/kg for the four AFs. Chun et al. (2007) reported LOQs of 0.15, 1.40, 1.30 and 2.5 µg/kg. Even if higher than in some other reports, LOQs obtained in our study were satisfactory, since they were more sensitive than the specified limits imposed by European Regulations (EC, 2010a).

The results of the in-house validation procedure demonstrated the conformity of the method of AFs analysis in almonds with provisions of Regulation (EC) No. 401/2006 (EC, 2006a). The recommended range for recovery rates is 70-100% for AFB_1 and AFG_1, and 50-120% for AFB_2 and AFG_2 for the AFs concentrations tested. The mean recovery rates obtained in our study were 93.7 and 96.8% for AFB_1 (for 6 and 2 µg/kg, respectively), 96.4 and 96.9% for AFB_2 (for 1.5 and 0.5 µg/kg, respectively), 86.0 and 103.0 for AFG_1 (for 6 and 2 µg/kg, respectively), and 92.9 and 105.6 for AFG_2 (for 1.5 and 0.5 µg/kg, respectively). RSD_r ranged from 0.7 to 11.3%, which also complied with the recommended values (which range from 22 to 33%). Similar results from both recovery rates and RSD_r were obtained by Trucksess et al. (1994). On the other hand, Campone et al. (2009) reported slightly lower recovery rates for similar spiking concentrations, ranging from 84 to 91%. Abdulkadar et al. (2000) tested almonds spiked with 10 µg/kg of AFB_1 and AFG_1 and 2.5 µg/kg of AFB_2 and AFG_2, and obtained average values ranging from 87 to 95%.

Under the described conditions, AFB_1, AFB_2, AFG_1 and AFG_2 were resolved with retention times between 15 and 25 min. Retention times can be reduced by increasing the organic solvent percentage (Campone et al., 2009). However, when analysing food sample extracts, it is common to have some level of background noise due to co-extractable materials which usually elute during the first minuts of the run. Therefore, the lower organic solvent percentage used, even though it elongates the time of run, is advantageous to allow the separation of the four AFs from the potential interfering components. HPLC chromatograms obtained from the blank and spiked almond extracts clearly showed that

there were no interfering peaks in the elution area of the four AFs, which suggests that the method employed is adequate and highly selective for AFs.

5.1.3.2 Aflatoxins detection in almond samples

A total of 4.97 µg/kg, corresponding mainly to AFB_1, was detected in only one of the 21 (5%) almond samples analysed. No AFGs were detected in any of the samples. European standards currently set admissible levels for almond kernels contamination with AFB_1 and total AFs (AFT, sum of B_1, B_2, G_1 and G_2) to 12 µg/kg and 15 µg/kg, respectively, for kernels that will be further subjected to sorting or physical treatment, or 8 µg/kg and 10 µg/kg, respectively, for kernels intended for direct consumption (EC, 2010a). The contaminated sample originated from storage almonds, which can be included in the first group. In either case, contamination was below the current admissible levels. Even if the more restrictive European legislation from 2006 was to be considered (EC, 2006b), where the levels for the first group were set to 5 and 10 µg/kg for AFB_1 and AFT, respectively, the contaminated sample was below the admissible levels.

Low levels of AF incidence in almonds had already been reported by others. Schade et al. (1975) found that only 14% of unsorted in-shell nuts from California were contaminated with AFs, generally at low levels. Abdel-Gawad & Zohri (1993) and Abdulkadar et al. (2000) analysed various nuts marketed in Saudi Arabia and Qatar (no origin reported), respectively, and found that none of the in-shell and shelled almond samples were contaminated. AFB_1 (95 ng/kg) and AFB_2 (15 ng/kg) were found in one sample of almonds from Spain by Jiménez & Mateo (2001). Only traces of AFs were associated with whole almonds from Morocco (Bottalico & Logrieco, 2001).

None of the field samples was found to be contaminated with AFs, even though almonds from Moncorvo were subjected to stressfull conditions in both years of field sampling. The only contaminated sample in our study corresponded to in-shell almonds from the initial period of storage. It would be expectable that, throughout this period, levels of contamination would increase. Saleemullah et al. (2006) studied the effect of storage on the AF contamination of almonds, and detected that the level of contamination was significantly affected by storage duration. In that study, contamination of AF-free almonds

inoculated with aflatoxigenic *A. flavus* increased to 7.5 µg/kg after 3 months of storage and to 12 µg/kg after 18 months, with moisture content increasing from 2.7% to 41.3%.

In terms of processor samples, we would expect that these samples would be more contaminated with AFs than those from field and storage, because of significantly higher levels of contamination with aflatoxigenic fungi, but no contamination was detected. Results of a survey on the occurrence of AFs in processed (peeled, sliced, diced, and ground) Italian almonds showed that a negligible AF risk, if any, was associated with processed products (principally ground almond; Bottalico & Logrieco, 2001). Opposite results were found in two surveys on processed California almonds (Schade et al., 1975; Schatzki, 1996), where AFs were found essentially on diced or ground material. This finding may be associated with the fact that processed nuts are considered low-quality products, since they usually integrate damaged almonds, either by lack of sorting or to hide damages.

In this study, a_W from storage and processor samples was always maintained below the safety value of 0.7. Aflatoxigenic isolates were able to persist or even grow but were not capable of producing AFs (Gqaleni et al., 1997; Arrus et al., 2005). Another factor that might be influencing the amount of AF in our samples is that simultaneous infection with other fungi, namely *A. niger*, *Rhizopus* spp., *Trichoderma* and *Penicillium* spp., can result in decreased AF levels (Wicklow et al., 1980; Mislivec et al., 1988; Nout, 1989; Doster et al., 1996; Aziz & Shahin, 1997; Bayman et al., 2002). In fact, no section *Nigri* isolates were detected in our AF contaminated sample. Furthermore, in samples where AFs were not detected, all nuts contaminated with section *Flavi* isolates were also contaminated with other fungi, namely *Penicillium* spp. and, with the exception of two storage samples, section *Nigri*.

It has also been shown that non-aflatoxigenic *A. flavus* have an effect of competitive exclusion towards aflatoxigenic isolates (Cotty & Bayman, 1993; Cotty, 1994). Except for storage samples (including the one contaminated), all other samples contaminated with aflatoxigenic isolates were also contaminated with a relevant proportion of non aflatoxigenic *A. flavus*. Also, a low number (2 isolates) and incidence (two in ten nuts) of *Aspergillus* section *Flavi* was detected as superficial contaminant of the AF contaminated sample, but the only two isolates were identified as *A. parasiticus*, a strong AFs producer. Doster et al. (1996) had also reported that all figs contaminated with *A. parasiticus* (present

in low numbers) were heavily contaminated with AFs (> 100 µg/kg), whereas figs contaminated with *A. flavus* (mainly atoxigenic) were free of AFs. One or all of these biological factors could have been responsible for the low incidence of AFs in our samples.

5.1.4 General quality and safety of Portuguese chestnuts and almonds

Our results suggest that chestnuts are superficially strongly contaminated with various fungi but internal contamination is usually limited to unidentified yeasts and a reduced number of filamentous fungi which are potentially toxigenic. In fact, *Penicillium* spp., which in our study were strongly associated with internal contamination, have been widely associated with nuts contamination with several types of mycotoxins (CAST, 2003). *Fusarium* spp., which are responsible for the production of a wide range of mycotoxins (CAST, 2003), have also been determined to be important in chestnut contamination. On the other hand, *Aspergillus* species were only found occasionally. There are few reports on chestnuts contamination with mycotoxins. Abdel-Gawad & Zohri (1993) analysed a wide range of mycotoxins in chestnuts strongly contaminated with *Fusarium*, *Penicillium* and *Aspergillus*, and detected AFB_1 and AFG_1. On the other hand, Overy et al. (2003) detected significant contamination of Canadian chestnuts with 5 mycotoxins, chaetoglobosins A and C, emodin, ochratoxin A and penitrem A, associated with the most prolific penicillia, but no AFs were detected.

As said, the production of mycotoxins is strongly dependent on the environmental conditions under which food products are produced and stored. Regional chestnuts are harvested during the rainy season, which could lead to the development of contaminating fungi and subsequent mycotoxin production, but they are immediately dried in a warm and aerated warehouse and fumigated. Treated chestnuts are then stored in 50 kg bags under controlled atmosphere with low temperature and high CO_2 concentration until they are further processed or shipped as fresh fruit. These conditions strongly deter fungal growth and mycotoxin production (e.g. Filtenborg et al., 2004; Giorni et al., 2008), mainly under high humidity or water activity of the fruit, as is the case with chestnuts. During processing, nuts go through various stages of selection (both mechanical and human), and low quality nuts (with visible insect or mould damage; naturally damaged shell, etc) are segregated and do not integrate final processed products.

Almonds from all over the world have been strongly associated with mycotoxigenic fungi, and especially the highly toxigenic sections *Flavi* and *Nigri* seem to be well adapted to this matrix. Also, they have been frequently associated with AF contamination. Almonds have not been a frequent subject of survey for mycotoxins other than AFs, but Zaied et al. (2010) have searched for OTA in almonds from Tunisian markets and found contamination with 61 µg/kg, proving that this may be a potential risk for almonds, as a consequence of the strong contamination with section *Nigri*. Other mycotoxins produced by field fungi are probably not an important issue in this type of nut.

However, almonds originating from Portugal seem to be produced, stored and processed in such a way that, even though allowing the contamination with those fungi, are not conducive to strong internal infection and AF contamination. Thus, it seems that those conditions are adequate for the production of almonds and by-products, and that there should not be major worries on the quality and safety of those products.

5.2 Molecular differentiation of aflatoxigenic and non-aflatoxigenic isolates

For the molecular differentiation of aflatoxigenic and non-aflatoxigenic isolates, we have selected the *aflD* gene, which is responsible for the conversion of norsolorinic acid (NOR) to averantin (AVN) in the middle of the AF biosynthetic pathway (Yu et al., 2004a), because its expression had been reported as showing a high correlation to aflatoxigenic ability (Scherm et al., 2005). The *aflQ* gene was specifically chosen because it is considered to be the only gene envolved in the final step of transforming *O*-methylsterigmatocystin (OMST) into AFB_1 (Yu et al., 2004a), a crucial step of the AF pathway that seems to be unique to aflatoxigenic species (Prieto & Woloshuk, 1997).

The presence of these two genes could not be correlated to AF producing ability: the genes were detected in all aflatoxigenic isolates, but the same happened for many of the non-aflatoxigenic isolates. Yin et al. (2009) reported that 24 of 35 *A. flavus* isolates containing no detectable AFs had the entire AF gene cluster, and only eleven atoxigenic isolates had different deletion patterns in the cluster.

Since multiplex RT-PCR for the 3 genes (*tub1*, *aflQ* and *aflD*) revealed some inconsistency in the amplification patterns, we chose to test *aflD* and *aflQ* (maintaining *tub1* as internal amplification control in all cases) expression separately. We found expression of *aflD* in both aflatoxigenic and non-aflatoxigenic isolates, and for that reason we chose not to analyse its expression for all the isolates. RT-PCR for *aflQ* showed a confusing, but consistent, band pattern. When using the primer pair ord1-gF/ord1-gR proposed by Sherm et al. (2005) for the amplification from RNA, we detected a band corresponding to the size of the expected amplification from DNA (719 bp) in all isolates and another band corresponding to the expected amplification from RNA (599 bp) in the toxigenic isolates. To exclude the possibility of DNA contamination of the RNA, we ran a PCR for the RNA samples and no amplification occured, confirming the efficacy of the DNase treatment. Furthermore, if any DNA contamination was to be present, two bands for the *tub1* gene would appear at sizes 1406 bp (DNA) and 1198 bp (RNA). Only the smaller band was present, further confirming the purity of the RNA samples. One of the possible explanations is that the 719 bp band obtained after RT-PCR of the *aflQ* gene may have resulted from defective splicing of the pre-mRNA molecule during processing (a

phenomenon known as alternative splicing), resulting in potential intron retention. Chang et al. (2010) estimated that 1.6% of the *A. flavus* genes are alternatively spliced. The number is far less than the estimates in higher organisms (e.g. up to 74% for human genome; Johnson et al., 2003) but close to the scale of the estimated 4.2% in the basidiomycetous yeast *Cryptococcus neoformans* (Galagan et al., 2005b). For instance, it has been reported that alternative splicing by intron retention is essential for amine-regulated gene expression in *Aspergillus oryzae* (Kubodera et al., 2003). In the hypothesis of alternative splicing, we can assume that an error in the RNA processing of the *aflQ* gene stopped the production of AFs, and that no alternative pathway existed to conclude the transformation of OMST into AFB_1.

