YEAST DIVERSITY RELATED WITH TOURIGA NACIONAL GRAPE VARIETY

F. L. Duarte[(1)], A. Teixeira[(1)], A. Costa[(1)], P. Ramos[(1)], D. Schuller[(2)], A. C. Gomes[(3)], M. F. Alemão[(1)]

[(1)]INIA - Dois Portos, Instituto Nacional de Recursos Biológicos
Quinta da Almoinha, 2565-191 Dois Portos, Portugal
filomena.duarte@inrb.pt

[(2)]CBMA - Molecular and Environmental Research Centre,
Department of Biology,University of Minho,
Braga, Portugal
dschuller@biouminho.pt

[(3)]Biocant - Centro de Inovação em Biotecnologia
Parque Tecnológico de Cantanhede, Núcleo 04, Lote 2
3060-197 Cantanhede, Portugal
acgomes@biocant.pt

ABSTRACT

The diversity of yeast species related with the grape variety Touriga Nacional was evaluated at four distinct vineyards of three Portuguese wine regions. Samples were collected 48 h after crushing, and when the loss of CO_2 reached 70 g/L. Restriction profiles analysis of 26S rDNA region with the endonucleases *Hae*III, *Cfo*I, *Hinf*I, *Apa*I and *Mse*I was used to characterize the five hundred and nine isolates obtained. A total of twenty yeast species were found. As expected higher species diversity was obtained for the initial sampling time. The most representative species was *Hanseniaspora uvarum*, which was detected in grape must from all the vineyards, followed by *Candida zemplinina*. Some species like *Pichia membranifaciens*, *Pichia kluyveri var kluyveri*, *Candida railenensis*, *Saccharomycopsis vini*, *Candida diversa,* among others, were only detected in grape must from one vineyard. None of the isolates belonged to the species *Saccharomyces cerevisiae*.

RÉSUMÉ

La diversité des espèces de levures en rapport avec le cépage Touriga Nacional a été évaluée sur quatre vignobles distincts de trois régions viticoles portugaises. Les levures ont été isolées 48 h après le broyage, et lorsque la perte de CO_2 a atteint 70 g/L. L'ánalyse de profils de restriction dune région du ADNr 26S avec les endonucléases *Hae*III, *Cfo*I, *Hinf*I, *Apa*I et *Mse*I a été utilisée pour caractériser les cinq cent neuf isolats obtenus. Un total de vingt espèces de levures a été trouvé. Comme prévu, plus grande diversité des espèces a été obtenue pour l'échantillonnage initial. L'espèce la plus représentative a été *Hanseniaspora uvarum*, qui a été détectée dans du moût de raisin de tous les vignobles, suivie par *Candida zemplinina*. Certaines espèces comme *Pichia membranifaciens*, *P. kluyveri var kluyveri*, *Candida railenensis*, *Saccharomycopsis vini*, *Candida diversa* entre autres ont été détectés dans les moûts de raisins d'un seule vignoble. Aucun des isolats n'appartenais à l'espèce *Saccharomyces cerevisiae*.

INTRODUCTION

Wine results from complex transformations of grapes that involve different groups of microorganisms. During alcoholic fermentation the non-*Saccharomyces* yeasts originating from grapes play an important role, influencing the structure, the complexity, the flavour and therefore the wine quality (Jolly *et al*. 2006; Ciani *et al*. 2010). Several factors can influence the ecology of non-*Saccharomyces* yeasts on grapes. The ripeness and the integrity of grape berries will largely determine the population numbers (Mortimer, Polsinelli, 1999; Fleet 2003). The use of pesticides also affects yeasts diversity on grapes, influencing both the number of different genera and the numbers within each genus (Guerra *et al*., 1999; Cordero-Bueso *et al*., 2011). The grape variety has been pointed out by several authors as affecting the yeast biota (Guerra *et al*., 1999; Raspor *et al*., 2006; Cordero-Bueso *et al*., 2011).
The aim of this work was to evaluate the diversity of yeast species related with the grape variety Touriga Nacional considered to be one of the best Portuguese red varieties, mainly for two years of harvest from four distinct vineyards of three Portuguese wine regions. Yeasts were isolated 48 h after crushing, and when the loss of CO_2 reached 70 g/L. Restriction profiles analysis of 26S rDNA region with the endonucleases *Hae*III, *Cfo*I, *Hinf*I, *Apa*I and *Mse*I was used to characterize the isolates and identification was done by comparing the profiles obtained with the existing wine yeasts restriction profiles database (Zanol *et al*., 2010). The sequencing of the D1/D2 domain of 26S rDNA of the isolates presenting restriction profiles without correspondence within the database was also determined.

