Lachancea dasiensis sp. nov., an ascosporeogenous yeast isolated from soil and leaves in Taiwan

Ching-Fu Lee, Cheng-Hsu Yao, Yi-Ru Liu, Chin-Wen Hsieh and Shuh-Sen Young

Correspondence

Ching-Fu Lee

leecf@mail.nhcue.edu.tw

Department of Applied Science, National Hsinchu University of Education, 521 Nanda Road, Hsinchu 300, Taiwan, ROC

A novel yeast species, *Lachancea dasiensis* sp. nov., is proposed in this paper based on two strains isolated from leaves and one strain from soil in Taiwan. The strains produce one or two spherical ascospores in each ascus, preceded by conjugation of individual cells. Genus assignment and distinction of the species from other recognized species of *Lachancea* are based on morphological and physiological characteristics and on phylogenetic analysis of nucleotide sequences of the D1/D2 domains of the large subunit (LSU) rRNA gene. Sequence analysis of the D1/D2 domains of the LSU rRNA gene reveals that the phylogenetically closest relatives of *L. dasiensis* sp. nov. are *Lachancea thermotolerans* and *Lachancea waltii*. The novel species could be differentiated from the latter two species based on electrophoretic karyotypes. The type strain of *Lachancea dasiensis* sp. nov. is SC5L02^T (=CBS 10888^T =BCRC 23139^T), which was isolated from a leaf of *Angiopteris lygodiiifolia* Rosenst. in Dasi, Taoyuan, Taiwan.

The species *Zygosaccharomyces cidri*, *Zygosaccharomyces fermentati*, *Kluyveromyces thermotolerans*, *Kluyveromyces waltii* and *Saccharomyces kluyveri* were clustered into an independent clade on the basis on combined analysis of multiple gene sequences (Kurtzman, 2003; Kurtzman & Robnett, 2003), indicating that they are closely related to each other, but to differing extents. A new genus, *Lachancea*, was proposed to accommodate these five species, and *Lachancea thermotolerans* was assigned as the type species (Kurtzman, 2003). Since the genus was described, only one further species has been reported, *Lachancea meyersii* (Fell *et al.*, 2004). The genus is characterized by the fermentation of at least one other sugar in addition to glucose, the production of one to four spherical ascospores after conjugation and the inability to assimilate nitrate.

Species of the genus *Lachancea* are cosmopolitan and inhabit many niches, including soil, plants, animals and processed foods or beverages (Kurtzman, 1998; Vaughan-Martini & Martini, 1998; Lachance, 1998; Fell *et al.*, 2004; Barnett *et al.*, 2000). The type strains of the six species were isolated from different habitats, such as processed foods or

beverages (Legakis, 1961; Naganishi, 1928; Filipov, 1932), holly exudates (Kodama & Kyono, 1974), *Drosophila pinicola* (Phaff & Knapp, 1956) and mangrove habitats (Fell *et al.*, 2004). During a survey of yeast diversity in soil and leaves in Taiwan, three novel yeast strains were isolated; these strains share identical sequences of the D1/D2 domain of the large-subunit (LSU) rRNA gene, the internal transcribed spacer (ITS) region and the small-subunit (SSU) rRNA gene and similar physiological characteristics, indicating that they are conspecific. The present paper proposes a novel species to accommodate these three strains, which are closely related to *Lachancea waltii* and *Lachancea thermotolerans*.

Strains SC5L02^T and SC6L01 were isolated from leaves of the fern *Angiopteris lygodiiifolia* Rosenst. and the palm *Arenga engleri* Beccari, respectively, in Dasi, Taoyuan, in 2006, while strain ES12S06 was isolated from a forest soil sample that was collected in Sinyi, Nantou, in 2007. Isolation of these strains was achieved by the method described by Lee *et al.* (2008). The yeasts were grown on YM agar at 24 °C for 3 days, followed by preservation in the freezer at –70 °C and/or on YM agar at 4 °C. The morphological, physiological and biochemical characteristics of the strains were determined by the methods described by Yarrow (1998). Strains used for examination of ascospores were incubated on McClary's acetate agar (Yarrow, 1998) for 28 days at 18 °C.

