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.

Kumiko Ueda-Nishimura and Kozaburo Mikata

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 10929\(^1\)), *Tetrapisispora* nanseiensis gen. nov., sp. nov. (type strain IF0 10899\(^1\)) and *Tetrapisispora* arboricola gen. nov., sp. nov. (type strain IF0 10925\(^1\)), 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* lodderae, *Kluyveromyces* phaffii, *Kluyveromyces* polyosporus 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 phenetically 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. phafii* 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. phafii* should be excluded from the genus *Kluyveromyces*. No other *K. phafii* strain or phylogenetically related species has been isolated since the *K. phafii* type strain was isolated from South African soil (van der Walt, 1963). For this reason, perhaps, *K. phafii* has not been transferred to another genus.

In this study, three new species closely related to *K. phafii* were isolated from Iriomote 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, *Tetrupisispora* gen. nov., is proposed in this paper to accommodate *K. phafii* 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 a 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× 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 ethidium bromide 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'-ATCTGTTGTAGCTCCAGT-3') and 18-R (5'-GATCCTTCGGCAGGTTCACC-3'). Diluted PCR products were used as a template for sequencing and 5' FITC-labelled primers were 400F (5'-TCCGGAGAGGGAGCCTGAGAACCG-3'), 550F (5'-GACGCGCGGTTAATCCGAC-3'), 950F (5'-TCAAGACGAAAGTTAGGGG-3'), 1200F (5'-AAAGGAATGAGGAAGGGGC-3'), 1300F (5'-TTGGTGGAGTGATTGTGCTG-3'), 1500F (5'-GATGCCCTTAGACGTTCGG-3') and 18-F for the sense strand and 400R (5'-TTCCAGGCTCCCCCTCCTCCGG-3'), 550R (5'-GAATATCCGGCGGCGTGTGGG-3'), 950R (5'-TCCCTAATTCCTCTTCTTGG-3'), 1200R (5'-GGTCCCTTCGGTCAATTC-3'), 1300R (5'-AGACAAATCTCCTCCACACAC-3'), 1500R (5'-CCAGACGTCCTAAGGCGCATACAG-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 i arose 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 i 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. phafii*, isolated from South African soil (van der Walt, 1963): fermentation of glucose and galactose, growth on glucose, galactose and glycerol 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 (2.0–0.2 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</th>
<th>Original no.</th>
<th>IFO 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>
<td></td>
</tr>
<tr>
<td>U96-107-4T</td>
<td>10899T</td>
<td>Soil</td>
<td>Ishigaki Island</td>
<td></td>
</tr>
<tr>
<td>Yk-305a2</td>
<td>1883</td>
<td>Soil</td>
<td>Yaku Island</td>
<td></td>
</tr>
<tr>
<td>U97-412-1T</td>
<td>10925T</td>
<td>Flower of <em>Elaeocarpus decipiens</em></td>
<td>Iriomote Island</td>
<td></td>
</tr>
<tr>
<td>U97-412-3</td>
<td>10926</td>
<td>Flower of <em>Elaeocarpus decipiens</em></td>
<td>Iriomote Island</td>
<td></td>
</tr>
<tr>
<td>U97-436-2</td>
<td>10927</td>
<td>Leaf of <em>Bruguiera conjugata</em></td>
<td>Iriomote Island</td>
<td></td>
</tr>
<tr>
<td>U97-437-2</td>
<td>10928</td>
<td>Leaf of <em>Rhizophora mucronata</em></td>
<td>Iriomote Island</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Photomicrographs of vegetative cells on YPD broth (upper) and asci on potassium acetate agar (lower). (a) U97-101-2T (*T. iriomotensis* IFO 10929T), (b) U96-107-4T (*T. nanseiensis* IFO 10899T), (c) U97-412-1T (*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 *K. phaffii* and all other known *Kluyveromyces* and *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 *K. phaffii*. To survey species identities of isolates, their DNA
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**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 10926); 7, U97-436-2 (*T. arboricola* IFO 10927); 8, U97-437-2 (*T. arboricola* IFO 10928).

