Pichia dushanensis sp. nov. and Hyphopichia paragotoi sp. nov., two sexual yeast species associated with insects and rotten wood

Yong-Cheng Ren, Si-Tong Liu, Ying Li and Feng-Li Hui

Seven yeast strains were isolated from the gut of insect larvae and decayed wood, which were collected from three localities near Nanyang, Henan Province, China. These strains were identified as two novel species through comparison of sequences in the D1/D2 domains of the large subunit (LSU) rRNA gene and other taxonomic characteristics. Pichia dushanensis sp. nov. was closely related to species in the Pichia clade and produced one to four spheroid ascospores in a deliquescent ascus. The D1/D2 sequence of P. dushanensis sp. nov. differed from its closest relative, Issatchenka (Picha) sp. NRRL Y-12824, by 3.6 % sequence divergence (16 substitutions and 4 gaps). The species also differed from its four closest known species, Candida rugopelliculosa, Pichia occidentalis, Pichia exigua and Candida phayaonensis, by 4.1–4.4 % sequence divergence (22–24 substitutions and 0–2 gaps) in the D1/D2 sequences. Hyphopichia paragotoi sp. nov. belonged to the Hyphopichia clade, and its nearest phylogenetic neighbours were Candida gotoi, Candida pseudohagii, Candida rhagii and Hyphopichia heimii with 3.2–4.2 % sequence divergence (16–21 substitutions and 1 gap) in the D1/D2 sequences. In comparison with previously established species, H. paragotoi sp. nov. formed one hat-shaped ascospore in a persistent ascus. The type strain of P. dushanensis sp. nov. is NYNU 14658T (=CICC 33049T=CBS 13912T), and the type strain of H. paragotoi sp. nov. is NYNU 14666T (=CICC 33048T=CBS 13913T).

Insects are common vectors of yeasts in nature. Diverse yeast species have been isolated from insects and their related substrates, including rotten wood, frass and galleries (Jindamorakot et al., 2007; Lachance et al., 2001, 2003, 2005; Nguyen et al., 2006; Suh et al., 2001, 2004, 2006, 2013). Specific yeast communities consisting mostly of yeasts belonging in few clades related to the yeast genera Metschnikowia, Kodamaea, Wickerhamiella and Starmerella have been observed in the digestive tract of beetles, drosophilids and bees, which visit ephemeral flowers (Lachance et al., 2001, 2003, 2005). Yeast endosymbionts have been found in the digestive tract of a variety of insects, including plant hoppers, aphids and beetles (Nardon & Grenier 1989; Noda & Omura, 1992; Suh et al., 2001). Most species in the Candida kruisii, Candida tanzawaensis and Pichia guillermondii clades have been isolated from the digestive tract of basidiocarp-feeding beetles and insect frass (Suh et al., 2004, 2006; Suh & Blackwell, 2004). Many known xylose-fermenting yeasts, such as the familiar genera Scheffersomyces and Spathaspora, are associated with wood-ingesting beetles and wood substrates (Nguyen et al., 2006; Cadete et al., 2013; Suh et al., 2013). Although the function of yeasts associated with insects has not been fully understood, some studies indicated that certain yeasts from these habitats might play important roles in their hosts, such as detoxification of food materials and supply of essential nutrients (Suh & Blackwell, 2004; Suh et al., 2013; Vega & Dowd, 2005).

In our study on yeasts associated with insects and their related substrates, we isolated several strains from the gut of insect larvae and decayed wood. Analysis of the sequences of the D1/D2 domains of the large subunit (LSU) rRNA gene showed that these strains represent two distinct species of the Pichia and Hyphopichia clades. In this paper, five strains (NUNU 14657, NUNU 14658T, NUNU 14659, NUNU 14774 and NUNU 14775) were regarded as representing a novel species of the genus Pichia, named Pichia dushanensis sp. nov., whereas the other two strains (NUNU 1464 and NUNU 14666T) were identified as representatives of a novel species of the genus Hyphopichia, named Hyphopichia paragotoi sp. nov.
Table 1. Strains and isolation sources of *Pichia dushanensis* sp. nov. and *Hyphopichia paragotoi* sp. nov.

