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ORIGINAL ARTICLE

Development of a multiplex real-time PCR assay for species-level identification within *Aspergillus* section *Fumigati*

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Abstract

Aspergillus fumigatus sensu lato comprises several cryptic species that are indistinguishable by morphology but differ in antifungal susceptibility and clinical relevance. Their frequent misidentification as *A. fumigatus* sensu stricto, has been associated with inappropriate therapy and poorer clinical outcomes. Reference identification relies on sequencing specific genes, which is costly and labor intensive. In this study, a multiplex RT-PCR assay combined with melting curve analysis was developed and validated for the rapid discrimination relevant cryptic species within *Fumigati* section. Primers targeting established sequencing loci were designed to specifically identify *A. fumigatus*, *A. lentulus*, *A. felis* complex and *A. udagawae*. Singleplex assays showed highly specific melting profiles with no cross-amplifications. In multiplex, specificity was maintained by supplementing the reaction with PCR enhancers, achieving excellent analytical performance (Area Under Curve 0.93 to 0.97), confirmed using 41 isolates. Method validation under a Portuguese surveillance program was performed for six months. The newly developed assay identifies *Fumigati* isolates to species level within 1 h and 40 min after DNA extraction, while sequencing required 1–2 days, with full concordance between methods. Additionally, manual and automated DNA extractions yield reliable results. This study reports, to the best of our knowledge, the first RT-PCR method enabling species-level identification within section *Fumigati*. The assay provides accurate and cost-effective identifications, while using a universal platform for surveillance settings, with potential for future expansion.

Keywords Cryptic species · Clinical mycology · Aspergillosis · Melting curve analysis · SYBR Green

Introduction

Aspergillus fumigatus (*A. fumigatus*) is the main cause of invasive aspergillosis, but several cryptic species within section *Fumigati*, including *A. lentulus*, *A. udagawae* and *A. felis*, have emerged as relevant pathogens. These species, although indistinguishable from *A. fumigatus,* have also gained increased relevance within One Health framework, as they are opportunistic pathogens affecting humans and animals, and are distributed in the environment (Lavanya et al. 2022; Fisher et al. 2024; Aneke et al. 2026). These species also display distinct virulence traits and higher susceptibility values to antifungal drugs, particularly *A. lentulus* and *A. udagawae* (Pinto et al. 2018; Mendonça et al. 2026). Such differences reinforce the need for identification methods that go beyond morphological discrimination, enabling timely and accurate surveillance across human, veterinary and environmental health settings, in a One Health approach (Sugui et al. 2015; Lamoth 2016; Geremia et al. 2024).

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Traditionally, the identification of *Aspergillus* species in clinical and epidemiological studies relies on morphological criteria. These include macroscopic features such as colony texture and conidial pigmentation, and microscopic structures such as conidiophores shape and arrangement of phialides (Samson et al. 2007; Sugui et al. 2015; Mendonça et al. 2022a, b). However, this approach only enables identification at genus and section levels, which ends up having several limitations when applied to cryptic species. In this scenario, macro and microscopic evaluation hampers reliable identification between *A. fumigatus* sensu stricto and its sibling species (Samson et al. 2007; Sugui et al. 2015; Lamoth 2016). To overcome these limitations, several molecular methods have been increasingly implemented, especially in surveillance programs. Sequencing of conserved loci such as β-tubulin, calmodulin, rodlet genes and *cyp51A* remains the gold standard for identification and discrimination within *A. fumigatus* sensu lato, yet this approach is not only time-consuming, but also resource-demanding (Alcazar-Fuoli et al. 2008; Balajee et al. 2009a; Lamoth 2016).

In 2013, the European Center for Disease Prevention and Control (ECDC) has recommended the establishment of epidemiological surveillance programs to collect data on *Aspergillus* spp. in both clinical and environmental settings (European Centre for Disease Prevention and Control 2013). In Portugal, a laboratory-based national surveillance program for *Aspergillus* has been in place since 2012, coordinated by the National Reference Laboratory for Mycology at the National Institute of Health Dr. Ricardo Jorge, Lisbon. Within this program *Aspergillus* isolates are referred from hospitals and other institutions, across the National Health Service for species identification and antifungal susceptibility testing. Species identification is routinely performed by sequencing of the calmodulin gene, which provides high specificity, but is relatively time-consuming and labor-intensive. The program aims to monitor the frequency and diversity of *Aspergillus* species and, in particular, to track azole resistance in *A. fumigatus*, thereby supporting evidence-based policies for aspergillosis management and antifungal awareness (Sabino et al. 2021). Additionally, several studies on specific occupational contexts have been developed aiming to assess the presence of *A. fumigatus* sensu stricto (Viegas et al. 2017, 2021; Sabino et al. 2025).

To address these limitations, we developed and validated a multiplex real-time PCR assay coupled with melting-curve analysis for the rapid identification of cryptic species within section *Fumigati*, specifically designed to be applied to DNA extracted from pure fungal cultures, as a support tool for surveillance-based identification. The method was implemented and tested in the context of the Portuguese *Aspergillus* Surveillance Program, during six months, aiming to provide a faster and cost-effective alternative to sequencing while maintaining full species-level identification of more relevant cryptic species.