MATERIAL AND METHODS

Yeasts isolation

Touriga Nacional grape samples were collected from two vineyards from Lisbon wine region (2005, 2006 and 2007), designated Dois Portos and Palhacana, one vineyard from Alentejo wine region (2006 and 2007), designated Montemor-o-Novo and one vineyard from Peninsula de Setubal wine region (2006), designated Azeitão. Three replica samples of approximately 2 Kg of grapes at the last stage of ripeness were aseptically collected at the same spots in different years, avoiding rotten grapes. The grapes, directly placed into sterile plastic bags, were transported to the laboratory within coolers with ice packs.
At the laboratory the sample weight was aseptically adjusted to 2 Kg, the grapes were hand crushed within the plastic bags and 400 mL of must was transferred to 500 mL laboratory glass bottles with a gas-permeable membrane screw cap. Bottles were incubated at 25 ºC without agitation. Samples were taken after 48 h and when must weight loss was 70 g/L, corresponding to the consumption of around two-thirds of the sugar content.
After appropriate dilutions samples were spread on plates containing YPD medium (yeast extract 1 % w/v, peptone 1 % w/v, glucose 2 % w/v, agar 2 % w/v) added with bacteria and filamentous fungi inhibitors, chloranphenicol (0,01 % w/v) and biphenyl (0,03 % w/v), respectively. Plates were incubated at 25 ºC for at least 48 h, afterwards 30 randomly chosen colonies were collected, isolated and kept at −80 ºC (glycerol 30 % v/v).

Identification of isolates by rDNA restriction profiles

To extract DNA, yeast cells were disrupted by vortexing with glass beads (0.5 mm Ø) in 500 µl lyses buffer (50 mM Tris-HCl, 250 mM NaCl, 50 mM EDTA and 0.3 % SDS). The cell lysate solution was appropriately diluted and then used for PCR amplification.

The amplification of the fragment of 26S rRNA gene, as well as the restriction analysis with enzymes *Mse*I, *Hinf*I, *Apa*I, *Hae*III and *Cfo*I was performed according to Zanol *et al*. (2010).
The identification of each isolate was achieved by using the restriction profiles library created with GelCompar II software version 5.1 (Applied Maths, Saint-Martens-Latem, Belgium) with the strains studied by Zanol *et al.* (2010). Similarities above 74 % were evaluated by visual comparison in order to confirm identification.
Sequencing of the D1/D2 domains of the 26S rRNA gene was carried out for profiles different from those existing in the library, using the procedure described by Baleiras-Couto *et al.* (2005).
Sequencing was also done for isolates presenting profiles identical for several species, in order to confirm identification. *Hanseniaspora uvarum* was differentiated from *H. guilliermondii* by testing growth at 37 ºC as described by Barata *et al.* (2008).

Statistical analysis

Classical ecology indexes were used to obtain the richness (S), the biodiversity (H') and the dominance (D) of the species studied according to Cordero-Bueso *et al*. (2011).

RESULTS AND DISCUSSION

Identification of isolates

Five hundred and nine isolates from Touriga Nacional grape variety were characterised by restriction profiles analysis of 26S rDNA, using the endonucleases *Mse*I, *Hinf*I, *Apa*I, *Hae*III and *Cfo*I. Their identification was achieved by means of the identification tool of software GelCompar II which compares the isolates profiles with those on a library created with the strains analysed by Zanol *et al.* (2010). The sequencing of the D1/D2 domains of 26S rDNA of the isolates presenting restriction profiles without correspondence within the database was also determined. Sequencing was also performed in order to confirm the identification provided by the restriction profiles. The results obtained are presented at Tab. 1a and Tab. 1b.

Table 1a – Results of the comparison of the isolates sequences with those present in GenBank from NCBI database - 48 h sampling time.

