Pichia garciniae sp. nov., isolated from a rotten mangosteen fruit (*Garcinia mangostana* L., Clusiaceae)

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Ascogenous yeasts were isolated from a decaying mangosteen fruit (*Garcinia mangostana* L., Clusiaceae). Based on colony morphology and RAPD analysis, the strains were grouped into four groups (G-I, G-II, G-III and G-IV). Phenotypic properties and analysis of the D1/D2 domain sequence of the 26S rRNA gene identified representative members of groups G-II, G-III and G-IV as *Pichia manshurica* (= *Pichia galeiformis*), *Pichia fermentans* and *Pichia anomala*, respectively. Representatives of group G-I, YS110^T and YS111, showed similar phenotypic traits, 99.9 % similarity in the sequence of the D1/D2 domain of the 26S rRNA gene and ITS1–5.8S rRNA gene–ITS2 sequence and 92 % DNA–DNA relatedness. Hence, YS110^T and YS111 are regarded as members of the same species. Based on D1/D2 domain and ITS region sequences, the nearest phylogenetic neighbours of YS110^T and YS111 were identified as *Pichia membranifaciens* and *P. manshurica*. However, YS110^T differs from these two nearest phylogenetic neighbours by >3 % in the D1/D2 domain sequence and by >18 % with respect to the ITS region sequence. In addition, YS110^T and YS111 differ from *P. membranifaciens* NRRL Y-2026^T and *P. manshurica* NRRL Y-27978^T with respect to a number of phenotypic traits. The strains show 52–57 % DNA–DNA relatedness with *P. membranifaciens* NRRL Y-2026^T and *P. manshurica* NRRL Y-27978^T. Strains YS110^T and YS111 are proposed as two strains of a novel species, for which the name *Pichia garciniae* sp. nov. is proposed. The type strain is YS110^T (=NRRL Y-48422^T =CBS 10758^T).

Yeasts of the genera *Rhodotorula*, *Cryptococcus*, *Sporobolomyces*, *Saccharomyces*, *Candida* and *Pichia*, amongst others, have been isolated from fresh and rotten fruits (Fleet, 2003; Rao *et al.*, 2007; Bhadra *et al.*, 2007; Slavikova *et al.*, 2007). These fruit-associated yeasts produce extracellular enzymes such as lipases, cutinases and pectinases and thus hasten the spoilage of fruits during storage and transportation (Fleet, 2003). A number of novel yeast strains have been isolated from both healthy and rotten fruits (Péter *et al.*, 2005; Tournas & Katsoudas, 2005; Rao *et al.*, 2007; Bhadra *et al.*, 2007). During the course of a study to identify yeasts associated with a decaying mangosteen fruit (*Garcinia mangostana* L., Clusiaceae), a number of yeast strains were isolated. Detailed polyphasic taxonomic analysis indicated that the

strains are representatives of the genus *Pichia*. Based on biochemical characteristics and the sequence of the D1/D2 domain of the 26S rRNA gene and the ITS1–5.8S rRNA gene–ITS2 region, strains YS110^T and YS111 represent a novel species of the genus *Pichia*.

Mangosteen fruits (about 1 kg) were purchased in May 2007 from a market in the city of Bangkok, Thailand, and transported to Hyderabad, Andhra Pradesh, India. Most of the fruits remained unspoiled for about a week after one of them showed visible signs of rotting. A small piece from the rotten fruit (approx. 1 g) was sliced using a sterile scalpel blade and suspended in 2 ml sterile distilled water by vigorous vortexing. The resulting suspension (100 µl) was then plated on YM agar plates (1⁻¹: 5 g peptone, 3 g yeast extract, 3 g malt extract, 10 g glucose and 15 g agar) supplemented with 50 µg chloramphenicol ml⁻¹ and the plates were incubated at 28 °C for 48 h. The abundance of yeasts in the decaying mangosteen fruit was 2.0–2.2 × 10² c.f.u. g⁻¹. A total of 32 yeast colonies appeared on YM-chloramphenicol plates, which could be grouped into four morphotypes based on the colour and shape of the colonies. Three representatives of each colony morphotype were purified by repeated streaking on YM agar plates

The GenBank/EMBL/DDBJ accession numbers for the 18S rRNA gene (partial)–ITS1–5.8S rRNA gene–ITS2–26S rRNA gene (partial) sequences of strains YS110^T and YS111 are respectively AM882679 and AM905031, and those of the D1/D2 domains of the 26S rRNA genes of strains YS119, YS118 and YS117 are AM882676–AM882678, respectively.