We detected a fragment corresponding to *aflQ* mRNA (599 bp) in all strong aflatoxigenic isolates, but not in the non-aflatoxigenic nor in the weak AFs producers (all *A. flavus*). Scherm et al. (2005) were able to detect *aflQ* expression in *A. parasiticus* strains only, and not in any of the *A. flavus* tested, even the aflatoxigenic ones. Our strong aflatoxigenic isolates, which showed a marked *aflQ* signal, are all classified as *A. parasiticus*. The fact that we were not able to detect *aflQ* expression in aflatoxigenic *A. flavus* is in accordance with those authors. It could result from the fact that they are very weak AFs producers, and gene expression is not detected, or because of incompatibility of the primers with *A. flavus aflQ* mRNA sequence. In fact, Sweeney et al. (2000) tested this primer pair for one strain of *A. parasiticus* only, but the sequence used for constructing the primers is reported to be the one corresponding to the *A. flavus aflQ* DNA sequence (=*ord1*, EMBL Accession no. U81806). Primers sequences are present in both *A. flavus aflQ* (=*ord1*; Prieto & Woloshuk, 1997) DNA and cDNA (EMBL Accession no. U81807) as well as in *A. parasiticus aflQ* (=*ordA*; Yu et al., 1998), so the non-detection of *aflQ* expression in *A. flavus* should not be the result of lack of complementarity between mRNA and primers sequences. However, we could detect a weak signal in the weak producer MUM 10.200, classified as *A. flavus*.

The primer pair ord1-gF/ord1-cR proposed by Degola et al. (2007) did not produce any amplicon. We later came to the knowledge that the primers sequences published by those authors were not correct, which justifies the complete absence of amplification.

In general terms, our results are contradictory to those reported by Scherm et al. (2005). These authors tested 9 structural and 2 regulation genes in 13 lab strains and

concluded that *aflD* expression had the best correlation between aflatoxigenicity and gene expression, and that *aflQ* expression did not show any consistency. Furthermore, they could not identify *aflQ* expression in any of the *A. flavus* strains, only in the aflatoxigenic *A. parasiticus* strains. In our study, the analysis of gene expression under inductive conditions showed a good correlation between *aflQ* expression and AF production ability, but that correlation was not observed for the *aflD* gene.

5.3 *Aspergillus* section *Flavi*

5.3.1 Identification of Portuguese isolates based on polyphasic approach

In the present study, we analysed, characterised and identified 352 isolates on the basis of 3 different types of methodologies: i) phenotypic (morphological and physiological) analysis; ii) DNA sequence analysis; and iii) protein mass spectral analysis by MALDI-TOF ICMS.

Several morphological features were tested as identification tools, but only colony colour on CYA and conidia ornamentation were used as part of the phenotypic scheme of species identification, because they were the only ones considered significantly informative. In fact, these features were very consistent within morphotypes and phenotypes, and showed to be highly associated (as confirmed by Cramer's test of association) also with species, after isolates' identification had been confirmed by molecular and spectral analysis.

Conidia ornamentation associated almost perfectly with colony colour (*Cramer's V* = 0.989, $P < 0.001$): all brown colonies showed conspicuously roughened conidia with thick walls (*A. tamarii* morphotype); all (except one) yellow-green colonies showed smooth conidia (*A. flavus* morphotype); and all dark-green colonies showed rough conidia (*A. parasiticus* morphotype). Based on this almost perfect association, we could be lead to the conclusion that the analysis of both features is redundant, and that only one of the features would be enough for morphotype identification. But the fact is that colony colour and conidia ornamentation are not always easily determined, and it is the association between both features that gives some confidence to this approach.

Conidial head seriation has also been referred to as a helpful feature for the differentiation of *A. flavus* and *A. parasiticus*, since the first is considered to be uniseriate and the latter biseriate (Klich, 2002a). But each isolate usually shows both uni- and biseriate heads (and so they are better classified as 'predominantly uniseriate' or 'predominantly biseriate'), and both species show uni- and biseriate isolates (Rodrigues et al., 2009). From our population, only 64 isolates were submitted to this analysis. From those, 63% of the *A. flavus* isolates were predominantly biseriate, whereas 94.1% of the *A.*

parasiticus were predominantly uniseriate. These results show the moderate association between conidial head seriation and species (*Cramer's* $V = 0.679$, $P < 0.001$), and consequently the weakness as identification tool.

Kozakiewicz (1989) reported sclerotia production as being a rare characteristic of *A. flavus* strains only. Furthermore, the size and shape of sclerotia have also been used to characterise and identify new species. Kurtzman et al. (1987) associated the unique shape of elongate (undetermined) sclerotia with the species *A. nomius*, and Cotty (1989) distinguished one atypical *A. flavus* by their small (< 400 µm) and numerous sclerotia (S-type). This atypical *A. flavus* has been raised to species and named *A. parvisclerotigenus* (Frisvad et al., 2005). Another species showing microsclerotia close to *A. flavus*, *A. minisclerotigenes*, has also been recently described (Pildain et al., 2008). In our survey, we found all identified species to have both sclerotia producers and non-producers. *A. flavus* sclerotia were present in 80.7% of the isolates, and were all of the L-type (> 400 µm), whereas 52.6% of the *A. parasiticus* isolates produced these structures. On the other hand, the two unidentified clusters of isolates related to *A. parasiticus* (phenotypes *A. parasiticus* II and III) had near 80% of sclerotia-producers. Also, 34.6% of the *A. tamarii* isolates showed rare white sclerotia on CYA plate. Goto et al. (1996) described the ability to produce dark brown and pyriform sclerotia by *A. tamarii* isolates cultivated in slants, but not by isolates cultivated in plate. In our study, sclerotia production was not considered an informative feature for isolate identification, even at the morphotype level, since the association between sclerotia production and species was low (*Cramer's* $V = 0.332$, $P < 0.001$).

We also tested the ability of 64 isolates to grow at 42 ºC in CZ. Kurtzman et al. (1987) refer that different species show different growth abilities under these culture conditions. We were not able to establish a relation between this feature and identified species.

Morphological characterisation was complemented with the extrolite profile of the isolates in order to (potentially) achieve species identification. Klich (2007) stated that not all *A. flavus* isolates produce AFs, and those that do usually produce only AFBs (and CPA), whereas almost all *A. parasiticus* isolates produce both AFBs and AFGs, but not CPA. Numerous studies have shown that the mycotoxigenic potential and profile of *A. flavus* is variable. In fact, this species has been frequently divided into groups

(chemotypes), depending on their toxigenic profile (Vaamonte et al., 2003; Razzaghi-Abyaneh et al., 2006; Giorni et al., 2007).

In the present study, isolates belonging to the *A. flavus* morphotype were divided into 4 phenotypes based on their extrolite profile. From those, 18% did not produce AFs or CPA under the tested conditions, 54% produced CPA only and 26% produced both AFBs and CPA. Other surveys conducted on peanuts, wheat, soybeans, maize and peanut-growing soils showed CPA producers varying from 73% to 99% (Blaney et al., 1989; Horn et al., 1996; Resnik et al., 1996; Vaamonde et al., 2003; Barros et al., 2005). Strains producing both CPA and AFBs were less frequent than in Argentinean peanuts (63%; Vaamonde et al., 2003), peanut-growing soils (80%; Barros et al., 2005) or corn-growing soils (75%; Razzaghi- Abyaneh et al., 2006), but more frequent than in other substrates (up to 15% in soybeans, wheat and maize; Resnik et al., 1996; Vaamonde et al., 2003).

In the molecular analysis, the isolates were divided into three major clusters, representing groups of species related to *A. flavus*, *A. parasiticus* and *A. tamarii*. In the *A. flavus* clade, the *A. flavus* I (non-aflatoxigenic) and *A. flavus* III (aflatoxigenic) isolates clustered with the closely related species *A. oryzae*, *A. thomii*, *A. kambarensis*, *A. fasciculatus* and *A. subolivaceus*, independently of their aflatoxigenic ability. Similarly to what had been previously reported (Pildain et al., 2008), the calmodulin gene was not able to clearly resolve these species. A distinct sub-clade related to *A. flavus*, *A. minisclerotigenes* and *A. parvisclerotigenus* was created with the *A. flavus* IV (MUM 10.203) isolate and with two *A. flavus* III isolates (MUM 10.204 and MUM 10.206). MUM 10.203 differs from the other isolates by its ability to produce small amounts of AFGs.

Several atypical *A. flavus* strains have been reported to produce both AFBs and AFGs, but, contrary to our isolates, they have been associated with the production of microsclerotia (S-type) and usually high levels of AFs (e.g. Saito & Tsuruta, 1993; Cotty & Cardwell, 1999; Geiser et al., 2000; Vaamonde et al., 2003; Pildain et al., 2008). These isolates included atypical S-type *A. flavus* isolates (named *A. flavus* S_{BG}; Cotty & Cardwell, 1999) as well as isolates later described as *A. parvisclerotigenus* (Frisvad et al., 2005) and *A. minisclerotigenes* (Pildain et al., 2008), to which our isolates did not cluster. The molecular analysis did not support the close genetic relation to any of those species. In respect to MUM 10.204 and MUM 10.206, we should emphasise that they were difficult to characterise, due to inconsistency in their aflatoxigenic profile (the production of AFGs

was dubious after 3 repetitions), and they were only presumptively integrated in the *A. flavus* III phenotype.

When analysed by MALDI-TOF ICMS, all *A. flavus* isolates clustered in a single clade, regardless of mycotoxigenic pattern. So, under the tested conditions, the method was not able to differentiate aflatoxigenic from non-aflatoxigenic isolates. Also, *A. oryzae* was not clearly differentiated from *A. flavus* from the spectral points of view. Even though *A. oryzae* and one non-aflatoxigenic *A. flavus* isolate (MUM 10.202) segregate from the major cluster of *A. flavus* isolates, we cannot undoubtedly state that they constitute different species. Li et al. (2000) stated the ability to differentiate *A. flavus* from *A. oryzae*, as well as aflatoxigenic from non-aflatoxigenic *A. flavus* strains, by MALDI-TOF ICMS based on the analysis of spore mass spectra, but only a few non-informative spectra were presented. No statistical analysis was performed and no dendrogram of relatedness was presented to prove that statement. Furthermore, the aflatoxigenic *A. flavus* strains used in that study produced both AFBs and AFGs, which, as already mentioned, is an atypical feature of *A. flavus*. In a later study (Lee et al., 2004), some of those atypical *A. flavus* strains were analysed by morphological and molecular (amplified fragment length polymorphisms, AFLP) analyses and were determined to be *A. parasiticus*.

Regarding the *A. parasiticus* morphotype, molecular analysis resulted in the segregation of the isolates into three different clades. The *A. parasiticus* I isolates and the only *A. parasiticus* IV isolate analysed (MUM 10.212) were clustered into the same clade as type-strains for *A. parasiticus*, *A. toxicarius*, *A. terricola* var. *americana*, *A. sojae* and *A. parasiticus* var. *globosus* (with these species being considered synonymous, at least for the partial calmodulin gene). These results would suggest that the two phenotypes are in fact different chemotypes of *A. parasiticus*. Another possibility would be that isolates *A. parasiticus* IV were *A. parasiticus* producing AFGs at undetectable levels. To our knowledge, there are no references on *A. parasiticus* isolates not producing AFGs or producing them at undetectable levels when AFBs are produced in high amounts. *A. parasiticus* isolates are consistently reported as strongly aflatoxigenic, producing both AFBs and AFGs, and only few studies have reported very low proportions (3-6%) of non-aflatoxigenic isolates within the species (Blaney et al., 1989; Doster et al., 1996; Horn et al., 1996; McAlpin et al., 1998; Tran-Dinh et al., 1999; Vaamonde et al., 2003). When the remaining *A. parasiticus* IV isolates were analysed by MALDI-TOF, MUM 10.212 was

also not differentiated from the typical *A. parasiticus* isolates (phenotype I), but all the other *A. parasiticus* IV isolates created an independent clade. These results suggest that these isolates might constitute a non-identified taxon closely related to *A. parasiticus*.

The isolates corresponding to the *A. parasiticus* II and *A. parasiticus* III phenotypes consistently formed two well-defined sub-clades in all data sets. *A. parasiticus* II isolates produced all AFs, but had the distinctive characteristic of producing more AFGs than AFBs. Besides that, the CPA HPLC chromatogram showed to be very distinctive from the other chromatograms. Also, *A. parasiticus* III isolates were differentiated from typical *A. parasiticus* by their clear ability to produce CPA. *A. parasiticus* II and *A. parasiticus* III are herein regarded as 2 putative unidentified species.

In the *A. tamarii* morphotype, only one chemotype (represented by the isolate MUM 10.217) was identified corresponding to the expected extrolite profile. The *A. tamarii* isolates clustered with the *A. tamarii* type-strain also in the molecular and spectral approaches, thus confirming the correct identification.

