Lipomyces orientalis sp. nov., a yeast species isolated from soil in Vietnam

Vu Nguyen Thanh

Correspondence

Vu Nguyen Thanh

thanh@firi.ac.vn

Department of Microbiology, Food Industries Research Institute, 301-Nguyen Trai, Thanh Xuan, Hanoi, Vietnam

Five strains of the novel ascogenous yeast species *Lipomyces orientalis* sp. nov. were isolated from soil samples collected in Vietnam. The yeast forms asci containing one to four ascospores. The ascospores of *L. orientalis* have warty surfaces and differ from the characteristic striated ascospores of *Lipomyces tetrasporus*. Phylogenetic analyses of D1/D2 26S rDNA and ITS sequences indicate that *L. orientalis* and *L. tetrasporus* are closely related, but they differ from each other by 6 nucleotides in the D1/D2 region and 16 nucleotides in the ITS region. The type culture is strain Lip 95^T (=CBS 10300^T=NRRL Y-27927^T).

Yeasts of the genus *Lipomyces* are true soil inhabitants and have a worldwide distribution. The genus *Lipomyces* and associated genera (*Dipodascopsis*, *Zygozoma*, *Myxozyma*) represent a unique branch in the evolution of the ascomycetes (van der Walt, 1992). Currently 11 species of *Lipomyces* are recognized, *Lipomyces doorenjongii*, *L. japonicus*, *L. kockii*, *L. kononenkoae*, *L. lipofer*, *L. mesembrius*, *L. spencermartinsiae*, *L. starkeyi*, *L. tetrasporus*, *L. yamadae* and *L. yarrowii* (van der Walt *et al.*, 1997; Smith, 1998; van der Walt *et al.*, 1999). Despite the worldwide distribution of the genus, some geographical and geochemical preferences have been recorded for some species. For example, *L. lipofer* is restricted to temperate zones (Canada, Russia) whereas *L. kononenkoae* and *L. spencermartinsiae* can be commonly found in ferric soils of the tropics (Mali, Nigeria, Trinidad) (Babjeva & Gorin, 1987). In a survey of the diversity of soil yeasts in Vietnam, strains representing a novel species of the genus *Lipomyces* were isolated.

A total of 52 soil samples from different locations in Vietnam were collected and analysed (see Supplementary Fig. S1 for locations of sampling sites). For isolation of lipomycetaceous yeasts, soil particles were sprinkled over agar plates containing nitrogen-depleted medium (l⁻¹: 20 g glucose, 20 g agar, 0.85 g KH₂PO₄, 0.15 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 0.1 g NaCl, 0.1 g CaCl₂·6H₂O, 0.5 mg H₃BO₃, 0.04 mg CuSO₄·H₂O, 0.1 mg KI, 0.2 mg FeCl₃·6H₂O, 0.4 mg MnSO₄·H₂O, 0.2 mg Na₂MoO₄·2H₂O, 0.4 mg ZnSO₄·

7H₂O). The plates were incubated at 25 °C for 2–3 weeks until characteristic slimy growth of *Lipomyces* was detected. Yeast colonies were purified on the nitrogen-depleted medium and then on malt/glucose agar (1 °Bx malt broth, 1 % glucose, 2 % agar). A protocol describing the preparation of the malt broth is available as supplementary material in IJSEM Online.

Lipomycetaceous yeasts were found in 24 of the 52 samples. From these, 73 strains were isolated. They all sporulated strongly on diluted malt/glucose agar after 3–4 weeks of incubation. The strains were divided into groups based on the mode of ascospore formation. Twenty-four strains were found to be of the ‘tetrasporus’ type (i.e. they formed asci containing one to four ascospores). The remaining strains produced asci containing mainly more than eight ascospores. Among the ‘tetrasporus’ group, 19 strains produced striated ascospores and the other five produced roughened ascospores (Fig. 1a). Closer examination by scanning electron microscopy (SEM) revealed that ascospores of the latter five strains had warty surfaces (Fig. 1b and Supplementary Fig. S2) and, thus, they differed morphologically from all known *Lipomyces* species.

