Use of molecular methods for the identification of yeast associated with table olives.

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Abstract

A molecular approach is used for the first time for the identification of yeast isolated from table olives. Our results validate those obtained in the past by the classical biochemical methodology. Yeast were isolated from both aerobically and anaerobically processed black table olives and also from canned seasoned green table olives. Molecular identification methodology used included restriction pattern analysis of both PCR-amplified 5.8S rRNA gene and internal transcribed spacers ITS_1 and ITS_2. For some species, sequence analysis of the 26S rRNA gene was necessary. These techniques have allowed the identification of three yeast species (*Issatchenkia occidentalis, Geotrichum candidum* and *Hanseniaspora guilliermondii*) which had not been described previously in table olives. S*accharomyces cerevisiae* and *Candida boidinii* were the most frequent species in green seasoned olives and processed black olives, respectively. The molecular study of total DNA variability among the *S. cerevisiae* strains isolated indicates a quite heterogeneous population, with at least four different restriction patterns.

1. Introduction

Table olives are a traditional fermented food of the Mediterranean countries. The International Olive Oil Council calculates that their world productions reached around 1,602,000 tones in 2003/2004 season. Yeast play a critical role in all olive fermentations, especially in directly brined green and natural black olives. The presence of yeast during the fermentation of green olives was reported in the earliest studies of this product. Thus, González-Cancho (1965) isolated yeast of the genera *Candida, Hansenula, Pichia, Torulopsis* and *Saccharomyces* from these fermentations. As lactic acid bacteria are partially inhibited in directly brined green and turning color olives due to the presence of toxic phenolic compounds, yeast play an essential role in such fermentations. Actually, Pelagatti (1978) and Marquina et al. (1992) isolated species of the genera *Candida, Debaryomyces, Kluyveromyces, Pichia, Rhodotorula* and *Saccharomyces* from them. On the other hand, Balatsouras (1967) found yeast of the genera *Trichosporum, Candida, Pichia, Kloeckera, Torulopsis* and *Debaryomyces* during the fermentation of directly brined natural black olives from Greek cultivars. Also, Borcakli et al. (1993) isolated species of *Debaryomyces* from Turkish cultivars. González-Cancho et al. (1975) identified *Saccharomyces cerevisiae* and *Pichia anomala* as the main species in Spanish natural black olive fermentations. When these olives were fermented in the presence of air the species present were *Candida saitoana, Debaryomyces hansenii, Pichia membranaefaciens* and *Williopsis saturnus* var. *mrakii* (Durán Quintana et al. 1986). We have no information of yeast isolations from seasoned green table olives, a product with great traditional value due to its natural elaboration.

Until present, the characterization of yeast associated with table olives has mainly been made through biochemical and morphological methods, using the taxonomic keys of Lodder (1970), Barnett et al. (1990), and Kurtzman and Fell (1998). More recently,

molecular methods were used for the identification of yeast from products like wine (López et al. 2003), orange juice (Heras-Vázquez et al. 2003), cheese (Vasdinyei and Deak 2003), yoghourt (Caggia et al. 2001), and "alpeorujo" (Giannoutsou et al. 2004), a residue from olive oil elaboration. The most relevant molecular methods used in the identification of yeast species are based on the variability of the ribosomal genes 5.8S, 18S and 26S (Cai et al. 1996, Jamens et al. 1996, Kurtzman 1992, Li 1997). The interest in ribosomal genes for species identification comes from the concerted fashion in which they evolve showing a low intraspecific polymorphism and a high interspecific variability (Li 1997). Previous results have demonstrated that the complex ITS regions (non-coding and variable) and the 5.8S rRNA gene (coding and conserved) are useful in measuring close fungus genealogical relationships since they exhibit far greater interspecific differences than the 18S and 26S rRNA genes (Cai et al. 1996, James et al. 1996, Kurtzman 1992). In this sense, it is very useful that restriction patterns generated with endonucleases *Cfo*I, *Hae*III, *Hinf*I and *ScrF*I from amplified 5.8S rRNA-ITSs (Esteve-Zarzoso et al. 1999) are available for a great amount of yeast species at www.yeast-id.com (Valencia University and CSIC, Spain). Nevertheless, we have no information that molecular techniques have ever been applied for the identification of yeast isolated from table olives.

