Candida olivae sp. nov., a novel yeast species from 'Greek-style' black olive fermentation

Aspasia A. Nisiotou, Efstathios Z. Panagou and George-John E. Nychas

Correspondence

Aspasia A. Nisiotou
anisiotou@aua.gr

Laboratory of Microbiology and Biotechnology of Foods, Department of Food Science and Technology, Agricultural University of Athens, Iera Odos 75, 118 55 Athens, Greece

Two yeast strains (FMCC Y-1^T and FMCC Y-2) were recovered during a survey of the yeast biota associated with fermenting black olives, collected from an olive tree (*Olea europaea* L. cv. 'Conservolea') orchard in Central Greece. Phylogenetic analysis based on rRNA gene sequences (18S, 26S, and 5.8S-ITS) indicated that the two strains represent a separate species within the *Candida membranifaciens* clade, in close relation to *Candida blattariae* NRRL Y-27703^T. Electrophoretic karyotyping and physiological analysis support the affiliation of the two strains to a novel species as *Candida olivae* sp. nov. The novel strains are conspecific with two *Candida* sp. strains previously isolated from the Mid-Atlantic Ridge hydrothermal fields [Gadanhó & Sampaio (2005). *Microb Ecol* **50**, 408–417], indicating that *Candida olivae* sp. nov. may occupy diverse ecological niches. FMCC Y-1^T (=CBS 11171^T =ATCC MYA-4568^T) is the type strain.

Naturally black olives, also referred to as 'Greek-style', are produced according to a traditional process involving spontaneous fermentation of drupes in brine. Fermentation is carried out by the indigenous yeast biota, which grows on the epicuticular wax of the olive skin and in the stomatal openings (Nychas *et al.*, 2002). Different yeasts with diverse phylogenetic affiliations have been isolated from fermenting olives, yet this ecosystem represents a highly unexplored habitat for yeast species (Arroyo-Lopez *et al.*, 2008).

Strains FMCC Y-1^T (=CBS 11171^T =ATCC MYA-4568^T) and FMCC Y-2 (=CBS 11172 =ATCC MYA-4569) were isolated during a survey of the yeast biota associated with fermenting black olives. Mature olives of the 'Conservolea' cultivar were collected from an olive tree orchard in Central Greece and transferred to the laboratory within 24 h. Fermentations were carried out in 6% NaCl brine supplemented with glucose (0.5% w/v). Brine samples were decimally diluted in Ringer's solution and 100 µl aliquots were spread onto Rose Bengal Chloramphenicol

agar (Oxoid). Colonies were restreaked onto YPD agar (1% yeast extract, 2% peptone, 2% glucose, 2% agar) and stored at –80 °C until further analysis. Morphological, physiological and biochemical characteristics were examined according to standard methods (Yarrow, 1998). Morphology of cells was observed under an Olympus BX50 microscope and images were acquired using an Olympus DP71 digital camera.

Genomic DNA was isolated according to Burke *et al.* (2000). The 18S rRNA gene, the D1/D2 domain of 26S rRNA gene and the 5.8S-internal transcribed spacer (ITS) rRNA region were amplified according to Ueda-Nishimura & Mikata (1999), Kurtzman & Robnett (1998) and Nisiotou & Gibson (2005), respectively. PCR products were purified by using the QIAquick PCR Purification kit (Qiagen). Both DNA strands were directly sequenced with an ABI 3730 XL automatic DNA sequencer by Macrogen (<http://www.macrogen.com>). BLAST searches of sequences from GenBank were carried out and CLUSTAL_X (1.83) software (<http://www-igbmc.u-strasbg.fr/BioInfo>) was used to perform sequence alignments. Phylogenetic analysis was conducted using the MEGA version 4 software package (Tamura *et al.*, 2007) after multiple alignment of data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining and maximum-parsimony methods were determined using bootstrap values based on 1000 replications.