RAPD profiles from 12 novel yeast isolates are available as supplementary material with the online version of this paper.

without the antibiotic and genomic DNA of the 12 isolates was extracted (Bhadra *et al.*, 2007) and purified (Makimura *et al.*, 1994). Following RAPD analysis using (GTG)₅ as a primer (Bhadra *et al.*, 2007), the 12 isolates could also be classified into four groups, G-I, G-II, G-III and G-IV, based on the banding pattern of the PCR products when resolved on a 1 % agarose gel (Supplementary Fig. S1, available in IJSEM Online). One or two representatives from each group, YS110^T and YS111 from G-I, YS117 from G-II, YS118 from G-III and YS119 from G-IV, were then subjected to detailed phylogenetic analysis.

For routine subculturing and maintenance, the strains were grown on/in YM agar/broth at 28 °C. Morphological, physiological and biochemical properties were determined as described by Yarrow (1998). All assimilation tests were performed twice and results were scored after both 1 and 3 weeks. An Axioplan microscope (Zeiss) was used to visualize the morphology of the vegetative cells and ascospores. Strains were grown on potato-dextrose-rose bengal agar (M938; HiMEDIA) (l⁻¹: 200 g potato infusion, 20 g glucose, 0.008 g rose bengal, 0.05 g chloramphenicol and 15 g agar) and Gorodkova agar (l⁻¹: 1 g glucose, 10 g peptone, 5 g NaCl and 18 g agar) and visualized after 3, 5, 7, 10 and 12 days for ascospore formation using a phase-contrast microscope under a ×100 oil-immersion objective. *Candida albicans* JCM 1542^T, *P. membranifaciens* NRRL Y-2026^T and *P. manshurica* NRRL Y-17978^T were used as reference strains for phenotypic studies.

Quinones were extracted and purified from cells grown in 250 ml YM broth for 3–5 days according to Yamada *et al.* (1989). The dark band of Co-Q observed under UV light was scraped and extracted with 2 ml acetone and identified using reversed-phase HPTLC (Thanh *et al.*, 2003) using Co-Q standards (Sigma-Aldrich). *Saccharomyces cerevisiae* NRRL Y-12632^T, *Pichia fermentans* NRRL Y-1619^T, *P. membranifaciens* NRRL Y-2026^T, *Debaryomyces hansenii* NRRL Y-17914^T and *Schizosaccharomyces pombe* NRRL Y-12796^T were used as reference strains for Co-Q analysis. The G+C content of the DNA was determined from melting point (*T*_m) curves (Sly *et al.*, 1986) obtained using a Lambda 2 UV-visible spectrophotometer (Perkin Elmer) equipped with Templab 2.0 software package (Perkin Elmer). DNA–DNA hybridization was performed using a DIG DNA labelling and detection kit (Roche Diagnostics) according to Bhadra *et al.* (2005).

The internal transcribed spacer (ITS) region including the 5.8S rRNA gene (ITS1–5.8S rRNA gene–ITS2) and the D1/D2 domain of 26S rRNA gene of isolates YS110^T, YS111, YS117, YS118 and YS119 were amplified using the primers ITS1 and NL4 and the amplicon was sequenced using the primers NL1, NL2A, NL3A, ITS3 and ITS4 (Lin *et al.*, 1995; Kurtzman & Robnett, 1998). Details of amplification and sequencing protocols are given elsewhere (Bhadra *et al.*, 2007). Sequences were corrected manually and aligned using CLUSTAL_X (Thompson *et al.*, 1997). Pairwise alignment studies were done using the ExPASy

Bioinformatics online software tools (<http://www.expasy.ch/tools/sim-prot.html>). Phylogenetic analyses were conducted using MEGA 3.1 (Kumar *et al.*, 2004) according to Rao *et al.* (2007). Bootstrap analysis (Felsenstein, 1985) was performed for 1000 replications. Reference sequences were retrieved from GenBank under the accession numbers indicated in the tree (Fig. 1).