The conventional methodology for biochemical yeast characterization requires evaluation of some 60-90 tests for a correct species identification. This process is complex, laborious and time consuming (Deak 1995). Molecular techniques are rapid, easy and more precise for yeast identification, eliminating part of the subjectivity that usually accompanies the output of the biochemical tests.

In this work, molecular methods for the identification of yeast species isolated from table olives has been used for the first time, as well as the analysis of total DNA restriction pattern to study the heterogeneity of the *S. cerevisiae* population. These techniques confer a

higher accuracy degree in the final identification than classical biochemical methods. Furthermore, because of their sensitivity and accuracy, these techniques can allow the identification of yeast species that may have not been described previously in this food fermentation using the classical techniques.

2. Material and Methods.

2.1. Processing of the raw material.

Green seasoned table olives (SO). Canned , seasoned green table olives from Aloreña cultivar, a variety of table olive of great acceptance in the Andalusian coast, were used. Elaboration and canning was carried out by a local producer (Aceitunas Bravo S.A., Alahurin el Grande, Málaga, Spain). Its elaboration included washing and smashing of the olives, which were finally seasoned with garlic, red pepper and a variety of mediterranean aromatic herbs and spices. The seasoned olives (0.9 kg) were canned into 1.6-L polyethilene containers and covered with 0.7 L of brine (4% (w/v) NaCl, 0.15% (w/v) citric acid and 0.017% (w/v) potassium sorbate). A total of 25 containers were collected directly from the manufacturer along the producing season (september to november 2003) and analysed.

Black table olives (BO). Hojiblanca variety green olives were used to fill 100-L containers and were covered with brine (4% (w/v) NaCl, 0.3% (w/v) acetic acid). At this step, both aerobic and anaerobic processes were carried out. For the anaerobic processing, no further treatment was applied. For the aerobic processing, a flow of air was supplied into the brines at a rate of 0.1-0.2 L per litre of containers capacity, 8 h/day. A total of three aerobic and two anaerobic containers were studied.

2.2. Microbiological analysis and statistical modeling. Brine samples and their appropriate decimal dilutions were plated using a Spiral System model DS (Interscience, Saint Nom La Breteche, France) on YM agar (Difco, Becton and Dickinson Company, Sparkes, MD, USA) supplemented with 0.005% (w/v) gentamicin sulphate and oxytetracycline (Oxoid LTD, Basingstoke, Hampshire, England) as selective agents for yeasts. All plates were incubated aerobically in the dark at 30ºC± 2 for 48h. Isolated yeast colonies were randomly picked out to carry out a quantitative analysis of the yeast populations, with one day as frequency of sampling. A total of 50 isolations for SO and 25 for BO were further studied. Colony forming units per ml (CFU/ml) were calculated and Ln (N/No) versus time was modelled according to a modification of the Gompertz equation proposed by Zwietering et al. (1990):

$$y=A*\exp\{-\exp[((\mu_{max}*e)/A)*(\lambda-t))+1]\}$$

where $y=Ln(N/N_0)$, N_0 is the initial microbial population; N the microbial population at time t, $A= \ln(N_\infty/N_0)$ is the maximum value reached with N_∞ as the asymptotic maximum population, μ_{max} is the maximum specific growth rate, and λ the lag phase period.

2.3 Yeast identification. Biochemical methods as well as molecular techniques were used in parallel for the identification of yeast species.

2.3.1. Biochemical and morphological methods. Sugar assimilation studies were carried out using the API 20 C AUX kit (Biomerieux, France). Sporulation was determined on McClary´s acetate agar medium (McClary et al. 1959) in the absence of a nitrogen source and acid stained with 1% (w/v) blue methylene and eosin, according to the procedure described by Lodder (1970). Cellular morphology was observed under the microscope after growth for 48 h in YM broth at 30º C.