CBS, Central Bureau voor Schimmel cultures; CICC, China Centre of Industrial Culture Collection, Beijing, China; NYNU, Microbiology Laboratory, Nanyang Normal University, Nanyang, Henan, China.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Location</th>
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<tr>
<td><em>Pichia dushanensis</em> sp. nov.</td>
<td></td>
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<tr>
<td>NYNU 14657</td>
<td>Rotten sawtooth oak wood</td>
<td>Dushan Forest Park, Nanyang, Henan Province, China</td>
</tr>
<tr>
<td>NYNU 14658^T (=CICC 33049^T=CBS 13912^T)</td>
<td>Gut of unidentified beetle larva collected from rotten sawtooth oak wood</td>
<td>Dushan Forest Park, Nanyang, Henan Province, China</td>
</tr>
<tr>
<td>NYNU 14674</td>
<td>Gut of unidentified beetle larva collected from rotten white oak wood</td>
<td>Dushan Forest Park, Nanyang, Henan Province, China</td>
</tr>
<tr>
<td>NYNU 14675</td>
<td>Gut of unidentified beetle larva collected from rotten cork oak wood</td>
<td>Dushan Forest Park, Nanyang, Henan Province, China</td>
</tr>
<tr>
<td>NYNU 14759</td>
<td>Gut of unidentified beetle larva collected from rotten cork oak wood</td>
<td>Baotianman Nature Reserve, Nanyang, Henan Province, China</td>
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<tr>
<td><em>Hyphopichia paragotoi</em> sp. nov.</td>
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<tr>
<td>NYNU 1464</td>
<td>Gut of unidentified beetle larva collected from rotten cork oak wood</td>
<td>Funiu Mountain Nature Reserve, Nanyang, Henan Province, China</td>
</tr>
<tr>
<td>NYNU 14666^T (=CICC 33048^T=CBS 13913^T)</td>
<td>Rotten cork oak wood</td>
<td>Dushan Forest Park in Nanyang, Henan Province, China</td>
</tr>
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</table>

Table 1 lists the strains described in this study. These strains were isolated from the gut of insect larvae and decayed wood, which were collected in Henan Province, Central China in June and July 2014. The yeasts were isolated from the insect gut by using a previously described method (Suh *et al.*, 2004, 2006; Suh & Blackwell, 2004). To isolate the yeasts from decayed wood, 1.0 g of sample was placed into 20 ml sterile yeast extract-malt extract (YM) broth (1 % glucose, 0.5 % peptone, 0.3 % yeast extract and 0.3 % malt extract; pH 5.4) supplemented with 0.02 % chloramphenicol in a 150 ml Erlenmeyer flask and then incubated at 25 °C for 3 days on a rotary shaker. Subsequently, 0.1 ml enrichment culture and appropriate decimal dilutions were spread on YM agar plates supplemented with 0.02 % chloramphenicol and then incubated at 25 °C for 3–4 days. Different yeast morphotypes were purified at least twice and then stored on YM agar slants at 4 °C or in 15 % glycerol at −80 °C.

Morphological observations and metabolic tests for standard yeast description were performed according to established methods (Kurtzman *et al.*, 2011). All assimilation tests were conducted twice in liquid media, and the results were recorded after incubation for 5 and 21 days. The starved inoculum was used in nitrogen assimilation tests. Sporulation tests were performed on YM agar, 5 % malt extract agar, corn meal agar and yeast carbon base supplemented with 0.01 % ammonium sulphate (YCBSA) agar (1.1 % yeast carbon base, 0.01 % ammonium sulphate and 1.8 % agar) in pure and mixed cultures at 18 and 25 °C for 4 weeks.

Genomic DNA was extracted using an Ezup Column Yeast Genomic DNA purification kit (Sangon Biotech) according to the manufacturer’s protocol. The D1/D2 domains of the LSU rRNA gene and the internal transcribed spacer (ITS) regions were amplified through PCR and then sequenced with primers NL1 and NL4 (Kurtzman & Robnett, 1998) or ITS1 and ITS4 (White *et al.*, 1990), respectively. Both DNA strands were sequenced and the reactions were carried out using a Dye Terminator Cycle sequencing kit (Applied Biosystems).