**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>

**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>–</td>
</tr>
<tr>
<td>2. U97-101-2T</td>
<td>10929T</td>
<td>8</td>
</tr>
<tr>
<td>3. U96-107-4T</td>
<td>10899T</td>
<td>9</td>
</tr>
<tr>
<td>4. Yk-305a2</td>
<td>1883</td>
<td>10</td>
</tr>
<tr>
<td>5. U97-412-1T</td>
<td>10925T</td>
<td>9</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>

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...
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 U97-412-1\(^T\), 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.si.spo’ra. G. pref. tetra four; L. n. pisi pea; L. fem. n. spora spore; M.L. n. *Tetrapisispora* four pea spores).
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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>Glucuronate</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 ellipsoidal, 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 diazotonic 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. [basisynom 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 μm, singulae aut binae, per gemmationem multilateralem reproducebantur. Cultura in agaro cum glucoso, peptono et extracto levidinis post unum mensem ad 24 °C, butyrosa, gliabra, nitida et eburnea. Pseudomycelium nullum. Asci per transformationem cellularum vegetatvarum diploidearum, 1–4 ascosporas continent. Ascosporae ovoideae aut reniformes. Ascosporae ex ascis liberantur et agglutinant.

Glucosum et galactosum fermentantur, at non L-sorbosum, maltosum, sucrosum, cellobiosum, trehalosum, lactosum, mellibusum, raffinosum nec d-xylosum. Glucosum, galactosum, glycerolum, d-glucono-1.5-lactosum et gluconicum assimilantur, at non L-sorbose, maltosum, sucrosum, cellobiosum, trehalosum, lactosum, mellibusum, raffinosum, meleztosum, 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.

Nitratus kalicus, ethylaminum, lysinum nec cadaverinum non assimilantur. Exiguum crescit sine vitaminis. Non crescer poetest in 0.01 % cycloheximido. Lente crescere poetest in 10% NaCl/5% glucosa. Augmentum in 34°C. Diazonium caeruleum B non respondens. G + C acidi 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.ri.o.moten'sis. L. adj. iriomotensis pertaining to Iriomote 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, meli-
Tetrapisispora gen. nov.

Tetrapisispora nanseiensis (nan.se.i.en’sis. L. adj. nan-seiensis 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-glucosono-1,5-lactone and galactose. No growth occurs on L-sorbose, maltose, sucrose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, starch, D-xylose, L-arabinose, D-ribosel, 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. 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 U97-101-2® was isolated in July 1997 from soil in Ootomi, Iriomote Island, Japan. Cultures of the type strain U97-101-2® (=IFO 10929®) have been deposited in the culture collection of the Institute for Fermentation, Osaka, Japan.

Latin diagnosis of Tetrapisispora nanseiensis sp. nov.

In medio liquidum cum glucosae, peptono et extracto levidinis post dies 2 ad 24 °C, cellulæ ovoidea vel cylindraciae, 1.0–3.0×3.0–6.0 μm, singulae aut binae, per gennationem multilateralem reproducentes. Cultura in agaro cum glucosae, peptono et extracto levidinis post unum mensem ad 24 °C, butyrosae, glabra, nitida et eburnea. Pseudomycelium nullum. Ascì per transformationem cellularum vegetatarvarum diploidearum, 1–4 ascospores continentes. Ascospores ovoidea. Ascì non rumpuntur.

Glucosum et galactosum fermentantur, at non l-sorbosum, maltosum, sucrosum, cellobiosum, trehalosum, lactosum, melibiosum, raffinosum nec d-xylosum. Glucosum, galactosum, glycerol, d-glucosono-1,5-lacton et gluconium assimilantur, at non l-sorbosum, maltosum, sucrosum, cellobiosum, trehalosum, lactosum, melibiosum, raffinosum, melezitosum, inulinsum, amyllum, d-xylosum, l-arabinosum, d-arabinosum, d-ribosum, l-rhamnosum, ethanolum, erythritolum, ribitolum, galactitolum, d-mannitolum, d-glucitol, α-methyl D-glucoside, salicinum, DL-lactatum, succinatum, citratum, inositolum, glucosaminum nec arbutinum.