2.3.2. Molecular techniques.

Amplification reactions. For the PCR amplification of the 5.8S rRNA gene and the intergenic spacers ITS_1 and ITS_2, the protocol described by Esteve-Zarzoso et al. (1999) was used. Briefly, an isolated yeast colony was collected using a sterile plastic tip and suspended in 98 µl PCR reaction mix containing 10 µL of 10x reaction buffer (Roche Molecular Biochemicals, Germany), 8 µL dNTP mix (50 µM each), 2 µL ITS_1 primer (0.5µM), 2 µL ITS_4 primer (0.5µM), and 76 µL de-ionized H_20. Primers ITS_1 and ITS_4 had the sequences 5-TCCGTAGGTGAACCTGCGG-3´ and 5´-TCCTCCGCTTATTGATATGC-3´ respectively. The mixture was heated at 94º C for 15 min to disrupt cells, and finally 2 µL Taq DNA polymerase (Roche Molecular Biochemicals, Germany) was added. Amplification was carried out in a PTC-100 thermocycler (M.J. Research, USA) using the following thermal cycling conditions: denaturation at 94º C for 1 min, annealing at 55.5 º C for 2 min, and elongation at 72 º C for 2 min. This was repeated for 40 cycles plus a final incubation step at 72 º C for 10 min. *Pichia anomala* , *Debaryomyces hansenii* and *Rhodotorula mucilaginosa* were used as controls for the amplification reactions and were previously isolated from table olives and identified. These control yeast belong to the current collection of microorganisms from the Food Biotechnology Department of Instituto de la Grasa (CSIC) at Seville, Spain.

Restriction analysis. Aliquots of the PCR-amplified products were separately digested with *Cfo*I, *Hae*III, *Hinf*I and *ScrF*I restriction enzymes (Roche Molecular Biochemicals, Germany). Reaction mixtures contained 2.5 µL 10x digestion buffer (supplied by the manufacturer), 6.5 µL de-ionized H_20, 1 µL restriction enzyme and 15 µL PCR product. The mixtures were incubated for 12 h at 37º C. Resulting DNA fragments were analysed by electrophoresis through 3% (w/v) agarose gels (SeaKem, Biowhittaker Molecular Applications, USA) in 1x TAE buffer. DNA molecular weight marker 1-kb+ DNA ladder (Life Technologies, USA) was used as standard. Restriction profiles generated were recorded

and compared with those contained in the data base at www.yeast-id.com (Valencia University and CSIC, Spain).

Sequence analysis of the 26S rRNA gene. In those cases where restriction analysis was not conclusive and for the confirmation of yeast species not described previously in table olives, sequence analysis of dominions D_1 and D_2 of the 26S rRNA gene was accomplished. For this purpose, PCR amplification of the 26S rRNA gene with the universal primers NL_1 (5'-GCATATCAATAAGCGGAGGAAAAG-3´) and NL_4 (5'-GGTCCGTGTTTCAAGACGG- 3´) (Kurtzman and Robnett 1998) was carried out and the resulting products sequenced. The corresponding sequences were finally compared with those found in the database at www.ebi.ac.uk/blast2/index.HTML. Amplification reaction was identical to the above described for the 5.8S rRNA gene except for the primers used (NL_1 and NL_4).

Restriction analyisis of total DNA from S. cerevisiae isolated from seasoned green olives. Isolated colonies from 10 different *S. cerevisiae* isolates were inoculated into 1 ml of YM broth and incubated for 12 h at 30º C. Cultures were centrifuged at 12000 rpm for 3 min and the pellets resuspended in sterile deionized water. Total DNA extraction was made by the protocol of Querol et al. (1992). Fifteen µL of the total DNA obtained was digested with the restriction enzyme *Hinf*I (Roche Molecular Biochemicals,Germany). Restriction reaction was set up as follows: 2.5 µL 10x buffer (supplied by the enzyme manufacturer), 2 µl RNase (RNAguard ®, Pharmacia Biotech, USA), 1 µl *Hinf*I , 5 µl deionized H_20, 15 µl DNA. The resulting fragments were separated by electrophoresis through 1% (w/v) agarose gels in 1x TAE buffer.

3. Results

In this work a total of 75 yeast isolated from different preparations of table olives were studied using biochemical and molecular tests in parallel. The amplification of the 5.8S ribosomal gene and the intergenic spacers ITSs showed bands ranging from 415 bp of *G.*

candidum to 850 bp of *S. cerevisiae* (Table 1). In all cases, the biochemical and morphological tests confirmed the results obtained by molecular methods, although biochemical identification alone would have not been conclusive.

