Full Paper

***Pichia porticicola* sp. nov., a novel ascomycetous yeast related to *Pichia acaciae* isolated from galleries of ambrosia beetles in Japan**

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Eleven strains of yeasts, isolated from galleries of ambrosia beetles in Japan, formerly identified as *Pichia acaciae* were found to have different sequences in the D1/D2 domain of the large subunit (LSU) rRNA gene. After detailed taxonomic studies including a DNA–DNA reassociation experiment, 11 strains were found to represent a novel species of the genus *Pichia*. It is described as *Pichia porticicola* sp. nov. (type strain NBRC 100302^T = CBS 11715^T). The eleven strains were isolated from various samples associated with different kinds of insects and trees which were collected in 11 prefectures, from the north to the south of Japan. This species might be a common species in galleries of ambrosia beetles in Japan.

Key Words—ambrosia beetles; novel yeast; *Pichia porticicola* sp. nov.

Introduction

Insect frass has been studied more than 75 years as a source of yeasts. It is now well known that this substrate is a good habitat of yeasts and many novel species of yeasts have been found in this kind of substrate (Phaff and Stamer, 1987). Banno et al. (1983) carried out extensive studies on ascomycetous yeasts isolated from galleries of ambrosia beetles in Japan. They isolated 385 yeast strains from 162 samples collected at 84 places in Japan, from Hokkaido in the north to Yaku Island in the south, between 1973 and 1977. One hundred and eighty-eight strains out of 385 produced ascospores and were identified as belonging to 18 species of the genera *Debaryomyces*, *Hansenula*, *Hormoascus*, *Kluyveromyces*, and *Pichia*. The strains have

been maintained in the Institute for Fermentation, Osaka (IFO), and but some of them were transferred to NITE Biological Resource Center (NBRC). Recently, as part of the quality control of yeast strains maintained in NBRC based on the sequence analysis of the LSU rRNA gene, two of them were described as a novel species of the genus *Ogataea* (Nakase et al., 2008), and some of the strains were found to represent several novel species related to *Pichia acaciae* (Van der Walt, 1966). In the present study, we show the taxonomic characterizations of 11 described strains as a novel species of the genus *Pichia*, *Pichia porticicola* sp. nov.

Materials and Methods

Strains examined. Eleven strains maintained at NBRC were used in the present study. The source and collection locality of the strains are shown in Table 1.

Examination of morphological and physiological characteristics. The morphology of vegetative cells grown in YPD broth for 3 days at 25°C was examined under a light microscope AX-70 (Olympus). Formation

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Table 1. The source and locality of strains are shown.

Strain No.	Source	Collection locality	DNA databank Accession No. D1/D2 domain of LSU rRNA gene	G+C content (mol%)	% Relatedness binding with labeled DNA from		
					NBRC 100302 ^T	NBRC 1681 ^T	NBRC 103638 ^T
<i>Pichia porticiicola</i>							
NBRC 100302 ^T	Gallery of <i>Indocryphtalus pubipennis</i> in <i>Quercus acutissima</i>	Wakayama Pref., Japan	AB427168	40.0	100	35	59
NBRC 100303	Gallery of <i>Xyleborus</i> sp. in <i>Machilus thunbergii</i>	Kagoshima Pref., Japan	AB427169	40.3	100	35	ND
NBRC 100304	Gallery of <i>Scolytoplatypus mikado</i> in <i>Fagus crenata</i>	Gifu Pref., Japan	AB427170	40.0	95	35	ND
NBRC 100305	Gallery of <i>Taenioglyphus</i> sp. in <i>Mallostus japonicus</i>	Miyazaki Pref., Japan	AB427171	40.0	86	30	ND
NBRC 100306	Gallery of <i>Scolytoplatypus mikado</i> in <i>Magnolia obovata</i>	Akita Pref., Japan	AB427172	40.0	89	35	ND
NBRC 100307	Gallery of <i>Xyleborus seiryorensis</i> in <i>Quercus</i> sp.	Kouchi Pref., Japan	AB427173	39.3	86	31	ND
NBRC 100308	Gallery of <i>Scolytoplatypus shogun</i> in <i>Machilus thunbergii</i>	Fukuoka Pref., Japan	AB427174	39.8	83	31	ND
NBRC 100309	Gallery of <i>Crossotarsus niponicus</i> in <i>Fagus crenata</i>	Kyoto Pref., Japan	AB427175	39.8	88	29	ND
NBRC 100310	Gallery of <i>Xyleborus</i> sp. in <i>Castanopsis cuspidata</i>	Ooita Pref., Japan	AB427176	40.0	86	32	ND
NBRC 100311	Gallery of <i>Xyleborus saxeseni</i> in <i>Alnus hirsuta</i>	Niigata Pref., Japan	AB427177	39.5	91	30	ND
NBRC 100312	Gallery of <i>Xyleborus</i> sp. in <i>Zelkova serrata</i>	Tottori Pref., Japan	AB427178	40.1	86	32	ND
<i>Pichia acaciae</i>							
NBRC 1681 ^T	Frass of <i>Buprestidae</i> larvae infesting <i>Acacia nilotica</i> var. <i>kraussiana</i>	South Africa	AB354212	41.2	35	100	56
<i>Pichia koratensis</i>							
NBRC 103638 ^T	Insect frass	Nakhonratchasima Prov., Thailand	DQ404484	40.7	55	45	100