Pairwise alignment analysis of the nucleotide sequences of the D1/D2 domains of the 26S rRNA genes of strains YS117, YS118 and YS119 showed 100 % similarity to those of *Pichia anomala* NRRL Y-366^T (GenBank accession no. U74592; not shown in Fig. 1), *P. fermentans* NRRL Y-1619^T and *P. manshurica* NRRL Y-17349 (the type strain of *Pichia galeiformis*), respectively. In the neighbour-joining phylogenetic tree constructed using D1/D2 sequences, YS117 grouped with *P. anomala* NRRL Y-366^T (data not shown), whereas YS118 and YS119 grouped with *P. fermentans* NRRL Y-1619^T and *P. manshurica* IFO 10726^T, respectively (Fig. 1). These strains also showed similar biochemical characteristics to their nearest phylogenetic relatives and produced ascospores after 5 days of incubation on potato-dextrose-rose bengal agar plates (not shown). Therefore, strains YS117, YS118 and YS119 are assumed to be strains of *P. anomala*, *P. manshurica* and *P. fermentans*, respectively.

The nucleotide sequences of the D1/D2 domain of the 26S rRNA gene and the ITS1–5.8S–ITS2 region of representative strains of G-I, YS110^T and YS111, were almost identical (99.9 % similarity). BLAST analysis of the nucleotide sequence of the D1/D2 domain of YS110^T indicated that the nearest phylogenetic neighbours are *P. membranifaciens* and *P. manshurica*. Strain YS110^T showed 97 % similarity with *P. manshurica* IFO 10726^T, 96.3 % similarity with *P. membranifaciens* NRRL Y-2026^T, 96.1 % similarity with *Pichia deserticola* NRRL Y-12918^T and 96 % similarity with *Candida ethanolica* NRRL Y-12615^T. The phylogenetic tree constructed using the neighbour-joining method based on D1/D2 sequences further strengthens the conclusion that YS110^T is most closely related to *P. membranifaciens*, with a bootstrap support of 99 % (Fig. 1). The nucleotide sequence of the ITS1–5.8S rRNA gene–ITS2 region of strain YS110^T shows 85.1 % similarity to that of *P. membranifaciens* CBS 209 (GenBank accession no. DQ104714) and 81.4 % similarity to that of *P. manshurica* IFO 10726^T (AB054036). Based on rRNA gene sequencing and electrophoretic karyotyping, Wu *et al.* (2006) reported that strains identified as *P. membranifaciens* could show 5–13 % variation in their ITS1–5.8S rRNA gene–ITS2 region nucleotide sequence. Therefore, a DNA–DNA relatedness study was conducted between strains YS110^T and YS111, *P. membranifaciens* NRRL Y-2026^T and *P. manshurica* NRRL Y-27978^T. Strains YS110^T and YS111 showed 92 % DNA–DNA relatedness with each other and both strains showed 52–57 % DNA–DNA relatedness with *P. membranifaciens* NRRL Y-2026^T and *P. manshurica* NRRL Y-27978^T.