In seasoned green table olives (SO) the initial population of yeast was $4\log_{10}$ cfu/ml, which progressively increased up to $6\log_{10}$ cfu/ml after 5 days of canning. The biological parameters of growth were calculated with the modified Gompertz equation, resulting a maximum specific growth rate (μ_{max}) of 0,074 $hours^{-1}$ (Standard Error [S.E.]= 0,005) and lag phase (λ) of 39,75 hours (S.E.=1,75) .The species identified and their frequencies are shown in Table 2. In the case of the isolate subsequently identified as *G. candidum,* sequencing of the 26S rRNA gene was necessary because of there were no reference to this species in the database used (www.yeast-id.com).Thus, after searching for DNA homology, it showed 98% identity to rRNA genes from *G. candidum*. With *Issatchenkia occidentalis* the sequence of the 26S gene was also necessary to confirm the identification made by RFLP 5.8S-ITS methods, because this yeast species had not been described previously in table olives. The results obtained showed 96% of identity to rRNA genes from that species. In the elaboration of black table olives (BO), an significative difference was observed between the aerobic process (AP) and the anaerobic process (FP) even with the same raw material. The initial populations were $3\log_{10}$ cfu/ml and $2\log_{10}$ cfu/ml for AP and FP, respectively.They reached their maximum populations 10 days after brining, with $7\log_{10}$ cfu/ml and $4\log_{10}$ cfu/ml for AP and FP, respectively. The biological parameters of growth were μ_{max}=0,060 $hours^{-1}$ (S.E.=0,009), λ=62,73 hours (S.E.=14,37) for AP, and μ_{max}=0,038 $hours^{-1}$ (S.E=0,0295) , λ=98,19 hours (S.E.=50,61) for FP. The yeast species identified are shown in Table 2. In this case sequencing of the 26S rRNA gene was not necessary for, although *G. candidum* was also isolated from this olive type, the similarity of its restriction pattern to that of the isolates from SO indicated that it is the same species. S*accharomyces cerevisiae* and *Candida boidinii*

were the most frequent species in green seasoned olives and processed black olives, respectively.

Figure 1 shows the restriction pattern of total DNA digested with *Hinf*I for 10 isolates of *S. cerevisiae* selected at random from SO. At least four different profiles can be observed. Profiles S1, S2 and S3 represent each 30% of the total, while profile S4 is only 10%. Major differences in the patterns can be observed at the top part of the gel, above the 2000 bp standard band (Figure 1). This is the area where restricted mitocondrial DNA shows better and is perfectly distinguishable from the background of restricted chromosomal DNA.

4. Discussion

Recent works have used exclusively biochemical methods for the identification of yeast presents in different foods, for example Thapa and Tamang (2004) in kodo ko jaanr, Kotzekidou (1997) in black table olives, Witthuhn et al. (2005) in kefir, and Lore et al. (2005) in suusac. Although extensively used till present, biochemical characterizations are not sufficiently reliable, since they can cause false identifications. This is due to the similar metabolism that related species may show. In addition, the subjectivity that accompanies the process of identification, due to the variability in the response that many species show in the tests of sugar assimilation and fermentation can lead to wrong results.

As it is shown in this paper, the combined use of molecular methods and biochemical tests have allowed the identification of three species of yeasts (*I. occidentalis, G. candidum* and *H. guilliermondii)* which had not been described previously in table olives. *I. occidentalis* and its anamorph state *C. sorbosa* are related with the pulp of tropical fruits (Trindade et al. 2002) as well as with vineyard and winery (Sabate et al. 2002). This species ferments glucose (the majoritary sugar in olive fruits) and, probably for this reason, can grow in anaerobic

conditions for several days after canning of table olives. On the other hand, *G. candidum* was identified by Giannoutsou et al. (2004) in alpeorujo, a residue of olive oil elaboration, and its ability to discolour black olive mill wastewater has been reported by Ayed et al. (2005). This species is also associated with contamination and flavour in cheese, as described by Kure et al. (2004). *G. candidum* metabolism is aerobic and, also probably for this reason, disappears few days after canning of table olives.The genus *Kloeckera* is the anamorph state for many species of the genus *Hanseniaspora*. In our case, we have always observed the sporulated species and this is why they have been assigned to the species *H. guilliermondii*. The anamorph state of this species, *Kloeckera apiculata* was isolated from pepper fermentations, a fermented product used by that time for table olive stuffing (González-Cancho et al. 1972). *Kloeckera sp* has been isolated by Balatsouras (1967) from black table olives.

