A new yeast genus, *Tetrapisispora* gen. nov.: *Tetrapisispora iriomotensis* sp. nov., *Tetrapisispora nanseiensis* sp. nov. and *Tetrapisispora arboricola* sp. nov., from the Nansei Islands, and reclassification of *Kluyveromyces phaffii* (van der Walt) van der Walt as *Tetrapisispora phaffii* comb. nov.

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Seven strains of three new yeast species were isolated from soil, flowers and leaves in the Nansei Islands, Japan. These isolates most closely resembled *Kluyveromyces phaffii* in physiological characteristics and nuclear DNA base composition (30-32 mol% G+C), but on the basis of DNA-DNA hybridization and electrophoretic karyotyping they were categorized into three new species different from *K. phaffii*. Phylogenetic analysis using 18S rRNA gene sequences showed that the three new species and *K. phaffii* were highly related to one another and phylogenetically separate from the members of other species. On the basis of phylogeny and physiological characters, it is proposed that the three new species represent novel taxa and should be designated *Tetrapisispora iriomotensis* gen. nov., sp. nov. (type strain IF0 10929T), *Tetrapisispora nanseiensis* gen. nov., sp. nov. (type strain IF0 10899T) and *Tetrapisispora arboricola* gen. nov., sp. nov. (type strain IF0 10925T), while *Kluyveromyces phaffii* becomes *Tetrapisispora phaffii* comb. nov.

**Keywords:** ascomycetous yeast, *Tetrapisispora* gen. nov., 18S rRNA gene

**INTRODUCTION**

The genus *Kluyveromyces* comprises ascomycetous fermentation yeasts characterized by smooth ascospores liberated from the asci (van der Walt, 1965). Molnár et al. (1996) showed that members of *Kluyveromyces* are homogeneous for the coenzyme Q-6. Poncet (1973) identified three phenetic lines by factor analysis. DNA base composition data (Vaughan-Martini & Martini, 1987) were compatible with the existence of three groups. The three devised groups, having low, medium and high DNA G+C contents, correspond respectively to groups A, B and C of Poncet (1973). In the fourth edition of 'The Yeasts' (Lachance, 1998), 15 species of *Kluyveromyces* are shown and the genus is divided into the three groups. Group A comprises *Kluyveromyces africanus*, *Kluyveromyces bacillisporus*, *Kluyveromyces blattae*, *Kluyveromyces delphensis*, *Kluyveromyces loderiae*, *Kluyveromyces phaffii*, *Kluyveromyces polyssporus* and *Kluyveromyces yarrowii*, species lacking in ethylamine and L-lysine assimilation, and groups B and C are made up of species able respectively to assimilate ethylamine and L-lysine strongly. The group-A species are phenoetically homogeneous by many criteria (Poncet, 1973; Kock et al., 1988). In view of the ascospore numbers and ploidy of natural isolates, Naumov (1987) proposed the reclassification of *K. phaffii* in a separate genus.

Previous phylogenetic analyses (Cai et al., 1996; James et al., 1997) showed that the genus *Kluyveromyces* is not monophyletic and that species of this genus are intermixed with members of other genera, particularly *Saccharomyces* and *Zygosaccharomyces*. Groups B...
and C of Kluyveromyces each constitute a monophyletic entity. K. blattae and K. phaffii of group A, which is phylogenetically heterogeneous, were found to form a distinct lineage and to display no specific association with the other species examined. Cai et al. (1996) proposed that both K. blattae and K. phaffii should be excluded from the genus Kluyveromyces. No other K. phaffii strain or phylogenetically related species has been isolated since the K. phaffii type strain was isolated from South African soil (van der Walt, 1963). For this reason, perhaps, K. phaffii has not been transferred to another genus.

In this study, three new species closely related to K. phaffii were isolated from Irinomote and Ishigaki Islands, in the subtropical zone in the south-west part of the Nansei Islands, and Yaku Island, in the temperate zone in the north-east part of the islands. A novel genus, Tetrapispora gen. nov., is proposed in this paper to accommodate K. phaffii and the three new species.

METHODS

Yeast isolation and identification. Yeasts were isolated from soil, leaves and flowers using enrichment culture. Enrichment broth medium contained 5% malt extract and 3% glucose and after autoclaving, its pH was adjusted to 3.7 and 50 mg l⁻¹ each of penicillin, streptomycin and chloramphenicol were added to repress bacterial growth. A piece or spoonful of a sample of substrate was inoculated into 10 ml medium in a 15-ml screw-cap test tube and incubated at 17 °C without shaking for isolation of fermentative yeast strains. On days 7 and 14 after inoculation, the fungal mat that appeared on the surface of the medium was removed and a loopful of the culture liquid was spread onto a YM agar plate. Yeast were identified by standard methods (van der Walt & Yarrow, 1984).