The sequences were compared pairwise through BLAST search (Altschul *et al.*, 1997) and aligned with the sequences of related species retrieved from the GenBank database by using the multiple alignment program CLUSTAL X (version 1.81) (Thompson *et al.*, 1997). Phylogenetic trees were reconstructed based on the LSU D1/D2 sequences by using MEGA software version 5.0 (Tamura *et al.*, 2011). Evolutionary distance data were calculated from Kimura’s two-parameter model (Kimura, 1980) in the neighbour-joining analysis. *Schizosaccharomyces pombe* CBS 356T was used as an outgroup. Confidence limits were estimated from the bootstrap analysis (1000 replicates) (Felsenstein 1985), and only values >50 % were recorded on the resulting trees. Reference sequences were retrieved from the GenBank database under the accession numbers indicated in the trees.

Sequence comparison, species delineation and ecology

Table 1 lists all the species described in this study. The species identified as a member of the genus *Pichia* was recovered from the gut of insect larvae (strains NYNU 14658^T, NYNU 14659, NYNU 14674 and NYNU 14675) and rotten wood (strain NUNU 14657). The five strains of the species had identical nucleotide sequences in both 18S rRNA and LSU rRNA genes and its sequence identity was >99.8 % with the type strains of *Pichia* sp. nov. and *Hyphopichia paragotoi* sp. nov. The species that is described in this study is genetically different from the type species of *Pichia* sp. nov. and *Hyphopichia paragotoi* sp. nov. To correctly describe the species, we compared the LSU D1/D2 sequences of all strains with the previously described species. The species was further confirmed by strain isolation and biogeographic distribution.

The species described in this study is genetically different from the type species of *Pichia* sp. nov. and *Hyphopichia paragotoi* sp. nov. The species that is described in this study is genetically different from the type species of *Pichia* sp. nov. and *Hyphopichia paragotoi* sp. nov. To correctly describe the species, we compared the LSU D1/D2 sequences of all strains with the previously described species. The species was further confirmed by strain isolation and biogeographic distribution.

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D1/D2 domain and ITS region. BLAST search using the D1/D2 sequences showed that this novel species was related to Issatchenkia (Pichia) sp. NRRL Y-12824, Candida rugopelliculosa CBS 6377T, Pichia occidentalis CBS 5459T, Pichia exigua NRRL Y-10920T and Candida phayaonensis CBS 12319T. The species differed from its closest relatives, which is the undescribed species Issatchenkia (Pichia) sp. NRRL Y-12824, by 3.6% sequence divergence (16 substitutions and 4 gaps) in the D1/D2 domain. The D1/D2 sequences from its four closest known species exhibited 4.1–4.4% sequence divergence (22–24 substitutions and 0–2 gaps). The ITS sequences were also sequenced for all strains of the proposed novel species. However, the ITS sequence of this yeast species could not be successfully aligned with the sequences of related species. In the phylogenetic tree based on the LSU D1/D2 sequences, the novel species was located in the Pichia clade and clustered with Issatchenkia (Pichia) sp. NRRL Y-12824 (Fig. 1). The position of this species within the Pichia clade remained unclear because of the low bootstrap value. In this group of species, the bootstrap value may increase through isolation of strains in the vicinity.

The phenotypic profile of this novel species fitted the description of the genus Pichia. One to four spherical ascospores were produced in a deliquescent ascus. Additionally, the five isolates of this novel species could be physiologically differentiated from their closest known species, C. rugopelliculosa (Lachance et al., 2011), in terms of growth in the presence of 1% acetic acid, ability to ferment trehalose and inability to assimilate D-glucosamine, DL-lactate and succinate. Hence, we concluded that the five isolates represented a novel species of the genus Pichia. The name Pichia dushanensis sp. nov. is proposed to categorize these isolates.

