Pichia myanmarensis sp. nov., a novel cation-tolerant yeast isolated from palm sugar in Myanmar

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Four halotolerant yeast strains, M21^T, M34-1, HS054 and D41, were isolated from various foods in South-East Asia. These isolates were most closely related to *Pichia anomala*, with which each strain had from zero to two differences in the 26S rDNA D1/D2 domain nucleotide sequence; for this reason, they were thought to be the same as, or sister species of, *P. anomala*. Of the four yeast isolates, only one strain, M21^T, had an 18S rDNA sequence that differed from those of *P. anomala* IFO 10213^T and the other three isolates, having 20 substitutions and two gaps. Strain M21^T showed lower cation (Li⁺) tolerance (≤ 0.3 M LiCl) than *P. anomala* IFO 10213^T or the other three strains (≤ 0.5 M LiCl). Furthermore, the DNA–DNA hybridization data indicated that M21^T was clearly distinct from *P. anomala* IFO 10213^T and the other three isolates. The ability of strain M21^T to assimilate D-arabinose distinguished it from *P. anomala* IFO 10213^T and the other three isolates; it also differed in that it was able to grow at 37 and 40 °C. Strain M21^T grew by multilateral budding, produced persistent asci, in which between one and four hat-shaped ascospores were formed, and contained ubiquinone Q-7. On the basis of this polyphasic characterization, strain M21^T represents a novel species within the Q-7-containing group of the genus *Pichia*, for which the name *Pichia myanmarensis* is proposed. The type strain is M21^T (=NBRC 11090^T = JCM 12922^T = CBS 9786^T).

We have studied the taxonomic diversity of yeasts isolated from general and traditional fermented foods in South-East Asia. All of the yeast isolates were identified, on a preliminary basis, from analyses of the 26S rDNA D1/D2 domain sequences. The cation (Li⁺) tolerance of each isolate was tested using its ability to grow in YM broth supplemented with LiCl. As a result of these surveys, four isolates were discovered whose 26S rDNA D1/D2 domain sequences were similar to that of *Pichia anomala* NRRL Y-366 (GenBank accession no. U74592). Of these, one isolate, M21^T, whose 26S rDNA D1/D2 sequence differed at two positions from that of *P. anomala* NRRL Y-366, showed cation (Li⁺) tolerance (≤ 0.3 M LiCl) that was lower than those of *P. anomala* IFO 10213^T and the other three isolates (≤ 0.5 M LiCl). Accordingly, other taxonomic characteristics of M21^T were determined and the strain was shown

to represent a novel species within the Q-7-containing group of the genus *Pichia*.

In Mandalay (Myanmar) in 2000, yeast strains M21^T and M34-1 were isolated from palm sugar collected from the floor of a storage room in a rum distillery and also from sap that had oozed out onto the skins of bananas being sold at a market. The strains were isolated by enrichment culture using 20 % (w/v) YM medium at 28 °C. Strains HS054 and D41 had been collected previously in a project supported by the Special Coordination Funds of the Japan Science and Technology Agency, between 1995 and 1999. Strain HS054 came from saccharified red rice (kao mark) in Surat Thani, Thailand, and had been kept in a laboratory in Thailand. The method used for isolating strain HS054 is unknown. Strain D41 was isolated from fermented glutinous rice (tape ketan) in Indonesia. The isolation was carried out as follows. Ringer's solution, in which the isolation source had been placed and incubated for 1 day, was streaked onto a medium containing 6 g malt extract, 1.5 g yeast extract, 350 g glucose, 350 g fructose and 6 g agar in 300 g water ($a_w = 0.76$). All isolates and *P. anomala* IFO 10213^T were cultivated in YM medium at 24 °C and stored in 15 % glycerol at –80 °C. The rRNA gene sequences were analysed as described previously (Nagatsuka *et al.*,

The GenBank/EMBL/DDBJ accession numbers for the 26S rDNA D1/D2 domain sequences of *P. anomala* strains D41, HS054 and M34-1 and *P. myanmarensis* M21^T are AB126675–AB126678. The accession numbers for the 18S rDNA sequences of *P. anomala* strains IFO 10213^T, D41, HS054 and M34-1 and *P. myanmarensis* M21^T are AB126679–AB126683.

