Full Paper

Ogataea paradorogensis* sp. nov., a novel methylotrophic ascomycetous yeast species isolated from galleries of ambrosia beetles in Japan, with a close relation to *Pichia dorogensis

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Two yeast strains isolated from galleries of ambrosia beetles in Japan and maintained in NITE Biological Resource Center (NBRC) as *Pichia pini* were found to represent a hitherto undescribed species. This species shows close relationship to *Pichia dorogensis* by the sequence analysis of the D1/D2 domain of 26S rDNA but is clearly differentiated from it by a DNA-DNA re-association experiment. It is described as *Ogataea paradorogensis* sp. nov. The vegetative cells and asci of this species are surrounded with distinct capsules like *P. dorogensis*. One to four hat-shaped ascospores, which tend to be liberated from the asci at maturation, are formed in the ascus.

Key Words—hat-shaped ascospores; novel capsule-forming yeast; novel insect-associated yeast; novel methylotrophic yeast; *Ogataea paradorogensis* sp. nov.

Introduction

Yamada et al. (1994) proposed the genus *Ogataea* for five nitrate-assimilating methylotrophic species of *Pichia* based on the analysis of the partial 18S and 26S rRNA sequences. Later, Yamada et al. (1995) included another two species in this genus as *Ogataea wickerhamii* and *Ogataea pini*. Mikata and Yamada (1995) transferred *Pichia kodamae* to the genus *Ogataea*. However, Kurtzman (1998) did not accept this genus in the *The Yeasts, a Taxonomic Study* (4th ed.). Morais et al. (2004) accepted this genus and listed

known species of the genus with description of a novel species, *O. falcaomoraensis*. Suh et al. (2006) accepted the genus *Ogataea* as a currently recognized genus in their study on the phylogenetics of Saccharomycetales. Péter et al. (2007a) described a methylotrophic yeast *Ogataea thermophila* as the teleomorph of *Candida thermophila* (Shin et al., 2001). Limtong et al. (2008) reported two novel methylotrophic species, *Ogataea chonburiensis* and *Ogataea nakhonphanomensis*, and proposed to transfer two methylotrophic species, *Pichia siamensis* and *Pichia thermomethanolica*, to the genus *Ogataea*. Péter et al. (2007b) described *Ogataea allantospora* isolated from the phylloplane. Three nitrate negative species, *O. pini*, *O. kodamae* and *O. falcaomoraensis* were included in *Ogataea* without emendation of the diagnosis of the genus to allow the inclusion of nitrate negative species. Péter et al. (2008) emended the diagnosis of the genus to allow the inclusion of nitrate negative species and de-

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scribed an additional nitrate negative species, *Ogataea nitratoaversa*. Recently, Ji and Bai (2008) described an additional new methylotrophic yeast *Ogataea ganodermae*. As the result, 16 species are currently recognized in this genus.

Banno et al. (1983) carried out extensive isolation studies on the yeasts living in galleries of the ambrosia beetles in Japan. They isolated 385 strains from 162 samples collected from various places in Japan and identified 188 strains of ascomycetous yeasts as 15 known species and 3 undescribed species of 5 genera. Among the 15 known species, four strains were identified as *Pichia pini*. In the course of a revision of the identification of yeast strains maintained in NITE Biological Resource Center (NBRC) based on sequence analyses of ribosomal RNA genes, 2 of 4 strains formerly identified as *P. pini* by Banno et al. (1983) were found to belong to an undescribed species. After detailed taxonomic studies, we concluded that this species should be described as a novel species of *Ogataea*, which is closely related to *Pichia dorogensis* described by Péter et al. (2003), a methanol-assimilating species. *Ogataea paradorogensis* sp. nov. is proposed for this species in the present paper as the 17th species of the genus.

Materials and Methods

Yeast strains. The two strains used in the present study, NBRC 100260 and NBRC 100261, were isolated from galleries of trees invaded by insects, *Xyleborus* sp., the former from *Fagus crenata* (Japanese beech), in Akita Pref. and the latter from *Machilus thunbergii* (red nanmu), in Kagoshima Pref. of Japan, by Banno et al. (1983) and have been maintained in the Institute for Fermentation, Osaka (IFO), then in NITE Biological Resource Center (NBRC).