By comparing the phenotypic aggregation of the isolates using colony colour on CYA, conidia ornamentation, CPA production and AFs production with the molecular identification achieved by the calmodulin sequence analysis, we could observe that the two approaches were in very good agreement. Similar conclusions had already been reported by various authors working with section *Flavi* and with other sections in the *Aspergillus* genus (e.g. Ito et al., 2001; Peterson et al., 2001; Samson et al., 2004b, 2007; Serra et al., 2006; Varga et al., 2007a, 2007b; Pildain et al., 2008; Zalar et al., 2008).

5.3.2 Identification of key taxonomic characters for species identification

Phenotypic features as markers for species identification

As previously referred, numerous studies have reported species identification of section *Flavi* isolates based on morphological and physiological characterisation, but none has clearly stated the level of accuracy of the features used. In our study we were able to determine which phenotypic characteristics were more reliable for species identification. In this analysis, colony colour on CYA, conidia ornamentation, CPA production, AFBs production and AFGs production showed high association with species. CPA, AFBs and AFGs production correlated perfectly or almost perfectly and significantly with phenotype,

meaning that all isolates included in each of the phenotypes showed the same extrolite profile.

Also, all isolates of each of the phenotypes had the same type of conidia ornamentation. Relative to colour on CYA, also used for isolate distribution into phenotypes, the association with phenotypes was very high but not perfect. In fact, there was one isolate that could not be assigned to a morphotype, because it showed smooth conidia but dark-green colour on CYA. After the mycotoxin profile was determined for that isolate, it was included in the *A. flavus* I phenotype. Other features like reverse on AFPA, fluorescence on CAM and aspergilla head seriation showed to be less reliable as identification characters, even though they had high level of association with the phenotypes. Sclerotia production on CYA was poorly associated with phenotype.

We consider that the determination of a reduced number of phenotypic features that result in reliable species identification is of major importance in fungal surveys and population studies, as well as in those cases where laboratories lack resources to extend the identification of large numbers of isolates to other methods, like molecular or spectral. By adopting the strategy described in the present study, phenotypic characterisation can become more reliable and less time consuming.

All isolates were screened for aflatoxigenic ability on CAM. It has been reported that CAM fluorescence does not always correspond to AF detection by chromatography (Abarca et al., 1988; Giorni et al., 2007; Scherm et al., 2005). The most striking example is given by Abarca et al. (1988), who reported that blue fluorescence on CAM was detected in only 4 out of ten aflatoxigenic *A. flavus* strains. In our study, presence of fluorescence on CAM was correlated with AFs production (determined by HPLC) at a very high level (*Cramer's* $V = 0.988$, $P < 0.001$), since this medium did not yield any false-positives and only two false-negatives were detected (percentage of agreement 99.4%). These two false-negatives showed to be weak AFs producers, as determined by HPLC. Furthermore, whenever present fluorescence assumed different colours: blue, violet or green.

Isolates producing AFBs only (AFB^+) also produced a violet fluorescence on CAM; isolates AFB^+ and AFG^+ generally produced a blue fluorescence, the exception being 5 of the 14 isolates producing AFGs at higher levels than AFBs, which produced a green fluorescence. To our knowledge, this is the first report on the association of different

fluorescence colour with different aflatoxigenic profiles. These results showed that CAM is a simple, rapid and reliable method for rapid screening of aflatoxigenic isolates.

Molecular markers for species identification

Fungal barcoding is being extensively studied and numerous reports have been released on the most appropriate DNA sequences to be used as universal molecular marker for fungal species identification. ITS has recently been proposed as the official primary barcoding marker for fungi (Deliberation of 37 mycologists from 12 countries at the Smithsonian's Conservation and Research Centre, Front Royal, Virginia, May 2007; see Bellemain et al., 2010). But the determination of a universal sequence that will serve all fungi has been contentious. In fact, each genus has its own specificities, an even within a genus some difficulties have been reported. For the *Aspergillus* genus, DNA sequences like the calmodulin and β-tubulin genes have been used extensively and have been proposed as more suitable barcodes.

In our study, we used the ITS region and the calmodulin gene to create molecular dendrograms that could be compared to phenotypic and spectral dendrograms. By comparing all sets of data, we concluded that the ITS region was not a sensitive genetic marker. Even though only 11 isolates were tested for this DNA region, it became clear that it did not achieve a level of resolution high enough to differentiate very closely related species, as was the case of our isolates. The calmodulin gene revealed higher sensitivity, and the molecular clustering was in agreement with the clustering obtained by the other techniques. Bearing in mind that our molecular studies were applied to a limited number of isolates and that it was not our goal to study barcoding sequences, our results strengthen the hypothesis that the ITS region is not suitable for the identification of closely related species, and that the calmodulin gene is a better candidate for the role of *Aspergillus* section *Flavi* barcoding.

It was also clear, not only from our molecular results but also from results reported elsewhere (Pildain et al., 2008) that very closely related species are difficult to discriminate based on a single DNA sequence. In fact, Samson & Varga (2009) recommend that, for species description, at least 2 gene sequences should be examined, using a technique called Multilocus approach, and various reports have been released demonstrating the usefulness of that approach (Ito et al., 2001; Peterson, 2001, 2008; Pildain et al., 2008)

MALDI-TOF validation for species identification

Even though major efforts are being put on the establishment of a fungal genetic barcode, DNA sequencing is a technique that, albeit accurate and sensitive (depending on the sequence to be used), is expensive and time consuming. In fact, to achieve a good quality DNA sequence, a succession of elaborate steps is required: fungal growth, DNA extraction, PCR amplification, PCR product purification, sequencing and a series of intermediate agarose gels to confirm the success of each step. Phenotypic identification is also extremely time consuming and requires the skills of a specialised mycologist. Furthermore, intra-specific variability and inter-specific similarity are obvious drawbacks to an accurate identification.

One of the aims of applying a polyphasic approach to our isolates was to determine the level of resolution and reliability of MALDI-TOF ICMS in the identification of very closely related species of *Aspergillus* section *Flavi*. MALDI-TOF ICMS is an innovative technique that has been extensively used in bacteria identification, but has only rarely been applied to fungal identification. Because the structures present in a fungal colony are more diverse and complex than in a bacterial colony, several reports have been published on method optimisation. In our study, a minute mix of hyphae and conidia taken from a colony with 4 to 5 days of growth was used as test material; this material was directly placed on the analysis plate, treated in-situ by brief seconds with a protein extracting solution, and directly analysed by the equipment. This procedure takes only a few minutes per sample. Data are then analysed with specific bioinformatics software for isolate identification. The major drawbacks of this technique are the initial cost of the equipment and the requirement for a specialised analyst for data analysis, but the advantages over other methods are striking: after the initial investment on the equipment and analyst training, the cost of each analysis is extremely low, in terms of both time and money expended.

Our results clearly demonstrate the utility of the methodology for discrimination between species and strains of fungi, including *Aspergillus*. MALDI–TOF MS-based fingerprinting is an objective and fast analytical methodology with the potential of strongly complementing current subjective and time-consuming identification techniques, which are mostly based on morphology and physiology. It is thus suitable for applications which have particular needs in high-throughput, highly accurate identification, and low sample

preparation. But results also reflected that the obtainable mass spectrum and its reproducibility are essential prerequisites for the successful classification and identification of fungal species. Various parameters have been reported to influence mass spectral data, such as the pre-treatment of the fungal sample (growth media, washing procedure), the applied matrix compound and solvent system as well as the MALDI sample preparation technique itself.

6 CONCLUSIONS AND PERSPECTIVES

As a result of the present study, several conclusions can be withdrawn. In terms of general and aflatoxigenic fungal contamination, we can consider the following:

- Chestnuts and almonds are different in their intrinsic biological, chemical and physical characteristics and they are produced under different environmental conditions. The conjugation of these factors resulted in different types of fungi identified as dominant contaminants. Chestnuts were more strongly associated with the so-called field fungi like *Alternaria*, *Cladosporium* and *Fusarium*, and almonds were predominantly contaminated with the storage fungi *Aspergillus* and *Penicillium*.

- Fungal contamination of both matrices was found to be predominantly superficial, as few fungi were able to effectively transpose the shell and infect the kernel. Yet, kernel contamination of almonds increased significantly after shelling.

- In almonds, fungi evolved from field to processing. Potentially toxigenic *Aspergillus* belonging to sections *Flavi* and *Nigri* became generally more significant and widespread throughout storage and processing, and were determined to be moderately associated.

- Numerous isolates belonging to section *Flavi* were detected in Portuguese almonds, and the majority of those isolates was found to be aflatoxigenic. *A. parasiticus*, which is the most aflatoxigenic of the species, was the most significant contaminant. This fact may constitute a problem in terms of food safety if storage and processing conditions are not effectively controlled.

- Portuguese almonds seem to be generally safe in terms of AF contamination, since only one storage sample was found to be contaminated with levels below the limits imposed by the latest legislation on the matter (Commission Regulation (EU) No 165/2010).

As is widely recognised, the presence of toxigenic moulds in a food product does not automatically mean the presence of mycotoxins, but rather that a potential for mycotoxin contamination exists. On the other hand, the absence of toxigenic moulds does not

guarantee that the food is free of mycotoxins, since the toxins may persist long after the moulds have disappeared. Knowledge of regional differences in the toxigenic and genetic diversity of *A. flavus* populations as well as knowledge of the association of these populations with the dominant culture in a region may help understand the population dynamics and also give important information that could be used in determination of the most effective control measures for reducing pre- and post-harvest AF contamination. Taking this in consideration, we consider that:

- Other surveys spanning different areas and stages of production need to be developed in Portuguese nuts. To our knowledge, this is the first study on contamination of Portuguese almonds and chestnuts with *Aspergillus* and aflatoxigenic fungi in particular, and it can only be regarded as a first contribution to the knowledge on this matter.

- Because they differ in intrinsic features as well as in producing and processing conditions, almonds and chestnuts need to be addressed separately when considering measures that will allow the control of infection by mycotoxigenic fungi and mycotoxins.

- Given the high incidence of *Aspergillus* sections *Flavi* and *Nigri* in almonds, toxins produced by these species, namely AFs and OTA, should be given particular attention in other studies of this nature. In the case of chestnuts, AF contamination was not studied, but the absence of isolates from aflatoxigenic species leads to the conclusion that AFs are probably not the major problem in terms of mycotoxin contamination of this matrix. Considering the major fungal contaminants and the production conditions, other toxigenic fungi and mycotoxins should be given more emphasis, as is the case of *Fusarium* spp. and related mycotoxins.

- At present, storage and processing conditions of Portuguese almonds seem to be adequate for the obtention of safe products. Drying almonds to a_W levels below 0.70 and the removal of nuts with visible damage from lots entering the processing stream are important steps towards having good quality products, even if it results in extra costs.

In terms of aflatoxigenic and non-aflatoxigenic isolates differentiation by molecular methods, this study headed to the following conclusions:

- The presence of two strategic genes of the AF biosynthetic pathway, *aflD* and *aflQ*, was not associated with the aflatoxigenic ability of the isolates. On the other hand, the analysis of their expression under inducive conditions showed a good correlation between *aflQ* expression and AF production ability, showing that this could be used as a molecular marker for differentiating aflatoxigenic from non-aflatoxigenic isolates.

- Since the *A. flavus* isolates used in this part of the study were all non-aflatoxigenic or produced low levels of AFs, we consider that more isolates from the species *A. flavus*, which is extremely variable in terms of AF production, need to be tested in order to guarantee the ability of *aflQ* expression to be used as a molecular marker for this characteristic.

Regarding the identification of *Aspergillus* section *Flavi* isolates, the following can be concluded:

- Morphological analysis has shown sensitive and reliable as a first approach for species identification only when complemented with the mycotoxin profile. The determination of the level of association between each phenotypic feature and the identified species, allowed us to reduce the number of features to be examined to those that really played an important role in identification, thus simplifying the classic phenotypic scheme of identification. Still, phenotypic characterisation was extremely time-consuming and not completely straightforward, and subjective results made accurate identification difficult to attain.

- In terms of molecular identification, the calmodulin gene showed to be more robust and reliable as genomic marker for this group of fungi than the ITS region, providing better DNA barcoding potential. Nevertheless, DNA sequence analysis was considered, under our laboratory conditions, time-consuming and expensive for the identification of a high number of isolates, even if only one sequence was analysed for each isolate.

- Using a single DNA sequence for isolate identification has shown to be accurate but unable to discriminate between isolates belonging to section *Flavi*, since this is a section composed of numerous very closely related species.

- MALDI-TOF ICMS results were congruent with those obtained by classic phenotypic and molecular approaches, thus confirming this technique as highly reliable for fungal identification. Furthermore, it was considered faster and less expensive in terms of labour and consumables when compared with the other techniques employed, which we consider an essential condition whenever high numbers of isolates are involved.

- MALDI–TOF MS-based fingerprinting methodology has herein been demonstrated to be sensitive and accurate for discrimination of section *Flavi* species, and can be regarded as an objective and fast analytical methodology with the potential of strongly complementing current subjective and time-consuming identification techniques. It is thus suitable for applications which have particular needs in high-throughput, highly accurate identification, and low sample preparation.