The D1/D2 domain of the LSU rRNA gene, the SSU rRNA gene and ITS fragments of strains SC5L02, SC6L01 and ES12S06 were amplified using primers NL1 and NL4

Abbreviations: ITS, internal transcribed spacer; LSU, large subunit; SSU, small subunit.

The GenBank/EMBL/DBJ accession numbers for the sequences of the D1/D2 domain of the LSU rRNA genes, ITS regions and SSU rRNA genes of strains SC5L02^T, SC6L01 and ES12S06 are respectively EU523636, EU547812 and FJ153149 (D1/D2), FJ153187, FJ153188 and FJ153189 (ITS) and FJ153104, FJ153105 and FJ153106 (SSU).

(Kurtzman & Robnett, 1998), P1 and NS8 (Suzuki & Nakase, 2002) and ITS1 and ITS4 (White *et al.*, 1990), respectively, with a Peltier thermal cycler (PTC-200; MJ Research) from genomic DNA extracted from yeast cells using a Biokit Genome DNA Extraction kit (Biokit Co.). Successful amplification was confirmed by agarose gel electrophoresis. Sequencing of the fragments was performed by using an automated DNA sequencer (Applied Biosystems 3730 DNA Analysis System). Both strands were sequenced, and the reactions were carried out using a Dye Terminator cycle sequencing kit (Applied Biosystems). The sequences were initially aligned using the multiple alignment program CLUSTAL_X 1.83 (Thompson *et al.*, 1997). A phylogenetic tree was constructed using the neighbour-joining method with the MEGA version 4.0 software package (Kumar *et al.*, 2004). *Kluyveromyces marxianus* CBS 834^T was used as an out-group. Bootstrap analysis was performed from 1000 boot-strap replications (Felsenstein, 1985).

To examine the electrophoretic karyotypes of the yeasts by PFGE, agarose plugs containing intact chromosomal DNA were prepared using the methods of Carle & Olson (1985) modified as follows. The examined strains were grown on YM agar slants at 25 °C for 24 h. The cells were then harvested and washed twice with 0.05 M EDTA (pH 7.5) and used to prepare intact yeast chromosomal DNA for PFGE. The washed cell pellets (about 150 mg) were resuspended in 300 µl SCE buffer [1 M sorbitol, 0.1 M trisodium citrate (pH 5.8) and 0.02 M EDTA (pH 7.5)] and mixed with 40 µl lyticase (Sigma) solution (1000 U ml⁻¹ in SCE). After the mixture was incubated at 37 °C for 30 min, it was mixed with 1 ml 1.2 % molten agarose (Amresco) in 125 mM EDTA (pH 7.5). The suspension was pipetted into moulds and allowed to solidify at 4 °C for 15 min, and the solidified plugs were then transferred into 2 ml LET buffer (0.45 M EDTA, pH 8.0, 10 mM Tris/HCl, pH 8.0, 7.5 % 2-mercaptoethanol) and incubated overnight at 37 °C. After being washed in 50 mM EDTA (pH 8.0), the plugs were incubated in 2 ml LEP solution [0.45 M EDTA, pH 8.0, 1 % *N*-lauroylsarcosine, 10 mM Tris/HCl, pH 8.0, 1 mg proteinase K ml⁻¹ (Merck)] and incubated at 50 °C for 24 h. The immersion solution was then decanted and the plugs were washed with three changes of 50 mM EDTA at pH 8.0 over a 24 h period at 50 °C. The washed agarose plugs were stored in 50 mM EDTA (pH 8.0) at 4 °C. Agarose blocks were cut to fit the wells of a 1 % agarose gel (Amresco) and the plug pieces were inserted and sealed in place with 0.8 % agarose. Gels were equilibrated in 0.5 × TBE (45 mM Tris/borate, 1 mM EDTA, pH 8.0) for 1 h before electrophoresis. Chromosomal DNA bands were separated on 1.0 % agarose gel in 0.5 × TBE buffer in a rotating-field electrophoresis apparatus (Rotaphor 6.0; Biometra). The electrophoresis program was completed in three steps with a total run time of 96 h: step 1, field switch time of 90–120 s for 8 h at 120 V; step 2, 120–360 s for 28 h at 120 V; step 3, 360–1200 s for 60 h at 60 V. The temperature of the running buffer was maintained at 14 °C throughout the