Differential characteristics for strains of *P. myanmarensis* and *P. anomala* are available in a supplementary table in IJSEM Online.

2002; Yamada *et al.*, 1999). Primers NL-1 and NL-4 (O'Donnell, 1993) were used to synthesize and sequence the D1/D2 domain fragments of the 26S rDNA. Primers P1 and P2 were used to synthesize the 18S rDNA fragments, while primers P1–P8 (Yamada *et al.*, 1999) were used to sequence the 18S rDNA. Phylogenetic trees were constructed on the basis of an alignment of 1642 nt 18S rDNA (1692 nt determined), excluding gaps, uncertain positions within the sequence retrieved from the database (designated n) and ambiguously aligned regions in considering the secondary structure of 18S rRNA (Gutell, 1993); nucleotide positions 131–135, 191–194, 231–234, 276–280, 491–496, 662–666, 678–682, 709–719, 1055–1064, 1354–1372 and 1490–1491, according to the *Saccharomyces cerevisiae* numbering system (James *et al.*, 1997) (Fig. 1), were excluded from the analysis. Ascospores were prepared for scanning electron microscopy (model JSM-5400; JEOL) using the method employed by Mikata & Nakase (1997). Physiological tests were performed using the standard protocols described by Yarrow (1998). The effect of osmotic pressure on growth was determined by a strain's ability to grow at high sugar concentrations (50 or 60 %, w/w, glucose; Yarrow, 1998). Cation tolerance was determined by a strain's ability to grow in YM broth (pH 6.2) supplemented with NaCl or LiCl at various concentrations, as previously described (Nagatsuka *et al.*, 2002). The quinone systems were determined by using HPLC (Yamada *et al.*, 1973; Kuraishi *et al.*, 1985). Nuclear DNA for determination of the DNA G+C content and for DNA–DNA hybridization was extracted and purified, using a modified version of the method of Marmur (1961), from cells treated with Zymolyase 20T (Seikagaku). Nuclear DNA was purified by equilibrium ultracentrifugation in CsCl/ethidium-bromide gradients according to the method of Hamamoto & Nakase (1995), using an ultracentrifuge (Hitachi CS-210) at 400 000 g for 5 h. The DNA G+C content was determined according to the HPLC method of Tamaoka & Komagata (1984). DNA–DNA hybridization and detection of biotinylated DNA were carried out as described previously (Nagatsuka *et al.*, 2002). Hybridization for isolate

M21^T was performed at 44 °C in a 2 × SSC buffer containing 50 % (v/v) formamide on an immunoplate (Nunc).

We determined 573 nt of the 26S rDNA D1/D2 domain sequence of each of the four isolates (M21^T, M34-1, HS054 and D41). The sequence of strain D41 was identical to that of *P. anomala* NRRL Y-366, whereas isolates M21^T, M34-1 and HS054 differed from *P. anomala* NRRL Y-366 at two, one and one position(s) of the 573 nt, respectively. Analysis of the 26S rDNA D1/D2 sequence therefore suggested that these four isolates belong to *P. anomala* or a closely related species, on the basis of the generalizations of Kurtzman & Robnett (1998). The 18S rDNA sequences of three of the isolates, M34-1, HS054 and D41, were identical to that of *P. anomala* IFO 10213^T, but the sequence of M21^T differed from the others by 20 substitutions and two gaps. The positions of the 20 substitutions were clustered and not scattered. Specifically, 10 of the substitutions were located in the V9 variable region (De Rijk *et al.*, 1992), while four others were located in the loop connecting the E21-2 and E21-5 helices in the V4 region. Isolate M21^T formed a well-supported (99 %) cluster with *P. anomala*, *Candida silvicultrix* and *Pichia sydowiorum* within a clade containing Q-7-containing species of *Pichia* and *Williopsis* (Fig. 1). These levels of sequence similarity show that isolate M21^T is clearly divergent from *C. silvicultrix* and *P. sydowiorum*.