DNA relatedness among *Pichia porticiicola* strains and type strains of *Pichia acaciae* and *Pichia koratensis*.

of pseudomycelium was investigated by the Dalmau plate technique on corn meal agar. YM agar and corn meal agar were used for the observation of ascospores. After confirmation of sufficient production of ascospores using a light microscope, the ascosporeogenous cells were treated with Zymolyase 100T (Seikagaku Kogyo Co.) solution (1 mg/ml) for 2 h at 37°C to digest the ascus wall, and washed twice with 0.1 M phosphate buffer (pH 7.2) by centrifugation. The free ascospores were fixed and dehydrated by the method described by Mikata and Nakase (1997). The preparations were examined under a scanning electron microscope JSM-6060 (JEOL, Ltd.) at a voltage of 15 kV. Physiological and cultural properties were examined according to the methods described in *The Yeasts, A Taxonomic Study*, 4th ed. (Yarrow, 1998).

Ubiquinone system. Characterization of the ubiquinone system was performed by the modified method of Mikata and Yamada (1999). Cells were grown in 1,000 ml Erlenmeyer flasks containing 400 ml of YPD broth on a rotary shaker at 120 rpm and 28°C and were harvested by centrifugation in the early stationary growth phase. The cells were washed three times with distilled water and freeze-dried. One gram of freeze-

dried cells was stirred for 60 min in a mixture of chloroform and methanol (2 : 1) overnight, and then cells were removed by filtration. The filtrate was evaporated to dryness. The residue was dissolved in 1 ml of acetone and was concentrated with nitrogen gas. The concentrate was dissolved in 0.3 ml of absolute ethanol and samples were analyzed by an LC-MS 8000 α (Shimadzu) equipped with an STR ODS-II column (150 $\times$ 2.0 mm) (Shinwa Chemical Industries, Ltd.).

DNA base composition and DNA–DNA hybridization. Yeast strains were inoculated in 200 ml Erlenmeyer flasks containing 50 ml of YPD broth on a rotary shaker at 100 rpm and 25°C overnight. Cells were harvested in the logarithmic growth phase and washed two times with 50 mM EDTA buffer (pH 7.5). The DNA was extracted and purified according to the method of Holm et al. (1986) as modified by Kaneko and Banno (1991). The DNA base composition was determined by HPLC as described by Tamaoka and Komagata (1984) using a DNA-GC kit (Yamasa Shoyu Co., Ltd., Chiba, Japan). DNA–DNA hybridization was performed by the photobiotin microplate-hybridization of Kaneko and Banno (1991). Hybridization was performed at 40°C in 2 $\times$ SSC buffer containing 50% (v/v) formamide

Fig. 1. Neighbor-joining tree for *Pichia porticicola* and related strains based on the D1/D2 domain sequences of the LSU rRNA gene.