Intact yeast chromosomal DNA for PFGE was prepared using the method described by Bai *et al.* (2000). Chromosomal DNA bands were separated in a contour-clamped homogeneous electric field electrophoresis apparatus (CHEF II, Bio-Rad) as described by Imanishi *et al.*

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the 26S rRNA gene, 5.8S-ITS and 18S rRNA gene sequences of strain FMCC Y-1^T are FJ715430, FJ715432 and FJ715434, respectively; those for the 5.8S-ITS gene sequences of *Candida blattariae* CBS 9876^T, FMCC Y-2, MARY 179 and MARY 223 are FJ715435, FJ715433, GQ258721 and GQ258722, respectively; those for the 26S rRNA gene sequences of strains FMCC Y-2 and MARY 223 are FJ715431 and GQ258724, respectively; and that for the 18S rRNA gene sequence of strain MARY 223 is GQ258723.

A bootstrap consensus neighbour-joining tree of combined D1/D2, ITS (including 5.8S) and 18S rRNA gene sequences is available with the online version of this paper.

(2007). The gel was stained in ethidium bromide solution (0.5 mg ml^{-1}) for 1 h, destained in distilled water and viewed under UV light (302 nm). *Saccharomyces cerevisiae* (YNN 295) chromosomal DNA (Bio-Rad) was used as a molecular size marker.

rRNA gene sequence comparisons of the 18S, 26S and 5.8S–ITS regions among isolates FMCC Y-1^T and FMCC Y-2 and other strains available in the GenBank database were performed. The novel strains shared identical sequences in the D1/D2 domain, which showed 100 % identity to those of two *Candida* sp. strains (strain MARY 179, AY731728; strain MARY 223, GQ258724, this study), isolated from hydrothermal fields of the Mid-Atlantic Rift (Gadanhó & Sampaio 2005). The novel strains differed from *Candida blattariae* NRRL Y-27703^T by 1.3 % (six non-contiguous substitutions and one indel in 532 nucleotides); and from *Candida membranifaciens* NRRL Y-2089^T by 1.5 % (six non-contiguous substitutions and two indels in 544 nucleotides). The 5.8S–ITS rRNA gene sequences of strains FMCC Y-1^T and FMCC Y-2 were identical to each other and differed from those of strains MARY 179 and MARY 223, which shared the same sequence, by a single nucleotide substitution in 590 nucleotides (0.2 %); from that of *C. blattariae* by 52 differences in 602 nucleotides (43 substitutions and nine indels; 8.6 %); and from *C. membranifaciens* by 45 differences in 596 nucleotides (35 substitutions and 10 indels; 7.5 %). Sequence comparisons of the 18S rRNA gene

revealed 21 (15 substitutions and six indels) nucleotide differences in 1670 nucleotides (1.3 %) and 17 substitutions in 1753 bp (1 %) between the novel species and *C. blattariae* and *C. membranifaciens*, respectively.

Phylogenetic trees based on D1/D2, ITS (including 5.8S rRNA) or 18S rRNA gene sequences were constructed to visualize interrelationships among the novel strains and other members of the *Candida membranifaciens* clade. In all cases the novel species, represented by the strain FMCC Y-1^T, was phylogenetically distinct from *C. blattariae* and other closely related species. No differences were detected between the neighbour-joining and maximum-parsimony methods, particularly as regards the position of strain FMCC Y-1^T. The phylogenetic placement of the new species, based on the neighbour-joining analysis of D1/D2 rRNA gene sequences, is shown in Fig. 1.

Chromosomal banding profiles of strains FMCC Y-1^T, FMCC Y-2, MARY 179 and MARY 223 were identical to each other but profoundly distinct from that of *C. blattariae* (Fig. 2). The four strains possessed three electrophoretic bands ranging in size from approximately 700 to 2200 kb. The bands detected may account for a higher overall chromosome number, because the 2200 kb band could be a doublet. The banding profile of *C. blattariae* CBS 9876^T consisted of four electrophoretic bands ranging from approximately 550 to 2200 kb.

