Yamadazyma terventina sp. nov., a yeast species of the Yamadazyma clade from Italian olive oils

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During an investigation of olive oil microbiota, three yeast strains were found to be divergent from currently classified yeast species according to the sequences of the D1/D2 domain of the gene encoding the rRNA large subunit (LSU) and the internal transcribed spacer region including the gene for 5.8S rRNA. Phylogenetic analysis revealed that these strains, designated CBS 12509, CBS 12510T and CBS 12511, represent a novel anascosporogenous species described herein as Yamadazyma terventina sp. nov; the type strain is DAPES 1924T (=CBS 12510T =NCAIM Y.02028T). This novel species was placed in the Yamadazyma clade, with Yamadazyma socolyti, Candida conglobata and Candida aaseri as closest relatives. Y. terventina differs from the above-mentioned species in the ability to strongly assimilate DL-lactate and weakly assimilate ethanol.

INTRODUCTION

Olive oil is obtained from the complete crushing of the fruits and from the successive separation of the oily fraction through centrifugation. During the crushing of the olives, micro-organisms present on the olives’ carposphere migrate into the oil together with suspended material, consisting of solid particles of fruit and micro-drops of vegetation water. In the olive oil habitat, the microbiological profile is quite different to that of healthy olive fruit’s microbiota. In the olive oil, some microbes from the olives’ carposphere succumb for a brief period whereas others, primarily yeasts, reproduce in a selective way, according to the chemical composition of the oil, and make up the typical microbiota of the olive oil (Ciafardini et al., 2004). In a recent study performed with chromogenic media, poly-varieties of extra virgin olive oil had up to three chromogenic yeast groups, while mono-varieties had no more than one (Zullo & Ciafardini, 2008). Studies have shown that some yeast species found in olive oil produce enzymes that may affect the oil’s quality in both positive and negative ways. The main yeast species isolated from the Italian extra virgin olive oils are classified as Candida wickerhamii, Candida boidinii, Candida diddensiae, Candida guillermondii, Barnettozyma (Williopsis) californica, Candida parapsilosis and Saccharomyces cerevisiae (Ciafardini & Zullo, 2002; Ciafardini et al., 2006; Zullo et al., 2010), while in a turbid Greek virgin olive oil Candida lisutiaeana, Candida famata and Rhodotorula mucilaginosa (Koidis et al., 2008) were also observed. Some of these yeast species are considered useful as they improve the sensory characteristics of the oil during preservation. In fact, some β-glucosidase- and esterase-producing strains of C. wickerhamii and S. cerevisiae are able to hydrolyse oleuropein into simpler, non-bitter compounds characterized by a high antioxidant activity (Ciafardini & Zullo, 2002). On the other hand, some lipase-producing strains of B. californica or S. cerevisiae can worsen olive oil quality through triglyceride hydrolysis. Cadez and co-workers (Cadez et al., 2012), recently described two novel yeast species (Candida adriatica and Candida molendinoloi) isolated from olive oil and its by-products. The present study describes a novel yeast species represented by three strains isolated from extra virgin olive oils from two different Italian regions.

METHODS

Yeast isolation. The yeasts studied were dimorphic forms isolated from commercial extra virgin olive oils during a survey including 23 producers located in Italy as described previously (Zullo et al., 2010). Three of these yeasts, designated DAPES (Collection of Department of Animal, Plant and Environmental Sciences) 1916, DAPES 1924 and DAPES 1931 in the previous study, could not be assigned to any known yeast species on the basis of the D1/D2 sequence and are described as representatives of a novel species in the present paper. The yeast strain DAPES 1916 was isolated from a commercial extra virgin olive oil of the ‘Taggiasca’ variety produced in Liguria (north Italy), whereas the
DAPES 1924 and DAPES 1931 strains were isolated from commercial extra virgin olive oil of the 'Leccino' variety, produced in the Trivento area (Molise region) located in the middle of Italy. The yeast strains were grown 5 days at 30 °C in malt extract-yeast extract-glucose-peptone (MYGP) agar as described by Kurtzman et al. (2011c) and then stored in 17% glycerol at −80 °C before any other study.

Sporulation and physiological tests. Ascospore formation was examined using McClary’s acetate agar and yeast extract-malt extract (YM) agar; mating activity was examined using McClary’s acetate agar. The cultures initially were grown for 5 days at 25 °C to induce sporulation and then were observed daily for ascospores for 1 week. The other physiological tests were carried out in accordance with the methods described in the 5th edition of 'The Yeasts: a Taxonomic Study' (Kurtzman, 2011a).

