Pseudozyma pruni sp. nov., a novel ustilaginomycetous anamorphic fungus from flowers in Taiwan

Guey-Yuh Liou, Yu-Hui Wei, Shie-Jea Lin, Chiou-Yen Wen and Fwu-Ling Lee

Correspondence

Fwu-Ling Lee
fll@firdi.org.tw

Bioresource Collection and Research Center (BCRC), Food Industry Research and Development Institute (FIRDI), PO Box 246, Hsinchu 30062, Taiwan, ROC

Two ustilaginomycetous anamorphic strains were isolated from flowers in Taiwan. Phylogenetic analysis based on the combined rRNA gene sequence of internal transcribed spacer 1 (ITS1)–5.8S–ITS2 and large-subunit D1/D2 domains indicated that the closest recognized species was *Pseudozyma fusiformata*. The results of DNA–DNA hybridization and physiological characteristics showed that the two strains represent a novel species within the genus *Pseudozyma*. The name *Pseudozyma pruni* sp. nov. is proposed, with FIRDI 005^T (=BCRC 34227^T =CBS 10937^T) as the type strain.

Most species of the genus *Ustilago* parasitize monocotyledonous hosts and are economically important fungi. A number of anamorphs of *Ustilago* have been classified into diverse genera (Boekhout *et al.*, 1998). These anamorphic species have been reclassified into the genus *Pseudozyma* Bandoni emend. Boekhout based on morphological, physiological and biochemical data and partial large-subunit (LSU) rRNA gene sequence analysis (Boekhout, 1995). *Pseudozyma* species are isolated most frequently from plant materials. Seven species were listed in the genus *Pseudozyma* by Boekhout & Fell (1998). More recently, six species were described by Sugita *et al.* (2003), Wang *et al.* (2006), Golubev *et al.* (2007) and Seo *et al.* (2007). During an investigation of yeast-like fungi in Taiwan, a novel species of the genus *Pseudozyma* was detected through rRNA gene sequencing of the internal transcribed spacer 1 (ITS1)–5.8S–ITS2 region and the large-subunit (LSU) D1/D2 domains, and confirmed by DNA–DNA hybridization.

Strains FIRDI 005^T (=BCRC 34227^T =CBS 10937^T) and 167b (=BCRC 34379) were isolated from flowers in Taiwan by using a method described previously (Wei *et al.*, 2005). Morphological and physiological characteristics were examined by using the methods described by

Yarrow (1998). Nuclear DNA that was intended for use in PCR was extracted by using a DNeasy Plant Mini kit (Qiagen).

The ITS region and LSU D1/D2 domains of the rRNA gene were defined by using primers ITS5/ITS4 (White *et al.*, 1990) and NL1/NL4 (O'Donnell, 1992). The reaction was performed in a GeneAmp PCR system 9700 (Applied Biosystems), using 30 cycles of the following steps: denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min following cycling. Sequencing reactions were performed by employing an ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing kit as directed by the manufacturer. Finally, the PCR products were sequenced by using an ABI PRISM 3730 DNA Analyzer.

Sequences of each strain were aligned by using CLUSTAL_X 1.83 (Thompson *et al.*, 1997). Ambiguously aligned regions were excluded from the phylogenetic analyses. Phylogenetic evaluation was performed with the PHYLIP 3.63 package (Felsenstein, 2004). Related teleomorphs were selected based on the study by Wang *et al.* (2006). Phylogenetic trees were constructed by using the neighbour-joining and maximum-parsimony methods. Confidence values for the phylogenetic trees were estimated from bootstrap analyses with 1000 replicates. The trees were viewed by using TreeView 1.6.6 (Page, 1996).

Isolation and purification of DNA for determination of the DNA base composition (expressed as DNA G + C content, mol%) and for determination of the levels of DNA relatedness were performed with a Genomic DNA Buffer Set and Genomic-tip 500/G (Qiagen). The purified DNA

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

The GenBank/EMBL/DDBJ accession numbers for the rRNA gene sequences of the ITS1–5.8S–ITS2 and LSU D1/D2 domains of *Pseudozyma pruni* FIRDI 005^T are EU379942 and EU379943, respectively.