Materials and methods

Identification of species-specific target regions and primer design

To enable species-level discrimination within *A. fumigatus* sensu lato, phylogenetic analyses were initially performed using the fungal barcode internal transcribed spacer (ITS) region, together with β-tubulin, calmodulin and *cyp51A*, which are commonly employed in sequencing-based identification workflows (Sugui et al. 2015; Lamoth 2016; Mendonça et al. 2022a, b). A total of 696 sequences from target and nine non-target species belonging to section *Fumigati* were gathered from National Center for Biotechnology Information (NCBI) and European Nucleotide Archive (ENA) databases. Sequence alignments were performed using MAFFT alignment (version 7), and Neighbor-Joining trees were constructed using MEGA (version 12) with 500 bootstrap replicates. Tree visualization and annotation were performed using the Interactive Tree of Life (iTOL) online tool (itol.embl.de/). Phylogenetic observations supported the selection of β-tubulin, calmodulin and *cyp51A* as suitable targets for species-level discrimination within section *Fumigati*, and primer design was subsequently performed within these regions using 581 sequences both from target and non-target species (Supplementarymaterial 1). After collecting all sequences alignments were made, separately for each gene, using MAFFT alignment (version 7), and were visualized using MEGA (version 12), to look for regions that were able to discriminate these species. Species-specific nucleotide differences identified near the 3' region were incorporated into the forward primers to maximize amplification specificity and prevent cross-reactivity among closely related species. In this design strategy, the reverse primer acted as a mostly conserved anchoring region, while the forward primer provided species-level specificity. This allowed the design of three primer pairs; β-tubulin primers to specifically identify *A. lentulus*, calmodulin primers to identify *A. udagawae*, and *cyp51A* primers to identify *A. felis*. As for *A. fumigatus*, primers were already designed from our previous work (Carvalho-Pereira et al. 2020; Mendonça et al. 2022a, b). Additionally, each primer pair was evaluated using Primer-BLAST (NCBI) against the nucleotide databases for fungi (taxID: 4751), bacteria (taxID: 2), *Aspergillus* (taxID: 5052), to assess potential cross-reactivity. OligoAnalyzer Tool (IDT), was further used to evaluate amplicon length and guanine/cytosine (GC) content.

Optimization and analytical validation of the multiplex RT-PCR assay

To ensure suitability for laboratories with different technical capacities, both manual (Mendonça et al. 2022a, b) and automated (EMAG® Nucleic Acid Extraction System; bioMérieux) DNA extraction methods were evaluated, to confirm equivalent performance. Additionally, a SYBR green chemistry was selected to ensure broad applicability across standard RT-PCR platforms while maintaining cost-effectiveness. Singleplex and multiplex reactions were initially optimized to assess melting temperature (T_m) specificity and reproducibility for each target species, using five independent replicates (Supplementary material 2). Analytical sensitivity was assessed by receiver operating characteristics (ROC) curve analysis using five DNA concentrations (5.33 to 0.33 ng/µL), as the test variable, and correct species-specific amplification (expected T_m value), as the positive outcome. Primer specificity was assessed using DNA from 23 target isolates, and 18 non-target species. The target isolates comprised cryptic *Fumigati* isolates collected within the Portuguese *Aspergillus* Surveillance Program, originating from different geographic regions, and including both clinical and environmental sources, previously characterized in a dedicated study (Sabino et al. 2021).

Application in blind samples – preliminary validation in surveillance context

A six-month proof-of-concept (POC) evaluation was conducted within the Portuguese *Aspergillus* Surveillance Program at the National Mycology Reference Laboratory to assess the applicability of the developed multiplex RT-PCR assay in a real-world setting. During routine processing, clinical and environmental samples are cultured in malt extract agar (MEA). Isolates are incubated for seven days at 30 °C, and a macro- and microscopic evaluation is performed to assign isolates to their respective section. Isolates assigned to section *Fumigati* were subjected to molecular identification by calmodulin sequencing, as previously described (Sabino et al. 2021). Two isolates exhibiting atypical phenotypic characteristics compared with the morphology commonly observed for *A. fumigatus* sensu stricto were selected for blind parallel testing. DNA extracted from pure cultures was analyzed using both reference sequencing workflow and the developed multiplex RT-PCR assay, allowing direct comparison of species identification and turnaround time.

Results

Phylogenetic analysis and target region selection

The ITS region is considered the universal fungal barcode and is commonly used for species-level identification (Mendonça et al. 2022a, b). However, within section *Fumigati*, ITS sequencing only enables identification at section level. Phylogenetic analysis of the ITS (Fig. 1a) region showed overlapping clustering between *A. fumigatus*, *A. lentulus*, *A. udagawae*, *A. felis* and related species, highlighting the conserved nature of this *locus* within cryptic *Fumigati* species.

In contrast, calmodulin, β-tubulin, and *cyp51A* phylogenetic trees (Fig. 1b–d, respectively) showed improved species-level clustering, with most cryptic species grouping into well-supported clades. Nevertheless, partial conservation between *A. felis* and *A. viridinutans*, as well as between *A. lentulus* and *A. fumisynnematus* was observed, particularly in the β-tubulin and calmodulin (Fig. 1b, c) regions. Despite this, these loci provided substantially greater discriminatory resolution than ITS, enabling the identification of species-specific regions suitable for primer design.

Species-specific primer design and multiplex optimization

Comparative sequence analysis revealed the presence of nucleotide polymorphism across all selected loci, enabling the identification of species-specific regions for primer design (Fig. 2). The primer design strategy preferentially incorporated discriminatory nucleotides near the 3' region of the forward primer to maximize amplification specificity, while reverse primers were designed within mostly conserved regions to ensure stable amplification anchoring.