Pulsed-field gel electrophoresis (PFGE). Agarose plugs containing yeast chromosomal DNA were prepared according to the method of Carle & Olson (1985). The gel was 1% agarose (Agarose NA; Pharmacia) in 0.5 x TBE buffer chilled at 14 °C, PFGE was performed using a CHEF DRII system (Bio-Rad) at 200 V for 15 h with a 60-70 s switch-time ramp and for 7 h with a 90-120 s switch-time ramp. After electrophoresis, the gel was stained with 0.5% ethidium bromide 1⁻¹ for 10 min at room temperature and successively destained in distilled water for times varying from 1 h up to 1 d.

Major ubiquinone analysis. The major ubiquinone was determined by HPLC according to the method described by Nakagiri (1991).

Genotypic analysis. Genomic DNA was extracted and purified using the procedure of Holm et al. (1986). The G+C content was determined by the HPLC method of Tamaoka & Komagata (1984). DNA similarity was measured by the microplate-hybridization method described by Kaneko & Banno (1991) and calculated as a mean of five measurements.

18S rDNA sequencing. 18S rDNA sequences were determined using the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham) following the manufacturer’s protocol. For amplification of 18S rDNA, PCR (Saiki et al., 1988) was performed for 30 cycles of denaturation at 94 °C for 0.5 min, annealing at 55 °C for 0.5 min and extension at 72 °C for 2.5 min with TaKaRa Taq DNA polymerase (Takara) using the primer pair 18-F (5'-ATCTGGTGTAGCTCTTCGAGG-3') and 18-R (5'-GATCCCTCAGGGTGTCACCC-3'). Diluted PCR products were used as a template for sequencing and 5'-FITC-labelled primers were 400F (5'-TCCGAGAGGGAGGCTGAAACAGG-3'), 550F (5'-GACGCCGGGTAAATTCCAGC-3'), 950F (5'-TCAAGAACGAAAGTATTCTCCG-3'), 1500F (5'-GATGCCCTTACGCTTCTCGG-3') and 18-F for the sense strand and 400R (5'-TTCTAGGCTCCCCTCCCGG-3'), 550R (5'-GAAATTACGGCGGTCTGCTGG-3'), 950R (5'-TCCCCAACTTCTGTTCCTTG-3'), 1500R (5'-GGTGCCCTTCCCGTCAATCCC-3'), 1300R (5'-AGACAAATCCTCACCAAC-3'), 1500R (5'-CCAGACGTCCTAAAGGCGATCACAG-3') and 18-R for the reverse strand.

Phylogenetic analysis. Sequence data were manually aligned to various 18S rDNA sequences of representatives of related genera obtained from GenBank. Positions that could not be compared among all sequences, corresponding to unknown bases, deletions and insertions, and regions difficult to align between each strain and outgroup were omitted from the phylogenetic analysis. A phylogenetic tree was constructed by Kimura’s two-parameter method (Kimura, 1980) and the neighbour-joining method (Saitou & Nei, 1987) using CLUSTAL w. Bootstrap values (Felsenstein, 1985) were calculated from 1000 replicates.

RESULTS

Morphology and physiology of isolated strains

Seven strains were isolated from soil, leaves of mangrove and flowers of back-mangrove (Table 1). In all isolates, asc was directly from diploid cells and one to four oval or reniform ascospores were formed. The spores of strain U97-101-2T were liberated from the ascus soon after formation and tended to agglutinate, whereas in the other strains, asc were persistent (Fig. 1). As monosporic cultures could sporulate, they were judged to be homothallic. In their physiological characteristics, the seven isolates were similar to K. phaffii, isolated from South African soil (van der Walt, 1963): fermentation of glucose and galactose, growth on glucose, galactose and glycerc and no utilization of most other compounds.