The numerals indicate the bootstrap values of 1,000 trials. Sequences were retrieved from the DDBJ database under the accession number indicated.

on an immunoplate (Nunc).

Sequencing of the D1/D2 domain of LSU rRNA gene and phylogenetic analysis. Genomic DNA was prepared using Dr. GentLE for Yeast (TaKaRa) according to the manufacturer's protocol. The D1/D2 domains of the LSU rRNA gene were amplified using primers NL-1 (5'-GCA TAT CAA GCG GAG GAA AAG-3') and NL-4 (5'-GGT CCG TGT TTG AAG ACG G-3'). The amplification was carried out in a reaction mixture containing TaKaRa Ex Taq (TaKaRa) by a Gene Amp PCR System 9700 (Applied Biosystems). PCR was performed according to the method previously described by Kurtzman and Robnett (1998). The sequences were determined using a BigDye terminator ver. 3.1 cycle sequencing kit (Applied Biosystems) and using an ABI 3130xl Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocol. The sequences determined in this study were deposited in the DDBJ database (Table 1). A phylogenetic tree was constructed from the evolutionary distance of Kimura (1980) by the neighbor-joining method (Saitou and Nei, 1987) using CLUSTAL X ver. 1.82 software (Thompson et al., 1997). Bootstrap values were calculated according to Felsenstein (1985) from 1,000 random trials. Reference sequences used for the phylogenetic study were obtained from the database and are shown in Fig.1.

Results and Discussion

In the phylogenetic tree based on the D1/D2 domain sequences of the LSU rRNA gene, all 11 strains had identical sequences and formed a cluster with *Pichia acaciae* and *Pichia koratensis* with high bootstrap support (Fig. 1), but in the nucleotide substitution the 11 strains differed by 6 nucleotides (1%) from *P. acaciae* NBRC 1681^T and by 5 nucleotides (1%) from *P. koratensis* NBRC 103638^T.

In the DNA-DNA hybridization experiment, the 11 strains showed low homology values of 29–59% with *P. acaciae* NBRC 1681^T and *P. koratensis* NBRC 103638^T. On the other hand, high homology values of 83–100% were demonstrated among the 11 strains, indicating that these strains belong to the same species.

In the phenotypic characteristics, the 11 strains in Table 1 can be discriminated from *P. acaciae* by the assimilation of succinic acid and D-gluconic acid, by the fermentation of galactose, and by the maximum growth temperature. The eleven strains and *P. korat-*

ensis can be discriminated from each other by the assimilation of L-sorbose and sucrose, the fermentation of galactose and sucrose, and the maximum growth temperature. The eleven strains produce hat-shaped ascospores similar to *P. acaciae*, but *P. koratensis* produces drum-shaped ascospores (Nakase et al., 2007), indicating that our 11 strains differ from *P. koratensis* morphologically.

On the basis of the data reported above, we concluded that our 11 strains represent a novel species of the genus *Pichia*, which we describe as *Pichia porticicola* sp. nov. The eleven strains assigned to *P. porticicola* were isolated from 11 different samples associated with different kinds of insects and trees which were collected from 11 prefectures, from the north to the south of Japan. This species is considered to be a common species in galleries of ambrosia beetles in Japan.

Pichia porticicola Ninomiya, Mikata, Nakagiri, Nakase & Kawasaki, sp. nov.

In liquido "YM", post dies 3 ad 25°C, cellulae globosae, subovoideae vel ovoideae, 2.0–4.5 × 2.0–6.0 µm, singulae, binae aut brevi-catenatae. Pellicula et sedimentum formantur. In agar "YM", post unum mensem ad 15°C, griseoalbida vel subflava, glabra, subnitida, mollis et margine erosa. Pseudomycelium formatur.