A maximum-parsimony phylogenetic tree based on the combined sequences of the rRNA gene ITS region and LSU D1/D2 domain of the novel species and related species, and a table showing the results of DNA–DNA hybridization, are available as supplementary material with the online version of this paper.

was hydrolysed to nucleosides as described by Tamaoka & Komagata (1984) and the hydrolysate was analysed by reverse-phase HPLC to determine the DNA base composition (mol% G+C). DNA–DNA relatedness values were determined by the photobiotin-labelling microplate method (Lee *et al.*, 1993, 1998). Hybridizations were performed at 47 °C for 24 h.

The rRNA gene ITS region and the LSU D1/D2 domain sequences of strains FIRDI 005^T and 167b were identical. The maximum-parsimony tree (see Supplementary Fig. S1, available in IJSEM Online) has the same topology as the neighbour-joining tree (Fig. 1). Phylogenetic analyses revealed that strain FIRDI 005^T belongs to the genus *Pseudozyma* and is located in the anamorphic clade of *Ustilago sensu stricto* (Stoll *et al.*, 2005; Wang *et al.*, 2006). The closest recognized species was *Pseudozyma fusiformata*, with strong bootstrap support based on combined alignments of the ITS region and the LSU D1/D2 domain of the

rRNA gene. In the LSU D1/D2 domain, strain FIRDI 005^T exhibited <2% divergence from seven species, namely *Pseudozyma flocculosa* (0.9%), *Pseudozyma graminicola* (1.4%), *P. fusiformata* (1.8%), *Pseudozyma shanxiensis* (1.8%), *Pseudozyma prolifica* (1.8%), *Pseudozyma rugulosa* (1.8%) and *Pseudozyma aphidis* (1.8%). In the ITS region, the sequence of strain FIRDI 005^T differed by 11.3% from that of *P. fusiformata*, whereas the differences in the sequences between strain FIRDI 005^T and other members of the genus *Pseudozyma* occurred over >14% of the sequence. The sequence difference of the ITS region within a single species is <1% in some species of the genus *Trichosporon* (Sugita *et al.*, 1999) and in *Pseudozyma antarctica* (Sugita *et al.*, 2003; Wei *et al.*, 2005). The sequence analyses support the distinction between strain FIRDI 005^T and the recognized species in the genus *Pseudozyma*.

Strain FIRDI 005^T differed from strain 167b in terms of colony morphology and physiological characteristics.

Fig. 1. Neighbour-joining phylogenetic tree of members of the genus *Pseudozyma* and related teleomorphs, based on the combined sequences of the ITS and LSU rRNA gene D1/D2 domain. The phylogenetic tree was rooted with *Cintractia axicola* and *Trichocintractia utriculicola* (Fell *et al.*, 2000). GenBank accession numbers for the sequences used in the analysis are given in parentheses (ITS/LSU rRNA gene D1/D2 domain; single accession numbers indicate sequences that cover both regions). Numbers on branches indicate the confidence level from 1000 bootstrap replicates. Only bootstrap values >50% are indicated. Bar, 0.1 substitutions per site.

Colonies of strain FIRDI 005^T were pale orange and friable, whereas colonies of strain 167b were white and velutinous. In terms of physiological characteristics, strain FIRDI 005^T differed from strain 167b in its ability to assimilate ribitol. Strain FIRDI 005^T differed from *P. fusiformata* in the assimilation of galactose, lactose and soluble starch and its growth at 37 °C. Strain FIRDI 005^T differed from *P. flocculosa* in the assimilation of lactose and ethanol and its growth at 37 °C (Table 1). Furthermore, the DNA G+C content of strain FIRDI 005^T was 56.2 mol%, very close to that of *P. fusiformata* (56.1 mol%). To confirm the relationship of strain FIRDI 005^T to *P. fusiformata* and *P. flocculosa*, a DNA–DNA hybridization experiment was performed (see Supplementary Table S1, available in IJSEM Online). Low DNA–DNA hybridization values (13–27 %) were observed between strain FIRDI 005^T, *P. fusiformata* BCRC 33869^T (=CBS 423.96^T) and *P. flocculosa* BCRC 33999^T (=CBS 167.88^T). Therefore, strain FIRDI 005^T is considered to represent a novel species of the genus *Pseudozyma*. This study indicated that the ITS region was a better molecular marker than the LSU D1/D2 domain for the identification of the genus *Pseudozyma* at the species level.