Examination of morphological, physiological, and biochemical characteristics. Most of the morphological, physiological, and biochemical characteristics were examined by the methods described by Yarrow (1998). The maximum growth temperature was determined in YM broth using metal block baths at 1°C intervals.

Ubiquinone system. Cells grown in YPD broth (2% glucose, 2% peptone, 1% yeast extract) on a rotary shaker at 120 rpm at 25°C for 3 days were harvested by centrifugation. The cells were washed three times with distilled water and freeze-dried. The isolation, pu-

rification and identification of ubiquinone homologues were done as described by Mikata and Yamada (1999).

Isolation and purification of nuclear DNA. Nuclear DNA was isolated and purified according to Holm et al. (1986).

DNA base composition. The DNA base composition was determined by HPLC after enzymatic digestion of DNA to deoxyribonucleosides as described by Tamaoka and Komagata (1984) using the DNA-GC Kit (Yamasa Shoyu Co., Ltd., Chiba, Japan).

DNA-DNA relatedness. DNA-DNA relatedness was examined by reassociation experiments using the photobiotin microplate-hybridization technique according to Kaneko and Banno (1991). Hybridization was performed at 40°C in 2× SSC buffer containing 50% (v/v) formamide on a microplate (Immunoplate, Nunc).

Sequencing and phylogenetic analysis. Genomic DNA was prepared using Dr. GenTLE for Yeast (TaKaRa) according to the manufacturer's protocol. The nucleotide sequences of the D1/D2 domain of 26S rDNA were directly determined according to the method reported by Kurtzman and Robnett (1998). The sequences determined in this study were deposited in the DDBJ database under the following accession numbers: NBRC 100260, D1/D2 domain of 26S rDNA (AB437092), ITS regions including 5.8S rDNA (AB437093); NBRC 100261, D1/D2 domain of 26S rDNA (AB437094), ITS regions including 5.8S rDNA (AB437095).

The sequences were aligned with those of undescribed species using CLUSTAL X ver. 1.83 (Thompson et al., 1997). The phylogenetic tree was constructed from the evolutionary distance data of Kimura (1980) using the neighbor-joining method (Saito and Nei, 1987). Sites where any gaps existed in any sequences were excluded. Bootstrap analyses (Felsenstein, 1985) were done based on 1,000 random samplings.

Results and Discussion

The two strains used in this study had identical sequences in the D1/D2 domain of the 26S rDNA and ITS regions including 5.8S rDNA. They are located in a clade including all of the species of *Ogataea* and several related *Pichia*, *Williopsis* and *Candida* in the NJ tree based on the D1/D2 sequences, and constituted a

subclade with *Pichia dorogensis* though the bootstrap value was low (Fig. 1). The two strains differed by six nucleotide substitutions (1.1%) from *P. dorogensis*, the nearest species, in the D1/D2 domain and for this reason are regarded as members of a separate species (Kurtzman and Robnett, 1998). In the DNA-DNA reassociation experiment, the two strains demonstrated high values of 100–103% with each other but low values of 6–10% were found between the type strain of *P.*

dorogensis (Table 1). This result clearly indicated that the two strains represent a single species different from *P. dorogensis*. Therefore, we conclude that the two strains formerly identified as *P. pini* (Banno et al., 1983) represent a novel species closely related to *P. dorogensis* and propose *Ogataea paradorogensis* sp. nov. for this species.

Ogataea paradorogensis resembles *P. dorogensis* not only in D1/D2 sequences but also in the pheno-

Fig. 1. Phylogenetic tree for *Ogataea paradorogensis* sp. nov. constructed by the neighbor-joining method based on the D1/D2 domain of 26S rDNA sequences.

The numerals represent the percentages from 1,000 replicate bootstrap resamplings (frequencies less than 50% are not shown). Sequences were retrieved from the DDBJ databases under the accession numbers indicated.

Table 1. DNA relatedness among *Ogataea paradorogensis* NBRC 100260 and NBRC 100261^T and the type strain of *Pichia dorogensis*.