The two isolates of the species putatively assigned to the genus Hyphopichia were also directly isolated from the gut of insect larvae (strain NYNU 1464) and rotten wood (strain NUNU 14666T). Both strains similarly possessed identical nucleotide sequences in both D1/D2 domain and ITS region. Analysis of the LSU D1/D2 sequences confirmed that this yeast belonged to the Hyphopichia clade and was phylogenetically related to Candida gotoi CBS 8531T, Candida pseudorhagii NRRL YB-2076T, Candida rhagii NRRL Y-2594T and Hyphopichia heimii NRRL Y-7502T (Fig. 2). The D1/D2 sequences of these two strains differed from their three closest known species by 3.2–4.2% sequence divergence (16–21 substitutions and 1 gap) in the D1/D2 domain and by 7.6–9.4% sequence divergence (9–15 substitutions and 12–23 gaps) in the ITS region.

The two strains of this novel species could be morphologically differentiated from their nearest phylogenetic neighbour, C. gotoi (Lachance et al., 2011), by their ability to produce one hat-shaped ascospore in a persistent ascus. Physiologically, the two strains were separated from C. gotoi in terms of positive assimilation of L-sorbosone, D-glucosamine, D-arabinose and D-gluconate, ability to ferment trehalose and raffinose, and inability to assimilate L-rhamnose, methyl 2-D-glucoside, soluble starch, erythritol and galactitol. The rRNA gene sequence and phenotypic comparisons indicated that these two strains represent a novel species, which differed from the currently recognized ones. The name Hyphopichia paragotoi sp. nov. is proposed for these two isolates.

C. rugopelliculosa, the closest known species of P. dushanensis sp. nov., has been isolated from a soybean protein factory in Japan (Lachance et al., 2011), whereas the other close relatives of this species, such as P. exigua, Pichia sporocuriosa, P. occidentalis and Candida pseudolambibica, are associated with insects, particularly boring insects (Kurtzman, 2011b; Lachance et al., 2011). C. gotoi, C. pseudorhagii, C. rhagii and H. heimii formed an independent cluster with H. paragotoi sp. nov. in the Hyphopichia clade; these species are generally located in similar ecological habitats, i.e. associated with insects and their related substrates (Kurtzman, 2011a; Lachance et al., 2011). In the present study, P. dushanensis sp. nov. and H. paragotoi sp. nov. were isolated from insect larvae and rotten wood from three different localities, thereby suggesting that these substrates are ecological niches of the two novel species.

**Description of Pichia dushanensis**

Pichia dushanensis (du.shan.en’is). N.L. fem. adj. dushanensis pertaining to Dushan Forest Park, the geographical origin of the type strain of the species).

After culture for 3 days in YM broth at 25 °C, cells appear ovoid to ellipsoidal (3.5–4 × 4–10.5 μm) and occur singly or in pairs (Fig. 3a). Budding is multilateral. After culture for 1 month at 25 °C, pellicle and sediments are formed. After culture for 6 days on YM agar at 25 °C, colonies appear smooth, flat with an elevated centre, white and have an entire edge. After culture for 2 weeks in Dalmau plates containing corn meal agar, pseudohyphae and true hyphae are not formed. One to four rough or smooth spherical ascospores are formed in a deliquescent ascus on cornmeal agar and YM agar after culture for 12 days at 25 °C (Fig. 3b). Cells ferment glucose and trehalose (weak), but not D-galactose, maltose, sucrose, lactose, cellobiose, raffinose or D-xylene. Cells also assimilate glucose, α-D-xylulose (weak) and ethanol, but not inulin, sucrose, raffinose, melezitose, galactose, lactose, maltose, melezitose, methyl 2-D-glucoside, soluble starch, cellobiose, salicin, L-sorbose, L-rhamnose, D-xylene, D-arabinose, D-arabinose, D-ribose, methanol, glycerol, erythritol, ribitol, galactitol, manitol, glutitol, myo-inositol, DL-lactate, succinate, citrate, D-glucanoate, D-glucosamine, 2-keto-D-gluculone, 5-keto-D-gluculone, arbutin, xylitol, L-arabinitol or D-glucuronate as sole carbon sources. Cells further assimilate ethylamine, L-lysine, cadaverine and D-tryptophan, but not nitrate, nitrite, creatine, creatinine, glucosamine and imidazole as sole nitrogen sources. Sensitive to 0.01% cycloheximide.