Physiological tests were carried out on the latter five strains using standard methods for yeast characterization (Yarrow, 1998). Sporulation of strains was obtained on 10-fold diluted malt/glucose agar (0.1 °Bx malt broth, 0.1 % glucose, 2 % agar). The morphology of vegetative cells and ascospore formation was studied using a light microscope (Eclipse E-600; Nikon). Photographs were taken with a digital video camera (TK-C1380E; JVC). For SEM, strongly sporulated biomass was collected and fixed with 10 % glutaraldehyde for 2 h. The biomass was then treated with increasing concentrations of acetone (25, 50, 75 and 100 %) and dried. The samples were coated with gold and viewed with scanning electron microscope (JSM-5410LV; JEOL) at 15 000 kV.

The GenBank/EMBL/DDBJ accession number for the 26S rDNA D1/D2 and ITS sequences of strain Lip 95^T are DQ275615 and DQ275616.

A protocol for the preparation of malt broth, a map showing the locations of the sampling sites, an electron micrograph of ascospores of strain Lip 95^T and a neighbour-joining tree based on D1/D2 sequences are available as supplementary material in IJSEM Online.

Fig. 1. (a) Asci and ascospores of *Lipomyces orientalis* sp. nov. Lip 95^T grown on diluted malt/glucose agar for 4 weeks at 25 °C. (b–c) Scanning electron micrographs of *Lipomyces orientalis* Lip 95^T (b) and *Lipomyces tetrasporus* Lip 5 (c) grown on diluted malt/glucose agar for 4 weeks. Bars, 5 µm (a) and 1 µm (b, c).

The five novel strains differed from the standard description of *L. tetrasporus* in three assimilation tests (assimilation of propane-1,2-diol, butane-2,3-diol and nitrite). These five lipomycetaceous strains were isolated from three soil samples collected at Thanh Hoa (strain MD66-1), Quang

Ninh (strains MD88-4 and MD 89-4) and Nha Trang (strains Lip 51 and Lip 95^T) (Table 1). The distances between these locations are roughly 260, 940 and 1000 km.

Extraction and purification of the coenzyme Q system were performed according to Yamada & Kondo (1973). Reversed-phase HPTLC was used for identification of coenzyme Q. Reversed-phase HPTLC plates (silica gel 60 for nano TLC; Merck) were prepared by dipping in n-hexane containing 5 % liquid paraffin. The plates were air-dried. Coenzyme Q samples and standards were spotted on the plates, which were then developed with methanol/isopropanol (1:1) saturated with liquid paraffin. Visualization was done by dipping the plates in 0.2 % KMnO₄ for 5 min. This procedure ensures good separation of CoQ-6, CoQ-7, CoQ-8, CoQ-9 and CoQ-10. To separate CoQ-10 and CoQ-10(H₂), a developing system containing acetone/acetonitrile (1:1) saturated with liquid paraffin was used. The major ubiquinone of the novel strains was Q-9.

For DNA extraction, strain Lip 95^T was grown on YMA (0.3 % yeast extract, 0.3 % malt extract, 0.5 % peptone, 1 % glucose, 2 % agar) for 3 days at 25 °C. One loopful of cells was transferred to a microfuge tube containing 1 ml 2 × SSC and heated at 99 °C for 10 min. Cells were collected and washed once with 1 ml sterile deionized water by centrifugation. To the cell pellet, about 75 µl glass beads (0.2–0.5 mm in diameter), 75 µl phenol/chloroform and 100 µl water were added. The tube was shaken at 1400 r.p.m. for 10 min and then centrifuged at 10 000 g for 10 min. The upper layer was transferred to a new microfuge tube and used directly as template for PCR. Due to the well-known inhibition effect of slimy substances of *Lipomyces* on the PCR (Nishimura *et al.*, 2002), care is needed regarding the age of culture and the cultivation medium.

The ITS-D1/D2 region was amplified using primers ITS1 and NL4 on a GeneAmp PCR System 9700 (PE Applied Biosystems). The PCR conditions were as follows: an initial denaturation step of 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 52 °C for 40 s and 72 °C for 60 s, with a final extension step at 72 °C for 7 min. The PCR product was purified by using a QIAEX II agarose gel extraction kit (Qiagen). The DNA was sequenced by the dideoxy chain-termination method using primers ITS1, ITS4, NL1 and NL4 described by Kurtzman & Robnett (1998) and Esteve-Zarzoso *et al.* (1999). A model 377 automated DNA sequencer (Applied Biosystems) was used. For phylogenetic analyses, the resulting sequences were compared with known sequences in GenBank using the web-based nucleotide-nucleotide BLAST search engine hosted by the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) (Altschul *et al.*, 1997). The most similar sequences were edited using the program BioEdit (Hall, 1999) and aligned using CLUSTAL X version 1.81 (Thompson *et al.*, 1997). The fixed-gap and floating-gap penalty values used were respectively 10 and 0.2. Phylogenetic trees were constructed from distance data transformed according to Kimura (1980), using the neighbour-joining method (Saitou & Nei, 1987) in

Table 1. Isolation sources of strains of *Lipomyces orientalis* sp. nov.

