**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 IFO 10929^T), *Tetrapisispora nanseiensis* gen. nov., sp. nov. (type strain IFO 10899^T) and *Tetrapisispora arboricola* gen. nov., sp. nov. (type strain IFO 10925^T), 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 polysporus* 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

Abbreviation: PFGE, pulsed-field gel electrophoresis.

The DDBJ accession numbers for the 18S rDNA sequences reported in this paper are AB016506–AB016509.

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 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, *Tetrapisispora* 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 × 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 mg ethidium bromide l⁻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'-ATCTGGTTGATCCTGCCAGT-3') and 18-R (5'-GATCCTTCCGCAGGTTCCACC-3'). Diluted PCR products were used as a template for sequencing and 5' FITC-labelled primers were 400F (5'-TCCGGAGAGGG-AGCCTGAGAAACG-3'), 550F (5'-GCAGCCGCGGT-AATTCCAGC-3'), 950F (5'-TCAAGAACGAAAGTTA-GGGG-3'), 1200F (5'-AAAGGAATTGACGGAAGG-GC-3'), 1300F (5'-TTGGTGGAGTGATTGTCTG-3'), 1500F (5'-GATGCCCTTAGACGTTCTGG-3') and 18-F for the sense strand and 400R (5'-TTCTCAGGCTCCC-TCTCCGG-3'), 550R (5'-GAATTACCGCGGCTGCT-GGC-3'), 950R (5'-TCCCCTAACTTTCGTTCTTG-3'), 1200R (5'-GGTGCCCTTCCGTCAATTCC-3'), 1300R (5'-AGACAACTACTCCACCAAC-3'), 1500R (5'-CCAGA-ACGTAAGGGCATCACAG-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, asci arose directly from diploid cells and one to four oval or reniform ascospores were formed. The spores of strain U97-101-2^T were liberated from the ascus soon after formation and tended to agglutinate, whereas in the other strains, asci were persistent (Fig. 1). As monosporic cultures could sporulate, they were judged to be homothallic. In their physiological characters, 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 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 U96-107-4^T from Ishigaki Island. Those of isolates U97-412-1^T, 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

Strain		Sample material	Locality
Original no.	IFO no.		
U97-101-2 ^T	10929 ^T	Soil	Iriomote Island
U96-107-4 ^T	10899 ^T	Soil	Ishigaki Island
Yk-305a2	1883	Soil	Yaku Island
U97-412-1 ^T	10925 ^T	Flower of <i>Elaeocarpus decipiens</i>	Iriomote Island
U97-412-3	10926	Flower of <i>Elaeocarpus decipiens</i>	Iriomote Island
U97-436-2	10927	Leaf of <i>Bruguiera conjugata</i>	Iriomote Island
U97-437-2	10928	Leaf of <i>Rhizophora mucronata</i>	Iriomote Island

Fig. 1. Photomicrographs of vegetative cells on YPD broth (upper) and asci on potassium acetate agar (lower). (a) U97-101-2^T (*T. iriomotensis* IFO 10929^T), (b) U96-107-4^T (*T. nanseiensis* IFO 10899^T), (c) U97-412-1^T (*T. arboricola* IFO 10925^T). Bars, 5 μm.

U97-412-1^T 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 U97-412-1^T, suggesting that these isolates were related but not identical. The pattern of U97-101-2^T 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

Fig. 2. PFGE analysis of *K. phaffii* and isolates. Lanes: m, *S. cerevisiae* SH 964 as molecular size marker; 1, *K. phaffii* IFO 1672^T; 2, U97-101-2^T (*T. iriomotensis* IFO 10929^T); 3, Yk-305a2 (*T. nanseiensis* IFO 1883); 4, U96-107-4^T (*T. nanseiensis* IFO 10899^T); 5, U97-412-1^T (*T. arboricola* IFO 10925^T); 6, U97-412-3 (*T. arboricola* IFO 10926); 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 1672^T and isolate U97-101-2^T showed low simi-

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.