Fig. 1. Bootstrap consensus neighbour-joining tree of D1/D2 rRNA gene sequences depicting the relationships between strain FMCC Y-1^T and closely related species of the genera *Candida* and *Pichia*. The respective GenBank accession numbers are given in parentheses. Numbers at branch points are bootstrap percentages derived from 1000 replicates. *Saccharomyces cerevisiae* NRRL Y-12632^T was the outgroup species in the analysis. Bar, 0.01 substitutions per nucleotide position. A tree based on the combined D1/D2, ITS (including 5.8S) and 18S rRNA gene sequences is presented in Supplementary Fig. S1, available in IJSEM Online.

Fig. 2. Electrophoretic karyotypes. Lanes: 1, strain FMCC Y-1^T; 2, *Saccharomyces cerevisiae* YNN 295; 3, strain FMCC Y-2; 4, *Candida blattariae* CBS 9876^T; 5, strain MARY 179; 6, *Saccharomyces cerevisiae* YNN 295; 7, strain MARY 223.

Strains FMCC Y-1^T, FMCC Y-2, MARY 179 and MARY 223 showed identical morphological and physiological characters, differing only in that the olive isolates could not assimilate ethanol. Their physiological profile clearly differentiated them from the closely related *C. blattariae* and from other *Candida* species of the *C. membranifaciens* clade (Fig. 3a, b; Table 1). The novel species could be distinguished from *C. blattariae* by its inability to ferment trehalose or assimilate L-rhamnose or DL-lactate, and by its ability to grow at 34 °C. In conclusion, based on physiological characterization and phylogenetic analysis, it is proposed that strains FMCC Y-1^T and FMCC Y-2 are conspecific with strains MARY 179 and MARY 223 and representatives of a distinct species within the genus *Candida*, *Candida olivae* sp. nov., with FMCC Y-1^T (=CBS 11171^T =ATCC MYA-4568^T) as the type strain.

Yeasts within the *C. membranifaciens* clade have been isolated from various habitats including plants, water and insects (Meyer *et al.*, 1998; Suh *et al.*, 2005). The novel strains were recovered during a survey of the yeast biota associated with spontaneously fermenting black olives. Strains were isolated from the initial stage of fermentation, i.e. immediately after the placement of olives in the brine, and they were not detected thereafter during the course. It is possible that *C. olivae* sp. nov. strains had been transmitted onto the surface of olives by insects, as this is a common route for fruit-surface yeast colonization. Accordingly, several members of the *C. membranifaciens* clade are insect-associated (Suh *et al.*, 2005). The fact that strains MARY 179 and MARY 223 were previously isolated from the Mid-Atlantic Rift hydrothermal fields (Gadanhó & Sampaio, 2005) indicates that the novel species may

Fig. 3. Vegetative cells (a) of strain FMCC Y-1^T grown in YM broth for 3 days at 25 °C. (b) Pseudohyphae formed under the coverglass of a Dalmat plate culture on cornmeal agar after 10 days at 25 °C. Bars, 10 µm.

occupy diverse ecological niches, not limited to certain environments or geographical localities.

Latin diagnosis of *Candida olivae* Nisiotou, Panagou et Nychas sp. nov.

In medio liquido YM post dies 3 ad 25 °C, cellulae ellipsoideae, cylindricae vel elongatae (1.54–3.65 × 5.10–8.29 µm), singulae, binae, et adhaerentes; pseudohyphae fiunt. Per gemmationem multipolarem reproducentes. Post 1 mensem sedimentum formatur. *In agar* YM post 1 mensem ad 25 °C, butyrosa, crenea, convexa, glabra, margo glabro vel undulato. *In agar* farina Zeae maydis confecto post 10 dies ad 25 °C, pseudohyphae fiunt. Ascosporae non fiunt. Glucosum et galactosum fermentantur at non sucrosum, maltosum, lactosum, raffinotum nec trehalosum. Glucosum, galactosum, sucrosum, maltosum, cellobiosum, trehalosum (lente), melezitotum, D-xylosum, L-arabinosum, D-arabinosum, D-ribosum (lente), D-glucosaminum (lente), N-acetyl-D-glucosaminum, glycerolum, erythritolum, adonitolum,

Table 1. Physiological characteristics of *Candida olivae* sp. nov. and closely related species

Strains: 1, *Candida olivae* sp. nov.; 2, *C. blattariae*; 3, *C. friedrichii*; 4, *C. membranifaciens*; 5, *C. diospyri*; 6, *C. buinensis*. Data for strains 3, 4 and 6 from Meyer *et al.* (1998). +, Positive; –, negative; D, delayed positive; V, variable; ND, no data available.