Source of unlabeled DNA		Mol% G+C	Relative DNA binding of labeled DNA from (%)	
			NBRC 101078 ^T	NBRC 100260
<i>Ogataea paradorogensis</i> sp. nov.	NBRC 100260	41.7	6	100
<i>Ogataea paradorogensis</i> sp. nov.	NBRC 100261 ^T	41.6	10	103
<i>P. dorogensis</i>	NBRC 101078 ^T	43.6	100	9

typic properties. Morphological and physiological properties of these two species are nearly the same including good production of cellular capsules. The only clear difference was found in the growth temperature, *O. paradorogensis* grew at 35°C but not at 36°C. Whereas *P. dorogensis* was reported to grow at 40°C (Péter et al., 2003). In the present study, the type strain of this *P. dorogensis* grow at 41°C but not at 42°C. Ascospores are abundantly produced on the usual growth media for yeasts such as YM agar and YM broth but poorly on the ME sporulation medium of Wickerham (Yarrow, 1998). Asci are usually formed after mother-daughter conjugation, but also after conjugation between independent cells. Hat-shaped ascospores (1–4, usually 2–4 per ascus) are produced in the ascus (Fig. 2C, D). Ascospores are liberated from the ascus at maturation and tend to agglutinate. Some ascospores look superficially spheroidal but are in fact hat-shaped. The two strains demonstrated good growth in carbon assimilation media containing 0.5–2% methanol but latent growth was observed in media containing 3% methanol.

Kurtzman and Robnett (1998) suggested that *P. methylvora*, *P. trehalophila*, *P. methanolica*, *P. nagani-*

shii, and *Williopsis salicorniae* should be included in the genus *Ogataea* as they are members of the same clade. They indicated also that a more robust dataset would be required to substantiate the circumscription of *Ogataea* as a phylogenetically sound genus. Morais et al. (2004) stated that the recently described *P. dorogensis*, *P. trehaloabstinens*, and *P. zsoitii* undoubtedly belong in the same natural group. Péter et al. (2007b) stated that the species of the genus *Ogataea* and related methylotrophic species of *Pichia* share extensive common properties. These ascosporeogenous species should therefore be included in the same genus. As a result, *P. siamensis* and *P. thermomethanolica* were transferred to *Ogataea* by Limtong et al. (2008) but other species, including *P. dorogensis*, not described as members of the genus *Ogataea*.

Pichia paradorogensis Nakase, Ninomiya, Kawasaki & Limtong, sp. nov.

In liquido “YM,” post dies 3 ad 25°C, cellulae globosae vel subovoideae, 3.5–10 × 3.5–10 µm, singulae, binae, brevi-catenatae vel racemosae (Fig. 2A). Sedimentum formatur. In agaro “YM,” post unum mensem ad 15°C, brunneoli-cinerea vel flavidi-cinerea, glabra,

Fig. 2. Morphology of vegetative cells of *Ogataea paradorogensis* sp. nov.

A: Vegetative cells of NBRC 100261^T grown in YM broth for 3 days at 25°C. B: Capsules produced around vegetative cells and an ascus of NBRC 100260 after 8 days' incubation in YM agar at 25°C (stained with Indian ink). C, D: Asci and ascospores of NBRC 100261^T produced on YM agar after 6 days' incubation at 25°C. Scale bars = 10 µm.

nitida, mollis vel mucosa, margine integer. Cellulae et asci circum cum capsulae sunt (Fig. 2B). Pseudomycelium non formatur. Asci formantur post conjugationem. Ascospores pileiformes, 1–4 in asco, $2.5\text{--}3.5 \times 3.5\text{--}6.5 \mu\text{m}$ (Fig. 2C, D). Fermentatio nulla. Glucosum, L-sorboseum (fortasse lente), cellobiosum, trehalosum, D-xylosum, L-arabinosum (lente vel nullum), D-arabinosum (lente vel nullum), D-ribosum, methanolum, ethanolum, glycerolum, erythritolum, ribitolum (fortasse lente), D-mannitolum, D-glucitolum, xylitolum, L-arabinitolum, arbutinum, salicinum, glucono- δ -lactonum (lente vel nullum), acidum D-gluconicum (lente vel lente & exiguum) et acidum citricum (lente vel lente et exiguum) assimilantur at non galactosum, sucrosum, maltosum, lactosum, melibiosum, raffinoseum, melezitoseum, inulinum, amyllum solubile, L-rhamnosum, D-glucosaminum, *N*-acetyl-D-glucosaminum, galactitolum, methyl- α -glucosidum, acidum 2-ketogluconinum, acidum 5-ketogluconicum, acidum DL-lacticum, acidum succinicum, acidum D-glucuronicum, acidum D-galacturonicum, inositolum, propanum-1,2-diolum, butanum-2,3-diolum nec hexadecanum. Kalium nitricum non assimilatur. Maxima temperatura crescentiae: $35\text{--}36^\circ\text{C}$. Ad crescentiam biotinum et thiaminum necessaria sunt. Proportio molaris guanini + cytosini in acido deoxyribonucleico: 41.6–41.7 mol% (per HPLC). Ubiquinonum majus: Q-7.