- By applying a polyphasic approach to the identification of section *Flavi* isolates, we were able to detect three groups of isolates that do not correspond to any known species, and the unidentified taxa are herein regarded as three potential new species. It is our conviction that the use of one of the identification approaches alone - phenotypic, molecular or spectral - would have not been enough for us to regard them as potential new species, and we stress the need for a polyphasic identification scheme when dealing with very closely related taxa.

It has been largely mentioned that taxonomic schemes of identification in *Aspergillus* section *Flavi* should be based on polyphasic approaches involving various types of features. In the present study, a broad type and number of characteristics - morphological, biochemical, molecular and spectral – was analysed for a large number of isolates. From those, we could identify key taxonomic characteristics which we propose should integrate polyphasic schemes of identification in section *Flavi*:

- Morphological: colony colour on CYA and conidia ornamentation;

- Metabolic: production of AFBs, AFGs and CPA and corresponding HPLC chromatograms;
- Molecular: analysis of the calmodulin gene sequence. On this matter, the concatenation with other DNA segments like the β-tubulin gene, the ITS region and others, although not tested in this study, has been demonstrated valuable.
- Spectral: mass spectra of intact cells tested under standardised conditions.

Since the know-how has been created, the technologies implemented, and the fungi gathered in our laboratory, we intend to implement similar taxonomic schemes for other sections of genus *Aspergillus* with relevance to science and industry, from which the most significant is section *Nigri*. For this matter, the near 1,000 *Aspergillus* isolates collected in this study will serve as good biological material.

The present work is far from being a finished business, and a lot of windows have been left open. Some of the perspectives of future work are the following:

- For the near future remains the corroboration of the three unidentified taxa as new species. On this matter, additional phenotypic and molecular work specifically directed to the unidentified isolates is currently under development.

- None of the identification schemes used in this study was able to differentiate the species *A. flavus* (potentially aflatoxigenic) and *A. oryzae* (non-aflatoxigenic). This is a question of major importance, given that *A. oryzae* is the most widely used fungus in the Oriental food industry. For that reason, we intend to deepen this work with MALDI-TOF ICMS, by analysing more *A. oryzae* isolates under varying conditions.

- Under the tested conditions, which were intended for species identification, MALDI-TOF ICMS was unable to differentiate between aflatoxigenic and non-aflatoxigenic isolates of *A. flavus*. Yet, aflatoxigenic ability is a phenotypic characteristic which depends greatly on fungal culture conditions. It is likely that, as happens for other methods, the differentiation of aflatoxigenic isolates by MALDI-TOF ICMS is dependent on the analysis of fungi under AF inducive

conditions. For the near future remains the intention of testing isolates under inductive and non-inducive conditions in order to determine the ability of MALDI-TOF ICMS on this particular issue.

- There is still the need for a comprehensive database of fungal fingerprint mass spectra to be established in order to achieve maximum accuracy of the method. Also, work is still to be done on the optimisation and standardisation of analyses. The high number of well characterised field isolates from *Aspergillus* section *Flavi* and other sections can serve as base material for achieving these goals;

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8 APPENDICES

Appendix I

Table AI.1 Significance of differences (P-value) for samples' a_w, as determined by the *Tamhane's T2* test.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. Storage 1 in-shell																
2. Storage 2 in-shell	0.537															
3. Storage 3 in-shell	0.006	0.000														
4. Storage 1 shelled	1.000	0.772	0.000													
5. Storage 2 shelled	0.727	1.000	0.000	0.950												
6. Storage 3 shelled	0.002	0.000	1.000	0.000	0.000											
7. C1	0.017	0.000	0.994	0.000	0.000	1.000										
8. C2	0.448	0.000	0.001	0.006	0.015	0.049	0.045									
9. D1	0.958	0.853	0.999	0.840	0.939	0.998	1.000	1.000								
10. D2	0.334	0.000	0.001	0.012	0.000	0.174	0.116	1.000	1.000							
11. F1 in-shell	0.011	0.000	1.000	0.000	0.007	1.000	1.000	0.036	1.000	0.499						
12. F1 shelled	0.061	0.220	1.000	0.050	0.494	1.000	1.000	0.999	1.000	1.000	1.000					
13. F2 kernel	0.170	0.000	0.001	0.005	0.000	0.379	0.021	0.958	1.000	0.783	0.402	1.000				
14. F2 shell	0.921	0.002	0.003	0.022	0.110	0.011	0.101	1.000	1.000	0.996	0.030	0.943	0.180			
15. F3 nutmeat	0.003	0.000	0.518	0.000	0.002	0.825	0.352	0.005	0.981	0.187	0.310	0.988	0.077	0.008		
16. F3 seed coat	0.005	0.000	0.000	0.001	0.004	0.000	0.000	0.000	0.494	0.019	0.000	0.140	0.001	0.002	0.000	

Table AI.2 Differences in frequency of fungi in chestnuts from the tree or from the ground, as determined by two-tailed *Fisher's exact* test.

	Genus			Section								
	Total	*Penicillium*	*Aspergillus*	*Aspergillus*	*Circumdati*	*Flavi*	*Flavipedes*	*Fumigati*	*Nidulantes*	*Nigri*	*Versicolores*	*Wentii*
Arv1/07 → Ch1/07												
In-shell	-	1.000	1.000	0.494	-	-	-	-	-	0.056	-	0.494
Shelled	**0.001**	1.000	0.361	1.000	-	-	-	-	-	0.361	-	-
All	**0.008**	1.000	0.405	1.000	-	-	-	-	-	**0.018**	-	0.497
Arv1/07 → Ch2/07												
In-shell	-	0.205	0.056	-	-	-	-	-	-	0.056	-	-
Shelled	0.120	0.758	0.117	1.000	-	-	-	-	-	0.117	-	-
All	0.114	0.195	**0.003**	1.000	-	-	-	-	-	**0.003**	-	-
Ch1/07 → Ch2/07												
In-shell	-	0.139	0.117	0.494	-	-	-	-	-	-	-	0.494
Shelled	**0.000**	0.758	1.000	-	-	-	-	-	-	1.000	-	
All	**0.000**	0.143	0.059	0.497	-	-	-	-	-	1.000	-	0.497

Values in bold, the frequencies were significantly different between samples, $P < 0.05$
Values not in bold, the frequencies were not significantly different between samples, $P > 0.05$
-, No statistics were computed because data were constant (frequency of infection 0% or 100%).

Table AI.3 Differences in frequency of fungi between in-shell and shelled nuts, as determined by two-tailed *Fisher's exact* test.

	Genus			Section								
	Total	*Penicillium*	*Aspergillus*	*Aspergillus*	*Circumdati*	*Flavi*	*Flavipedes*	*Fumigati*	*Nidulantes*	*Nigri*	*Versicolores*	*Wentii*
Chestnut, field												
Arv1/07	**0.000**	**0.003**	1.000	1.000	-	-	-	-	-	0.714	-	-
Ch1/07	**0.000**	**0.006**	0.361	0.494	-	-	-	-	-	1.000	-	0.494
Ch2/07	**0.026**	**0.000**	-	-	-	-	-	-	-	-	-	-
All	**0.000**	**0.000**	0.411	1.000	-	-	-	-	-	1.000	-	0.498
Almond, field												
2007	**0.000**	**0.000**	**0.000**	**0.000**	1.000	0.242	0.494	-	-	**0.026**	**0.006**	0.242
2008	**0.000**	**0.000**	**0.000**	**0.000**	0.242	**0.000**	0.494	-	1.000	**0.000**	0.242	**0.003**
All	**0.000**	**0.000**	**0.000**	**0.000**	0.121	**0.000**	0.121	-	1.000	**0.000**	**0.000**	**0.000**
Almond, storage												
Storage 1	**0.001**	**0.000**	**0.000**	**0.000**	0.487	0.231	0.487	1.000	-	-	**0.000**	**0.006**
Storage 2	**0.000**	**0.000**	**0.000**	**0.000**	0.231	**0.008**	1.000	-	-	**0.008**	**0.000**	**0.000**
Storage 3	**0.000**	**0.000**	**0.000**	**0.000**	-	0.106	-	-	-	1.000	**0.003**	**0.000**
All	**0.000**	**0.000**	**0.000**	**0.000**	**0.003**	**0.000**	**0.015**	1.000	1.000	**0.000**	**0.000**	**0.000**
Almond, processor												
A	-	-	-	-	0.211	0.650	-	-	-	0.582	1.000	**0.023**
F	-	-	-	-	0.211	**0.003**	-	-	-	**0.000**	0.303	**0.001**
All	-	-	-	-	**0.020**	**0.008**	-	-	-	**0.001**	0.451	**0.000**

Values in bold, the frequencies were significantly different between samples, $P < 0.05$
Values not in bold, the frequencies were not significantly different between samples, $P > 0.05$
-, No statistics were computed because data were constant (frequency of infection 0% or 100%).

Table AI.4 Differences in frequency of fungi between almond samples collected in 2007 and 2008, as determined by two-tailed *Fisher's exact* test.

	Genus			Section								
	Total	*Penicillium*	*Aspergillus*	*Aspergillus*	*Circumdati*	*Flavi*	*Flavipedes*	*Fumigati*	*Nidulantes*	*Nigri*	*Versicolores*	*Wentii*
In-shell	-	**0.000**	**0.013**	0.358	0.616	**0.021**	1.000	-	1.000	**0.002**	0.197	0.118
Shelled	0.823	0.197	-	-	-	-	-	-	-	-	-	-
All	0.842	**0.025**	0.082	0.404	0.621	**0.028**	1.000	-	1.000	**0.005**	0.212	0.132

Values in bold, the frequencies were significantly different between samples, $P < 0.05$
Values not in bold, the frequencies were not significantly different between samples, $P > 0.05$
-, No statistics were computed because data were constant (frequency of infection 0% or 100%).

Table AI.5 Differences in frequency of fungi between almonds and chestnuts (only for 2007 tree collected samples), as determined by two-tailed *Fisher's exact* test.

	Genus			Section								
	Total	*Penicillium*	*Aspergillus*	*Aspergillus*	*Circumdati*	*Flavi*	*Flavipedes*	*Fumigati*	*Nidulantes*	*Nigri*	*Versicolores*	*Wentii*
In-shell	-	**0.001**	**0.000**	**0.000**	1.000	0.242	0.494	-	-	1.000	**0.006**	0.242
Shelled	0.652	0.714	0.117	1.000	-	-	-	-	-	0.242	-	-
All	0.686	**0.039**	**0.006**	**0.005**	1.000	0.246	0.497	-	-	0.792	**0.007**	0.246

Values in bold, the frequencies were significantly different between samples, $P < 0.05$
Values not in bold, the frequencies were not significantly different between samples, $P > 0.05$
-, No statistics were computed because data were constant (frequency of infection 0% or 100%).

Table AI.6 Differences in frequency of fungi between storage samples A and B, as determined by two-tailed *Fisher's exact* test.

	Genus			Section								
	Total	*Penicillium*	*Aspergillus*	*Aspergillus*	*Circumdati*	*Flavi*	*Flavipedes*	*Fumigati*	*Nidulantes*	*Nigri*	*Versicolores*	*Wentii*
A1-B1												
In-Shell	-	-	-	-	0.474	1.000	1.000	-	-	-	1.000	1.000
Shelled	1.000	1.000	1.000	1.000	-	-	-	1.000	-	-	1.000	0.474
All	1.000	1.000	1.000	1.000	0.487	1.000	1.000	1.000	-	-	1.000	0.501
A2-B2												
In-Shell	-	-	-	-	1.000	-0.070	1.000	-	-	1.000	0.303	1.000
Shelled	0.350	0.350	0.303	0.582	-	1.000	-	-	-	-	-	-
All	0.501	0.501	0.514	0.748	1.000	0.273	1.000	-	-	1.000	0.514	1.000
A3-B3												
In-Shell	-	-	-	-	-	0.582	-	-	-	-1.000	0.179	1.000
Shelled	1.000	1.000	1.000	0.628	-	-	-	-	-	-	1.000	-
All	1.000	1.000	1.000	0.741	-	0.605	-	-	-	1.000	0.155	1.000
A-B												
In-Shell	-	-	-	-	0.353	0.771	1.000	-	-	1.000	0.084	1.000
Shelled	1.000	1.000	0.779	1.000	-	1.000	-	1.000	-	-	0.492	0.492
All	1.000	1.000	0.848	1.000	0.364	1.000	1.000	1.000	-	1.000	0.131	0.701

Values in bold, the frequencies were significantly different between samples, $P < 0.05$
Values not in bold, the frequencies were not significantly different between samples, $P > 0.05$
-, No statistics were computed because data were constant (frequency of infection 0% or 100%).

Table AI.7 Differences in frequency of fungi in almond samples from field until the end of storage, as determined by two-tailed *Fisher's exact* test.