The rest of the yeast species identified in this study had been described in table olives previously, confirming the molecular tests the identifications made in the past exclusively by biochemical methods. *S. cerevisiae* is a fermentative yeast and its presence in table olives has been recorded since the first studies of this product (González-Cancho et al. 1965 and 1975). The anaerobic conditions of canning or processing are favourable for the growth of this yeast as confirmed by the abundant gas production. *Z .bailii* and *C. diddensiae* had been previously isolated from black table olives by Kotzekidou (1997) and Durán-Quintana (1976) respectively, but never from seasoned green olives. *C. boidinii* was isolated by Santa Maria (1958) from alpechín, denominating it as *C. olivarium*. This yeast was also isolated, together with *G. candidum* and *Saccharomyces sp.*, from alpeorujo by Giannoutsou et al. (2004). Alpeorujo and alpechín are residues from the process of elaboration of olive oil from turning-colour and black olives. Thus, it is not surprising the similarity between the yeast populations in alpeorujo and processed black table olives. The presence of *C. boidinii* in table olives was mentioned by Durán-Quintana et al. (1976, 1986), who isolated it only from natural black

olive fermentations of the Hojiblanca cultivar, while it was absent in Lechín and Verdial varieties. The presence of pink yeasts (*Rhodotorula* sp. mainly*)* have been reported in turning-color olives by Pelagatii (1978) and Marquina et al. (1992), who observed its capacity to produce olive softening. Finally, *Dekkera bruxellensis* was also isolated previously by Kotzekidou (1997) from black table olives.

In the majority of cases, species of the same and related genera show similar sizes for the amplified 5.8S rRNA gene (Esteve-Zarzoso et al. 1999). In our case, *Rhodotorula glutinis* (640 bp) and *R. graminis* (660 bp) showed similar amplified patterns but, in contrast, the species of genus *Candida* showed very variable sizes for the respective amplified fragments (*C. diddensiae* 630 bp; *C. holmii* 750 bp; *C. sorbosa* 450 bp; *C. boidinii* 750 bp). The genus *Candida* includes yeast species that cannot be classified in other asexual ascomycetes, resulting thus a very heterogeneous genus.

In this study we have shown that the analysis of the restriction patterns generated by digestion with *Hinf*I of total DNA from *S. cerevisiae* is a good method to differentiate strains, as previously suggested by Querol et al. (1992) for various yeast species. In our case, it is difficult to make conclusions about the percentages of the different *S. cerevisae* strains from the product because of the low number of colonies which were analysed, although it might show the heterogeneity of the *S. cerevisiae* populations in table olives. New tests will be necessary to check if some type of ecological succession exists. The biochemical tests for this species showed the existence of two diferent profiles for maltose assimilation, although the rest of the biochemical tests could not explain the existence of the four different strains showed by the molecular methods. The enzyme *Hinf*I cuts the nuclear DNA at many sites, rendering a great number of bands which are perfectly distinguishable from those from mitochondrial DNA, which offers much less restriction sites (López et al. 2003). The study of the mtDNA of yeast species has been made previously by Sabate et al. (2002) in vineyard and

winery and Santamaria et al. (2005) in spontaneous alcoholic fermentations. This last author reported the presence of a great number of *S. cerevisiae* strains in these fermentations. Other authors have satisfactorily used this technique to characterize wild yeast strains of the *Zygosaccharomyces* genus (Guillamón et al. 1997).

This study represents an update as well as current approach to the knowledge of the behaviour of yeast populations and species present in directly brined table olives, with a higher accuracy degree in the final identification and good correlation between biochemical and molecular tests.

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Table 1. Restriction pattern analysis of PCR-amplified 5.8S rRNA-ITSs of yeast species isolated from table olives.