Latin diagnosis of *Pseudozyma pruni* G.-Y. Liou, Y.-H. Wei & F.-L. Lee sp. nov.

*In agar*o malti post dies 5 ad 20 °C, cellulae vegetativae ellipsoideae, cylindratae aut fusiformae, 1.1–3.5 × 3.1–10.0 µm. Pseudomycelia formantur. Fermentatio nulla. Assimilantur glucosum, galactosum, L-sorbosum (infirmum), D-ribosum, D-xylosum, L-arabinosum, D-arabinosum (lente), L-rhamnosum (infirmum), sucrosus, maltosus, trehalosus, α-methyl-D-glucosidus, cellobiosus, salicinum, arbutinum, melibiosus, lactosus, raffinosis, melezitosis, inulinum (infirmum), amyllum solubile, glycerolum, erythritolum, ribitolum (variabile), xylitolum (lente), L-arabinitolum (lente), D-glucitolum, D-mannitolum, inositolum, D-glucono-δ-lactonum, 2-ketogluconatum (infirmum), D-gluconatum (infirmum), D-glucuronatum, galacturonatum (infirmum), DL-lactatum (infirmum), succinatum, citratum (infirmum), ethanolum, propanum 1,2 diolum, butanum 2,3 diolum (infirmum), quina-tum, kalium nitricum, natrium nitrosus, ethylaminum (infirmum), L-lysinum, cadaverinum (infirmum), creatinum (infirmum), creatininum (infirmum), D-glucosaminum, imidazolium (infirmum), ad crescentiam vitaminum non necessarium est, crescit in medio 0.01 % cycloheximidio addito. Non assimilantur galactitolum, methanolum, saccharatum,

Table 1. Physiological characteristics that differentiate species of the genus *Pseudozyma*

Taxa: 1, *P. pruni* sp. nov. FIRDI 005^T and 167b; 2, *P. antarctica* CBS 214.83^T and CBS 516.83; 3, *P. aphidis* CBS 517.83^T; 4, *P. flocculosa* CBS 167.88^T; 5, *P. fusiformata* CBS 6951^T; 6, *P. graminicola* VKM Y-2938^T; 7, *P. hubeiensis* WS 6.4^T; 8, *P. jejuensis* OL71^T; 9, *P. parantarctica* DMST 15422^T; 10, *P. prolifica* CBS 319.87^T; 11, *P. rugulosa* CBS 170.88^T; 12, *P. shanxiensis* SH 64^T; 13, *P. thailandica* DMST 15423^T; 14, *P. tsukubaensis* CBS 6389^T. The data for *P. pruni* sp. nov. were from this study; the remaining data were taken from Boekhout & Fell (1998), Golubev *et al.* (2007), Seo *et al.* (2007), Sugita *et al.* (2003) and Wang *et al.* (2006). +, Positive; –, negative; L, latent; s, slow positive; w, weakly positive; v, variable.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Assimilation of:														
Galactose	+	+	+	s	–	s	+	s	+	s	+	+	+	s
D-Arabinose	s	+	+	s	+	–	+	–	+	+	+	v	–	L
L-Rhamnose	w	v	+	–	–	–	–	–	+	–	+	–	–	–
Methyl α-D-glucoside	+	+	+	+	+	–	+	–	+	+	+	+	+	+
Cellobiose	+	+	s	s	s	w	+	L	+	+	s	–	+	+
Melibiose	+	–	+	s	v	+	+	–	+	–	s	+	+	–
Lactose	+	+	+	–	–	s	+	+	+	+	–	+	–	+
Soluble starch	+	+	+	s	–	s	w	+	+	+	+	+, w	+	+
Erythritol	+	+	+	+	s	w	+	s	s	+	s	–	+	+
D-Glucitol	+	+	+	+	+	s	+	+	+	+	+	+, w	+	–
Inositol	+	+	+	+	+	+	–	–	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	s	+	+	+	+	+	+, w	+	–
Ethanol	+	+	–	–	+	+	–	–	–	+	+	+	+	+
Saccharate	–	–	+	–	–	–	–*	–	+	–	+	+	–	–
Ethylamine	w	+	+	–	+	–	+	–	–	+	+	+	–	+
Lysine	+	+	+	+	+	+	+	+	+	+	+	+	+	–
Cadaverine	w	+	+	+	+	+	+	+	+	+	+	+	–	+
Growth:														
Without vitamins	+	–	–	+	+	+	+	+	w	+	–	+	–	s
At 37 °C	+	v	+	–	–	–	w	+	+	–	+	+	+	+
At 40 °C	–	–	–	–	–	–	–	–	–	–	–	+	–	–

*Data from this study.