	Genus			Section								
	Total	*Penicillium*	*Aspergillus*	*Aspergillus*	*Circumdati*	*Flavi*	*Flavipedes*	*Fumigati*	*Nidulantes*	*Nigri*	*Versicolores*	*Wentii*
Field → Storage 1												
In-Shell	-	-	**0.048**	**0.000**	0.639	0.359	0.581	-	1.000	**0.000**	**0.000**	**0.008**
Shelled	0.400	0.517	**0.000**	0.091	-	-	-	0.308	1.000	-	0.308	0.091
All	0.333	0.846	**0.013**	**0.000**	0.643	0.392	0.586	0.308	1.000	**0.000**	**0.000**	**0.004**
Storage 1 → Storage 2												
In-Shell	-	-	-	-	1.000	0.082	1.000	-	-	**0.008**	0.407	0.320
Shelled	0.341	0.731	1.000	0.661	-	1.000	-	1.000	-	-	1.000	0.487
All	0.453	0.453	1.000	0.821	1.000	0.066	1.000	-	-	**0.012**	0.498	0.815
Storage 2 → Storage 3												
In-Shell	-	-	-	-	0.231	0.176	1.000	-	-	**0.044**	0.191	0.731
Shelled	0.748	0.731	0.731	0.716	-	1.000	-	-	-	-	1.000	-
All	0.808	0.815	0.815	0.818	0.241	0.139	1.000	-	-	0.057	0.474	0.815
Field → Storage 3												
In-Shell	-	-	**0.048**	**0.000**	0.547	0.757	1.000	-	1.000	**0.001**	**0.000**	**0.001**
Shelled	0.098	0.517	**0.000**	**0.000**	-	-		-	-	-	0.308	-
All	0.147	0.846	**0.004**	**0.000**	0.552	0.775	1.000		1.000	**0.004**	**0.000**	**0.004**

Values in bold, the frequencies were significantly different between samples, $P < 0.05$
Values not in bold, the frequencies were not significantly different between samples, $P > 0.05$
-, No statistics were computed because data were constant (frequency of infection 0% or 100%).

Table AI.8 Differences between processor's samples A and B, as determined by two-tailed *Fisher's exact* test.

	Genus			Section					
	Total	*Penicillium*	*Aspergillus*	*Aspergillus*	*Circumdati*	*Flavi*	*Nigri*	*Versicolores*	*Wentii*
A in-shell vs A shelled	-	-	-	-	0.211	0.650	0.582	1.000	**0.023**
A in-shell vs B	-	-	-	-	0.582	0.370	0.211	**0.001**	0.070
A shelled vs B	-	-	-	-	1.000	1.000	1.000	**0.001**	1.000

Values in bold, the frequencies were significantly different between samples, $P < 0.05$
Values not in bold, the frequencies were not significantly different between samples, $P > 0.05$
-, No statistics were computed because data were constant (frequency of infection 0% or 100%).

Table AI.9 Differences between samples C and D, as determined by two-tailed *Fisher's exact* test.

	Genus			Section				
	Total	*Penicillium*	*Aspergillus*	*Aspergillus*	*Flavi*	*Nigri*	*Versicolores*	*Wentii*
C1 vs D1	-	-	-	-	0.451	0.127	1.000	**0.048**
C2 vs D2	-	-	-	-	**0.041**	**0.008**	0.106	1.000
C vs D	-	-	-	-	**0.023**	**0.001**	0.568	0.105
C1 vs C2	-	-	-	-	0.333	1.000	**0.020**	**0.015**
D1 vs D2	-	-	-	-	1.000	0.487	1.000	**0.000**
1 vs 2	-	-	-	-	0.453	0.781	0.083	**0.000**

Values in bold, the frequencies were significantly different between samples, $P < 0.05$
Values not in bold, the frequencies were not significantly different between samples, $P > 0.05$
-, No statistics were computed because data were constant (frequency of infection 0% or 100%).

Table AI.10 Differences between different stages of processing of samples F, as determined by two-tailed *Fisher's exact* test.

	Genus			Section						
	Total	*Penicillium*	*Aspergillus*	*Aspergillus*	*Circumdati*	*Flavi*	*Flavipedes*	*Nigri*	*Versicolores*	*Wentii*
In-shell F1 vs shelled F1	-	-	-	-	0.211	**0.003**	0.211	**0.000**	0.303	**0.001**
In-shell F1 vs shell F2	-	-	-	-	1.000	-	0.211	1.000	0.370	1.000
In-shell F1 vs kernel F2	-	-	-	-	1.000	1.000	0.095	0.532	0.700	**0.017**
Shelled F1 vs kernel F2	-	-	-	-	0.140	**0.000**	1.000	**0.000**	**0.045**	0.204
Kernel F2 vs shell F2	-	-	-	-	1.000	1.000	1.000	0.532	0.700	**0.045**
Kernel F2 vs nutmeat F3	-	-	0.487	0.487	**0.047**	**0.000**	1.000	**0.000**	**0.001**	**0.003**

Values in bold, the frequencies were significantly different between samples, P < 0.05
Values not in bold, the frequencies were not significantly different between samples, P > 0.05
-, No statistics were computed because data were constant (frequency of infection 0% or 100%).

Table AI.11 Differences in frequency of fungi of almonds in relation to origin, as determined by two-tailed *Fisher's exact* test.

	Genus			Section						
Moncorvo (A3/B3) vs Faro (F1)	Total	*Penicillium*	*Aspergillus*	*Aspergillus*	*Circumdati*	*Flavi*	*Flavipedes*	*Nigri*	*Versicolores*	*Wentii*
In-shell	-	-	-	-	**0.030**	**0.000**	**0.030**	**0.000**	0.709	0.210
Shelled	**0.004**	**0.000**	**0.001**	**0.000**	-	**0.030**	-	0.333	1.000	0.333
All	**0.011**	**0.001**	**0.003**	**0.002**	**0.033**	**0.000**	**0.033**	0.000	1.000	0.261

Values in bold, the frequencies were significantly different between samples, P < 0.05
Values not in bold, the frequencies were not significantly different between samples, P > 0.05
-, No statistics were computed because data were constant (frequency of infection 0% or 100%).

Table AI.12 Differences in frequency of fungi of almonds between field, storage and processor samples, as determined by two-tailed *Fisher's exact* test

	Genus			Section								
	Total	*Penicillium*	*Aspergillus*	*Aspergillus*	*Circumdati*	*Flavi*	*Flavipedes*	*Fumigati*	*Nidulantes*	*Nigri*	*Versicolores*	*Wentii*
Field → Storage 1												
In-Shell	-	-	**0.048**	**0.000**	0.639	0.359	0.581	-	1.000	**0.000**	**0.000**	**0.008**
Shelled	0.400	0.517	**0.000**	0.091	-	-	-	0.308	1.000	-	0.308	0.091
Storage 1 → Storage 3												
In-Shell	-	-	-	-	0.487	1.000	0.487	-	-	1.000	**0.014**	0.748
Shelled	0.752	1.000	1.000	0.235	-	-	-	1.000	-	-	1.000	0.487
Storage 3 → D1												
In-Shell	-	-	-	-	-	**0.000**	-	-	-	**0.000**	0.191	0.480
Shelled	**0.000**	**0.000**	**0.000**	**0.000**	-	**0.000**	-	-	-	**0.000**	0.182	**0.000**
Field → D1												
In-Shell			**0.048**	**0.000**	0.547	**0.000**	1.000	-	1.000	**0.001**	0.095	**0.000**
Shelled	**0.003**	**0.000**	**0.000**	**0.000**		**0.000**	-	-	-	**0.000**	**0.002**	**0.000**

Values in bold, the frequencies were significantly different between samples, $P < 0.05$
Values not in bold, the frequencies were not significantly different between samples, $P > 0.05$
-, No statistics were computed because data were constant (frequency of infection 0% or 100%).

Table AI.13 Differences in number of isolates of *Aspergillus* section *Flavi* between field, storage and processor samples, as determined by two-tailed *Mann-Whitney* test

		Morphotype				
	Flavi Total	*A. flavus*	*A. parasiticus*	*A. tamarii*	AF+	*A. flavus* AF+
Stage of production (Moncorvo, 2008)						
Field vs Storage 1	0.109	0.547	0.060	1.000	0.214	0.652
Field vs Processor	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
Storage 3 vs Processor	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
Origin (Moncorvo vs Faro)						
In-shell	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.037**
Shelled	**0.001**	1.000	**0.000**	1.000	**0.000**	1.000
All	**0.000**	**0.000**	**0.000**	**0.002**	**0.000**	**0.000**
Type of processing (Faro)						
In-shell vs shelled	**0.000**	**0.001**	**0.000**	0.211	**0.000**	0.474
In-shell vs kernel	**0.001**	0.068	**0.001**	0.095	**0.003**	0.494
In-shell vs shell	0.283	0.846	**0.026**	0.270	0.089	0.266
Shelled vs kernel	**0.000**	**0.003**	**0.004**	1.000	**0.000**	0.064
Shelled vs nutmeat	**0.008**	1.000	**0.008**	1.000	**0.008**	1.000
Shelled vs seed coat	0.181	1.000	**0.031**	1.000	0.031	1.000
kernel vs nutmeat	**0.000**	**0.000**	**0.000**	1.000	**0.000**	**0.008**
nutmeat vs seed coat	0.231	1.000	1.000	1.000	1.000	1.000

Values in bold, the frequencies were significantly different between samples, $P < 0.05$
Values not in bold, the frequencies were not significantly different between samples, $P > 0.05$

Appendix II

Table AII.1 List of *Aspergillus* section *Flavi* isolates

Code	Origin	Sclerotia on CYA25[a]	Seriation on CYA25[b]	Conidia on CYA25	Diameter on CZ42[c]	Reverse on AFPA	Colony colour on CYA25	Fluorescence on CAM	AFG_2 on YES[d]	AFG_1 on YES[d]	AFB_2 on YES[d]	AFB_1 on YES[d]	CPA on CYA[e]	Phenotype	Species (Phenotypic + Molecular + MALDI-TOF)
Controls															
MUM92.01		> 400	b/u	smooth	1.8	orange	yellow-green	violet	-	-	-	+/-	+		*A. flavus*
MUM92.02		> 400	u	rough	n.d.	orange	dark-green	blue	+	+	+	+	-		*A. parasiticus*
05UAsBr01 (MUM 10.220)		> 400	b	smooth	n.d.	orange	yellow-green	-	-	-	-	-	-		*A. flavus*
01UAs55			b	smooth	n.d.	orange	yellow-green	-	-	-	-	-	-		*A. flavus*
NRRL427			n.d.	thick/rough	n.d.	brown	brown	-	-	-	-	-	+		*A. tamarii*
NRRL429			n.d.	thick/rough	n.d.	brown	brown	-	-	-	-	-	+		*A. tamarii*
NRRL3353			n.d.	rough	n.d.	cream	dark-green	blue	++	+++	+	++	-		*A. nomius*
NRRL13137			n.d.	rough	n.d.	cream	dark-green	blue	++	+++	+	++	-		*A. nomius*
Field strains															
07AAsp05	Moncorvo	> 400	b/u	rough	1.4	orange	dark-green	blue	+	+	+/-	+	-	*A. parasiticus* I	*A. parasiticus*
07AAsp37 (MUM 10.200)	Moncorvo	-	b/u	smooth	1.4	orange	yellow-green	violet	-	-	+	++	+	*A. flavus* III	*A. flavus*
07AAsp43 MUM 10.201)	Moncorvo	-	u/b	rough	1.5	orange	dark-green	blue	+	+	+/-	+	-	*A. parasiticus* I	*A. parasiticus*
08AAsp34	Moncorvo	> 400	u	rough	0.8	orange	dark-green	blue	+	+++	+	++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp35 (MUM 10.202)	Moncorvo	> 400	b	smooth	2.7	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
08AAsp36 (MUM 10.225)	Moncorvo	> 400	u/b	rough	0.5	orange	dark-green	blue	+	+++	+	++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp37 (MUM 10.203)	Moncorvo	> 400	b	smooth	2	orange	yellow-green	-	-	+/-	-	+/-	+	*A. flavus* IV	*A. flavus*
08AAsp38	Moncorvo	-	u	rough	1.3	orange	dark-green	blue	+	+++	+	++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp39	Moncorvo	-	u	rough	2.2	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp41	Moncorvo	> 400	b	smooth	2	orange	yellow-green	-	-	+/-	-	+/-	+	*A. flavus* IV	*A. flavus*

(continues)

Table AII.1 (continued)