Analysis of yeast proteins. Yeast proteins were studied using the SDS-PAGE technique. The yeast proteins were extracted according to the Kushnirov protocol (Kushnirov, 2000) with some modifications. Briefly, yeast strains were grown on MYGP agar medium at 30 °C. The cultures initially were grown for 5 days at 25 °C to induce sporulation and then were observed daily for ascospores for 1 week. After 10 min of incubation at room temperature (hereinafter RT), the cells were centrifuged 5 min at 6000 × g and resuspended in 5 ml NaOH (0.2 M aqueous solution). After 5 min of incubation at room temperature, the yeast cells were pelleted as described previously, then resuspended in 250 μl sample buffer (0.06 M Tris/HCl pH 6.8, 2.5% glycerol, 2.5% SDS, 5% β-mercaptoethanol, 0.003 g bromophenol blue), vortexed for 1 min and boiled for 5 min. The treated cells were centrifuged and 20 μl supernatant for each strain was taken for electrophoretic analysis. Five microlitres of standard weight ladder were loaded into the first well of a 10% acrylamide mini-gel (Bio-Rad Mini Protean cell), followed by the samples, loaded in the subsequent lanes, and analyzed as described by Laemmli (1970).

Molecular identification. Genomic DNA was extracted from a representative of each single morphology (Bolano et al., 2001; Cardinali et al., 2001). Amplification of the internal transcribed spacer (ITS)1, 5.8S, ITS2 and D1/D2 domains of the 26S rRNA gene was performed using primer ITS5 (5'-GGAAGTAAAGTCGTAACAAGG) and LR6 (5'-CGCCAGTTCTGTCTTACC) as indicated by Boekhout et al. (2003) with EuroTag enzyme (EuroClone) and a PTC-100 Peltier Thermal Cycler (MJ Research). Amplicons were purified with the GFX PCR DNA purification kit (GE Healthcare) while the electrophoresis was performed on 1.5% agarose gel (Gellyphor; EuroClone). Amplicons were sequenced using a ABI PRISM 3730xl with primer ITS1 (5’- TCCGTTAGGTAACCTGCGG) and NL4 (5’-GTCGCTGTTTCCAGACGG). Electrophoretogram trimming, contig assembly and BLAST search were performed with Geneious 5 (Drummond et al., 2011).

Fig. 1. Evolutionary relationships of taxa related to Y. terventina. Phylogram of the Yamadazyma clade showing the most parsimonious tree based on the concatenation of the sequence from the D1/D2 region of the LSU rDNA and the ITS1, 5.8S ribosomal gene, and ITS2 sequence. The relationships of strains CBS 12509, CBS 12510 and CBS 12511 were reconstructed using the maximum-parsimony method set with the Subtree-Pruning-Regrafting (SPR) tree inference option. Gaps and missing data were treated as complete deletion. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Type strain sequences were retrieved from GenBank and CBS databases (first number identifies ITS sequence, the second is the D1/D2 sequence). Nucleotide sequences were concatenated and then aligned with MUSCLE (Edgar, 2004). Phylogenetic analyses were conducted in MEGA5 (Tamura et al., 2011). Schizosaccharomyces pombe was used as outgroup. Bar, number of changes over the whole sequence.
Sequence alignment was then carried out by Muscle (Edgar, 2004). Phylogenetic analysis was conducted in MEGA5 (Tamura et al., 2011).

RESULTS

The large subunit (LSU; 26S) rRNA D1/D2 domain, ITS1, 5.8S rDNA and ITS2 sequences (see Fig. 1) of the above-mentioned strains were previously compared in GenBank by a BLAST search. The similar sequences, previously identified, were included in the phylogenetic analysis, which confirmed the currently accepted Yamadazyma clade composition and structure (Groenewald et al., 2011; Kurtzman & Suzuki, 2010; Kurtzman et al., 2011b).

According to the phylogenetic reconstruction, the novel species proposed herein was placed in a well-supported clade, including members of the genera Yamadazyma and Candida, globally named the Yamadazyma clade (Fig. 1). Due to the collocation of the novel species in the Yamadazyma clade, the novel species has been named Yamadazyma terventina and not Candida terventina, according to the 'one fungus – one name' recommendation (Hawksworth, 2011). The closest relatives were Candida conglobata (42 substitutions equivalent to 3.33 % difference), Candida aaseri (50 substitutions equivalent to 3.97 % difference), a synonym of the butter contaminant Candida butyri (Pfüller et al., 2011) isolated from butter and at low frequency in cases of mastitis (Spanamberg et al., 2008) and Yamadazyma scolyti (61 substitutions equivalent to 4.09 % difference).

Table 1. Comparison of the assimilation and fermentation profile of selected substrates by species phylogenetically closely related to Yamadazyma terventina

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<td>37 °C Glucose fermentation</td>
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Sequence alignment was then carried out by Muscle (Edgar, 2004). Phylogenetic analysis was conducted in MEGA5 (Tamura et al., 2011).