Within the β-tubulin region (Fig. 2a), the selected target sequence for *A. lentulus* showed substantial nucleotide variability relative to most non-target species, particularly at the 3' region. However, only a single nucleotide difference was observed between *A. lentulus* and *A. fumisynnematus*. Similar patterns were observed for calmodulin and *cyp51A* (Fig. 2b, c), where discriminatory nucleotide variations located near the 3' region enabled differentiation of the target species from all non-target species, minimizing the likelihood of cross-reactivity.

In silico validation of the designed primers through Primer-BLAST showed no off-target amplicons when using the designed sequences against fungi, bacteria and *Aspergillus* databases, supporting the specificity of the primers' design. Since species discrimination in the developed assay relies on T_m analysis, amplicon size and GC content were considered critical parameters during primer design. The previously validated *A. fumigatus* specific primer pair (Carvalho-Pereira et al. 2020; Mendonça et al. 2022a, b), targeting a non-coding region, generated an amplicon of 189 base pairs (bp) with a GC content of 36.0%. For *A. lentulus*, the selected β-tubulin region generated an 86 bp amplicon with 53.5% GC content, whereas

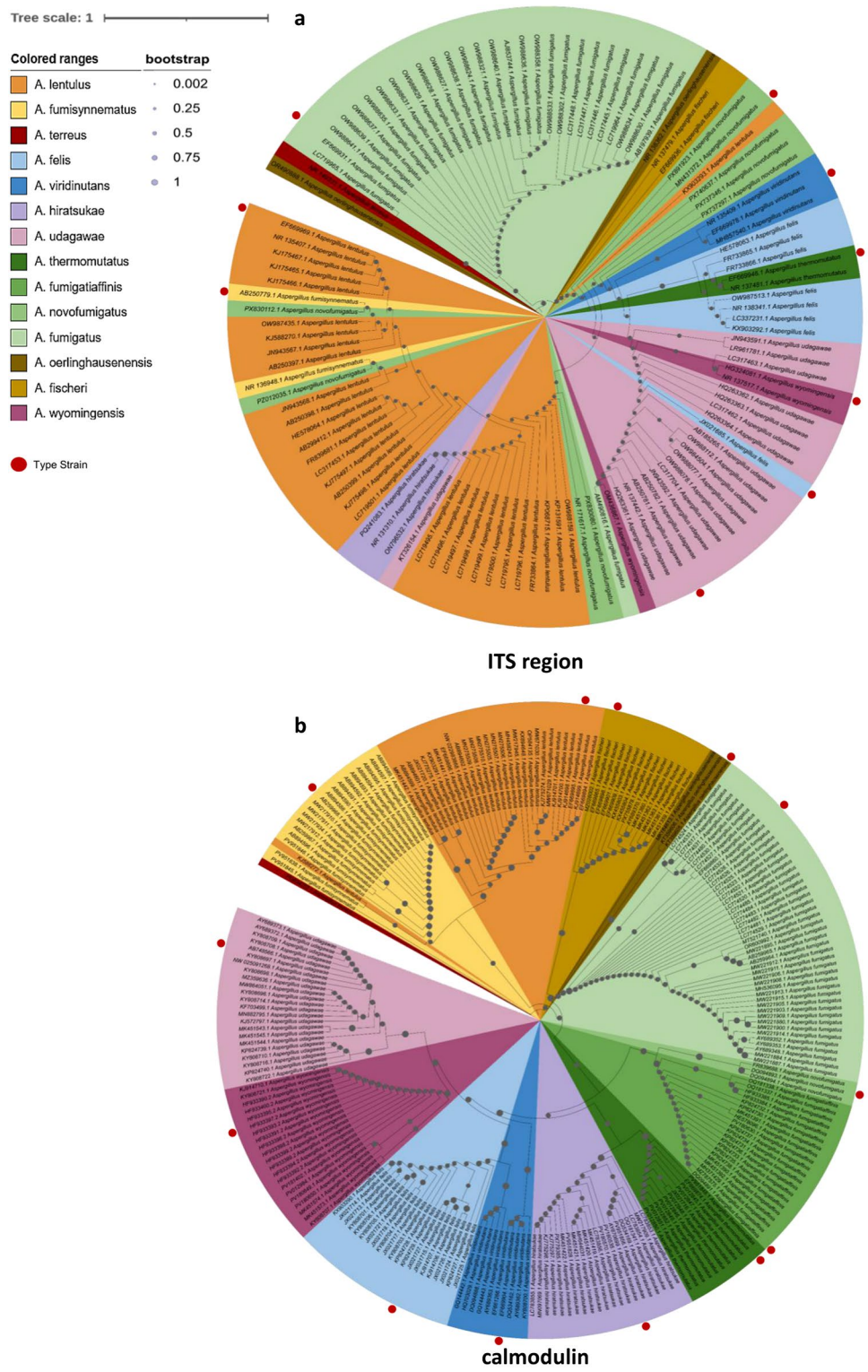
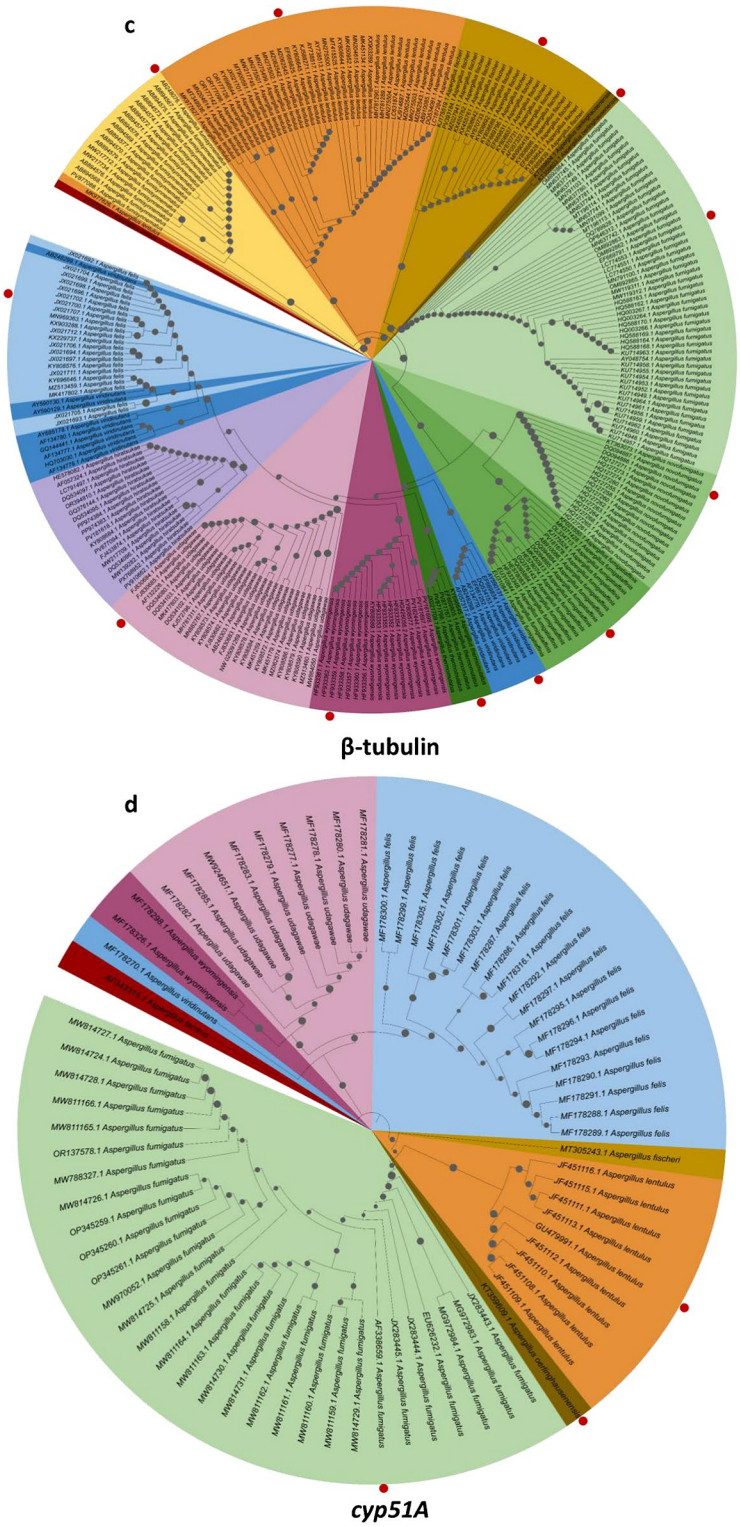