We compared the cation tolerance and osmotolerance of the four isolates and *P. anomala* IFO 10213^T. All strains grew in 10 % NaCl plus 5 % glucose and 0, 1 and 2 M NaCl broth but not in 3 M NaCl. *P. anomala* IFO 10213^T and three of the isolates, M34-1, HS054 and D41, grew in broth containing 0.5 M LiCl and 50 % glucose agar, but isolate M21^T did not. M21^T clearly differed from *P. anomala* and the other three isolates in terms of its tolerance of cation (Li⁺) toxicity or osmotic pressure. The DNA relatedness values for the five strains (Table 1) demonstrated that M21^T is distinct from *P. anomala* IFO 10213^T, and that the other three isolates, M34-1, HS054 and D41, are members of *P. anomala*. The DNA G+C contents of M21^T and of *P. anomala*, including strains M34-1, HS054 and D41, were

Fig. 1. Phylogenetic positions of *Pichia myanmarensis* sp. nov., based on the 18S rDNA sequence. The phylogenetic tree was constructed by the neighbour-joining method, based on 1642 nt, using Kimura's two-parameter distance correction. Numerals indicate bootstrap values, expressed as percentages of 1000 samples. Bar, distance corresponding to 1 nt change per 100 nt positions.

Table 1. Levels of DNA relatedness for *P. anomala* and *P. myanmarensis*

Strain	Relative binding (%) with labelled DNA from:				
	IFO 10213 ^T	D41	HS054	M34-1	M21 ^T
<i>P. anomala</i> IFO 10213 ^T	100	93.6	113.1	100	38.9
<i>P. anomala</i> D41	74.5	100	94.8	97.0	33.7
<i>P. anomala</i> HS054	78.2	92.3	100	100.5	30.6
<i>P. anomala</i> M34-1	72.3	93.2	90.1	100	29.5
<i>P. myanmarensis</i> M21 ^T	25.9	27.2	24.6	30.5	100

32.2–33.2 and 33.6–34.8 mol%, respectively. In the other physiological and biochemical tests, isolate M21^T showed features similar to those of *P. anomala* IFO 10213^T, including the other three isolates, but differed in terms of D-arabinose assimilation and growth at 37 and 40 °C. In all isolates, the major ubiquinone was Q-7, as in *P. anomala*. In M21^T, persistent asci arose from diploid cells, with each ascus forming between one and four hat-shaped ascospores on ME agar (Fig. 2), cornmeal, acetate and YM agar after incubation at 24 °C for 3 days. Thus, on the basis of the phenotypic, phylogenetic and genomic distinctiveness of strain M21^T, we conclude that this isolate should be classified as a novel species within the Q-7-containing group of the genus *Pichia*. We propose the name *Pichia myanmarensis*, with M21^T (=NBRC 11090^T=JCM 12922^T=CBS 9786^T) as the type strain.

Latin diagnosis of *Pichia myanmarensis* Nagatsuka, Kawasaki et Seki sp. nov.

In medio liquido YM post dies 3 ad 24 °C, cellulae sphaeroidae vel ovoideae (1.9–4.1 × 2.1–6.1 µm), singulae aut binae, per gemmationem multipolarem reproducentes. In medio liquido fermentatione sedimentum et pellicula tenuis formantur. In agar YM post dies 3 ad 24 °C, cultura lineariz albida vel cremea et nitida cum margine glabra et convexa sublimitas. In