Electrophoretic karyotypes of the isolates

Chromosomal DNA banding patterns of the isolates were examined by PFGE (Fig. 2). All seven isolates showed most of the chromosomes displayed in a wide range of sizes (20-0-0.2 Mb), in a similar way to Saccharomyces cerevisiae. The pattern of isolate Yk-305a2 from Yaku Island was similar to that of isolate U96-107-4T from Ishigaki Island. Those of isolates U97-412-1T, U97-412-3, U97-436-2 and U97-437-2 were also similar to each other. However, slight differences were found within each of these groups.
Table 1. Isolated strains and their origins

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Original no.</th>
<th>Sample material</th>
<th>Locality</th>
</tr>
</thead>
<tbody>
<tr>
<td>U97-101-2T</td>
<td>10929T</td>
<td>Soil</td>
<td>Iriomote Island</td>
</tr>
<tr>
<td>U96-107-4T</td>
<td>10899T</td>
<td>Soil</td>
<td>Ishigaki Island</td>
</tr>
<tr>
<td>Yk-305a2</td>
<td>1883</td>
<td>Soil</td>
<td>Yaku Island</td>
</tr>
<tr>
<td>U97-412-1T</td>
<td>10925T</td>
<td>Flower of \textit{Elaeocarpus decipiens}</td>
<td>Iriomote Island</td>
</tr>
<tr>
<td>U97-412-3</td>
<td>10926</td>
<td>Flower of \textit{Elaeocarpus decipiens}</td>
<td>Iriomote Island</td>
</tr>
<tr>
<td>U97-436-2</td>
<td>10927</td>
<td>Leaf of \textit{Bruguiera conjugata}</td>
<td>Iriomote Island</td>
</tr>
<tr>
<td>U97-437-2</td>
<td>10928</td>
<td>Leaf of \textit{Rhizophora mucronata}</td>
<td>Iriomote Island</td>
</tr>
</tbody>
</table>

![Photomicrographs of vegetative cells on YPD broth (upper) and asci on potassium acetate agar (lower).](image)

(a) U97-101-2T (\textit{T. iriomotensis} IFO 10929T), (b) U96-107-4T (\textit{T. nanseiensis} IFO 10899T), (c) U97-412-1T (\textit{T. arboricola} IFO 10925T).

Bars, 5 μm.

U97-412-1T and U97-412-3 were thought to originate from an identical strain, since they were isolated from the same sample. The pattern of U97-412-3 had an extra, minor band between the first and second major bands in comparison with that of U97-412-1T, suggesting that these isolates were related but not identical. The pattern of U97-101-2T was unlike all the others. Therefore, the seven isolates were categorized into three groups by their PFGE patterns, which differed also from those of type strains of \textit{K. phaffii} and all other known \textit{Kluyveromyces} and \textit{Saccharomyces} species (data not shown).

DNA base composition and DNA–DNA hybridization

The G+C contents of the DNA of all isolates were 30.4–32.3 mol%, close to the 32.7 mol% of \textit{K. phaffii}. To survey species identities of isolates, their DNA
K. Ueda-Nishimura and K. Mikata

**Fig. 2.** PFGE analysis of *K. phaffii* and isolates. Lanes: m, *S. cerevisiae* SH 964 as molecular size marker; 1, *K. phaffii* IFO 1672T; 2, U97-101-2T (T. iriomotensis IFO 10929T); 3, Yk-305a2 (T. nanseiensis IFO 1883); 4, U96-107-4T (T. nanseiensis IFO 10899T); 5, U97-412-1T (T. arboricola IFO 10925T); 6, U97-412-3 (T. arboricola IFO 10927); 7, U97-436-2 (T. arboricola IFO 10927); 8, U97-437-2 (T. arboricola IFO 10928).

similarity values to each other and to *K. phaffii* were examined by the microplate-hybridization method. The results are shown in Table 2. *K. phaffii* type strain IFO 1672T and isolate U97-101-2T showed low similarity to all tested strains. DNA similarity values of U96-107-4T and Yk-305a2 were 79.2 and 97.0% and those of U97-412-1T to U97-412-3, U97-436-2 and U97-437-2 were 99.7, 105.2 and 116.6%. These data showed that U96-107-4T and Yk-305a2 should be considered to be conspecific, as should U97-412-1T, U97-412-3, U97-436-2 and U97-437-2. The DNA similarity values of the isolates thus confirmed the three distinct groups revealed by PFGE patterns (Fig. 2).

**Phylogeny**

18S rDNA sequences of the isolates were determined by the direct method. Although U96-107-4T from Ishigaki Island and Yk-305a2 from Yaku Island were the same species, a substitution was found between the

**Table 3. Identities and numbers of substitutions between 18S rDNA sequences**

The upper-right figures show percentage sequence identity; lower-left figures show numbers of base substitutions. Boldface type indicates isolates from the same species.