Isolate	Frag. Size (bp)	Similar type strain (accession nº)	Similarity (%)
TN3205/05	440	*Candida fermentati* (AY187283.1)	99.7
TN3228/05	624	*Aureobasidium pullulans* (DQ321374)	99.8
TN6138/06	439	*Aureobasidium pullulans* (DQ321374)	99.3
TN6152/06	543	*Aureobasidium pullulans* (DQ321374)	99.4
TN6158/06	484	*Aureobasidium pullulans* (DQ321374)	99.3
TN2131/06	525	*Cryptococcus flavescens* (AB035042)	97.7
TN2132/06	678	*Cryptococcus flavescens* (AB035042)	98.1
TN2134/06	548	*Lachancea thermotolerans* (U69581)	97.0
TN4343/06	415	*Candida zemplinina* (AY160761)	99.7
TN1156/06	477	*Candida zemplinina* (AY160761)	100.0
TN4334/06	553	*Issatchenkia hanoiensis* (AY163900)	99.8
TN4337/06	532	*Issatchenkia terricola* (U76345)	99.6
TN1131/06	407	*Issatchenkia terricola* (U76345)	99.2
TN1160/06	465	*Saccharomycopsis vini* (U40133)	99.1
TN1157/07	579	*Candida oleophila* (U45793.1)	99.4
TN2144/07	584	*Candida railenensis* (U45800.1)	99.4
TN2259/07	568	*Pichia kluyveri var kluyveri* (U75727.1)	99.4
TN2357/07	573	*Candida californica* (EF550230.1)	100.0
TN6260/07	509	*Rhodosporidium babjevae* (AF070420.1)	99.0

Table 1b – Results of the comparison of the isolates sequences with those present in GenBank from NCBI database – 70 g/L weight loss sampling.

Isolate	Frag. Size (bp)	Similar type strain (accession nº)	Similarity (%)
TN1171/07	473	*Zygosaccharomyces bisporus* (U72162.1)	99.3
TN1173/07	378	*Zygosaccharomyces bisporus* (U72162.1)	99.7
TN1177/07	528	*Candida diversa* (U71064.1)	100.0
TN1179/07	528	*Candida diversa* (U71064.1)	100.0
TN1186/07	523	*Candida diversa* (U71064.1)	99.6
TN1272/07	542	*Issatchenkia terricola* (U76345)	99.8
TN2271/07	427	*Zygosaccharomyces bailii* (U72161.1)	99.5
TN2274/07	547	*Hanseniaspora uvarum* (U84229.1)	100.0
TN2279/07	561	*Hanseniaspora uvarum* (U84229.1)	99.8
TN2379/07	470	*Candida zemplinina* (AY160761.1)	100.0
TN6172/07	558	*Hanseniaspora guilliermondii* (U84230.1)	100.0
TN6469/07	520	*Hanseniaspora uvarum* (U84229.1)	100.0
TN6481/07	353	*Hanseniaspora uvarum* (U84229.1)	99.1
TN6570/07	563	*Hanseniaspora uvarum* (U84229.1)	100.0
TN6680/07	553	*Hanseniaspora guilliermondii* (U84230.1)	100.0

Three isolates (TN2131/06, TN2132/06 and TN2134/06) could not be assigned to any species as its sequence presented a similarity lower than 99 % with the type strain of any species at the database. A good correspondence between sequencing and restriction profiles was obtained as previously observed by Baleiras-Couto *et al*. (2005) and Zanol *et al*. (2010).

Yeasts diversity

For Dois Portos vineyard a great diversity of species was found, with a total of 12 species detected (Tab. 2). Among samples the species richness varied from two to six, the initial samples (48 h) presenting higher richness when compared with the same samples after 70 g/L of weight loss. Higher dominance index was inversely observed for sampling at two-thirds sugar consumption. Some species were only found at the initial sampling time like *Hanseniaspora guilliermondii, Metschnikowia pulcherrima, Zygoascus hellenicus* and *Candida oleophila*. Other species were only isolated after a higher enrichment time like *Zygosaccharomyces bisporus* and

Table 2-Yeast diversity detected at Dois Portos vineyard.

Sampling time	48 h				70 g/L	
Year	2005	2006	2007		2007	
Sample number	11	11	11	12	11	12
H. guilliermondii	0	0	2	0	0	0
H. uvarum	0	8	10	17	0	1
I. terricola	0	3	10	6	1	1
C. zemplinina	0	16	6	3	20	22
M. pulcherrima	0	0	0	4	0	0
Z. hellenicus	0	0	1	0	0	0
C. oleophila	0	0	1	0	0	0
Z. bisporus	0	0	0	0	3	0
C .diversa	0	0	0	0	5	0
C. fermentati	9	0	0	0	0	0
A. pullulans	1	0	0	0	0	0
S. vini	0	1	0	0	0	0
Species richness	2	4	6	4	4	3
Biodiversity (H')	0.33	1.04	1.75	1.14	0.91	0.31
Dominance (D)	0.82	0.42	0.27	0.39	0.52	0.84