Code	Origin	Sclerotia on CYA25[a]	Seriation on CYA25[b]	Conidia on CYA25	Diameter on CZ42[c]	Reverse on AFPA	Colony colour on CYA25	Fluorescence on CAM	AFG_2 on YES[d]	AFG_1 on YES[d]	AFB_2 on YES[d]	AFB_1 on YES[d]	CPA on CYA[e]	Phenotype	Species (Phenotypic + Molecular + MALDI-TOF)
08AAsp42 (MUM 10.204)	Moncorvo	-	b	smooth	2.5	orange	yellow-green	violet	-	-	+/-	+	+	*A. flavus* III	*A. flavus*
08AAsp43	Moncorvo	-	b	smooth	2.1	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
08AAsp66	Moncorvo	> 400	u/b	rough	0.5	orange	dark-green	blue	+	+++	+	++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp67 (MUM 10.205)	Moncorvo	> 400	b/u	rough	2.2	orange	dark-green	green	++	++	+	+	(?)	*A. parasiticus* II	**New sp. 1**
08AAsp68	Moncorvo	-	u	rough	2.6	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp72	Moncorvo	-	u	rough	2.7	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp76	Moncorvo	> 400	b/u	smooth	1.5	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
08AAsp77	Moncorvo	-	b	smooth	2.9	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
08AAsp83	Moncorvo	> 400	u	rough	1.6	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp101	Moncorvo	-	u/b	rough	1.6	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp103	Moncorvo	-	u	rough	1.7	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp105	Moncorvo	> 400	b/u	smooth	3	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
08AAsp106	Moncorvo	> 400	b/u	smooth	3	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
08AAsp108	Moncorvo	-	u	rough	2.8	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp109	Moncorvo	> 400	b	smooth	2.7	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
08AAsp110	Moncorvo	> 400	u/b	rough	1.7	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp111	Moncorvo	-	b/u	rough	3.1	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp112	Moncorvo	> 400	u/b	smooth	2.6	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
08AAsp113	Moncorvo	> 400	u/b	smooth	2.9	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
08AAsp115	Moncorvo	> 400	b	smooth	2	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
08AAsp116 (MUM 10.206)	Moncorvo	-	b	smooth	2	orange	yellow-green	violet	-	-	+/-	+	+	*A. flavus* III	*A. flavus*
08AAsp117	Moncorvo	> 400	u	rough	1.8	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp119	Moncorvo	-	b	thick/rough	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
08AAsp158	Moncorvo	-	u/b	rough	2.3	orange	dark-green	blue	-	-	++	+++	-	*A. parasiticus* IV	**New sp. 3**
08AAsp159	Moncorvo	-	b	smooth	2	orange	yellow-green	violet	-	-	+/-	+	+	*A. flavus* III	*A. flavus*
08AAsp176	Moncorvo	> 400	b	thick/rough	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
08AAsp177	Moncorvo	> 400	u	rough	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*

(continues)

Table AII.1 (continued)

Code	Origin	Sclerotia on CYA25[a]	Seriation on CYA25[b]	Conidia on CYA25	Diameter on CZ42[c]	Reverse on AFPA	Colony colour on CYA25	Fluorescence on CAM	AFG_2 on YES[d]	AFG_1 on YES[d]	AFB_2 on YES[d]	AFB_1 on YES[d]	CPA on CYA[e]	Phenotype	Species (Phenotypic + Molecular + MALDI-TOF)
08AAsp178	Moncorvo	> 400	u/b	rough	n.d.	orange	dark-green	blue	+	+++	+	++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp179 (MUM 10.207)	Moncorvo	> 400	b	slightly rough	n.d.	orange	yellow-green	violet	-	-	+/-	+++	+	*A. flavus* III	*A. flavus*
08AAsp180	Moncorvo	> 400	b/u	rough	n.d.	orange	dark-green	blue	+++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp181	Moncorvo	> 400	b/u	rough	n.d.	orange	dark-green	blue	+++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp182	Moncorvo	> 400	b/u	rough	n.d.	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp183 (MUM 10.208)	Moncorvo	> 400	u	rough	n.d.	orange	dark-green	blue	++	+++	++	+++	+	*A. parasiticus* III	**New sp. 2**
08AAsp184	Moncorvo	> 400	b	rough	n.d.	orange	dark-green	blue	+++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp185	Moncorvo	> 400	b	rough	n.d.	orange	dark-green	blue	+++	+++	+	+++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp223	Moncorvo	> 400	u/b	rough	n.d.	orange	dark-green	blue	+++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp224	Moncorvo	> 400	u/b	rough	n.d.	orange	dark-green	blue	+++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp225 (MUM 10.209)	Moncorvo	-	b/u	smooth	n.d.	orange	yellow-green	violet	-	-	++	+++	+	*A. flavus* III	*A. flavus*
08AAsp226	Moncorvo	> 400	u/b	rough	n.d.	orange	dark-green	blue	+++	+++	+	+++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp252 (MUM 10.210)	Moncorvo	-	b/u	rough	n.d.	cream	dark-green	blue	+++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp253	Moncorvo	-	u/b	rough	n.d.	orange	dark-green	blue	+++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
08AAsp273	Moncorvo	> 400	u/b	rough	n.d.	orange	dark-green	blue	++	+++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp01	Moncorvo	-	u/b	rough	n.d.	orange	dark-green	blue	++	+++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp02	Moncorvo	-	u/b	rough	n.d.	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp03	Moncorvo	-	b	smooth	n.d.	orange	yellow-green	violet	-	-	-	++	+	*A. flavus* III	*A. flavus*
09AAsp04	Moncorvo	-	u/b	rough	n.d.	orange	dark-green	blue	++	+	+++	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp05	Moncorvo	> 400	b	smooth	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp06	Moncorvo	> 400	u/b	rough	n.d.	orange	dark-green	blue	++	+	+++	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp07	Moncorvo	-	u/b	rough	n.d.	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp08	Moncorvo	> 400	b	smooth	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp26	Moncorvo	> 400	u/b	rough	n.d.	orange	dark-green	blue	++	+	+++	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp68	Moncorvo	> 400	u/b	rough	n.d.	orange	dark-green	blue	++	+	+++	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp69	Moncorvo	-	u	rough	n.d.	orange	dark-green	blue	++	+	+++	++	-	*A. parasiticus* I	*A. parasiticus*

(continues)

Table AII.1 (continued)

Code	Origin	Sclerotia on CYA25[a]	Seriation on CYA25[b]	Conidia on CYA25	Diameter on CZ42[c]	Reverse on AFPA	Colony colour on CYA25	Fluorescence on CAM	AFG_2 on YES[d]	AFG_1 on YES[d]	AFB_2 on YES[d]	AFB_1 on YES[d]	CPA on CYA[e]	Phenotype	Species (Phenotypic + Molecular + MALDI-TOF)
09AAsp70	Moncorvo	> 400	u/b	rough	n.d.	orange	dark-green	blue	++	+	+++	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp117	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	++	++	++	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp118	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	++	++	++	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp119	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp120	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp140	Moncorvo	-	n.d.	n.d.	n.d.	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp141	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp142	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp143	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	-/+	++	+	*A. flavus* III	*A. flavus*
09AAsp144	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp145	Moncorvo	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+/-	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp146 (MUM 10.211)	Moncorvo	> 400	n.d.	rough	n.d.	cream	dark-green	blue	+	++	+/-	+	(?)	*A. parasiticus* II	**New sp. 1**
09AAsp147	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp148	Moncorvo	n.d.	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp149	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp150	Moncorvo	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp151	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp152 (MUM 10.221)	Moncorvo	> 400	n.d.	rough	n.d.	orange	dark-green	green	++	++	+	+	(?)	*A. parasiticus* II	**New sp. 1**
09AAsp153 (MUM 10.243)	Moncorvo	n.d.	n.d.	n.d.	n.d.	cream	dark-green	blue	+	++	+	+	(?)	*A. parasiticus* II	**New sp. 1**
09AAsp154	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp155	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp156	Faro	-	n.d.	thick/rough	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp157	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp185	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp186	Faro	n.d.	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+++	+/-	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp187 (MUM 10.212)	Faro	n.d.	n.d.	rough	n.d.	orange	dark-green	violet	+/-	+/-	++	++	-	*A. parasiticus* I	*A. parasiticus*

(continues)

Table AII.1 (continued)

Code	Origin	Sclerotia on CYA25[a]	Seriation on CYA25[b]	Conidia on CYA25	Diameter on CZ42[c]	Reverse on AFPA	Colony colour on CYA25	Fluorescence on CAM	AFG_2 on YES[d]	AFG_1 on YES[d]	AFB_2 on YES[d]	AFB_1 on YES[d]	CPA on CYA[e]	Phenotype	Species (Phenotypic + Molecular + MALDI-TOF)
09AAsp188	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+	+	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp189	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	++	+++	+	*A. flavus* III	*A. flavus*
09AAsp190	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp191	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp192	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp193	Moncorvo	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp194	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp195	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp196	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	+	++	+	*A. flavus* III	*A. flavus*
09AAsp197	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp198	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp199	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp200	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp201 (MUM 10.222)	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	(?)	*A. parasiticus* II	**New sp. 1**
09AAsp202	Moncorvo	-	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp203	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	+	++	+	*A. flavus* III	*A. flavus*
09AAsp204	Moncorvo	> 400	n.d.	n.d.	n.d.	cream	dark-green	blue	+	++	+	+	(?)	*A. parasiticus* II	**New sp. 1**
09AAsp205	Faro	n.d.	n.d.	smooth	n.d.	orange	dark-green	-	n.d.	n.d	n.d.	n.d.	-	n.d.	*A. parasiticus*
09AAsp206	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp207	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	+	+++	+	*A. flavus* III	*A. flavus*
09AAsp208	Moncorvo	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp209	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+/-	++	-/+	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp210	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp211	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+	+	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp212	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp213	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp214	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp215	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+++	+	+++	-	*A. parasiticus* I	*A. parasiticus*

(continues)

Table AII.1 (continued)

Code	Origin	Sclerotia on CYA25[a]	Seriation on CYA25[b]	Conidia on CYA25	Diameter on CZ42[c]	Reverse on AFPA	Colony colour on CYA25	Fluorescence on CAM	AFG_2 on YES[d]	AFG_1 on YES[d]	AFB_2 on YES[d]	AFB_1 on YES[d]	CPA on CYA[e]	Phenotype	Species (Phenotypic + Molecular + MALDI-TOF)
09AAsp216	Moncorvo	-	n.d.	thick/rough	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp217	Moncorvo	-	n.d.	thick/rough	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp218	Moncorvo	-	n.d.	thick/rough	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp219	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp220	Moncorvo	-	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp221	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp222	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp223	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp224	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp225	Faro	n.d.	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+	+	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp226	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp227	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp228	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp229	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp230	Moncorvo	> 400	n.d.	rough	n.d.	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp231	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp232	Faro	n.d.	n.d.	rough	n.d.	orange	dark-green	violet	-	-	++	+++	-	*A. parasiticus* IV	**New sp. 3**
09AAsp233 (MUM 10.244)	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	++	++	+	+	(?)	*A. parasiticus* II	**New sp. 1**
09AAsp234	Faro	n.d.	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+/-	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp235 (MUM 10.224)	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	violet	-	-	++	+++	-	*A. parasiticus* IV	**New sp. 3**
09AAsp236	Moncorvo	-	n.d.	rough	n.d.	orange	dark-green	blue	+	+	+	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp237	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp238	Moncorvo	> 400	n.d.	rough	n.d.	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp239 (MUM 10.245)	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	green	++	++	+	+	(?)	*A. parasiticus* II	**New sp. 1**
09AAsp240 (MUM 10.213)	Moncorvo	n.d.	n.d.	rough	n.d.	cream	dark-green	blue	+/-	++	+/-	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp241	Moncorvo	> 400	n.d.	rough	n.d.	orange	dark-green	violet	-	-	+	++	-	*A. parasiticus* IV	**New sp. 3**
09AAsp242	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*

(continues)

Table AII.1 (continued)

Code	Origin	Sclerotia on CYA25[a]	Seriation on CYA25[b]	Conidia on CYA25	Diameter on CZ42[c]	Reverse on AFPA	Colony colour on CYA25	Fluorescence on CAM	AFG_2 on YES[d]	AFG_1 on YES[d]	AFB_2 on YES[d]	AFB_1 on YES[d]	CPA on CYA[e]	Phenotype	Species (Phenotypic + Molecular + MALDI-TOF)
09AAsp243	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp244	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp245	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp246	Faro	n.d.	n.d.	n.d.	n.d.	orange	dark-green	violet	-	-	+	++	-	*A. parasiticus* IV	**New sp. 3**
09AAsp247	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp248	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp249	Moncorvo	-	n.d.	rough	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp250	Moncorvo	> 400	n.d.	rough	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp251	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp252	Moncorvo	-	n.d.	rough	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp253	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp254	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp255	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp256	Moncorvo	> 400	n.d.	smooth	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp257	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp258	Faro	-	n.d.	n.d.	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp259	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	-/+	++	+	*A. flavus* III	*A. flavus*
09AAsp260 (MUM 10.214)	Faro	> 400	n.d.	rough	n.d.	orange	dark-green	green	++	++	+	+	(?)	*A. parasiticus* II	**New sp. 1**
09AAsp261 (MUM 10.246)	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	green	++	+++	+/-	+	(?)	*A. parasiticus* II	**New sp. 1**
09AAsp262	Moncorvo	n.d.	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp263	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	violet	-	-	+	+++	-	*A. parasiticus* IV	**New sp. 3**
09AAsp264	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp265	Faro	n.d.	n.d.	n.d.	n.d.	orange	dark-green	blue	+/-	++	-/+	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp266 (MUM 10.215)	Faro	n.d.	n.d.	rough	n.d.	cream	dark-green	blue	+/-	++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp267	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+/-	++	+/-	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp268	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp269	Faro	n.d.	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*