agar farinae *Zea mays* post dies 7 ad 24 °C, pseudomycelium nullum. Asci formati sine conjugatione. In agar ME, farinae *Zea mays*, aceti et YM post dies 3 ad 24 °C, ascospores petasae (1.1–1.5 × 1.6–2.3 µm), 1–4 in quoque asco. Glucosum, galactosum (occulutus), sucrosum, maltosum (occulutus) et raffinose (infirme) fermentatur, sed non lactosum et trehalosum. Glucosum, galactosum, sucrosum, maltosum, cellobiosum (tarde), trehalosum, raffinose (tarde), melezitotum, amyllum solubile, D-xylosum (tarde), L-arabinosum (infirme), D-arabinosum (tarde), D-ribosum, ethanolum, glycerolum, erythritolum, ribitolum, D-mannitolum, D-glucitolum, methyl α-D-glucosidum, salicinum, acidum D-gluconicum, acidum DL-lacticum, acidum succinicum, et acidum citricum assimilantur, sed non L-sorbose, lactosum, melibiosum, inulinum, L-rhamnosum, D-glucosaminum, N-acetyl-D-glucosaminum, methanolum, galactitolum nec inositolum. Kalium nitricum, kalium nitracum, ethylaminum, L-lysinum et cadaverinum assimilantur. Crescens in medio sine vitamino. Augmentum in 37 et 40 °C (infirme). Non crescens in 0.01 et 0.1 % cycloheximido. Crescens in 10 % natrio chlorido/5 % glucoso. Non crescens in 50 % glucoso et 0.5 M litio chlorido/liquido YM. Crescens in 0.3 M litio chlorido/liquido YM. Ureum non hydrolysat. Diazonium caeruleum B non respondens. Amyllum non formatur. Systema coenzymatis Q-7 adest. Guaninum et cytosinum acidi deoxyribonucleati 32.2–33.2 mol% (per HPLC).

Typus stirpis M21^T isolatus ex phoenix saccharon, Mandalay Pref., Myanmar. In collectione zymotica Centraalbureau voor Schimmelcultures, Trajectum ad Rhenum, sub no. CBS 9786^T, typus stirps deposita est.

Description of *Pichia myanmarensis* Nagatsuka, Kawasaki & Seki sp. nov.

Pichia myanmarensis [my.an.mar.en'sis. N.L. fem. adj. *myanmarensis* pertaining to Myanmar (Burma), where the yeast was originally isolated].

Growth in YM agar: after 3 days at 24 °C, cells are spherical to ovoid (1.9–4.1 × 2.1–6.1 µm), appearing singly or in pairs. Budding is multipolar (Fig. 2a). Sediment and thin pellicles are formed on the surface of the fermentation medium. Growth on YM agar medium: after 3 days incubation at 24 °C, the streak culture is white to cream in colour, smooth and glossy; it has an entire margin and convex elevation. Dalmau plate culture on cornmeal

Fig. 2. (a) Micrograph of *P. myanmarensis* M21^T, showing vegetative cells and sporulated cells grown on ME agar for 3 days at 24 °C. Bar, 5 µm. (b) Scanning electron micrograph of an ascospore of *P. myanmarensis* M21^T. Bar, 1 µm.