electrophoresis. After electrophoresis, the gels were stained with 0.5 µg ethidium bromide ml⁻¹ for 30 min and destained with tap water for 30 min and then photographed under UV illumination. Intact chromosomal DNAs of *Saccharomyces cerevisiae* YPH 80 purchased from Biometra were used as DNA markers.

Sequence comparison

Reference sequences used in this paper were retrieved from GenBank under the accession numbers indicated in Fig. 1. Strains SC5L02^T, SC6L01 and ES12S06 were found to share identical sequences in the D1/D2 domain of the LSU rRNA gene, the ITS and the SSU rRNA gene, indicating that these strains are conspecific. In the neighbour-joining phylogenetic tree based on the D1/D2 domains of the LSU rRNA gene (Fig. 1), the three strains clustered with *L. thermotolerans* and *L. waltii*, confirming that the novel species is a member of *Lachancea*. The three strains differed from their phylogenetically closest relatives, the type strains of *L. thermotolerans* and *L. waltii*, by 9 and 11 nucleotide substitutions in the D1/D2 domains of the LSU rRNA gene (Fig. 1), 26 in the ITS region and 8 and 9 in the SSU rRNA gene, respectively. These results clearly indicate that these strains are representatives of a novel species of *Lachancea*.

Electrophoretic karyotyping

Previous studies have shown that all species of *Lachancea* appear to have an identical number of chromosomes (eight) and they show species-specific patterns of electrophoretic karyotypes according to the range of chromosome sizes (Naumova *et al.*, 2007). In the present paper, only seven chromosomal DNA bands were resolved for strains SC6L02 and ES12S06 under the conditions described above; the band with a stronger relative intensity may correspond to a doublet (Fig. 2, lanes 4–6). Eight chromosomes were assumed to be present in these strains. Strains SC5L02^T, SC6L01 and ES12S06 were found to be quite similar in their electrophoretic karyotypes, and the patterns were different from those of the type strains of *L. thermotolerans* and *L. waltii* (Fig. 2). The results described above support the conclusion that strains SC5L02^T, SC6L01 and ES12S06 represent a novel species closely related to *L. thermotolerans* and *L. waltii*.

Taxonomy

Strains SC5L02^T, SC6L01 and ES12S06 exhibited similar physiological and morphological characteristics. They reproduce asexually by multilateral budding and sexually by producing one or two spherical ascospores per ascus after conjugation between individual cells (Fig. 3). The morphology of these strains does not allow them to be differentiated easily from other recognized species, whereas these strains can be distinguished from other *Lachancea* species in fermentation, assimilation reactions and cycloheximide resistance (Table 1). These strains can be

Fig. 1. Neighbour-joining phylogenetic tree, based on the sequence of the D1/D2 domain of the LSU rRNA gene, showing the relationships of strains of *Lachancea dasiensis* sp. nov. and related species of the *Lachancea* clade. *Kluyveromyces marxianus* CBS 834^T was used as an outgroup. Bootstrap values of 50% or above are given at branch nodes and are based on 1000 replicates. All taxa are represented by type strains. Bar, 0.01 substitutions per nucleotide position.

differentiated from *L. thermotolerans* in cycloheximide resistance and from *L. waltii* in the fermentation of D-galactose and maltose and assimilation of many carbon compounds (Table 1). Based on the evidence described above, a novel species, *Lachancea dasiensis* sp. nov., is proposed to accommodate these strains.