Nitras 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% glucosae. Augmentum in 34 °C. Diazonium caerullanum B non respondens. G+C acidii deoxyribonucleatii 30 mol% per HPLC. Systema co-enzymatis Q-6 adest.

Typus depositus in collectione Institute for Fermentation, Osaka, Japonia (IFO 10899®).

Description of Tetrapisispora arboricola sp. nov.

In medio liquidum cum glucosae, peptono et extracto levidinis post dies 2 ad 24 °C, cellulæ ovoidea vel sphaericæ, 2.0–4.0×2.0–6.0 μm, singulae aut binae, per gennationem multilateralem reproducentes. Cultura in agaro cum glucosae, peptono et extracto levidinis post unum mensem ad 24 °C, butyrosae, glabra, nitida et eburnea. Pseudomycelium nullum. Ascì per transformationem cellularum vegetatarvarum diploidearum, 1–4 ascospores continentes. Ascospores ovoidea vel reniformes. Ascì non rumpuntur.

Glucosum et galactosum fermentantur, at non l-
sorbosum, maltosum, sucrosum, cellobiosum, trehalosum, lactosum, mellibiosum, raffinosum nec D-xylosyl. Glucosum, galactosum, trehalosum et glycerolum assimilantur, at non L-sorbosum, maltosum, sucrosum, cellobiosum, lactosum, mellibiosum, raffinosum, melezitosum, inulinum, amyllum, D-xylosyl, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, ethanolum, erythritolium, ribitolum, galactitolum, D-mannitollum, D-glucitolum, α-methyl D-glucosidum, salicinum, D-glucono-1,5-lactonum, acidum lacticum, acidium D-arabinozum, D-ribosum, a-rhamnosum, ethnoluum, ziti tosum, in din urn, amyllum.

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Nitras kalicus, ethylaminum, lysinum nee cadaverinum non assimilantur. Non crescit sine vitaminis. Non acidi deoxyribonucleati crescere potest in Osaka, in cultura potest in arboricola. After 1 month at 24°C, the 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 2.0-4.0 × 2.0-6.0 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 cornmeal agar. Asci are persistent. In 1965, van der Walt combined T. phafii (= K. phafii) and T. iriomoensis, asci ruptured soon after formation; in the other six strains (T. nanseiei, T. arboricola), ascis 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 and Yk-305a2, and more than two substitutions were found between strains of different species (Table 3).

T. phafii (= K. phafii) and the three new species were similar in their morphological and physiological characters, but differed in the type of ascus deliquescence; in T. phafii (= K. phafii) and U97-101-2T (T. iriomoensis), ascis ruptured soon after formation; in the other six strains (T. nanseiei, T. arboricola), ascis 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. phafii (= K. phafii) and the three new species displayed a particularly close genealogical affinity with each other, and the T. phafii (= K. phafii) cluster exhibited no specific association with any other species examined (Fig. 3).

On the basis of polyphasic taxonomic data presented
in this study. *K. phaffii* and three new species clearly represent a novel taxonomic group equivalent to a new genus, *Tetrapisispora* 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 multisporus species, (ii) reinstating the name *Zygofabospora* for haplontic, four-spored species and (iii) creating a third genus for diplontic species, which at the time included only *K. phaffii*. *T. phaffii (= K. phaffii)* was originally described as *Fabospora 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 asci rupture on maturation. However, the new species, *T. arboricola*, ascii 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. 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. blattae* is distinct from other species of *Kluyveromyces*. Cai et al. (1996) reported that the 18S rDNA sequence identity between *T. phaffii (= K. phaffii)* and *K. blattae* was 96-0%; nevertheless, *T. phaffii (= K. phaffii)* was most similar to *K. yarrowii* (97.0%) and *K. blattae* was most similar to *Zygosaccharomyces florentinus* (96.5%). The aforementioned results showed that *T. phaffii (= K. phaffii)* had a loose association with *K. blattae*. The loose association of *K. blattae* with the *Tetrapisispora* 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. blattae* is also only distantly related to the genus *Tetrapisispora*. Therefore, *K. blattae* should not be included in the genus *Tetrapisispora*.

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