Characteristic	1	2	3	4	5	6
Fermentation of:						
Galactose	D	+	V	V	+	+
Sucrose	–	–	–	+	–	–
Raffinose	–	–	–	+	–	–
Trehalose	–	D	ND	+	ND	–
Assimilation of:						
L-Sorbose	–	–	+	+	+	+
Melibiose	–	–	+	+	–	–
Raffinose	–	–	+	+	–	–
D-Arabinose	+	+	V	+	+	+
L-Rhamnose	–	+	–	V	+	–
Ethanol	V	+	+	+	+	+
D-Gluconate	+	+	–	+	ND	+
DL-Lactate	–	D	V	+	–	–
L-Lysine	+	+	+	V	+	+
Growth at 34 °C	+	–*	V	V	+	–

*Determined at 35 °C.

D-mannitolum, *D-glucitolum* α -methyl-*D-glucosidum*, *salicinum*, *D-gluconatum*, *acidum succinicum*, *acidum citricum*, *L-arabinitolum et arbutinum assimilantur at non L-sorbosum*, *lactosum*, *melibiosum*, *raffiniosum*, *inulinum*, *amylum solubile*, *L-rhamnosum*, *methanolum*, *DL-acidum lacticum*, *nec inositolum*. *Ethylaminum*, *L-lysinum et cadaverinum assimilantur at non natrum nitrosum nec kalium nitricum*. *Ad crescentiam vitaminarum externarum necessariae sunt*. In 10% *natrium chloridum*/5% *glucoso crescentiae*. *Maxima temperatura crescentiae*: 34 °C. *Materia amyloidea iodophila non formantur*. *Ureum non hydrolysat*. *Crescit in medio cum 50% (w/v) glucoso*. *Typus stirps CBS 11171^T isolatus ex fermentum oliva, depositus in collectione Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands*.

Description of *Candida olivae* Nisiotou, Panagou & Nychas sp. nov.

Candida olivae (o.li'vae. L. gen. n. *olivae* of/from an olive).

In YM broth (Yarrow, 1998), after 3 days at 25 °C, the cells are ellipsoidal, cylindrical, elongate (1.54–3.65 × 5.10–8.29 µm) and occur singly, in pairs, in chains or aggregated in clusters (Fig. 3a). Pseudohyphae may be present. Budding is multilateral. After 1 month at 25 °C a flocculent sediment is present. On YM agar (Yarrow, 1998), after 1 month at 25 °C, the streak culture is membranous, cream-coloured, flat, and rough; the margin is undulating. After 10 days Dalmau plate culture on cornmeal agar at 25 °C, pseudohyphae (Fig. 3b) are present. No ascospores are

produced after 5 weeks at 15 and 25 °C from individual strains on agar or strains crossed on YM, malt extract, 5% malt extract, Gordokowa, cornmeal or half strength cornmeal agar. Glucose and galactose are fermented; sucrose, maltose, lactose, raffinose and trehalose are not fermented. Glucose, galactose, sucrose, maltose, cellobiose, trehalose (delayed), melezitose, D-xylose, L-arabinose, D-arabinose, D-ribose (delayed), D-glucosamine (delayed), N-acetyl-D-glucosamine, glycerol, adonitol, erythritol, D-mannitol, D-sorbitol, methyl α -D-glycoside, salicin, D-gluconate, succinate, citrate, xylitol, L-arabinitol and arbutin are assimilated; L-sorbose, lactose, melibiose, raffinose, inulin, soluble starch, L-rhamnose, methanol, DL-lactate and inositol are not assimilated. Ethylamine hydrochloride, L-lysine and cadaverine are assimilated; sodium nitrite and potassium nitrate are not assimilated. Growth in vitamin-free medium is negative. Growth in 10% sodium chloride plus 5% glucose in nitrogen base is positive. Maximum growth temperature is 34 °C. Starch-like compounds are not produced. Urease activity is negative. Growth in the presence of 50% (w/v) glucose is positive.