Strain	Other collection numbers	Source of isolation
Lip 51	CBS 10301, NRRL Y-27928	Mixed forest soil, hillside (bottom part), 50 m from water edge, Bai San island, Nha Trang
Lip 95 ^T	CBS 10300 ^T , NRRL Y-27927 ^T	Mixed forest soil, hillside (bottom part), 50 m from water edge, Bai San island, Nha Trang
MD66-1	CBS 10302, NRRL Y-27929	Grassland soil, hillside (bottom part), Cam Thuy, Thanh Hoa
MD88-4	CBS 10303, NRRL Y-27930	Mixed forest soil, hillside (bottom part), 100 m from water edge, Cat Ba island, Quang Ninh
MD 89-4	CBS 10304, NRRL Y-27931	Mixed forest soil, hillside (bottom part), 100 m from water edge, Cat Ba island, Quang Ninh

CLUSTAL X. Bootstrap analyses were performed from 1000 random resamplings (Felsenstein, 1985). The phylogenetic tree was displayed using TreeExplorer version 1.21 (which can be downloaded from <http://evolgen.biol.metro-u.ac.jp/pub/MolEvol/TE212.zip>). Sequences from *Stephanoascus smithiae* strains NRRL Y-17849 (GenBank accession no. U76531) and CBS 5657 (AJ606463) were used as the outgroups for the D1/D2 and ITS trees, respectively.

ITS and 26S rDNA D1/D2 sequences were obtained for strain Lip 95^T. In the phylogenetic tree constructed based on D1/D2 sequences, Lip 95^T clustered with *L. kononenkoae*, *L. spencermartinsiae*, *L. starkeyi* and *L. tetrasporus* but formed an out-branch (see Supplementary Fig. S3 in IJSEM Online). In the D1/D2 region, Lip 95^T was closest to *L. tetrasporus* but differed from the latter at six positions (five substitutions and one insertion). In the ITS tree, Lip 95^T clustered with *L. tetrasporus* (Fig. 2). They differed from each other at 16 positions in the ITS region. Based on the distinctive phenotypic characteristics and phylogenetic position amongst established taxa, a novel species of *Lipomyces*, *Lipomyces orientalis* sp. nov., is proposed to accommodate the new isolates.

Latin diagnosis of *Lipomyces orientalis* Thanh sp. nov.

In YMA ad 25 °C post dies tres cellulae singulae, binae, ovoidae, 4–9 × 5–12 µm. In agaro malti cultura aquosa mucosescens, partim crenea, impellucida, glabra, nitida, margine integro. In agaro farinae Zeae maydis confecto post decemduum in 25 °C hyphae et pseudohyphae nullae. In medio liquido post unum mensem ad 25 °C, sedimentum formatur. Asci plerumque affixi, saccati vel tortioter tubilares, pariete tarde deliquescente. Ascosporae maturae ellipsoideae, succinae, verrucosae, 2.2–2.7 × 3.2–3.9 µm, 1–4 in asco, conglutinantes ubi liberatae. Fermentatio nulla. D-Glucosum, D-galactosum, L-sorboseum, D-xylosum, L-arabinozum (exiguum vel non), D-arabinozum (exiguum), L-rhamnosum (fortasse exiguum), sucrosum, maltosum, α,α-trehalosum (exiguum), methyl-α-D-glucosidum, cellobiosum (fortasse exiguum), salicinum (fortasse exiguum), arbutinum (exiguum), melibiosum (fortasse exiguum), lactosum (exiguum vel non), raffinoseum, melezitoseum, inulinum, amyllum solubile (exiguum), erythritolum, ribitololum (lente et exiguum), xylitololum, D-glucitololum, D-mannitololum, galactitololum, D-glucono-1,5-lactonum (exiguum), 2-keto-D-gluconatum, 5-keto-D-gluconatum (exiguum), D-gluconatum

Fig. 2. Neighbour-joining tree depicting the relationships between *Lipomyces orientalis* and neighbouring taxa. The tree was constructed from nucleotide divergence in the ITS region using the program CLUSTAL X. Bootstrapping was done with 1000 replications; values above 50% are given. *Stephanoascus smithiae* CBS 5657^T was used as the outgroup. GenBank accession numbers are given after strain names.