Previous studies have shown that strains with >1 % substitution in the D1/D2 domain sequence usually

Fig. 1. Phylogenetic tree drawn using the neighbour-joining method with Kimura's two-parameter model based on the D1/D2 domain sequence of the 26S rRNA gene, depicting the relationships of *Pichia garciniae* sp. nov. strains YS110^T and YS111 with related species of the genera *Pichia*, *Candida* and *Issatchenkia*. *Zygosaccharomyces fermentati* NRRL Y-1559^T was used as an outgroup. Bootstrap values calculated as percentages from 1000 replications are indicated at branch nodes. Sequences that were obtained in this study are indicated in bold. Bar, 2 substitutions per 100 nucleotide positions.

represent separate species (Kurtzman & Robnett, 1997, 1998; Lu *et al.*, 2004; Suh & Blackwell, 2004). Strains YS110^T and YS111 differ by 3–3.7% in their D1/D2 sequence and 14.9–18.6% in their ITS1–5.8S rRNA gene-ITS2 region sequence from the type strains of *P. membranifaciens* and *P. manshurica*. The DNA–DNA relatedness of YS110^T and YS111 with the type strains of *P. membranifaciens* and *P. manshurica* was <70%. Therefore, based on phylogenetic analysis and DNA–DNA relatedness studies, strains YS110^T and YS111 should be regarded as strains of a novel species of the genus *Pichia*. Strains YS110^T and YS111 do not differ significantly in their phenotypic properties from strains of *P. membranifaciens* as reported by Barnett *et al.* (2000) but, when YS110^T and YS111 were compared directly in this study with the type strain of *P. membranifaciens*, NRRL Y-2026^T, differences were clearly visible (Table 1). The strains produce two to four ascospores after 3–5 days of incubation on potato-dextrose-rose bengal agar and Gorodkova agar (Fig. 2). Thus, based on phenotypic differences, phylogenetic analysis and DNA–DNA relatedness studies, strains YS110^T and YS111 represent a novel species of *Pichia*, for which the name *Pichia garciniae* sp. nov. is proposed.

Latin diagnosis of *Pichia garciniae* Bhadra et Shivaji sp. nov.

In medio agar YM post dies 3 ad 28 °C, cellulae vegetativae globosae aut oblongae (4–7 × 3.5–4 µm), cellulae singulae, binae et aggregatae. Per gemmationem multipolarem reproductes. Post 1 mensem ad 28 °C pellicula et sedimentum formantur. *In agar* YM post dies 15 ad 28 °C, colonia butyrosa, glabra. Ascospores 2–4 per ascum. D-Glucosum non-fermentatur. Assimilantur D-glucosum, D-glucosaminum (infirme), glycerolum, xylitolum et succinatum; non-assimilantur D-galactosum, L-sorbose, D-ribosum, D-xylosum, L-arabinosum, D-arabinosum, L-rhamnosum, sucrosus, maltosus, cellobiosus, salicinum, melibiosus, lactosus, raffinosis, melezitosis, amylosus, erythritolum, ribitolum, myo-inositolum, D-gluconatum, D-glucuronatum, citratum, propane-1,2-diolum et butane-2,3-diolum. Assimilantur ethylaminum, L-lysinum, cadaverinum et glucosaminum; non-assimilantur nitratum, nitritum, creatininum et imidazolium. Crescit et medio 0.01% cycloheximido addito. Ureum non-hydrolysat. Diazonium caeruleum B non-respondens. Ubiquinone majus Q7. G + C acidi deoxyribonucleati 48.5 mol%. Typus YS110^T (=NRRL Y-48422^T =CBS 10758^T), designat stirpem typicam. Isolata ex gut mangosteen fructu (*Garcinia mangostana* L., Clusiaceae), depositata in CBS, Hollandia.

Table 1. Phenotypic characteristics of strains YS110^T and YS111, *P. membranifaciens* and *P. manshurica*

Data were obtained in this study unless indicated. All strains assimilate D-glucose but not D-galactose, L-sorbose, D-ribose, D-xylose, L-arabinose, D-arabinose, L-rhamnose, sucrose, maltose, cellobiose, salicin, melibiose, lactose, raffinose, melezitose, starch, erythritol, ribitol, *myo*-inositol, D-gluconate, D-glucuronate, propane-1,2-diol or butane-2,3-diol as sole carbon sources. All strains assimilate ethylamine, L-lysine, cadaverine and glucosamine but not nitrate, nitrite, creatinine or imidazole as sole nitrogen sources. All strains are sensitive to 0.1 % cycloheximide and do not grow in medium containing 1 % (v/v) methanol. None of the strains form starch-like compounds or hydrolyse urea and all strains give a negative diazonium blue B reaction. +, Positive; w, weakly positive, D, variable; –, negative.