<table>
<thead>
<tr>
<th>Strain</th>
<th>IFO no.</th>
<th>Identities (%) and numbers of substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1. <em>K. phaffii</em></td>
<td>1672T</td>
<td>100</td>
</tr>
<tr>
<td>2. U97-101-2T</td>
<td>10929T</td>
<td>99·5</td>
</tr>
<tr>
<td>3. U96-107-4T</td>
<td>10899T</td>
<td>8</td>
</tr>
<tr>
<td>4. Yk-305a2</td>
<td>1883</td>
<td>9</td>
</tr>
<tr>
<td>5. U97-412-1T</td>
<td>10925T</td>
<td>10</td>
</tr>
<tr>
<td>6. U97-436-2</td>
<td>10927</td>
<td>9</td>
</tr>
<tr>
<td>7. U97-437-2</td>
<td>10928</td>
<td>9</td>
</tr>
</tbody>
</table>

**Table 2. Similarity and G+C content of DNA of isolated strains**

Hybridization was performed at 40 °C. Boldface type indicates isolates of the same species; ND, not determined.

<table>
<thead>
<tr>
<th>Strain</th>
<th>IFO no.</th>
<th>G + C content (mol%)</th>
<th>DNA similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1. <em>K. phaffii</em></td>
<td>1672T</td>
<td>32·7</td>
<td>100</td>
</tr>
<tr>
<td>2. U97-101-2T</td>
<td>10929T</td>
<td>32·3</td>
<td>33·2</td>
</tr>
<tr>
<td>3. U96-107-4T</td>
<td>10899T</td>
<td>30·4</td>
<td>29·4</td>
</tr>
<tr>
<td>4. Yk-305a2</td>
<td>1883</td>
<td>31·2</td>
<td>21·6</td>
</tr>
<tr>
<td>5. U97-412-1T</td>
<td>10925T</td>
<td>31·2</td>
<td>34·2</td>
</tr>
<tr>
<td>6. U97-412-3</td>
<td>10926</td>
<td>31·4</td>
<td>ND</td>
</tr>
<tr>
<td>7. U97-436-2</td>
<td>10927</td>
<td>32·3</td>
<td>ND</td>
</tr>
<tr>
<td>8. U97-437-2</td>
<td>10928</td>
<td>32·0</td>
<td>ND</td>
</tr>
<tr>
<td>9. <em>S. cerevisiae</em></td>
<td>10217T</td>
<td>38·0</td>
<td>ND</td>
</tr>
</tbody>
</table>
18S rDNA sequences of the two isolates (Table 3). This was located in a variable region that was ignored in the analysis because of difficulty of alignment among all used sequences, and thus no phylogenetic distinction could be detected. The sequences for U97-42-1, U97-436-2 and U97-437-2 from Iriomote Island were identical. The three isolated species and K. phaffii were found to be closely related to one another, with a 100% bootstrap value and levels of 18S rDNA sequence identity of >99.4% (Table 3), and were phylogenetically separate from the other Kluyveromyces species and non-Kluyveromyces species examined (Fig. 3). Their closest relative was K. blattae, which also formed a distinct line of descent that was quite separate from the other species.

DNA–DNA hybridization tests suggested that the new isolates and K. phaffii represented three different species. On the basis of these facts, the novel genus Tetrapisispora gen. nov. is proposed. Differential characteristics for Tetrapisispora species are listed in Table 4.

**Latin diagnosis of Tetrapisispora gen. nov.**


**Description of Tetrapisispora Ueda-Nishimura et Mikata gen. nov.**

Tetrapisispora (Te.tr.a.pi.sí.spo’rá. G. pref. tetra four; L. n. pisi pea; L. fem. n. spora spore; M.L. n. Tetrapisispora four pea spores).
Table 4. Distinguishing characteristics of Tetrapisispora species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>T. phaffii</th>
<th>T. iriomotensis</th>
<th>T. nanseiensis</th>
<th>T. arboricola</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assimilation of:</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Gluconate</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-/+</td>
</tr>
<tr>
<td>Growth on vitamin-free medium</td>
<td>-</td>
<td>+w</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth on 0.01 % cycloheximide</td>
<td>-</td>
<td>-</td>
<td>+S/−</td>
<td>−</td>
</tr>
<tr>
<td>Growth on 10% NaCl+5% glucose</td>
<td>−</td>
<td>+S</td>
<td>−</td>
<td>+S</td>
</tr>
<tr>
<td>Ascus deliquescence</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

The genus belongs to the Saccharomycetaceae. Cells are ellipsoid, ovoid to cylindrical, reproducing by multilateral budding. No pseudomycelium is formed. The genus is homothallic and diploid. Asci arise by transformation of diploid vegetative cells and are ruptured or persistent on maturation, containing 1–4 ascospores. Ascospores are ovoid to reniform and smooth. Glucose and galactose are fermented vigorously. Glucose, galactose and glycerol are assimilated. Nitrate, ethylamine, lysine and cadaverine are not assimilated. The diazonium blue B reaction is negative. Ubiquinone system is Q-6. G+C content of DNA is 30–33 mol%.