(continues)

Table AII.1 (continued)

Code	Origin	Sclerotia on CYA25[a]	Seriation on CYA25[b]	Conidia on CYA25	Diameter on CZ42[c]	Reverse on AFPA	Colony colour on CYA25	Fluorescence on CAM	AFG_2 on YES[d]	AFG_1 on YES[d]	AFB_2 on YES[d]	AFB_1 on YES[d]	CPA on CYA[e]	Phenotype	Species (Phenotypic + Molecular + MALDI-TOF)
09AAsp270	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp271	Moncorvo	-	n.d.	thick/rough	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp272	Moncorvo	-	n.d.	n.d.	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp273	Moncorvo	-	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp274	Moncorvo	-	n.d.	n.d.	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp275	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp276	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp277	Faro	-	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp278	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp279	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp280	Faro	-	n.d.	n.d.	n.d.	brown	brown-green	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp281	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp287	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp288	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp289	Moncorvo	> 400	n.d.	rough	n.d.	orange	dark-green	blue	+	+++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp290	Moncorvo	> 400	n.d.	smooth	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp291	Moncorvo	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+	+	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp292	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp297	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp298 (MUM 10.223)	Moncorvo	> 400	n.d.	rough	n.d.	orange	dark-green	blue	-/+	+++	-/+	+	(?)	*A. parasiticus* II	**New sp. 1**
09AAsp299 (MUM 10.247)	Moncorvo	> 400	n.d.	rough	n.d.	orange	dark-green	blue	+	++	-/+	-/+	(?)	*A. parasiticus* II	**New sp. 1**
09AAsp300	Moncorvo	> 400	n.d.	smooth	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp301	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	+	+	+	*A. flavus* III	*A. flavus*
09AAsp302	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp303	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp304 (MUM 10.216)	Moncorvo	> 400	n.d.	rough	n.d.	orange	dark-green	yellow	+/-	+	+	++	-	*A. parasiticus* I	*A. parasiticus*

(continues)

Table AII.1 (continued)

Code	Origin	Sclerotia on CYA25[a]	Seriation on CYA25[b]	Conidia on CYA25	Diameter on CZ42[c]	Reverse on AFPA	Colony colour on CYA25	Fluorescence on CAM	AFG_2 on YES[d]	AFG_1 on YES[d]	AFB_2 on YES[d]	AFB_1 on YES[d]	CPA on CYA[e]	Phenotype	Species (Phenotypic + Molecular + MALDI-TOF)
09AAsp305 (MUM 10.248)	Faro	> 400	n.d.	rough	n.d.	orange	dark-green	blue	+	++	+	++	+	*A. parasiticus* III	**New sp. 2**
09AAsp306	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	-/+	++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp307	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp308	Moncorvo	> 400	n.d.	smooth	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp309	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+/-	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp310	Moncorvo	> 400	n.d.	smooth	n.d.	orange	yellow-green	violet	-	-	+/-	+	+	*A. flavus* III	*A. flavus*
09AAsp311	Moncorvo	> 400	n.d.	smooth	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp312	Moncorvo	> 400	n.d.	smooth	n.d.	orange	yellow-green	violet	-	-	+	++	+	*A. flavus* III	*A. flavus*
09AAsp313	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	-	+/-	+	*A. flavus* III	*A. flavus*
09AAsp314	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	+/-	+++	+	*A. flavus* III	*A. flavus*
09AAsp315	Moncorvo	> 400	n.d.	smooth	n.d.	orange	yellow-green	violet	-	-	++	+++	+	*A. flavus* III	*A. flavus*
09AAsp316	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp317	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp318	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+	+	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp319	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp320	Moncorvo	-	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp321	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	+	++	+	*A. flavus* III	*A. flavus*
09AAsp322	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp323	Faro	n.d.	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp324	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	violet	-	-	+/-	+	+	n.d	*A. flavus*
09AAsp325	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp326	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp327	Moncorvo	> 400	n.d.	smooth	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp328	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp329	Moncorvo	> 400	n.d.	smooth	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp330	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+++	+/-	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp331	Moncorvo	-	n.d.	rough	n.d.	orange	dark-green	blue	+	+	+	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp332	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+	+	+	-	*A. parasiticus* I	*A. parasiticus*

(continues)

Table AII.1 (continued)

Code	Origin	Sclerotia on CYA25[a]	Seriation on CYA25[b]	Conidia on CYA25	Diameter on CZ42[c]	Reverse on AFPA	Colony colour on CYA25	Fluorescence on CAM	AFG_2 on YES[d]	AFG_1 on YES[d]	AFB_2 on YES[d]	AFB_1 on YES[d]	CPA on CYA[e]	Phenotype	Species (Phenotypic + Molecular + MALDI-TOF)
09AAsp333	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+	+	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp334	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp335	Moncorvo	-	n.d.	n.d.	n.d.	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp336	Faro	n.d.	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+	+	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp337	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp338	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp339	Moncorvo	-	n.d.	n.d.	n.d.	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp340	Moncorvo	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp341	Moncorvo	n.d.	n.d.	thick/rough	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp342	Faro	+	n.d.	thick/rough	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp343	Moncorvo	n.d.	n.d.	n.d.	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp349	Moncorvo	> 400	n.d.	n.d.	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp378	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp379	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	+	+++	+	*A. flavus* III	*A. flavus*
09AAsp380	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp381	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp382	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp383	Faro	n.d.	n.d.	n.d.	n.d.	orange	dark-green	blue	+/-	++	-/+	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp384	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp385	Faro	-	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	-/+	+	+	*A. flavus* III	*A. flavus*
09AAsp386	Faro	> 400	n.d.	n.d.	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp387	Faro	> 400	n.d.	n.d.	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp388	Faro	-	n.d.	n.d.	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp389	Moncorvo	-	n.d.	thick/rough	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp390	Moncorvo	-	n.d.	thick/rough	n.d.	cream/orange	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp391	Faro	-	n.d.	thick/rough	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp392 (MUM 10.217)	Faro	-	n.d.	thick/rough	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp393	Moncorvo	-	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*

(continues)

Table AII.1 (continued)

Code	Origin	Sclerotia on CYA25[a]	Seriation on CYA25[b]	Conidia on CYA25	Diameter on CZ42[c]	Reverse on AFPA	Colony colour on CYA25	Fluorescence on CAM	AFG_2 on YES[d]	AFG_1 on YES[d]	AFB_2 on YES[d]	AFB_1 on YES[d]	CPA on CYA[e]	Phenotype	Species (Phenotypic + Molecular + MALDI-TOF)
09AAsp394	Moncorvo	> 400	n.d.	n.d.	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp395	Moncorvo	> 400	n.d.	n.d.	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp396	Moncorvo	-	n.d.	n.d.	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp397	Moncorvo	-	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp398	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	++	++	++	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp399	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	-/+	+	-/+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp400	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	+	++	+	*A. flavus* III	*A. flavus*
09AAsp401	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp402	Moncorvo	-	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp403	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp404	Moncorvo	-	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp405	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp406	Moncorvo	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp407	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	++	++	++	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp408	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	-/+	+	+	*A. flavus* III	*A. flavus*
09AAsp409	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	++	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp410	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp411	Moncorvo	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp412	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp413	Moncorvo	n.d.	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp414	Moncorvo	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp415	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp416	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp417	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+/-	++	-/+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp418	Moncorvo	> 400	n.d.	thick/rough	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp419	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	+	+	+	*A. flavus* III	*A. flavus*
09AAsp420	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp421	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*

(continues)

Table AII.1 (continued)

Code	Origin	Sclerotia on CYA25[a]	Seriation on CYA25[b]	Conidia on CYA25	Diameter on CZ42[c]	Reverse on AFPA	Colony colour on CYA25	Fluorescence on CAM	AFG_2 on YES[d]	AFG_1 on YES[d]	AFB_2 on YES[d]	AFB_1 on YES[d]	CPA on CYA[e]	Phenotype	Species (Phenotypic + Molecular + MALDI-TOF)
09AAsp422	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp423	Moncorvo	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp424	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp425	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp426	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	++	++	++	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp427	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	-/+	++	-/+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp428	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp429	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp473	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+/-	+	+/-	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp474	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp475	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	++	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp476	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	++	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp477 (MUM 10.249)	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	++	++	+	*A. parasiticus* III	**New sp. 2**
09AAsp478 (MUM 10.218)	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	+	+	+	*A. flavus* III	*A. flavus*
09AAsp479	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	++	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp480	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	+	+	+	*A. flavus* III	*A. flavus*
09AAsp481	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp482	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	+	++	+	*A. flavus* III	*A. flavus*
09AAsp483	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	++	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp484	Faro	-	n.d.	thick/rough	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp485	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp486	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp487 (MUM 10.250)	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	-/+	-/+	(?)	*A. parasiticus* II	**New sp. 1**
09AAsp488 (MUM 10.251)	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	++	++	+	*A. parasiticus* III	**New sp. 2**
09AAsp489	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	++	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp490	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	++	++	+	++	-	*A. parasiticus* I	*A. parasiticus*

(continues)

Table AII.1 (continued)

Code	Origin	Sclerotia on CYA25[a]	Seriation on CYA25[b]	Conidia on CYA25	Diameter on CZ42[c]	Reverse on AFPA	Colony colour on CYA25	Fluorescence on CAM	AFG_2 on YES[d]	AFG_1 on YES[d]	AFB_2 on YES[d]	AFB_1 on YES[d]	CPA on CYA[e]	Phenotype	Species (Phenotypic + Molecular + MALDI-TOF)
09AAsp491	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp492	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	+	++	+	*A. flavus* III	*A. flavus*
09AAsp493	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp494 (MUM 10.219)	Faro	> 400	n.d.	rough	n.d.	orange	dark-green	blue	+/-	++	+	++	+	*A. parasiticus* III	**New sp. 2**
09AAsp495	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp496	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp497	Faro	-	n.d.	n.d.	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp498	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp515	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp516	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp517	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp518	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	+	++	+	*A. flavus* III	*A. flavus*
09AAsp519	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp520	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp521	Faro	-	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	+	++	+	*A. flavus* III	*A. flavus*
09AAsp522	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp523	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp524	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp525	Faro	-	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	+	++	+	*A. flavus* III	*A. flavus*
09AAsp526	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp527	Faro	-	n.d.	n.d.	n.d.	brown	brown	-	-	-	-	-	+	*A. tamarii*	*A. tamarii*
09AAsp528	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	-	*A. flavus* I	*A. flavus*
09AAsp529	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	++	+++	+	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp530	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp531	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp532	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp533	Moncorvo	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp534	Faro	n.d.	n.d.	n.d.	n.d.	orange	dark-green	blue	+	+++	+	++	-	*A. parasiticus* I	*A. parasiticus*

(continues)

Table AII.1 (continued)

Code	Origin	Sclerotia on CYA25[a]	Seriation on CYA25[b]	Conidia on CYA25	Diameter on CZ42[c]	Reverse on AFPA	Colony colour on CYA25	Fluorescence on CAM	AFG_2 on YES[d]	AFG_1 on YES[d]	AFB_2 on YES[d]	AFB_1 on YES[d]	CPA on CYA[e]	Phenotype	Species (Phenotypic + Molecular + MALDI-TOF)
09AAsp537	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+/-	++	-/+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp538	Faro	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp539	Faro	n.d.	n.d.	n.d.	n.d.	orange	dark-green	violet	-	-	+	++	-	*A. parasiticus* IV	**New sp. 3**
09AAsp540	Moncorvo	-	n.d.	n.d.	n.d.	orange	yellow-green	violet	-	-	-/+	++	+	*A. flavus* III	*A. flavus*
09AAsp541	Moncorvo	> 400	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp542	Moncorvo	n.d.	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp543	Moncorvo	n.d.	n.d.	n.d.	n.d.	orange	yellow-green	-	-	-	-	-	+	*A. flavus* II	*A. flavus*
09AAsp544	Faro	n.d.	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp545	Faro	n.d.	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp546	Moncorvo	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+/-	++	-/+	+	-	*A. parasiticus* I	*A. parasiticus*
09AAsp547	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp548	Faro	n.d.	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp549	Faro	n.d.	n.d.	n.d.	n.d.	orange	dark-green	blue	++	+++	++	+++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp550	Faro	-	n.d.	n.d.	n.d.	orange	dark-green	blue	+	++	+	++	-	*A. parasiticus* I	*A. parasiticus*
09AAsp551	Faro	> 400	n.d.	n.d.	n.d.	orange	dark-green	blue	+/-	++	-/+	+	-	*A. parasiticus* I	*A. parasiticus*