Fig. 1 Phylogenetic analyses of ITS (**a**), calmodulin (**b**), β-tubulin (**c**) and *cyp51A* (**d**) regions from species belonging to *Aspergillus* section *Fumigati*. Neighbor-Joining trees were constructed using sequences retrieved from NCBI and ENA databases, with 500 bootstrap replicates. The ITS region showed limited discriminatory power among cryptic *Fumigati* species, whereas β-tubulin, calmodulin and *cyp51A* enabled improved species-level clustering, supporting their selection for species-specific primer design. Species are color coded as indicated in the colored ranges section. Bootstrap values ranged from 0.002 to 1.0, as indicated. Type strains are indicated by a red circle. In the *cyp51A* phylogeny, the highlighted sequences correspond to reference strains for the *cyp51A* gene

the calmodulin target selected for *A. udagawae* generated a 230 bp amplicon with 52.5% GC content. Finally, the *A. felis* cyp51A target generated a 173 bp amplicon with 51.0% GC content. Primer sequences and predicted amplicon characteristics are summarized in Table 1.

Singleplex reactions established species-specific T_m values for the target species, as shown in Fig. 3a. DNA from 23 target isolates showed highly consistent T_m values, without overlapping, which are presented as the mean and standard deviation from five independent replicates, namely *A.*

Fig. 1 (continued)



fumigatus (78.8 ± 0.24 °C; Coefficient Variation = 0.30%), *A. lentulus* (82.2 ± 0.22 °C; CV = 0.28%), *A. udagawae* (85.4 ± 0.22 °C; CV = 0.26%), *A. felis* (84.2 ± 0.29 °C; CV = 0.34%), as shown in Supplementarymaterial 3. By testing DNA from 41 independent isolates, no unspecific amplifications were detected, even when using DNA from other *Fumigati* non-target isolates (Supplementary material 4). Multiplex condition was optimized by supplementing the reaction with 0.8 M betaine, which minimized non-specific primer interactions. Still, a downshift of the T_m values

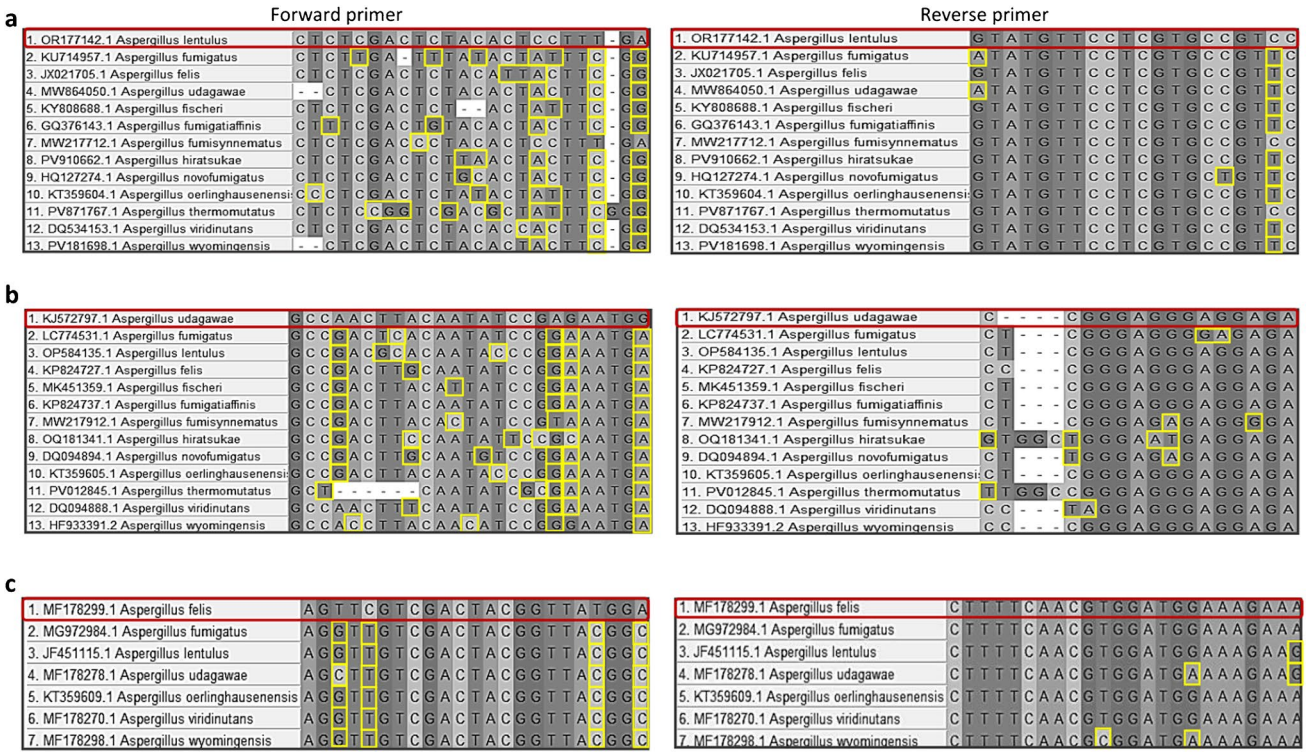


Fig. 2 Representative sequence alignments showing the regions selected for species-specific primer design within *Apergillus* section *Fumigati*. β-tubulin alignments used for specific identification of *A. lentulus* (**a**). Calmodulin alignment used for specific identification of *A. udagawae* (**b**). *Cyp51A* alignment used for specific identification of *A. felis* (**c**). Forward primers were designed within regions containing discriminatory nucleotide polymorphism, particularly near the 3' end, whereas reverse primers targeted more conserved regions. Nucleotide differences between target and non-target species are highlighted in yellow

Table 1 Primer sequences designed (Invitrogen). *Aspergillus fumigatus* primer sequences are included in the Portuguese Patent number PT 115216 (Carvalho-Pereira et al. 2020; Mendonça et al. 2022a, b)

Species	Target Region	Primer Sequence (5' to 3')	Amplification Length (bp)	GC Content (%)
A. fumigatus	Non-coding Region	**Fw:** GCC CTC TTC CGT TAT TCC TT **Rev:** GCG CAT TGA TAG CTA CCT CAG GC	189	36.0
A. lentulus	β-tubulin	**Fw:** CTC TCG ACT CTA CAC TCC TTT GA **Rev:** GG ACG GCA CGA GGA ACA TAC	86	53.5
A. udagawae	calmodulin	**Fw:** GCC AAC TTA CAA TAT CCG AGA ATG G **Rev:** TCT CCT CCC TCC CGG	230	52.5
A. felis	*cyp51A*	**Fw:** AGT TCG TCG ACT ACG GTT ATG GA **Rev:** TTT CTT TCC ATC CAC GTT GAA AAG	173	51.0

Abbreviations:**Fw** refers to forward primer; **Rev** refers to reverse primer

was observed, attributed to the intrinsic mechanisms of betaine (Mendonça et al. 2022a, b). In multiplex, the method ensured consistent and reliable melting profiles, although slightly different than in singleplex (Fig. 3b). Under multiplex conditions, analytical sensitivity was further assessed by ROC curve analysis (Fig. 3c), using DNA concentrations from 5.33 to 0.33 ng/µL. All target species showed excellent analytical performance, with area under the curve (AUC) values ranging from 0.93 to 0.97, and 95% confidence intervals consistently above 0.83.