![Fig. 2. Light microscopic morphology of cells of Y. terventina CBS 12509, CBS 12510\textsuperscript{T} and CBS 12511. (a) Budding cells; (b) pseudomycelia. Bars, 5 μm.](image-url)
to 4.84 % difference). Members of the clade rather distant to the novel species were *Yamadazyma triangularis*, a yeast of clinical relevance, isolated in lung tissue and considered as a potential opportunistic pathogen (99 substitutions, equivalent to 7.86 % difference) (Kurtzman et al., 2011a; Smith & Batenburg-Van der Vege, 1986) and *Yamadazyma philogaea* (73 substitutions, equivalent to 5.79 % difference) (Robiglio et al., 2011; van der Walt & Johannsen, 1975).

The three yeast strains, derived from different extra virgin olive oils produced both in the north and in the middle of Italy, were characterized by identical ITS and D1/D2 sequences, but could be differentiated by some physiological traits independently from their geographical origin. In fact, d-glucosamine was assimilated only by the strain CBS 12510T, whereas D-ribose was used by CBS 12510T and CBS 12511 (Table 1). Another difference was that the type strain could form pseudomycelium on YEPDA (1 % yeast extract, 1 % peptone, 2 % glucose, 1.8 % agar; Difco), Dalmau plates and MYGP agar-olive oil medium (O/N growth), whereas the other two strains produced pseudomycelium only on Dalmau culture or MYGP agar-olive oil medium after 15 days incubation. It was the presence of this more complex morphology that induced us to designate CBS 12510T as type strain. The electrophoretic profiles of the yeast proteins (SDS-PAGE) extracted from the three yeast strains showed the same pattern in a range between 30 and 90 kDa (data not shown), further supporting the fact that the three strains belong to the same species. The data reported above indicate that this species presents some degree of phenotypic variability in spite of the identity of the three molecular markers that are currently widely used to differentiate yeasts at the species level. However, the assimilation and fermentation profiles of the proposed species differ for several traits from those of the closest relatives of the clade (Table 1).

*Y. terventina* does not assimilate D-arabinose (also found for *C. aaseri* and *Y. triangularis*) or ethylamine (also found for *C. friedrichii*); the strains give instead variable responses for the assimilation of d-ribose, which is actively used by almost all closely related species. Furthermore, *Y. terventina* is the only species to assimilate DL-lactate actively.

On Dalmau slide cultures with cornmeal agar or rice extract agar after 5 days at 25 °C, pseudomycelium is formed under the cover glass and without cover glass (Fig. 2b). Sporulation does not occur on McClary’s acetate agar and YM agar at 17 °C and 25 °C after 10 days. Glucose is fermented. Glucose, galactose, l-sorbose, sucrose, cellobiose, x,x-trehalose, melezitose, maltose, D-xyllose, L-arabinose, L-rhamnose, erythritol, adonitol, D-mannitol, D-sorbitol, salicin, D-glucono-1,5-lactone, DL-lactate, succinate, citrate, D-glucosamine, L-lysine, malic acid and N-acytelyglucosamine are assimilated. Other carbon compounds tested in this study, including, D-ribose, ethanol, glycerol, methyl x-D-glucoside and 2-keto-D-gluconate are weakly assimilated. D-Glucuronate, myo-inositol, raffinose, D-arabinose, melibiose, lactose, inulin, starch, dulcitol, methanol, nitrate, nitrite and ethylamine are not assimilated. Growth on 50 % glucose, 10 % NaCl and 12.5 % NaCl is negative. Growth occurs on 5 % NaCl, but not in the presence of 0.1 p.p.m. cycloheximide. Growth occurs at 25 °C but not at 4, 37 or 42 °C. No starch-like substance is produced. Urea hydrolysis and the Diazonium blue B reaction are negative. Lipase activity is positive. Proteinase activity verified on skim milk agar and on growth medium supplemented with azocasein; in the former all three strains show a positive reaction, while only the type strain shows proteolytic activity in the latter. The cultures of the three strains representing the novel species in this study did not show any mating activity when mixed in pairs.

The type strain, DAPES 1924T (=CBS 12510T=NCAIM Y.02028T), was isolated from an extra virgin olive oil produced in the ‘Trivento’ area (Molise region) located in the middle of Italy. MycoBank no. is MB 800582. Two further reference strains of *Y. terventina* are DAPES 1916 (=CBS 12509=NCAIM Y.02027) and DAPES 1931 (=CBS 12511=NCAIM Y.02029).

**REFERENCES**


**Description of Yamadazyma terventina**

Ciafardini, Zullo, Antonielli, Corte, Roscini & Cardinale sp. nov.

*Yamadazyma terventina* (ter.ven’ti.na. N. L. fem. adj. *terventina* of or pertaining to Terventum, referring to the Latin name of the town in the Molise region, middle of Italy, where the yeasts have been isolated for the first time).

After growth in YM broth at 25 °C for 3 days, the cells are ellipsoidal (2–4 x 4–6 μm) and occur singly or in pairs (Fig. 2a). Asexual reproduction occurs by multilateral budding. Sediment is not present. After 5 days at 25 °C on YM agar, streak cultures present round colonies with both sharp and jagged edges, and a colour varying from glossy to matt white.