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**Fig. 1.** Phylogenetic tree based on the D1/D2 domains of the LSU rRNA gene sequences showing the positions of *Pichia dushanensis* sp. nov. with respect to closely related species. *Schizosaccharomyces pombe* CBS 356T was used as an outgroup. Bootstrap values >50% are given at nodes based on 1000 replications. Bar, 2% sequence difference.
and does not grow in 5% glucose medium containing 10% NaCl. Growth is observed at 37 °C, but not at 40 °C. Growth is observed in the presence of 1% acetic acid. Acid formation on chalk agar is negative. Does not form starch-like compounds and does not hydrolyse urea. The diazonium blue B reaction is also negative.

The type strain NYNU 14658^T was isolated from the gut of insect larvae collected from Dushan Forest Park in Nanyang, Henan Province, China. The living culture from the type strain is maintained in the lyophilized state as strain CBS 13912^T in the collection of the Yeast Division of the Central Bureau voor Schimmel cultures, Utrecht, the Netherlands and as strain CICC 33049^T in the China Centre of Industrial Culture Collection, Beijing, China. The MycoBank number of the strain is MB 811939.

**Description of Hyphopichia paragotoi Hui, Ren, Liu & Li sp. nov.**

*Hyphopichia paragotoi* (pa-ra-go-to'i. Gr. prep. para beside, alongside of, near, like; N.L. gen. n. gotoi a specific epithet; N. L. gen. n. paragotoi like gotoi, referring to its phylogenetic closeness to *Candida gotoi*).

After 3 days of culture in YM broth at 25 °C, cells appear ovoid to cylindrical (2.5–5.5 × 2.5–6.5 μm) and occur singly or in pairs (Fig. 3c). Budding is multilateral. Sediment forms after 1 month, but no pellicle is observed. After 3 days of culture on YM agar at 25 °C, the streak culture is butyrous, elevated with smooth surface, white and has an entire margin. After 12 days of culture in Dalmau plates containing cornmeal agar at 25 °C, pseudohyphae are formed but true hyphae are not formed. One hat-shaped ascospore, which is formed in a persistent ascus, may be unconjugated or conjugated with independent cells (Fig. 3d). Ascospores are observed on cornmeal and YCBAS agars after 8 days of culture at 25 °C. Ferments glucose, D-galactose, sucrose, trehalose and raffinose, but not maltose, lactose, cellulbiose or D-xylose. Assimilates glucose, sucrose, raffinose, D-galactose, α,α-trehalose, maltose, melezitose, cellubiose, salicin, L-sorbose, D-xylose, L-arabinose, D-arabinose, D-ribose, ethanol, glycerol, ribitol, D-mannitol, D-glucitol, D-lactate, succinate, citrate, D-gluconate, D-glucosamine, 2-keto-D-gluconate, 5-keto-D-gluconate, arbutin, xylitol and L-arabinitol. No growth occurs in inulin, melibiose, lactose, methyl α-D-glucoside, soluble starch, L-ramnose, methanol, erythritol, galactitol, myo-inositol or D-gluconate. Assimilation of nitrogen compounds is positive for ethylamine, L-lysine, cadaverine and D-tryptophan, but negative for nitrate, nitrite, creatine, creatinine, glucosamine and imidazole. The maximum temperature for growth is 37 °C. No growth is observed in the presence of 1% acetic acid and 0.01% cycloheximide. Growth in 5% glucose medium containing 10% NaCl is negative. Starch-like compounds are not produced. The diazonium blue B and urease reactions are also negative.

The type strain NYNU 14666^T was isolated from the gut of insect larvae collected from Dushan Forest Park in Nanyang, Henan Province, China. The living culture from the type strain is maintained in the lyophilized state as strain CBS 13913^T in the collection of the Yeast Division of the Central Bureau voor Schimmel cultures, Utrecht, the Netherlands.
and as strain CICC 33048<sup>T</sup> in the China Centre of Industrial Culture Collection, Beijing, China. The MycoBank number of the strain is MB 811940.

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**References**