Fig. 2. *Pseudozyma pruni* FIRDI 005^T. (a) Blastoconidia in 5% malt extract broth at 20 °C for 5 days; (b) blastoconidia in slide culture on 5% malt extract agar at 20 °C for 5 days. Bars, 10 µm.

D-galactonatum. In medio 0.1% cycloheximidio addito non crescit. In medio 50% glucosum continente non crescit. Augmentum fiunt in temperatura 37 °C. Urea finditur. Amylum non formantur. Diazonium caeruleum B positivum. Proportio molaris guanini + cytosine in acido deoxyribonucleico: 56.2 mol% (per HPLC).

Typus: isolatus ex flos Prunus mume Sieb. & Zucc. ad Tianwei, Changhua, Taiwan, FIRDI 005^T (=BCRC 34227^T =CBS 10937^T), depositus in BCRC, FIRDI, Hsinchu, Taiwan.

Description of *Pseudozyma pruni* G.-Y. Liou, Y.-H. Wei & F.-L. Lee sp. nov.

Pseudozyma pruni (pru'ni. L. n. *prunus* a plum tree, also a botanical genus name; L. gen. n. *pruni* of *Prunus*, from which the type strain was isolated).

After 5 days growth on 5% malt extract agar at 20 °C, colonies are smooth to irregularly wrinkled, somewhat friable or velutinous, pale orange or white and have entire or somewhat eroded margins. Cells are ellipsoidal, cylindrical or fusiform and variable in size (1.1–3.5 × 3.1–10.0 µm), some have denticles up to 4.2 µm, they contain oil droplets (Fig. 2) and pseudohyphae are present. Fermentation is negative. The following compounds are assimilated: glucose, galactose, L-sorbose (weak), D-ribose, D-xylose, L-arabinose, D-arabinose (slow), L-rhamnose (weak), sucrose, maltose, trehalose, methyl α-D-glucoside, cellobiose, salicin, arbutin, melibiose, lactose, raffinose, melezitose, inulin (weak), soluble starch, glycerol, erythritol, ribitol (variable), xylitol (slow), L-arabinitol (slow), D-glucitol, D-mannitol, inositol, D-glucono-δ-lactone, 2-ketogluconate (weak), D-gluconate (weak), D-glucuronate, D-galacturonate (weak), DL-lactate (weak), succinate, citrate (weak), ethanol, propane 1,2 diol, butane 2,3 diol (weak), quinate, potassium nitrate, sodium nitrite, ethylamine (weak), L-lysine, cadaverine (weak), creatine (weak), creatinine (weak), D-glucosamine, imidazole (weak) and 0.01% cycloheximide. Vitamins are not necessary for growth. Galactitol, methanol, saccharate, D-galactonate and 0.1% cycloheximide are not assimilated. No growth is observed in the presence of 50% glucose. Maximum growth temperature is 37 °C. Urease activity is positive. Starch-like substance is not produced. Diazonium blue B reaction is positive. G+C content of nuclear DNA: 56.2 mol% (by HPLC).

The type strain, FIRDI 005^T (=BCRC 34227^T =CBS 10937^T), was isolated from flowers of *Prunus mume* Sieb. & Zucc. that were collected at Tianwei, Changhua, Taiwan, on 17 April 1998.

Acknowledgements

This work was supported by a grant (contract no. 96-EC-17-A-17-R7-0525) from the Ministry of Economic Affairs in Taiwan. We thank Drs T.-Y. Liu, C.-C. Liao and G.-F. Yuan of FIRDI for their encouragement and revision of the manuscript. Additionally, we thank Dr L.-L. Liaw, FIRDI, for her assistance with DNA sequencing.