Fig. 3. Vegetative cells of *Lipomyces orientalis* Lip 95^T grown on YMA for 3 days at 25 °C. Bar, 5 µm.

(exiguum), *succinatum* (exiguum), *citratum* (exiguum), et *ethanolum* (exiguum) assimilantur, at non D-glucosaminum, D-ribosum, glycerolum, myo-inositolum, D-galacturonatum, DL-lactatum, methanolum, propane-1,2-diolum, nec butane-2,3-diolum. Natrium nitrosum, L-lysinum, glucosaminum, et imidazolium assimilantur, at non kalium nitricum. Materia amyloidea formatur. Ureum non hydrolysatur. Non crescit in 10 % NaCl/5 % glucoso. Crescere sine vitaminis. Crescit in 30 °C, non crescit in 35 °C. Crescit in medio 1 mg cycloheximido ml⁻¹ addito. Ubiquinonum majus: Q-9. Typus: Lip 95^T (= CBS 10300^T = NRRL Y-27927^T). Isolata a terra, ex Bai San, Nha Trang, Vietnam. Depositata in collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum.

Description of *Lipomyces orientalis* Thanh sp. nov.

Lipomyces orientalis (o.ri.en.tal'is. L. masc. adj. *orientalis* from the East, referring to the fact that the species was first isolated from soil in Vietnam).

On YMA after 3 days at 25 °C, cells are ovoidal, 4–9 × 5–12 µm, and occur singly or in pairs (Fig. 3). The streak culture on malt agar is mucoid, partly hyaline, partly creamish-opaque, smooth and glistening, with an entire margin. In Dalmat plate cultures on cornmeal agar after 10 days at 25 °C, neither hyphae nor pseudohyphae are formed. In YM broth after 1 month at 25 °C, sediment is present. Asci are attached as a rule, saccate to irregularly tubular or contorted, with slowly deliquescent walls. Mature ascospores are ellipsoid, amber-coloured, warty, 2.2–2.7 × 3.2–3.9 µm, one to four per ascus, and conglomerative when liberated (Fig. 1b). Does not ferment D-glucose. Assimilates D-glucose, D-galactose, L-sorbose, D-xylose, L-arabinose (weakly or not at all), D-arabinose (weakly), L-rhamnose (maybe weakly), sucrose, maltose, α,α-trehalose (weakly), methyl α-D-glucoside, cellobiose (maybe weakly), salicin

(maybe weakly), arbutin (weakly), melibiose (maybe weakly), lactose (weakly or not at all), raffinose, melezitose, inulin, starch (weakly), erythritol, ribitol (delayed and weakly), xylitol, D-glucitol, D-mannitol, galactitol, D-glucono-1,5-lactone (weakly), 2-keto-D-gluconate, 5-keto-D-gluconate (weakly), D-gluconate (weakly), succinate (weakly), citrate (weakly) and ethanol (weakly). Does not assimilate D-glucosamine, D-ribose, glycerol, myo-inositol, D-galacturonate, DL-lactate, methanol, propane-1,2-diol or butane-2,3-diol. Assimilates nitrite, L-lysine, glucosamine and imidazole. Does not assimilate nitrate. Produces starch-like substances. Urease reaction is negative. Does not grow in YNB medium (Difco) containing 10 % NaCl and 5 % glucose. Grows in vitamin-free medium. Does not grow at 35 °C, but grows at 30 °C. Growth in medium containing 0.1 % cycloheximide is positive. The major ubiquinone is Q-9.

The type strain, Lip 95^T (= CBS 10300^T = NRRL Y-27927^T), as well as strains Lip 51 (= CBS 10301 = NRRL Y-27928), MD 66-1 (= CBS 10302 = NRRL Y-27929), MD 88-4 (= CBS 10303 = NRRL Y-27930) and MD 89-4 (= CBS 10304 = NRRL Y-27931), were isolated from uncultivated soil in Vietnam and have been deposited in the Yeast Collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, and the Agricultural Research Service Yeast Collection, US Department of Agriculture, Peoria, IL, USA.

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