Candida diversa. *Z. bisporus* was only detected in one of the samples and has been rarely isolated from grapes and wines (Barata *et al*., 2008). On the contrary, *Hanseniaspora uvarum, Issatchenkia terricola* and *Candida zemplinina* were detected on both sampling times. This latter species has also been detected during different stages of grape must fermentation by several authors (Baleiras-Couto *et al.* 2005; Ocón *et al*., 2010).
Palhacana grapes presented even higher diversity, with a total of 16 yeast species detected (Table 3). The species richness among samples varied from 2 to 6 and like observed for Dois Portos was lower for samples corresponding to weight loss of 70 g/L. *H. uvarum* was detected in almost all samples, with higher percentage at the initial sampling. At two-thirds sugar consumption, as observed for Dois Portos results, a high predominance of *C. zemplinina* was detected, except for one of the samples where *Zygosaccharomyces bailii* prevailed. Different values for the Biodiversity index were observed within each sampling time and was higher for one of the samples collected at 70 g/L weight loss, inversely to Dois Portos samples.

Table 3-Yeast diversity detected at Palhacana vineyard.

Sampling time	48 h				70 g/L		
Year	2006	2007			2007		
Sample	21	21	22	23	21	22	23
H.guilliermondii	0	0	0	0	0	1	0
H. uvarum	15	19	20	21	5	7	0
I. terricola	0	2	4	1	0	4	1
C. zemplinina	0	0	0	0	18	0	28
M. pulcherrima	0	3	0	0	0	0	0
P. membranifaciens	0	0	1	1	0	0	0
Z. hellenicus	0	2	0	0	3	0	0
C. oleophila	0	1	0	1	0	0	0
C. railenensis	0	3	0	0	0	0	0
C. cf. flavescens	3	0	0	0	0	0	0
P. kluyveri var kluyveri	0	0	3	0	0	0	0
I. orientalis	0	0	1	0	0	0	0
C. californica	0	0	0	1	0	0	0
L. cf. thermotolerans	4	0	0	0	0	0	0
Z. bailii	0	0	0	0	0	11	0
A. pullulans	0	0	0	2	0	0	0
Species richness	3	6	5	6	3	4	2
Biodiversity (H')	0.84	1.75	1.00	0.88	0.82	1.16	0.15
Dominance (D)	0.52	0.43	0.51	0.62	0.53	0.35	0.93

In relation to the grapes from Alentejo vineyard several isolates could not be recovered from storage and richness was very low, with only one yeast species detected for the majority of the samples. Biodiversity and Dominance indexes were not presented for samples with low number of isolates. At the earlier sampling time a great predominance of *Aureobasidium pullulans* was found for this vineyard, inversely to the previous vineyards, where only 3 isolates of this filamentous fungus, that presents a yeast-like phase, were found. This fungus has been commonly isolated from grapes (Raspor *et al*., 2006; Di Maro *et al.,* 2007; Ocón *et al.,* 2010). The only species detected at two-thirds sugar fermentation were *H. uvarum* and *H. guilliermondii*. Low biodiversity and high dominance index were observed for this vineyard yeast biota. Its localisation far from any winery and from other vineyards may contribute to the low yeast diversity observed.

Table 4-Yeast diversity detected at Montemor-o-Novo vineyard.

Sampling time	48 h			70 g/L				
Year	2006	2007		2007				
Sample	61	61	62	61	62	64	65	66
H. guilliermondii	0	0	1	25	27	0	0	29
H. uvarum	0	0	2	1	1	23	30	0
A. pulullans	22	7	3	0	0	0	0	0
R. babjevae	0	0	1	0	0	0	0	0
Species richness	1	1	4	2	2	1	1	1
Biodiversity (H')	0	-	-	0.16	0.15	0	0	0
Dominance (D)	1.00	-	-	0.93	0.93	1.00	1.00	1.00

Higher species richness was found for Azeitão vineyard. *Issatchenkia hanoiensis* was only detected at this vineyard. This species has been scarcely isolated from grapes and musts, being detected at fermentations from Castelão and Catalanese grape varieties (Baleiras-Couto *et al*. 2005, Di Maro *et al*., 2007).

Table 5-Yeast diversity detected at Azeitão vineyard.