References

- Boekhout, T. (1995). *Pseudozyma* Bandoni emend. Boekhout, a genus for yeast-like anamorphs of *Ustilaginales*. *J Gen Appl Microbiol* **41**, 359–366.
- Boekhout, T. & Fell, J. W. (1998). *Pseudozyma* Bandoni emend. Boekhout and a comparison with the yeast state of *Ustilago maydis* (De Candolle) Corda. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 790–797. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Boekhout, T., Bandoni, R. J., Fell, J. W. & Kwon-Chung, K. J. (1998). Discussion of teleomorphic and anamorphic genera of heterobasidiomycetous yeasts. In *The Yeasts, a Taxonomic Study*, 4th edn, pp. 609–625. Edited by C. P. Kurtzman & J. W. Fell. Amsterdam: Elsevier.
- Fell, J. W., Boekhout, T., Fonseca, A., Scorzett, G. & Statzell-Tallman, A. (2000). Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int J Syst Evol Microbiol* **50**, 1351–1371.
- Felsenstein, J. (2004). PHYLIP (phylogenetic inference package), version 3.6. Distributed by the author. Department of Genome Sciences and Department of Biology, University of Washington, Seattle, WA, USA.
- Golubev, W., Sugita, T. & Golubev, N. (2007). An ustilaginomycetous yeast, *Pseudozyma graminicola* sp. nov., isolated from the leaves of pasture plants. *Mycoscience* **48**, 29–33.
- Lee, F.-L., Lee, C.-F., Okada, S., Uchimura, T., Komagata, K. & Kozaki, M. (1993). *Candida galacta* comb. nov., a new combination for *Candida apis* var. *galacta*. *Int J Syst Bacteriol* **43**, 183–184.
- Lee, F.-L., Fu, H.-M. & Hsu, W.-H. (1998). DNA hybridization and electrophoretic study of some *Candida* species. *Int J Syst Bacteriol* **48**, 1463–1466.
- O'Donnell, K. (1992). Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). *Curr Genet* **22**, 213–220.

- Page, R. D. M. (1996).** TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.
- Seo, H.-S., Um, H.-J., Min, J., Rhee, S.-K., Cho, T.-J., Kim, Y.-H. & Lee, J. (2007).** *Pseudozyma jejuensis* sp. nov., a novel cutinolytic ustilaginomycetous yeast species that is able to degrade plastic waste. *FEMS Yeast Res* **7**, 1035–1045.
- Stoll, M., Begerow, D. & Oberwinkler, F. (2005).** Molecular phylogeny of *Ustilago*, *Sporisorium*, and related taxa based on combined analysis of rDNA sequences. *Mycol Res* **109**, 342–356.
- Sugita, T., Nishikawa, A., Ikeda, R. & Shinoda, T. (1999).** Identification of medically relevant *Trichosporon* species based on sequences of internal transcribed spacer regions and construction of a database for *Trichosporon* identification. *J Clin Microbiol* **37**, 1985–1993.
- Sugita, T., Takashima, M., Poonwan, N., Mekha, N., Malaithao, K., Thungmuthasawat, B., Prasarn, S., Luangsook, P. & Kudo, T. (2003).** The first isolation of ustilaginomycetous anamorphic yeasts, *Pseudozyma* species, from patients' blood and a description of two new species: *P. parantarctica* and *P. thailandica*. *Microbiol Immunol* **47**, 183–190.
- Tamaoka, J. & Komagata, K. (1984).** Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- 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.
- Wang, Q.-M., Jia, J.-H. & Bai, F.-Y. (2006).** *Pseudozyma hubeiensis* sp. nov. and *Pseudozyma shanxiensis* sp. nov., novel ustilaginomycetous anamorphic yeast species from plant leaves. *Int J Syst Evol Microbiol* **56**, 289–293.
- Wei, Y.-H., Lee, F.-L., Hsu, W.-H., Chen, S.-R., Chen, C.-C., Wen, C.-Y., Lin, S.-J., Chu, W.-S., Yuan, G.-F. & Liou, G.-Y. (2005).** *Pseudozyma antarctica* in Taiwan: a description based on morphological, physiological and molecular characteristics. *Bot Bull Acad Sinica (Taiwan)* **46**, 223–229.
- 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 to Methods and Applications*, pp. 315–322. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego: 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.