The type species is Tetrapisispora phaffii (van der Walt) Ueda-Nishimura et Mikata comb. nov. [basionym Fabospora phaffii van der Walt (1963)]. Synonyms: Kluyveromyces phaffii (van der Walt) van der Walt (1965) nom. inval.; Kluyveromyces phaffii (van der Walt) van der Walt (1971). The type strain of Tetrapisispora phaffii is CBS 4417T (=IFO 1672T, =ATCC 24235T, =NRRL Y-8282T), isolated by van der Walt (1963) from soil. Other species accepted in the genus are Tetrapisispora iriomotensis sp. nov., Tetrapisispora nanseiensis sp. nov. and Tetrapisispora arboricola sp. nov.

Latin diagnosis of Tetrapisispora iriomotensis sp. nov.

In medio liquido cum glucoso et peptono et extracto levidinis post dies 2 ad 24 °C, cellulae ovoideae vel ellipsiodeae, 2.0–5.0×5.0–8.0 μm, singulae aut binae, per gernomatim multilateralem reproductenses. Cultura in agaro cum glucoso, peptono et extracto levidinis post unum mensum ad 24 °C, butyrosa, glabra, nitida et erubens. Pseudomycelium nullum. Asci per transformationem cellularum vegetativarum diplodiarum, 1–4 ascosporas continent. Ascospora ovoideae aut reniformes. Ascosporiae ex ascis liberantur et agglutinant.

Glucosum et galactosum fermentantur, at non L-sorbosum, maltosum, sucrosum, cellobiosum, trehalosum, lactosum, mellibiosum, raffinosum nec D-xylosum. Glucosum, galactosum, glycerolum, D-glucono-1,5-lactosum et gluconicum assimilantur, at non L-sorbosum, maltosum, sucrosum, cellobiosum, trehalosum, lactosum, mellibiosum, raffinosum, melezitosum, inulimum, amyllum, D-xylosum, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, ethanolum, erythritolum, ribitolum, galactitolum, D-mannitolum, D-glucitolum, α-methyl D-glucosidum, salicinum, acidum lacticum, acidum succinicum, acidum citricum, inositolum, glucosaminum nec arbutinum.

Nitras kalicus, ethylaminum, lysinum nec cadaverinum non assimilantur. Exiguum crescit sine vitaminis. Non crescere potest in 0.01% cycloheximido. Lente crescere potest in 10 % NaCl/5% glucosa. Augmentum in 34 °C. Diazonium caerulin B non respondens. G+C acid i deoxyribonucleati 32 mol% per HPLC. Systema coenzymatis Q-6 adest.

Typus depositus in collectione Institute for Fermentation, Osaka, Japonia (IFO 10929T).

Description of Tetrapisispora iriomotensis sp. nov.

Tetrapisispora iriomotensis (i.r.i.o.m.o.ten'sis. L. adj. iriomotensis pertaining to Irionote Island, Japan, where the yeast was originally isolated). In YPD broth, after 48 h growth at 24 °C, the cells were ovoid to ellipsoidal and 2.0–5.0×5.0–8.0 μm, single or in pairs. Sediment is present. Budding is multipolar. After 1 month at 24 °C, YPD agar streak culture is butyrous, smooth, glistening and cream-coloured. Branching hyphae or pseudohyphae are not formed in Dalmatian plate cultures on corn meal agar. Oval asci containing 1–4 ovoid to reniform ascospores are formed directly from diploid cells after incubation for 1 week at 24 °C on potassium acetate agar, YM agar and cornmeal agar. The ascospores are liberated from the ascus soon after formation and tend to agglutinate.

Glucose and galactose are fermented. L-Sorbose, maltose, sucrose, cellobiose, trehalose, lactose, melli-
biose, raffinose and D-xylose are not fermented. The following carbon compounds are assimilated: glucose, galactose, glycerol, D-glucono-1,5-lactone and gluconate. No growth occurs on L-sorbose, maltose, sucrose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, ethanol, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, α-methyl D-glucoside, salicin, DL-lactate, succinate, citrate, inositol, glucosamine or arbutin. The following nitrogen compounds are not assimilated: nitrate, ethylamine, lysine and cadaverine. Weak growth occurs in vitamin-free medium. No growth occurs in the presence of 0.01% cycloheximide. Slow growth occurs in the presence on 10% NaCl/5% glucose. Growth occurs at 34°C, but not at 37°C. The diazonium blue B reaction is negative. Production of starch-like substances is negative. G+C content of DNA is 32 mol% by HPLC. Ubiquinone system is Q-6.