Latin diagnosis of *Lachancea dasiensis* Lee sp. nov.

In medio liquido cum glucoso et peptono et extracto levidinis post dies 3 ad 25 °C, cellulae ovoideae vel ellipsoideae (2.7–5.2 × 3.0–6.3 µm), singulae aut binae, per gemmationem reproducentes. Post 1 mensem sedimentum formatur. Cultura in agarum cum glucoso et peptono et extracto levidinis post dies 7 ad 25 °C, crenea, butyrosa, glabra et nitida. In agarum farinae Zea mays post dies 10 ad 25 °C, mycelium et

pseudomycelium nulla. Asci formantur ex conjugatione inter cellulas. Asci continentes 1–2 ascoporas sphaericae (2.9–3.2 µm). Glucosum, galactosum, maltosum, methyl α-D-glucosidum, sucrosum, melibiosum (infirmum), melezitotum et raffinotum fermentantur, at non α,α-trehalosum, lactosum, cellobiosum, inulinum, amyllum, nec D-xylosum. Glucosum, galactosum, sucrosum, maltosum, α,α-trehalosum (infirmum), methyl α-D-glucosidum, melibiosum, raffinotum, melezitotum, D-glucitolum, D-mannitolum, et D-glucono-1,5-lactotum assimilantur, at non L-sorbotum, D-glucosaminum, D-

Fig. 2. Electrophoretic karyotypes of strains of *L. dasiensis* sp. nov. and related strains. Lanes: 1, *Saccharomyces cerevisiae* YPH80; 2, *L. thermotolerans* NRRL Y-8284^T; 3, *L. waltii* BCRC 22066^T; 4, *L. dasiensis* SC5L02^T; 5, *L. dasiensis* SC6L01; 6, *L. dasiensis* ES12S06.

Fig. 3. Morphology of *L. dasiensis* SC5L02^T as determined by light microscopy. (a) Budding cells of the strain in YM broth for 3 days at 25 °C. (b-d) Ascospores produced on McClary's acetate agar after 28 days at 18 °C. Bars, 10 µm.

Table 1. Comparison of phenotypic characteristics of *L. dasiensis* sp. nov. and other recognized species of the genus

Species: 1, *L. dasiensis* sp. nov. (data from this study); 2, *L. thermotolerans* (data from Lachance, 1998); 3, *L. waltii* (Lachance, 1998); 4, *L. meyersii* (Fell *et al.*, 2004); 5, *L. cidri* (Vaughan-Martini & Martini, 1998); 6, *L. fermentati* (Vaughan-Martini & Martini, 1998); 7, *L. kluyveri* (Kurtzman, 1998). +, Positive; –, negative; w, weak; v, variable; D, delayed growth; s, slow growth.

Characteristic	1	2	3	4	5	6	7
Fermentation of:							
D-Galactose	+	v	–	–	+	+	+
Maltose	+	v	–	v	+	+	–
α,α -Trehalose	–	s	–	w	+	+	–
Melibiose	w	–	–	–	+	v	+
Assimilation of:							
D-Galactose	+	+	–	–	+	+	+
L-Sorbose	–	v	+	–	s	+	v
Maltose	+	+	–	+	+	+	+
α,α -Trehalose	w	+	–	+	+	+	+
Methyl α -D-glucoside	+	+	–	+	+	+	+
Melibiose	+	v	–	–	+	v	+
Melezitose	+	+	–	+	–	+	v
Inulin	–	v	–	v	+	v	–
Glycerol	–	v	–	D	+	v	v
2-Keto-D-gluconate	–	v	–	+	+	+	–
DL-Lactate	–	–	–	–	+	+	+
Succinate	–	v	–	–	+	+	–
Ethanol	–	s	+	D	+	+	+
Growth with:							
0.01 % Cycloheximide	+	–	+	–	+	+	–
10 % NaCl	–	v	–	+	+	v	–