-: not detected
n.d.: not determined
[a] size, in µm: average of 15 sclerotia
[b] u: uniseriate; b: biseriate; u/b: predominantly uniseriate; b/u: predominantly biseriate
[c] average of 3 colonies, in cm
[d] ++: strong signal; +: medium signal; +/-: weak signal; -/+: very weak signal
[e] (?) difficult to determine

Appendix III

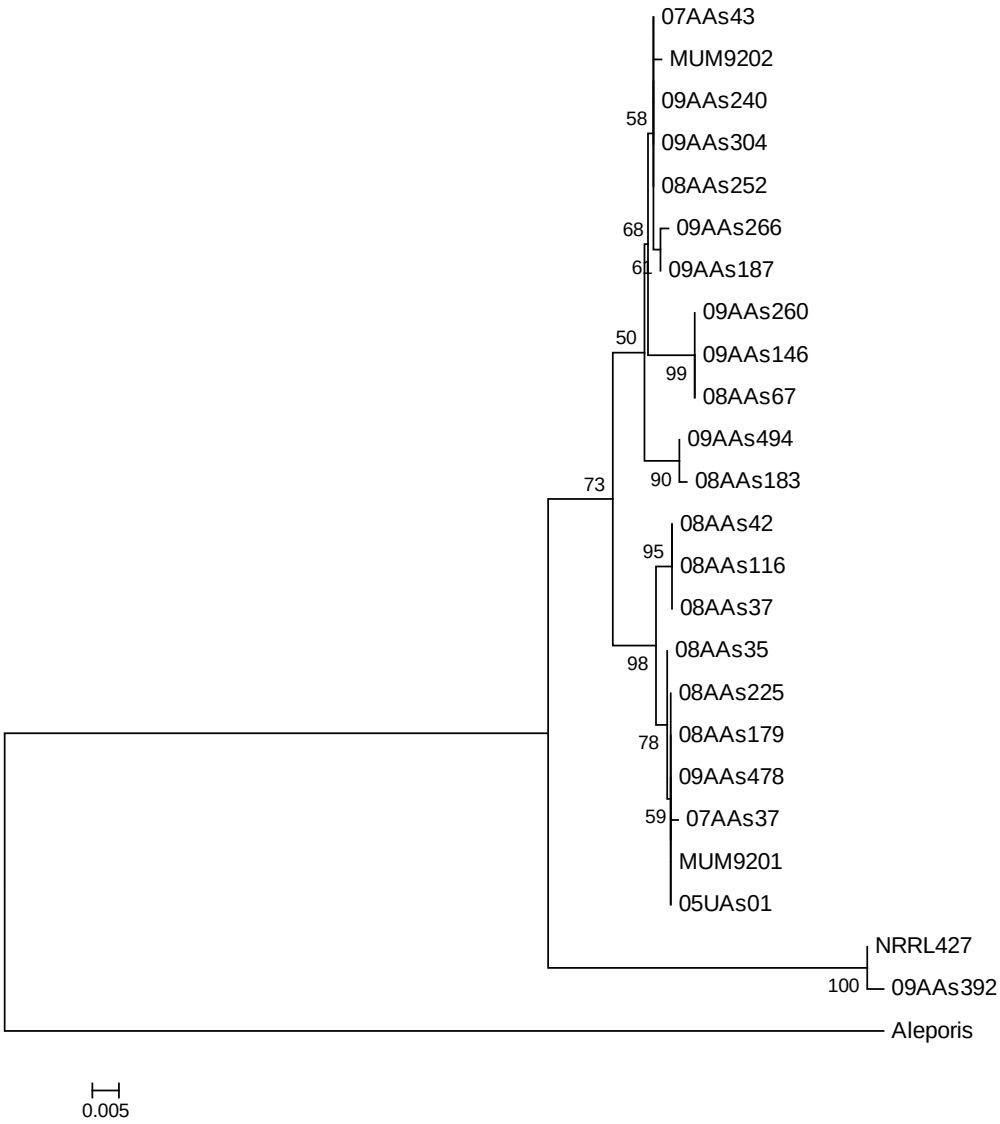

Figure AIII.1 Phylogram of evolutionary relationships of 25 taxa. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 730 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

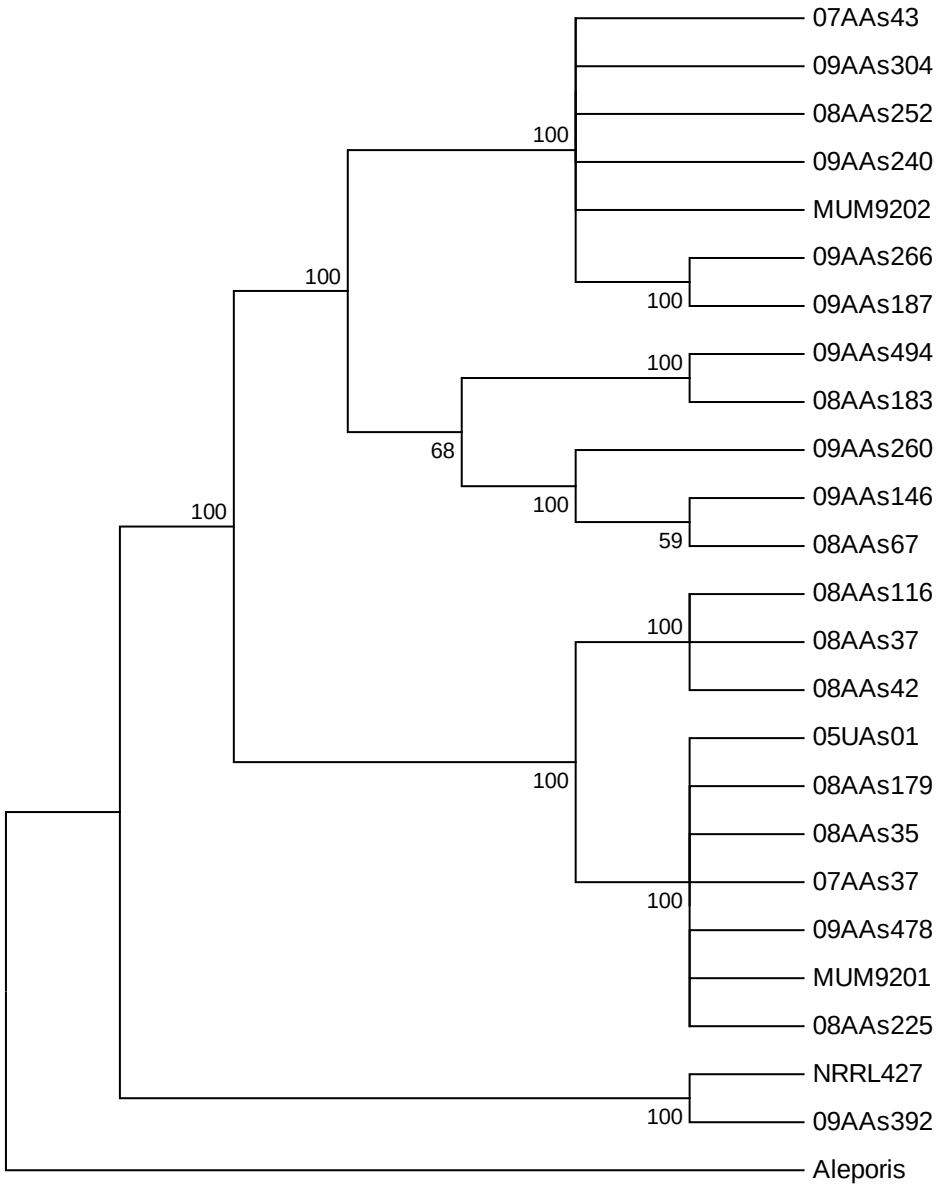

Figure AIII.2 Evolutionary relationships of 25 taxa. The evolutionary history was inferred using the Maximum Parsimony method. The consensus tree inferred from 4938 most parsimonious trees is shown. Branches corresponding to partitions reproduced in less than 50% trees are collapsed. The consistency index is 0.960000 (0.922222), the retention index is 0.964286 (0.964286), and the composite index is 0.925714 (0.889286) for all sites and parsimony-informative sites (in parentheses). The percentage of parsimonious trees in which the associated taxa clustered together are shown next to the branches. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 4 in which the initial trees were obtained with the random addition of sequences (10 replicates). The codon positions included were 1st+2nd+3rd+Noncoding. All alignment gaps were treated as missing data. There were a total of 730 positions in the final dataset, out of which 70 were parsimony informative. Phylogenetic analyses were conducted in MEGA4.

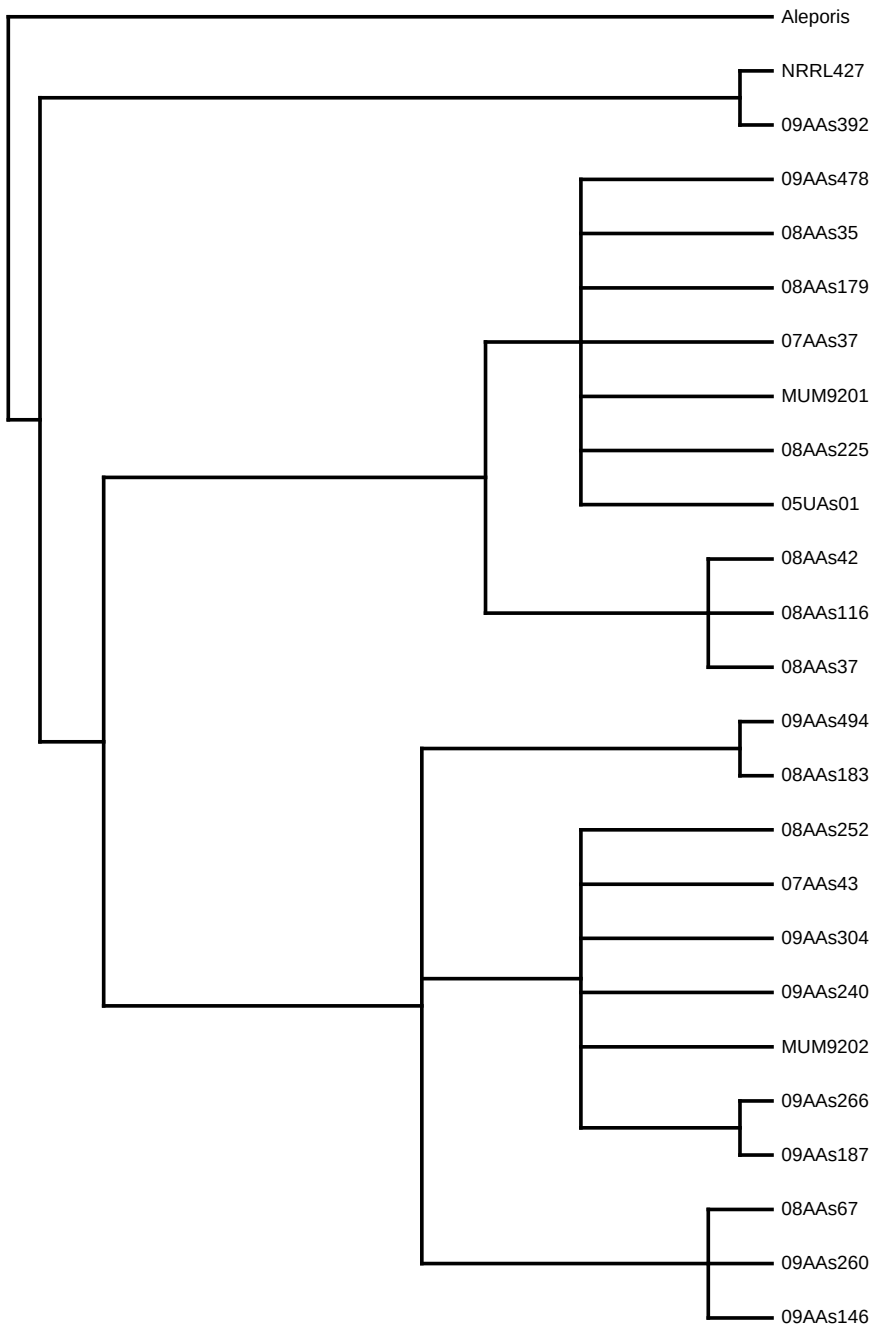

Figure AIII.3 Phylogram of relatedness obtained by Maximum Likelihood analysis using PAUP4.0.