Sampling time	48 h
Year	2006
Sample	43
H. uvarum	19
C. zemplinina	3
I. terricola	6
I. hanoiensis	1
Species richness	5
Biodiversity (H')	0.95
Dominance (D)	0.48

Remarkably *Saccharomyces cerevisiae* was not detected for any of the 22 samples analysed what might be related with the fact that healthy, undamaged grapes were preferentially collected.

CONCLUSIONS

Variation of the yeast biota detected on different years and vineyards was observed. The year of 2007 presented higher yeast biodiversity. Higher yeast biodiversity was also found at the initial sampling time than at sampling after two-thirds sugar consumption. The most representative species was *Hanseniaspora uvarum*, which was detected in grape must from all the vineyards, followed by *Candida zemplinina*. Some species like *Pichia membranifaciens*, *P. kluyveri var kluyveri*, *C. railenensis*, *Saccharomycopsis vini*, *C. diversa* among others were only detected in grape must from one vineyard. *Saccharomyces cerevisiae* was not detected for any of the 22 samples analysed. At late fermentation the predominantly detected species was *H. guilliermondii*, also followed by *C. zemplinina*. Replicate samples were similar in relation to the most frequent species.

ACKNOWLEDGMENTS

This work was partially carried out within the following projects: FCOMP-01-0124-FEDER, which is financed with funds from FEDER through the "Programa Operacional Factores de Competitividade" – COMPETE, and from FCT – "Fundação para a Ciência e a Tecnologia" (internal FCT ref. PTDC/AGR-ALI/098292/2008 and ref. PTDC/AGR-ALI/098292/2008; European Community´s Seventh Framework Programme (FP7/2007-2013) under grant agreement nº 232454.

BIBLIOGRAPHY.

Baleiras-Couto M.M., Reizinho R.G., Duarte F.L., 2005. Partial 26S rDNA restriction analysis as a tool to characterise non-*Saccharomyces* yeasts present during red wine fermentations. *Int. J. Food Microbiol*., 102: 49-56.

Barata A., Gonzélez S., Malfeito-Ferreira M., Querol A., Loureiro V., 2008. Sour rot-damaged grapes are sources of wine spoilage yeasts. *FEMS Yeast Res.*, 8: 1008-1017.

Ciani M., Comitini F., Mannazzu I. M., Domizio P., 2010. Controlled mixed culture fermentation: a new perspective on the use of non-*Saccharomyces* yeasts in winemaking. *FEMS Yeast Res*., 10: 123-133.

Cordero-Bueso G., Arroyo T., Serrano A., Tello J., Aporta I., Vélez M.D., Valero E., 2011. Influence of the farming system and vine variety on yeast communities associated with grape berries. *Int. J Food Microbiol*. 145:132-139.

Fleet G.H., 2003. Yeast interactions and wine flavour (review article). *Int. J. Food. Microbiol*. 86: 11-22.

Guerra, E., Sordi G., Mannazzu, I., Clementi F., Fatichenti, F., 1999. Presenza di lieviti vinari su uve marchigiane sottoposte a diversi trattamenti antiparassitari. Ital. J. Food Sci. 11: 221-230.

Mortimer R., Polsinelli M., 1991. On the origins of wine yeast. *Res. Microbiol.*, 150: 199-204.

Jolly N.P., Augustyn O.P.H., Pretorius I.S., 2006. The role and use of Non-*Saccharomyces* yeasts in wine production. *S. Afr. J. Enol. Vitic*., 27: 15-39.

Di Maro E., Ercolini D., Coppola S., 2007. Yeast dynamics during spontaneous wine fermentation of the Catalanesca grape. *Int. J. Food Microbiol*., 117: 201-210.

Ocón E., Guitierrez A., Garijo P., Tenorio C., Lopez I., Lopez R., Santamaria P., 2010. Quantitative and qualitative analysis of non-*Saccharomyces* yeasts in spontaneous alcoholic fermentations. *Eur. Food Res. Technol*. 230: 885-891.

Raspor P., Milek D., Polanc J., Mozina S., Cadez N., 2006. Yeasts isolated from three varieties of grapes cultivated in different locations of the Dolenjska vine-growing region, Slovenia. Int. J. Food Microbiol., 109: 97-102.

Zanol G., Baleiras-Couto M.M., Duarte F., 2010. Restriction profiles of 26S rDNA as a molecular approach for wine yeasts identification. *Ciência Téc. Vitiv.*, 25: 75-85.