Application in the surveillance context

During six months of routine surveillance in the context of the Portuguese *Aspergillus* Surveillance Program, coordinated by the Reference Unit for Parasitic and Fungal Infections, two

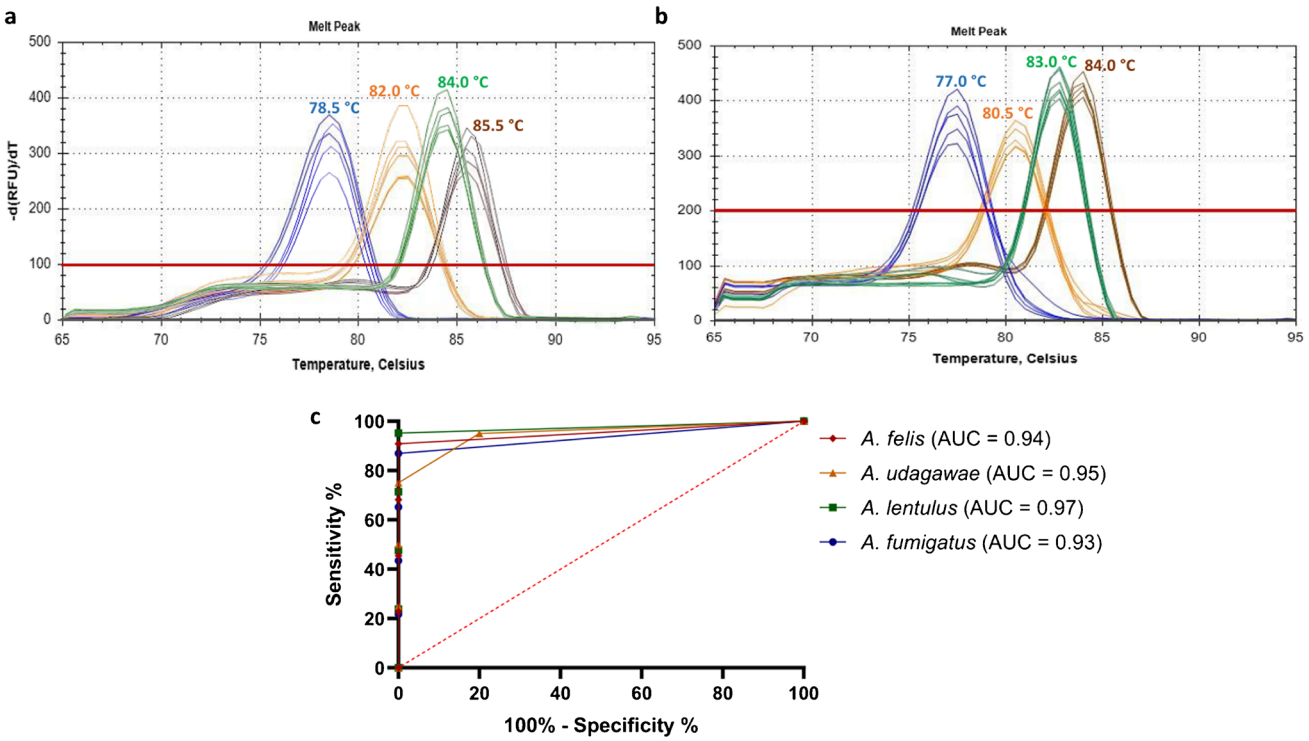


Fig. 3 Representative melting curve profiles obtained for *A. fumigatus* sensu lato (**a-b**). T_m values obtained in singleplex with 1.33 ng/µL gDNA: *A. fumigatus* 78.5 °C; *A. lentulus* 82.0 °C; *A. felis* 84.0 °C; *A. udagawae* 85.5 °C (**a**). T_m values obtained in multiplex with 0.66 ng/µL gDNA after betaine addition: *A. fumigatus* 77.0 °C; *A. lentulus* 80.5 °C; *A. felis* 83.0 °C; *A. udagawae* 84.0 °C. (**b**) Each melting curve represents one independent replicate (n = 5), used to calculate mean T_m, standard deviation and coefficient variation (**a-b**). ROC curves showing analytical performance of the multiplex assay, generated from five DNA concentrations. Areas under the curve are indicated for each species (**c**)

isolates exhibiting atypical morphology (VA515 and VA532) were identified. The workflow shown in Fig. 4a was subsequently applied to both isolates, which had initially been assigned to section *Fumigati* based on macro and microscopic characteristics observed after fungal culture growth.

Typical *A. fumigatus* sensu stricto isolates generally display abundant conidial production and characteristic greenish pigmentation after a few days of incubation in MEA. However, colony morphology within section *Fumigati* may vary considerably depending on the isolate and growth conditions. In this context, isolates VA515 and VA532 showed cotton-like colony texture, reduced sporulation and pale pigmentation after seven days of incubation (Fig. 4b, c), differing from the morphology commonly observed for *A. fumigatus* sensu stricto. These atypical phenotypic characteristics prompted further molecular characterization using both sequencing of the calmodulin gene, and the developed multiplex RT-PCR assay.