The type strain, FMCC Y-1^T (=CBS 11171^T =ATCC MYA-4568^T), was isolated from fermenting olives in Athens, Greece.

Acknowledgements

This research was supported by TRUEFOOD – ‘Traditional United Europe Food’, an Integrated Project financed by the European Commission under the 6th Framework Programme for RTD (contract no. FOOD-CT-2006-016264). José Paulo Sampaio is gratefully acknowledged for providing strains MARY 179 and MARY 223. We thank Marc-André Lachance for critical comments and helpful suggestions.

References

- Arroyo-López, F. N., Querol, A., Bautista-Gallego, J. & Garrido-Fernández, A. (2008). Role of yeasts in table olive production. *Int J Food Microbiol* 128, 189–196.
- Bai, F.-Y., Liang, H.-Y. & Jia, J.-H. (2000). Taxonomic relationships among the taxa in the *Candida guilliermondii* complex, as revealed by comparative electrophoretic karyotyping. *Int J Syst Evol Microbiol* 50, 417–422.
- Burke, D., Dawson, D. & Stearns, T. (2000). Yeast genomic DNA: glass bead preparation. In *Methods in Yeast Genetics, a Cold Spring Harbor Laboratory Course Manual*, pp. 113–114. Cold Spring Harbor NY: Cold Spring Harbor Laboratory.
- Gadanhó, M. & Sampaio, J. P. (2005). Occurrence and diversity of yeasts in the Mid-Atlantic Ridge hydrothermal fields near the Azores Archipelago. *Microb Ecol* 50, 408–417.
- Imanishi, Y., Ueda-Nishimura, K. & Mikata, K. (2007). Two new species of *Kazachstania* that form ascospores connected by a belt-like intersporal body: *Kazachstania zonata* and *Kazachstania gamospora*. *FEMS Yeast Res* 7, 330–338.
- Kurtzman, C. P. & Robnett, C. J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S)

ribosomal DNA partial sequences. *Antonie Van Leeuwenhoek* **73**, 331–371.

Meyer, S. A., Payne, R. W. & Yarrow, D. (1998). *Candida* Berkhout. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 454–573. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.

Nisiotou, A. A. & Gibson, G. R. (2005). Isolation of culturable yeasts from market wines and evaluation of the 5.8S-ITS rDNA sequence analysis for identification purposes. *Lett Appl Microbiol* **41**, 454–463.

Nychas, G.-J. E., Panagou, E. Z., Parker, M. L., Waldron, K. W. & Tassou, C. C. (2002). Microbial colonization of naturally black olives during fermentation and associated biochemical activities in the cover brine. *Lett Appl Microbiol* **34**, 173–177.

Suh, S.-O., Nguyen, N. H. & Blackwell, M. (2005). Nine new *Candida* species near *C. membranifaciens* isolated from insects. *Mycol Res* **109**, 1045–1056.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.

Thompson, J. D., Higgins, D. G., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. J. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Ueda-Nishimura, K. & Mikata, K. (1999). A new yeast genus, *Tetrapisispora* gen. nov.: *Tetrapisispora iriomotensis* sp. nov., *Tetrapisispora nanseiensis* sp. nov. and *Tetrapisispora arboricola* sp. nov., from the Nansei Islands, and reclassification of *Kluyveromyces phaffii* (van der Walt) van der Walt as *Tetrapisispora phaffii* comb. nov. *Int J Syst Bacteriol* **49**, 1915–1924.

Yarrow, D. (1998). Methods for the isolation, maintenance and identification of yeasts. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 77–100. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.