The type strain U97-101-2T was isolated in July 1997 from soil in Ootomi, Iriomote Island, Japan. Cultures of the type strain U97-101-2T (IFO 10929T) have been deposited in the culture collection of the Institute for Fermentation, Osaka, Japan.

Latin diagnosis of *Tetrapisispora nanseiensis* sp. nov.


Nitra calicis, ethylaminum, lysinum nec cadaverinum non assimilantur. Non crescit sine vitaminis. Exiguum crescere potest in 0.01% cycloheximido. Non crescere potest in 10% NaCl/5% glucosum. Augmentum in 34°C. Diazonium caeruleum B non respondens. G+C acidi deoxyribonucleati 30 mol% per HPLC. Systema coenzymatis Q-6 adest.

Typus depositus in collectione Institute for Fermentation, Osaka, Japonia (IFO 10890T).

Description of *Tetrapisispora nanseiensis* sp. nov.

*Tetrapisispora nanseiensis* (nan.sei.en'sis. L. adj. nanseiensis pertaining to the Nansei Islands in Japan, where the yeasts were originally isolated).

In YPD broth, after 48 h of growth at 24°C, the cells were ovoid to cylindrical and 1.0-3.0×3.0-6.0 µm, single or in pairs. Sediment is present. Budding is multipolar. After 1 month at 24°C, YPD agar streak culture is butyrous, smooth, glistening and cream-coloured. Branching hyphae or pseudohyphae are not formed in Dalmau plate cultures on corn meal agar. Ovoid asci containing 1-4 oval ascospores are formed directly from diploid cells after incubation for 1 week at 24°C on potassium acetate agar, YM agar and cornmeal agar. Asci are persistent.

Glucose and galactose are fermented. L-Sorbose, maltose, sucrose, cellobiose, trehalose, lactose, melibiose, raffinose and D-xylose are not fermented. The following carbon compounds are assimilated: glucose, galactose, glycerol, D-glucono-1,5-lactone and gluconate. No growth occurs on L-sorbose, maltose, sucrose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, ethanol, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, α-methyl D-glucoside, salicin, DL-lactate, succinate, citrate, inositol, glucosamine or arbutin. The following nitrogen compounds are not assimilated: nitrate, ethylamine, lysine and cadaverine. Growth occurs in vitamin-free medium. Slow growth occurs in the presence of 0.01% cycloheximide, but not 0.1%. No growth occurs in the presence on 10% NaCl/5% glucose. Growth occurs at 34°C, but not at 37°C. The diazonium blue B reaction is negative. Production of starch-like substances is negative. G+C content of DNA is 30 mol% by HPLC. Ubiquinone system is Q-6.

Strain U96-107-4T (IFO 10899T) was isolated in October 1996 from soil in Ban-na, Ishigaki Island, Japan, and strain Yk-305a2 (IFO 1883) was isolated in 1975 from soil in Yaku Island, Japan. Cultures of the type strain U96-107-4T (IFO 10899T) have been deposited in the culture collection of the Institute for Fermentation, Osaka, Japan.

Latin diagnosis of *Tetrapisispora arboricola* sp. nov.

*In medio liquid cum glucoso, peptono et extracto levidinis post dies 2 ad 24°C, cellulae ovoideae vel sphaericae, 2.0-4.0×2.0-6.0 µm, singulae aut binae, per germinationem multilateralem reproducteuntur. Cultura in agaro cum glucoso, peptono et extracto levidinis post unum mensem ad 24°C, butyrosa, glabra, nitida et eburnea. Pseudomyccelium nullum. Asci per transformationem cellularum vegetatavarum diploidearum, 1-4 ascoparum continentes. Ascoparum ovoideae vel reniformes. Asci non rumpuntur. Glucosum et galactosum fermentantur, at non L-
sorosum, maltosum, sucosum, cellulosum, trehalosum, lactosum, mellibiosum, raffinosum nec D-xylosyl. Glucosum, galactosum, trehalosum et glycerolum assimilantur, at non L-sorosum, maltosum, sucosum, cellulosum, lactosum, mellibiosum, raffinosum, melitzosum, indinum, amyllum, D-xylosyl, L-arabinosum, D-arabinosinum, D-ribosum, L-rhamnosum, ethanolum, erythritolum, ribitolum, galactitolum, D-mannitolum, D-glucitolum, α-methyl D-glucosidum, salicinum, D-arabitosum, D-ribosum, ~-rhamnosum, ethnol, ziz tosum, in din urn, amyllum.

cellobiosum, lactosum, melibiosum, rafinosum, melezitose, xylosum.

erithritolum, ribitolum, galactitolum, D-mannitolum, gluconicum, gluconatum, acidum citricum, inositolum, gluconicum, acidum lacticum, acidum gluconicum, glucitolum, D-glacturonicum, acidum succinicum, acidum citricum, inositolum, gluconicum, glucosaminum nec arbutinum.