Following DNA extraction, the multiplex assay provided results for identification in less than two hours. Isolate VA532, despite its atypical colony morphology, was identified as *A. fumigatus* with a characteristic T_m at 77.0 °C, while isolate VA515 was identified as *A. udagawae* yielding a melting peak of 84.0 °C (Fig. 4d). Sequencing analysis within this workflow identified VA532 as *A. fumigatus* (coverage 94.0%, percent identity 100.0%: accession code PZ404157), and isolate VA515 as *A. udagawae* (coverage 93.0%, percent identity 99.81%: accession code PZ404156), validating the multiplex assay (Supplementarymaterial 4). However, sequencing-based identification required an additional 1–2 days following DNA extraction.

Discussion

Accurate identification of *A. fumigatus* sensu lato is essential for effective aspergillosis management, and for monitoring emerging resistance species across human, animal and environmental health sectors (Sabino et al. 2014, 2021; Sabino and Veríssimo 2022). The development of this method is directly aligned with the recommendation of the ECDC, establishing epidemiological surveillance programs to monitor *Aspergillus*

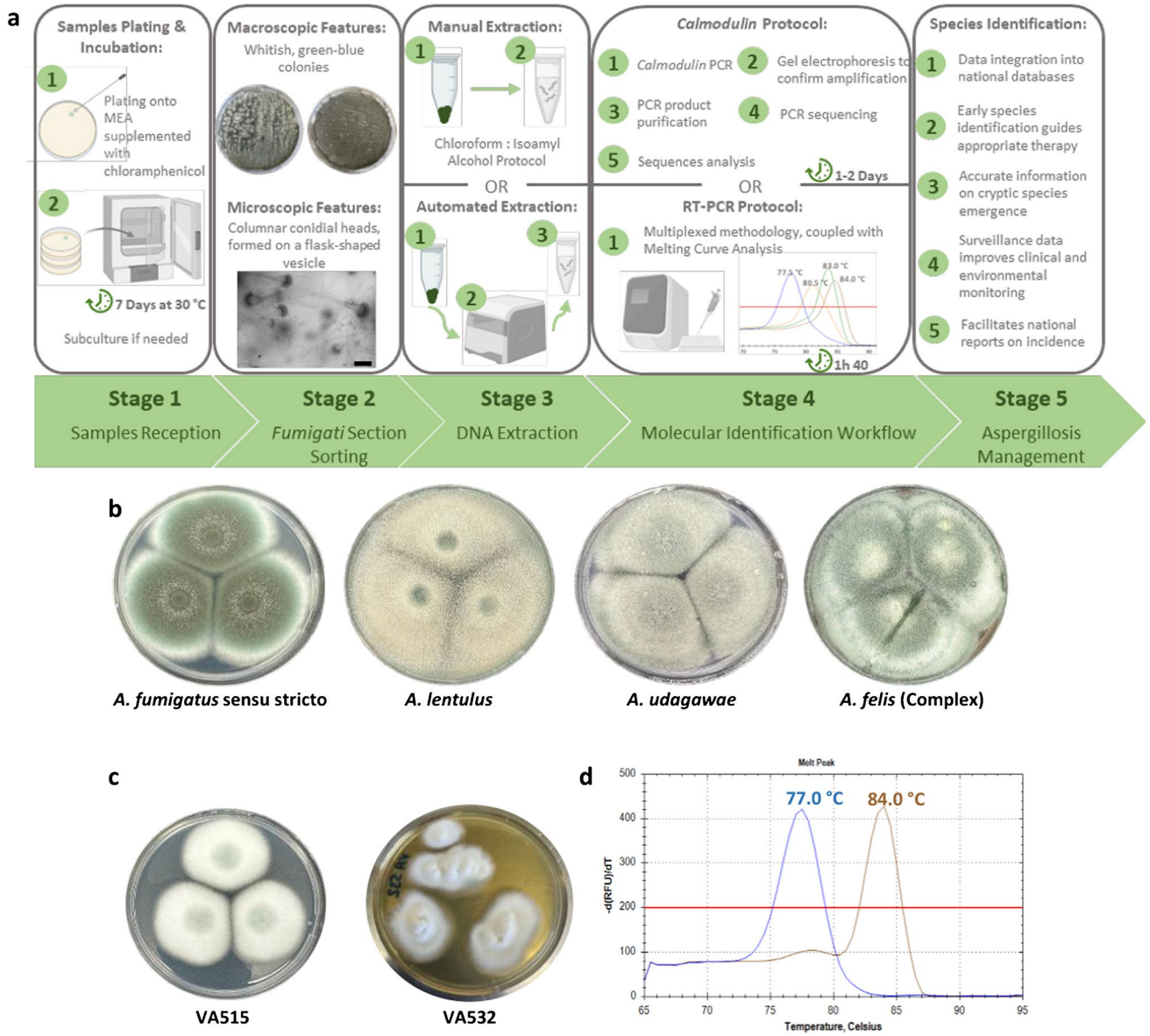


Fig. 4 Identification workflow of the blind fungal isolates, and the observed colony morphologies (**a-d**). The pipeline includes samples culturing, morphological sorting (macro and microscopic features), DNA extraction, and molecular identification by sequencing of calmodulin gene, or multiplex panel. Bar represents 25 µm, 400x. Created with BioRender.com (**a**). Typical colonies are greenish-blue, with abundant sporulation after 7 days of incubation. Atypical morphologies have a cotton-like texture, and whitish-green colonies, with poor sporulation (**b-c**). Representative melting curve profiles obtained by multiplex with 0.66 ng/µL gDNA for isolate VA532 identified as *A. fumigatus* with a T_m of 77.0 °C, and isolate VA515 identified as *A. udagawae* with a T_m of 84.0 °C (**d**)