agar: after 7 days incubation at 24 °C, pseudohyphae are not formed. Formation of ascospores: vegetative cells are transformed directly into persistent asci, each containing between one and four hat-shaped ascospores (1.1–1.5 × 1.6–2.3 µm) (Fig. 2). Sporulation is observed on ME, cornmeal, acetate and YM agar after incubation for 3 days at 24 °C. The phenotypic characteristics of the species are shown in a supplementary table in IJSEM Online. Glucose, galactose (latent), sucrose, maltose (latent) and raffinose (weak) are fermented, but lactose and trehalose are not. Glucose, galactose, sucrose, maltose, cellobiose (slow), trehalose, raffinose (slow), melezitose, soluble starch, D-xylose (slow), L-arabinose (weak), D-arabinose (slow), D-ribose, ethanol, glycerol, erythritol, ribitol, D-mannitol, D-glucitol, methyl α -D-glucoside, salicin, D-gluconate, DL-lactate, succinate and citrate are assimilated as sole carbon sources, whereas L-sorbose, lactose, melibiose, inulin, L-rhamnose, D-glucosamine, N-acetyl-D-glucosamine, methanol, galactitol and inositol are not assimilated. Nitrate, nitrite, ethylamine, L-lysine and cadaverine are assimilated as sole nitrogen sources. Grows in vitamin-free medium. Grows at 37 °C, and grows weakly at 40 °C. No growth in the presence of 0.01 or 0.1 % cycloheximide. Grows in 10 % NaCl plus 5 % glucose. Does not grow on 50 % (w/w) glucose/yeast-extract agar, or in 0.5 M LiCl/YM broth. Grows in 0.3 M LiCl/YM broth. Urease-negative. The diazonium blue B reaction is negative. Tests negative for the production of starch-like substances. The major ubiquinone system is Q-7. The G+C content of the nuclear DNA is 32.2–33.2 mol%, as determined by HPLC.

The type strain M21^T (=NBRC 11090^T=JCM 12922^T=CBS 9786^T) was isolated from palm sugar on the floor of a storage room in a rum distillery in Mandalay, Myanmar, in 2000.

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References

- De Rijk, P., Neefs, J.-M., Van de Peer, Y. & De Wachter, R. (1992). Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Res* **20** (Suppl.), 2075–2089.
- Gutell, R. R. (1993). Collection of small subunit (16S- and 16S-like) ribosomal RNA structures. *Nucleic Acids Res* **21**, 3051–3054.
- Hamamoto, M. & Nakase, T. (1995). Ballistosporeous yeasts found on the surface of plant materials collected in New Zealand. 1. Six new species in the genus *Sporobolomyces*. *Antonie van Leeuwenhoek* **67**, 151–171.
- James, S. A., Cai, J., Roberts, I. N. & Collins, M. D. (1997). A phylogenetic analysis of the genus *Saccharomyces* based on 18S rRNA gene sequences: description of *Saccharomyces kunashirensis* sp. nov. and *Saccharomyces martiniae* sp. nov. *Int J Syst Bacteriol* **47**, 453–460.
- Kuraishi, H., Katayama-Fujimura, Y., Sugiyama, J. & Yokoyama, T. (1985). Ubiquinone systems in fungi I. Distribution of ubiquinones in the major families of ascomycetes, basidiomycetes, and deuteromycetes, and their taxonomic implications. *Trans Mycol Soc Jpn* **26**, 383–395.
- 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.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J Mol Biol* **3**, 208–218.
- Mikata, K. & Nakase, T. (1997). Surface structure of ascospores of genus *Nadsonia* Sydow. *Microbiol Cult Coll* **13**, 97–102.
- Nagatsuka, Y., Kawasaki, H., Limtong, S., Mikata, K. & Seki, T. (2002). *Citeromyces siamensis* sp. nov., a novel halotolerant yeast isolated in Thailand. *Int J Syst Evol Microbiol* **52**, 2315–2319.
- O'Donnell, K. (1993). *Fusarium* and its near relatives. In *The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics*, pp. 225–233. Edited by D. R. Reynolds & J. W. Taylor. Wallingford, UK: CAB International.
- Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- Yamada, Y., Okada, T., Ueshima, O. & Kondō, K. (1973). Coenzyme Q system in the classification of the ascosporeogenous yeast genera *Hansenula* and *Pichia*. *J Gen Appl Microbiol* **19**, 189–208.
- Yamada, Y., Kawasaki, H., Nagatsuka, Y., Mikata, K. & Seki, T. (1999). The phylogeny of the cactophilic yeasts based on the 18S ribosomal RNA gene sequences: the proposals of *Phaffomyces antillensis* and *Starmera caribaea*, new combinations. *Biosci Biotechnol Biochem* **63**, 827–832.
- 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.