Holotypus. NBRC 100261^T (=CBS 10978^T) in statu lyophilo, cultura viva ex ligno pulvere ab insecto efferenti, Kagoshima Pref., Japonia isolata et in collectione culturarum in 'NITE Biological Resource Center (NBRC),' Kisarazu, Chiba, Japonia, conservatus.

Growth in YM broth: After 2 or 3 days at 25°C , cells are spheroidal to short ovoidal, occur singly, in pairs, in short chains or in small clusters, often bear several buds, $3.5\text{--}10 \times 3.5\text{--}10 \mu\text{m}$ (Fig. 2A). Sediment is produced. After 1 month at 15°C , an incomplete ring and sediment are present.

Growth on YM agar: After 1 month at 15°C , the streak culture is pale brownish-grey to pale yellowish-grey, smooth, shiny, soft to mucous and has an entire margin. Vegetative cells and asci from YM agar after several days' incubation are surrounded with capsules (Fig. 2B).

Slide culture on corn meal agar: Mycelium and pseudomycelium are not produced.

Ascospore formation: The ascus is usually produced after mother-daughter conjugation, but also after conjugation between independent cells. Hat-

shaped ascospores (1–4, usually 2–4 per ascus) are produced in the ascus, $2.5\text{--}3.5 \times 3.5\text{--}6.5 \mu\text{m}$ (Fig. 2C, D). In some cases, ascospores may appear spheroidal. At maturation, ascospores are liberated from the ascus and tend to agglutinate.

Fermentation: Absent.

Assimilation of carbon compounds:

Glucose	+
Galactose	–
L-Sorbose	+ (may be latent)
Sucrose	–
Maltose	–
Cellobiose	+
Trehalose	+
Lactose	–
Melibiose	–
Raffinose	–
Melezitose	–
Inulin	–
Soluble starch	–
D-Xylose	+
L-Arabinose	+ (latent) or –
D-Arabinose	+ (latent) or –
D-Ribose	+
L-Rhamnose	–
D-Glucosamine	–
<i>N</i> -Acetyl-D-glucosamine	–
Methanol	+
Ethanol	+
Glycerol	+
Erythritol	+
Ribitol	+ (may be latent)
Galactitol	–
D-Mannitol	+
D-Glucitol	+
Xylitol	+
L-Arabinitol	+
Methyl- α -D-glucoside	–
Arbutin	+
Salicin	+
Glucono- δ -lactone	+ (latent) or –
D-Gluconic acid	+
	(latent or latent & weak)
2-Ketogluconic acid	–
5-Ketogluconic acid	–
DL-Lactic acid	–
Succinic acid	–
Citric acid	+
	(latent or latent & weak)

D-Glucuronic acid	—
D-Galacturonic acid	—
Inositol	—
Propane-1,2-diol	—
Butane-2,3-diol	—
Hexadecane	—

Assimilation of nitrogen compounds:

Potassium nitrate	—
Sodium nitrite	—
Ethylamine	+
L-Lysine	+
Cadaverine	+

Vitamins required: Biotin and thiamine.

Production of starch-like substances: Negative.

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

0.1% cycloheximide resistance: Positive (latent & weak).

Maximum growth temperature: 35–36°C.

Liquefaction of gelatin: Negative.

Acid production on chalk agar: Negative.

Diazonium blue B color reaction: Negative.

Urease: Negative.

G+C content of nuclear DNA: 41.6–41.7 mol% (by HPLC).

Major ubiquinone: Q-7.

Type strain: NBRC 100261^T is chosen as the type strain of this species because ascospore formation is better than NBRC 100260. It was isolated from galleries of a tree, *Machilus thunbergii*, invaded by an insect, *Xyleborus* sp., collected in Kagoshima Pref., Japan. This strain was also deposited at Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands as CBS 10978^T.

Etymology: The specific epithet “*paradorogensis*” was chosen since this species is closely related to *Pichia dorogensis*.

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