Glucosum, galactosum (variabile), maltosum (variabile), trehalosum et amyllum solubile (variabile) fermentatur at non sucrosum, lactosum, raffinose nec inulinum. Glucosum, galactosum, maltosum, cellobiosum, trehalosum, lactosum (variabile), amyllum solubile, D-xylosum (variabile), L-arabinosum (variabile), D-ribosum (lente), D-glucosaminum (lente), N-acetyl-D-glucosaminum, ethanolum, glycerolum, erythritolum, ribitolum, D-mannitolum, D-glucitolium, α-methyl-D-glucosidum (lente), arbutinum, acidum D-gluconicum, salicinum et acidum citricum (variabile) assimilantur at non L-sorbosum, sucrosum, melibiosum, raffinose, melezitolum, inulinum, D-arbinosum, L-rhamnosum, methanolum, galactitolium, acidum DL-lacticum, acidum 2-ketogluconicum, acidum 5-ketogluconicum, acidum succinicum nec inositolum. Ethylaminum, L-lysinum et cadaverium assimilatur at non nitricum. Maxima temperatura crescentiae: 36–37°C. Ad crescentiam vitaminum necessarium sunt. Proportio molaris guanini + cytosini in acido deoxyribonucleico: 39.3–40.3 mol% (per HPLC). Ubiquinonum majus: Q-9.

Holotypus. NBRC 100302^T (= CBS 11715^T), isolatus

Fig. 2. Morphology of *Pichia porticicola* NBRC 100302^T.

A and B: Vegetative cells grown in YM broth for 3 days at 25°C, C: Pseudomycelium produced on corn meal agar after 7 days at 25°C, D to G: Ascospores produced on corn meal agar after 7 days at 25°C. Scale bars of A to F: 5 µm, G: 1 µm.

ex pulverize ligno insecto, Wakayama Pref., Japonia, in statu lyophilo in collectione culturarum in 'NITE Biological Resource Center (NBRC)', Kisarazu, Chiba, Japonia.

Growth in YM broth: After 3 days at 25°C, cells are proliferated by multilateral budding, globose, ovoidal to long ovoidal or ellipsoidal, $2.0\text{--}4.5 \times 2.0\text{--}6.0$ µm, single, in pairs or in short-chains; sometimes pseudomycelia are observed (Fig. 2B). After 1 month at 15°C, an incomplete ring, fragile pellicle and sediment are formed.

Growth on YM agar: After 1 month at 15°C, the streak culture is grayish white, smooth, dull, soft and has an entire margin.

Slide culture on corn meal agar: After 7 days at 25°C pseudomycelia are developed (Fig. 2C).

Ascospore formation: After 7 days at 25°C on corn meal agar, vegetative cells are transformed directly into persistent asci, each containing one to four hat-shaped ascospores (Fig. D to G).

Fermentation: Glucose, galactose (variable), maltose (variable), trehalose, and soluble starch (variable) are fermented, but sucrose, lactose, raffinose and inu-

lin are not.

Assimilation of carbon compounds: Glucose, galactose, maltose, cellobiose, trehalose, lactose (variable), soluble starch, D-xylose (variable), L-arabinose (variable), D-ribose (latent), D-glucosamine (latent), *N*-acetyl-D-glucosamine, ethanol, glycerol, erythritol, ribitol, D-mannitol, D-glucitol, α -methyl-D-glucoside (latent), arbutin, D-gluconic acid, salicin and citric acid (variable) are assimilated, but L-sorbose, sucrose, melibiose, raffinose, melezitose, inulin, D-arbinose, L-rhamnose, methanol, galactitol, DL-lactic acid, 2-keto-gluconic acid, 5-ketogluconic acid, succinic acid and inositol are not.

Assimilation of nitrogen compounds: Ethylamine, L-lysine and cadaverine are assimilated, but potassium nitrate is not.

Vitamin-free medium: Negative.

Growth in 10% NaCl + 5% glucose: Positive.

Maximum growth temperature: 36–37°C.

G+C content of nuclear DNA: 39.3–40.3 mol% (by HPLC).

Major ubiquinone: Q-9.

Type strain: NBRC 100302^T, isolated from the gallery of a tree, *Quercus acutissima*, infested by an insect, *Indocryphalus pubipennis*, in Wakayama Pref., is the holotype strain of this species. It is maintained at NITE Biological Resource Center (NBRC) by freezing and liquid drying. This strain was also deposited at Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands as CBS 11715^T.

Etymology: Since all strains of this species were isolated from galleries of trees bored by insects, the specific epithet “porticicola” (dweller of small gallery) was chosen.

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