ribosum, D-xylosum, L-arabinosum, D-arabinosum, L-rhamnosum, cellobiosum, salicinum, arbutinum, lactosum, inulinum, amyllum, glycerolum, erythritolum, ribitolum, xylitolum, L-arabinitolum, galactitolum, myo-inositolum, acidum 2-keto-D-gluconicum, acidum 5-keto-D-gluconicum, acidum D-gluconicum, acidum D-glucuronicum, acidum D-galacturonicum, acidum DL-lacticum, acidum succinicum, acidum citricum, methanolum, ethanolum, propane-1,2-diolum, butane-2,3-diolum nec N-acetylglucosaminum. L-Lysinum et ethylaminum assimilatur, at non nitrosum, nitricum, creatinum, nec cadaverinum. Ad crescentiam vitamina externa necessaria sunt. Crescere potest in 50 % glucosum et 0.1 % cycloheximido. Non crescit in substrato 10 % sal/5 % glucosum continente. Non crescit in 60 % glucosum addito. In 30 °C crescere potest at non in 35 °C. Materia amyloidea iodophila non formatur. Ureum non hydrolysat. Diazonium caeruleum B est negativum.

Typus stirpis SC5L02^T (=CBS 10888^T =BCRC 23139^T) isolatus ex folio, Dasi, Taoyuan, Taiwan. Bioresources Collection and Research Center (BCRC), Food Industry Research and Development Institute, Taiwan, et Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands, deposita est.

Description of *Lachancea dasiensis* Lee sp. nov.

Lachancea dasiensis (da.si.en'sis. N.L. fem. adj. *dasiensis* pertaining to Dasi, Taoyuan, Taiwan, from where the yeast was originally isolated).

After growth in YM broth at 25 °C for 3 days, cells are ovoidal to ellipsoidal, 2.7–5.2 × 3.0–6.3 µm, and occur singly or in pairs (Fig. 3a). Vegetative reproduction is by multilateral budding. Sediment is present. After 7 days at 25 °C on YM agar, streak cultures are creamy, butyrous, smooth and glistening. On Dalmat plate cultures on cornmeal agar after 10 days at 25 °C, neither pseudomycelium nor true mycelium is formed under the cover glass. Sporulation occurs on McClary's acetate agar and V8 agar, but not on YM agar, malt agar or cornmeal agar after incubation at 18 °C for 28 days. Asci may result from conjugation between independent cells (Fig. 3b). Ascospores are spheroidal (2.9–3.2 µm in diameter) with one or two per ascus (Fig. 3c, d). Glucose, galactose, maltose, methyl α -D-glucoside, sucrose, melibiose (weak), melezitose and raffinose are fermented, but α,α -trehalose, lactose, cellobiose, inulin, starch and D-xylose are not fermented. Glucose, galactose, sucrose, maltose, α,α -trehalose (weak), methyl α -D-glucoside, melibiose, raffinose, melezitose, D-glucitol, D-mannitol and D-glucono-1,5-lactone are assimilated; L-sorbose, D-glucosamine, D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, cellobiose, salicin, arbutin, lactose, inulin, starch, glycerol, erythritol, ribitol, xylitol, L-arabinitol, galactitol, myo-inositol, 2-keto-D-gluconate, 5-keto-D-gluconate, D-glucuronate, D-glucuronic acid, DL-lactate, succinate, citrate, methanol, ethanol, propane-1,2-diol, butane-1,2-diol and N-acetylglucosamine are not assimilated. Ethylamine and L-lysine are assimilated; nitrate, nitrite, creatine and cadaverine are not assimilated. Growth in vitamin-free medium is negative. Growth on 50 % glucose is positive. Growth on 60 % glucose and on 10 % NaCl plus 5 % glucose is negative. Growth occurs in the presence of 0.1 % cycloheximide. Growth occurs at 30 °C but not at 35 °C. No starch-like substance is produced. Acid production on chalk agar is negative. Urease hydrolysis and diazonium blue B reaction are negative.