Property	Strains YS110 ^T and YS111	<i>P. membranifaciens</i>		<i>P. manshurica</i> NRRL Y-27978 ^T
		NRRL Y-2026 ^T	Barnett <i>et al.</i> (2000)	
Fermentation of D-glucose	–	D	D, –	–
Assimilation of:				
D-Glucosamine	w	w	+, –	–
Inulin	–	–	–	w
Glycerol	+	+	+, –	–
Xylitol	+	–	+, –	–
Succinate	+	D	+, –	–
Citrate	–	–	+, –	–
Growth in:				
Acetic acid (1 %)	–	–	+, –	–
Cycloheximide (0.01 %)	+	–	D, –	–
D-Glucose (50 %)	–	w	+, –	–

Description of *Pichia garciniae* Bhadra & Shivaji sp. nov.

Pichia garciniae (gar.ci'ni.ae. N.L. gen. fem. n. *garciniae* of *Garcinia*, a botanical genus name, referring to the isolation

of the type strain from rotting fruit of mangosteen, *Garcinia mangostana* L.).

After 3 days on YM agar at 28 °C, cells are spherical or ovoid (4–7 × 3.5–4 µm) and occur singly, in pairs or in groups (Fig. 2). Budding is multilateral. After 1 month at 28 °C, sediment and pellicle formation is observed. On YM agar medium after 15 days at 28 °C, the culture is butyrous, pale brownish in colour, smooth with a rough margin. Pseudohypha formation is not observed on YM agar or cornmeal agar plates after 10–21 days of incubation at 28 °C. After 3–5 days of incubation at 28 °C on Gorodkova agar and potato-dextrose-rose bengal agar, conjugation is followed by formation of two to four hat-shaped ascospores per cell. Asci deliquescent. Cells do not ferment D-glucose. Assimilates D-glucose, D-glucosamine (weakly), glycerol, xylitol and succinate but does not assimilate D-galactose, L-sorbose, D-ribose, D-xylose, L- or D-arabinose, L-rhamnose, sucrose, maltose, cellobiose, salicin, melibiose, lactose, raffinose, melezitose, starch, erythritol, ribitol, *myo*-inositol, D-gluconate, D-glucuronate, citrate, propane-1,2-diol or butane-2,3-diol as sole carbon sources. Assimilates ethylamine, L-lysine, cadaverine and glucosamine but does not assimilate nitrate, nitrite, creatinine or imidazole as sole nitrogen sources. Does not grow in vitamin-free media. Strains are sensitive to 0.1 % cycloheximide and do not grow in medium containing 1 % (v/v) acetic acid or methanol. Resistant to 0.01 % cycloheximide and does not grow in 50 % glucose. Does not form starch-like compounds and does not hydrolyse urea and the diazonium blue B reaction is negative.

Fig. 2. Phase-contrast micrographs of *Pichia garciniae* sp. nov. YS110^T showing conjugating cells (a), automicetic cells bearing protuberances and released ascospores (b, c). Cells were grown at 28 °C for 3–5 days on potato-dextrose-rose bengal agar. Bars, 6 µm.

Coenzyme Q7 is the major ubiquinone. The G + C content of the DNA of the type strain is 48.5 mol%.

The type strain is YS110^T (=NRRL Y-48422^T =CBS 10758^T), isolated from a decaying mangosteen fruit (*Garcinia mangostana* L., Clusiaceae).

Acknowledgements

The yeast strains were isolated as part of a project funded by the Council of Scientific and Industrial Research, New Delhi, India, to S.S. to characterize methylotrophic yeasts from the environment. We are also thankful to Professor C. Kurtzman of the ARS Culture Collection, Peoria, IL, USA, for providing us with type strains. Our special thanks go to Professor M.-A. Lachance for helping us to select and improve the photomicrographs of ascospores.

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