Yeast species	PCR product (bp)	Restriction enzyme*			
		*Cfo*I	*Hae*III	*Hinf*I	*ScrF*I
G. candidum	415	415	415	200+115+100	-
I. occidentalis	450	230 + 100 + 60 + 60	310+70+70	250+120+100	-
D. bruxellensis	485	255+140+90	375+95	270+215	-
C. diddensiae	630	280+170+150	425+130+70	315+315	-
R. glutinis	640	320+240+80	430+210	340+225+75	-
R. graminis	660	320+290	400+215	230+215+150	-
C. holmii	750	375+300	500+250	325+250+150	-
C. boidinii	750	350+310+90	710	390+190+160	-
H. guilliermondii	775	320+310+105	775	385 +200 +160+80	-
Z. bailii	790	320 + 270 +90+90	790+90	330 + 210 +160+60	-
S. cerevisiae	850	375 + 325 +150	495 +230 +125	375 +365 +110	400 + 320 +120

*Restriction enzymes used to generate appropriated patterns from the PCR-amplified products. Figures are expressed as bp.

Table 2. Frequency of yeast species isolated from table olives.

Yeast species	Olive type[a]		
	SO	AP	FP
Saccharomyces cerevisiae	58%	nd	28%
Issatchenkia occidentalis	20%	nd	nd
Geotrichum candidum	10%	7%	nd
Zygosaccharomyces bailii	5%	nd	nd
Candida diddensiae	5%	nd	nd
Candida holmii	2%	nd	nd
Candida boidinii	nd	70%	27%
Hanseniaspora guilliermondii	nd	15%	9%
Rhodotorula glutinis	nd	8%	9%
Dekkera bruxellensis	nd	nd	18%
Rhodotorula graminis	nd	nd	9%

[a]SO (Seasoned green table olives), AP (Aerobic processed black table olives), FP (anaerobic processed black table olives).

nd: not detected

Figure legend.

Figure 1. Restriction pattern analysis of total DNA from different *Saccharomyces cerevisiae* strains isolated from seasoned green table olives (SO) digested with *Hinf*I. The four different restriction patterns found are indicated as S1, S2, S3 and S4, respectively, at the top of the corresponding lane. Molecular weigths of relevant DNA bands of the standard (1kb+ DNA Ladder, Life Technologies) are indicated.

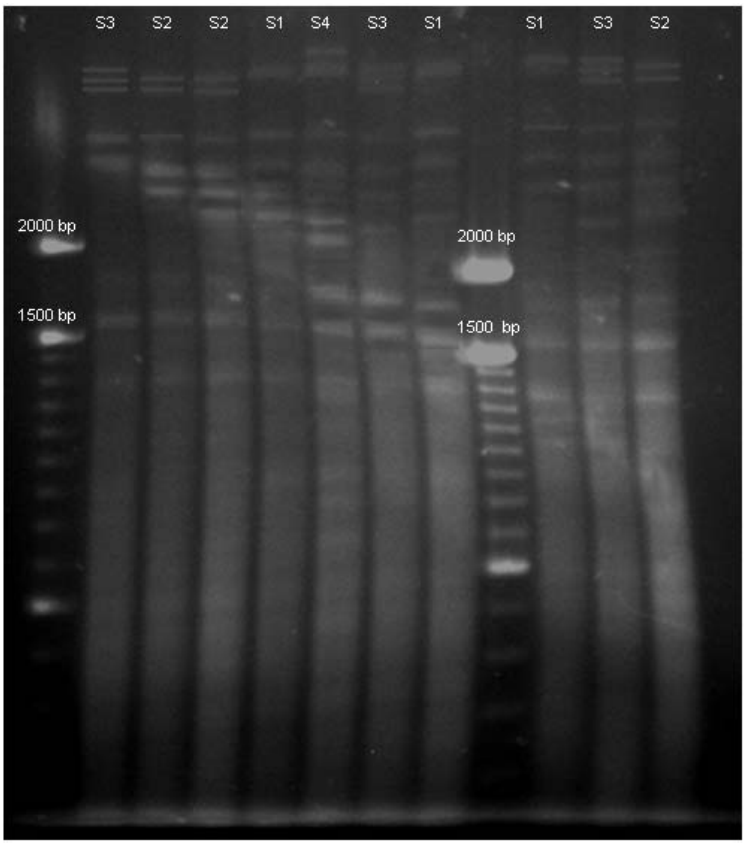

Figure 1