The type strain, strain SC5L02^T (=CBS 10888^T =BCRC 23139^T), was isolated from a leaf of *Angiopteris lygodiifolia* Rosenst. in Dasi, Taoyuan, Taiwan, in 2006.

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References

- Barnett, J. A., Payne, R. W. & Yarrow, D. (2000). *Yeasts: Characteristics and Identification*, 3rd edn. Cambridge: Cambridge University Press.
- Carle, G. F. & Olson, M. V. (1985). An electrophoretic karyotype for yeast. *Proc Natl Acad Sci U S A* **82**, 3756–3760.

- Fell, J. W., Statzell-Tallman, A. & Kurtzman, C. P. (2004).** *Lachancea meyersii* sp. nov., an ascosporogenous yeast from mangrove regions in the Bahama Islands. *Stud Mycol* **50**, 359–363.
- Felsenstein, J. (1985).** Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Filipov, G. S. (1932).** Porca fruktovyh konservov, vyzvannaja drozhevym gribkom. *Tr Gent Nauchnoissled Bohim Inst Pisc Vkus Prom Narkomsnaba Sojuza SSR* **2**, 26–32 (in Russian).
- Kodama, K. & Kyono, T. (1974).** Ascosporogenous yeasts isolated from tree exudates in Japan. *J Ferment Technol* **52**, 1–9.
- Kumar, S., Tamura, K. & Nei, M. (2004).** MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Kurtzman, C. P. (1998).** *Zygosaccharomyces* Barker. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 424–436. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Kurtzman, C. P. (2003).** Phylogenetic circumscription of *Saccharomyces*, *Kluyveromyces* and other members of the Saccharomycetaceae, and the proposal of the new genera *Lachancea*, *Nakaseomyces*, *Naumovia*, *Vanderwaltozyma* and *Zygorulasporea*. *FEMS Yeast Res* **4**, 233–245.
- 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.
- Kurtzman, C. P. & Robnett, C. J. (2003).** Phylogenetic relationships among yeasts of the ‘*Saccharomyces* complex’ determined from multigene sequence analyses. *FEMS Yeast Res* **3**, 417–432.
- Lachance, M. A. (1998).** *Kluyveromyces* van der Walt emend. van der Walt. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 227–247. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Lee, C. F., Liu, C. H., Young, S. S. & Chang, K. S. (2008).** *Kazachstania jiaenicus* sp. nov., an ascomycetous yeast species isolated from soil in Taiwan. *FEMS Yeast Res* **8**, 114–118.
- Legakis, Ph. A. (1961).** *A contribution to the study of the yeast flora of apples and apple wine*. Thesis, University of Athens, Greece.
- Naganishi, H. (1928).** The study of five new species of yeast. *J Zymol* **6**, 1–12.
- Naumova, E. S., Serpova, E. V. & Naumov, G. I. (2007).** Molecular systematics of *Lachancea* yeasts. *Biochemistry (Mosc)* **72**, 1356–1362.
- Phaff, H. J. & Knapp, E. P. (1956).** The taxonomy of yeasts found in exudates of certain trees and other natural breeding sites of some species of *Drosophila*. *Antonie van Leeuwenhoek* **22**, 117–130.
- Suzuki, M. & Nakase, T. (2002).** A phylogenetic study of ubiquinone-7 species of the genus *Candida* based on 18S ribosomal DNA sequence divergence. *J Gen Appl Microbiol* **48**, 55–65.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Vaughan-Martini, A. & Martini, A. (1998).** *Saccharomyces* Meyen ex Rees. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 358–371. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990).** Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a Guide for Methods and Applications*, pp. 315–322. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. New York: Academic Press.
- 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.