species in both clinical and environmental settings (European Centre for Disease Prevention and Control 2013). Despite these recommendations, reports of *Aspergillus* surveillance remain limited across Europe. This gap likely reflects the limited capacity of the reference laboratories to process large number of isolates using sequencing-based identification, which remains time-consuming and costly. The multiplex RT-PCR method presented here addresses this limitation by offering a high-throughput, rapid, and cost-effective alternative for routine surveillance workflows, by using a platform that most heath facilities acquired for COVID-19 monitoring.

Compared with other available identification methods, such as MALDI-TOF, and commercial PCR kits, this SYBR Green-based assay offers a balance between accessibility and affordability. While MALDI-TOF is limited by databases coverage, currently enabling reliable identification of only *A. lentulus*

among cryptic species, and commercial kits typically detect *A. fumigatus* sensu stricto (Rath and Steinmann 2018; Shao et al. 2022), the method presented here bridges these gaps.

Although the proof-of-concept included a limited number of isolates, this reflects the natural occurrence of cryptic isolates in Portugal. The assay successfully identified *A. fumigatus* (VA532) and *A. udagawae* (VA515), in less than two hours, with full concordance with sequencing results. Importantly, atypical morphology alone should not be interpreted as indicative of cryptic species, as colony characteristics within section *Fumigati* may vary considerably between isolates and according to antifungal exposure (Samson et al. 2007; Houbraken et al. 2016). This was observed for VA532, which displayed atypical morphology despite being identified as *A. fumigatus* sensu stricto. These findings demonstrate the suitability of the assay for rapid molecular identification and real-time monitoring of section *Fumigati*.

While the assay was designed to target four species, it does not encompass the entire section *Fumigati*, and validation against additional cryptic species remains needed. Nevertheless, the selected targets represent the most frequently reported cryptic *Fumigati* species in surveillance and clinical studies across Europe and United States (Alcazar-Fuoli et al. 2008; Balajee et al. 2009b; Alastruey-Izquierdo et al. 2018), reinforcing the assay's practical focus. Moreover, only *A. hiratsukae* and *A. oerlinghausenensis* isolates were available for non-target specificity testing within section *Fumigati* throughout the Portuguese surveillance program, active since 2012, reflecting the low natural occurrence of other cryptic species in this setting. Although potential cross-reactivity between *A. lentulus* β-tubulin primer pair and *A. fumisynnematus* cannot be completely excluded, this is unlikely to represent a major practical limitation due to the low reported prevalence of *A. fumisynnematus* and its different antifungal susceptibility profile compared to *A. lentulus* (Alcazar-Fuoli et al. 2008).

Importantly, the assay was specifically designed for use with DNA extracted from fungal cultures, rather than direct testing of primary clinical samples. Isolation of the organisms remains the gold standard for *proven* aspergillosis diagnosis and is mandatory for antifungal susceptibility testing (Mendonça et al. 2022a, b; Pagano and Fernández 2025), which is critical for appropriate management of cryptic aspergillosis. Although the assay demonstrated the ability to detect low DNA concentrations (0.33 ng/µL), this analytical sensitivity is unlikely to represent a limitation in culture-based workflows, where fungal biomass is readily available. Future adaptations could further expand the assay according to the epidemiology of each geographical region, including additional primers targeting other cryptic species, or resistance-associated markers (Burks et al. 2021).

Conclusion

This study presents a multiplex RT-PCR assay coupled with melting curve analysis for discrimination of the most prevalent cryptic species in section *Fumigati*, designed for use with DNA extracted from pure fungal cultures. Phylogenetic analyses supported the selection of β-tubulin, calmodulin and *cyp51A* as suitable targets for species-level discrimination within this section, instead of the usual fungal hallmark ITS region. The assay showed highly reproducible T_m values for the target species (SD < 0.29 °C, and CV from 0.26 to 0.34%), with an analytical performance of AUC > 0.93. The method also showed equal performance between manual and automated DNA extraction methods, enabling species-level identification in less than two hours, with full concordance to sequencing results in the POC. By providing a rapid and cost-effective alternative to sequencing-based workflows, this method may support routine molecular identification and surveillance of *A. fumigatus* sensu lato.

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Author contributions All authors contributed to the study conception and design. Data collection, experiments and analysis were performed by Alexandre Mendonça, and data validation performed by Ricardo Franco-Duarte, Raquel Sabino, Paula Sampaio. Supervision and project administration was performed by Ricardo Franco-Duarte, Raquel Sabino and Paula Sampaio. Resources were provided by Cristina Veríssimo, Raquel Sabino and Paula Sampaio. The first draft of the manuscript was written by Alexandre Mendonça and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability All data supporting the findings of this study are included within the article and its Supplementary Information files. In addition, fasta sequences for the species used in the specificity assays can be found in: https://doi.org/10.6084/m9.figshare.32681076.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication All authors of this article have read and approved the final version submitted.

Competing interests The author(s) declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Generative AI Statement The author(s) declare that generative AI was used to correct English grammar and improve text readability.

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