Nitras kalicus, ethylaminum, lysinum nec cadaverinum non assimilantur. Non crescit sine vitaminis. Non crescer potest in coenzymatis. Crescere potest in culture is butyrous, smooth, glistening and cream-coloured. Branching hyphae or pseudohyphae are not formed directly from diploid cells after incubation in YPD broth, after 48 h growth at 24 °C, the cells were ovoid to spherical and 2-0-40 x 2-0-60 μm, single or in pairs. Sediment is present. Budding is formed in Dalmau plate cultures on cornmeal agar. Oval asc containing 1-4 ovoid to reniform ascospores were formed directly from diploid cells after incubation for 1 week at 24 °C on potassium acetate agar, YM agar and cornmeal agar. Asci are persistent.

Glucose and galactose are fermented. L-Sorbose, maltose, sucrlose, cellobiose, trehalose, lactose, melibiose, raffinosose and D-xylosyl are not fermented. The following carbon compounds are assimilated: glucose, galactose, trehalose and glycerol. No growth occurs on L-sorbose, maltose, sucrlose, cellobiose, lactose, melibiose, raffinosose, mezitose, inulin, starch, D-xylosyl, L-arabinose, D-arabinose, D-ribose, L-rhamnosyl, ethanol, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, α-methyl D-glucoside, salicinum, D-glucuno-1,5-lactone, acidum lacticum, acidum succinicum, acidum citricum, inositolum, gluconicum, glucosaminum nec arbutinum.

Typus depositus in collectione Institute for Fermentation, Osaka, Japonia (IFO 10925T).

Description of Tetrapisispora arboricola sp. nov.

Tetrapisispora arboricola (ar.bo.ric.o.la. L. fem. n. arboricola tree-dweller, relating to the original isolation of the species).

In YPD broth, after 48 h growth at 24 °C, the cells were ovoid to spherical and 2-0-40 × 2-0-60 μm, single or in pairs. Sediment is present. Budding is multipolar. After 1 month at 24 °C, YPD agar streak culture is butyrous, smooth, glistening and cream-coloured. Branching hyphae or pseudohyphae are not formed in Dalmau plate cultures on cornmeal agar. Oval asc containing 1-4 ovoid to reniform ascospores are formed directly from diploid cells after incubation for 1 week at 24 °C on potassium acetate agar, YM agar and cornmeal agar. Asci are persistent.

Glucose and galactose are fermented. L-Sorbose, maltose, sucrrose, cellobiose, trehalose, lactose, melibiose, raffinosose and D-xylosyl are not fermented. The following carbon compounds are assimilated: glucose, galactose, trehalose and glycerol. No growth occurs on L-sorbose, maltose, sucrlose, cellobiose, lactose, melibiose, raffinosose, mezitose, inulin, starch, D-xylosyl, L-arabinose, D-arabinose, D-ribose, L-rhamnosyl, ethanol, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, α-methyl D-glucoside, salicinum, D-glucuno-1,5-lactone, acidum lacticum, acidum succinicum, acidum citricum, inositolum, gluconicum, glucosaminum nec arbutinum.

Strains U97-412-1T (= IFO 10925T) and U97-412-3 (= IFO 10926), from flowers of Elaeocarpus decipiens, U97-436-2 (= IFO 10927), from a leaf of Bruguierea conjugata, and U97-437-2 (= IFO 10928), from a leaf of Rhizophora mucronata, were isolated in July 1997 in a mangrove area of Iriomote Island, Japan. Cultures of the type strain U97-412-1T (= IFO 10925T) have been deposited in the culture collection of the Institute for Fermentation, Osaka, Japan.

DISCUSSION

The Nansei Islands stretch for about 1200 km between the Japanese mainland in the temperate zone and Taiwan in the subtropical zone. They are the habitat of many native animals and plants and are often thought of as the 'Oriental Galapagos'. The three new species had not been isolated from any other place surveyed on the Japanese mainland. The fact that strains related to T. phaffii (= K. phaffii) were isolated from Ishigaki and Iriomote Islands, located in the south of the Nansei Islands, and Yaku Island, lying about 1200 km to the north, suggests that these strains may be distributed widely throughout the Nansei Islands.

In this study, new species were isolated from normal substrates by enrichment culture using screw-cap culture tubes, suggesting that this method is suitable for the isolation of fermenting yeasts.

Vaughan-Martini et al. (1993) reported that low DNA relatedness corresponds to completely different chromosomal patterns in the case of Saccharomyces. They also reported that similar but not identical karyotypes did not guarantee conspecificity. However, DNA-DNA hybridization tests in this study showed that strains with similar but not identical karyotypes were conspecific: U96-107-4T and Yk-305a2, and U97-412-1T, U97-412-3, U97-436-2 and U97-437-2. The 18S rDNA sequences showed no more than one substitution between conspecific strains U96-107-4T and Yk-305a2, and more than two substitutions were found between strains of different species (Table 3).

T. phaffii (= K. phaffii) and the three new species were similar in their morphological and physiological characters, but differed in the type of ascus deliquescence; in T. phaffii (= K. phaffii) and U97-101-2T (T. iriomotensis), ascii ruptured soon after formation; in the other six strains (T. nanseiei, T. arboricola), ascii were persistent. In 1965, van der Walt combined Kluyveromyces species with others on the basis that ascus deliquescence correlated better than spore number with other characteristics of the species (van der Walt, 1965). On this basis, U97-101-2T belongs to the genus Kluyveromyces and the other isolates to a different genus. However, T. phaffii (= K. phaffii) and the three new species displayed a particularly close genealogical affinity with each other, and the T. phaffii (= K. phaffii) cluster exhibited no specific association with any other species examined (Fig. 3).
in this study, \( K. \text{phaffii} \) and three new species clearly represent a novel taxonomic group equivalent to a new genus, \( Tetrapipsispora \) gen. nov.

A new proposal to rearrange the genus \( Kluyveromyces \) came from Naumov (1986, 1987), who reviewed some of the evidence published to that point and advocated (i) confining \( Kluyveromyces \) to the multispored species, (ii) reinstating the name \( Zygosaccharomyces \) for haplontic, four-spored species and (iii) creating a third genus for diplontic species, which at the time included only \( K. \text{phaffii} \). \( T. \text{phaffii} (= K. \text{phaffii}) \) was originally described as \( Fabospora \text{phaffii} \) by van der Walt (1963). Therefore, the third genus might be the genus \( Fabospora \), which was defined by Kudrjanzev (1960) as diplontic, four-spored species in which the ascii rupture on maturation. However, the new species, \( T. \text{naneiensis} \) and \( T. \text{arboricola} \), asci of which were persistent, were not assigned to the genus \( Fabospora \). The type species of \( Fabospora \) was not defined (Kudrjanzev, 1960); furthermore, all the other species, \( Fabospora \) \( macedoniensis \), \( Fabospora \) \( fragilis \) and \( Fabospora \) \( marxiana \), have been classified into \( Kluyveromyces \) \( marxianus \) (Lachance, 1998). To prevent taxonomic confusion, \( Fabospora \) should not be adopted as the genus of \( T. \text{phaffii} \) and three new species.

The results of mating studies (Johannsen, 1980), DNA reassociation analysis (Fuson et al., 1987), rDNA restriction mapping (Shen et al., 1994) and the rDNA analysis (Fig. 3) likewise indicate that \( K. \text{blattae} \) is distinct from other species of \( Kluyveromyces \). Cai et al. (1996) reported that the 18S rDNA sequence identity between \( T. \text{phaffii} (= K. \text{phaffii}) \) and \( K. \text{blattae} \) was 96·0±%; nevertheless, \( T. \text{phaffii} (= K. \text{phaffii}) \) was most similar to \( K. \text{varrowii} \) (90·7±%) and \( K. \text{blattae} \) was most similar to \( Zygosaccharomyces \) \( florentinus \) (95·6±%). The aforementioned results showed that \( T. \text{phaffii} (= K. \text{phaffii}) \) had a loose association with \( K. \text{blattae} \). The loose association of \( K. \text{blattae} \) with the \( Tetrapipsispora \) cluster was not statistically significant, as shown by the relatively low bootstrap value of 54±6% omitted from Fig. 3. In terms of morphological characters, \( K. \text{blattae} \) is also only distantly related to the genus \( Tetrapipsispora \). Therefore, \( K. \text{blattae} \) should not be included in the genus \( Tetrapipsispora \).

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REFERENCES


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