Strain	IFO no.	Identities (%) and numbers of substitutions				
		1	2	3	4	5
1. <i>K. phaffii</i>	1672 ^T	—	99.5	99.5	99.4	99.5
2. U97-101-2 ^T	10929 ^T	8	—	99.5	99.4	99.4
3. U96-107-4 ^T	10899 ^T	9	9	—	99.9	99.8
4. Yk-305a2	1883	10	10	1	—	99.8
5. U97-412-1 ^T	10925 ^T	9	10	3	4	—
6. U97-436-2	10927	9	10	3	4	0
7. U97-437-2	10928	9	10	3	4	0

larity to all tested strains. DNA similarity values of U96-107-4^T and Yk-305a2 were 79.2 and 97.0% and those of U97-412-1^T to U97-412-3, U97-436-2 and U97-437-2 were 99.7, 105.2 and 116.6%. These data showed that U96-107-4^T and Yk-305a2 should be considered to be conspecific, as should U97-412-1^T, 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 U96-107-4^T from Ishigaki Island and Yk-305a2 from Yaku Island were the same species, a substitution was found between the

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.

Strain	IFO no.	G + C content (mol%)	DNA similarity (%)				
			1	2	3	4	5
1. <i>K. phaffii</i>	1672 ^T	32.7	100	15.6	17.9	26.9	32.6
2. U97-101-2 ^T	10929 ^T	32.3	33.2	100	24.3	35.0	30.5
3. U96-107-4 ^T	10899 ^T	30.4	29.4	18.8	100	97.0	29.9
4. Yk-305a2	1883	31.2	21.6	17.8	79.2	100	20.5
5. U97-412-1 ^T	10925 ^T	31.2	34.2	25.4	33.3	40.1	100
6. U97-412-3	10926	31.4	ND	24.1	ND	ND	99.7
7. U97-436-2	10927	32.3	ND	36.7	ND	ND	105.2
8. U97-437-2	10928	32.0	ND	37.7	ND	ND	116.6
9. <i>S. cerevisiae</i>	10217 ^T	38.0	ND	19.1	ND	ND	29.7

Fig. 3. Phylogenetic tree showing the relationship of the strains isolated to species of the genera *Kluyveromyces*, *Saccharomyces*, *Zygosaccharomyces* and *Torulaspora*. The tree is based on 18S rDNA sequence data and was constructed by using the neighbour-joining method. Bootstrap values were calculated from 1000 trees and values < 60% were omitted. GenBank, EMBL and DDBJ accession numbers are shown in parentheses. Bar, sequence dissimilarity of 0.25%. The outgroup species was *Candida albicans*.

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.

Genus ad Saccharomycetaceae pertinet. Cellulae ellipsoideae, ovoideae vel cylindricae, per gemmationem multilateralem reproducentes. Pseudomycelium nullum. Genus homothallicum et diploideum. Asci per transformationem cellularum vegetativarum diploidearum et rumpuntur aut non rumpuntur, 1–4 ascosporas continentes. Ascosporae ovoidae aut reniformes. Nitras kalicus, ethylaminum, lysinum nec cadaverinum non assimilantur. Diazonium caeruleum B non respondens.

Systema coenzymatis Q-6 adest. G+C acidi deoxyribonucleati 30–33 mol%. Species typica: Tetrapisispora phaffii (van der Walt) Ueda-Nishimura et Mikata comb. nov.

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

Tetrapisispora (Te.trä.pi.si.spo'ra. 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

+s, Slow growth; +w, weak growth.

Characteristic	<i>T. phaffii</i>	<i>T. iriomotensis</i>	<i>T. nanseiensis</i>	<i>T. arboricola</i>
Assimilation of:				
Trehalose	—	—	—	+
Gluconate	+	+	+	—/+
Growth on vitamin-free medium	—	+w	—	—
Growth on 0.01 % cycloheximide	—	—	+s/—	—
Growth on 10 % NaCl + 5 % glucose	—	+s	—	+s
Ascus deliquescence	+	+	—	—

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. [basonym *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 4417^T (= IFO 1672^T, = ATCC 24235^T, = NRRL Y-8282^T), 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 ellipsoideae, 2.0–5.0 × 5.0–8.0 µm, singulae aut binae, per gemmationem multilateralem reproducentes. Cultura in agar cum glucoso, peptono et extracto levidinis post unum mensem ad 24 °C, butyrosa, glabra, nitida et eburnea. Pseudomycelium nullum. Asci per transformationem cellularum vegetativarum diploidearum, 1–4 ascosporas continentes. Ascosporae ovoidae aut reniformes. Ascosporae ex ascis liberantur et agglutinant.

Glucosum et galactosum fermentantur, at non L-sorbosum, maltosum, sucrosum, cellobiosum, trehalo-

sum, lactosum, mellibiosum, raffinsum nec D-xylosum. Glucosum, galactosum, glycerolum, D-glucono-1,5-lactonum et gluconicum assimilantur, at non L-sorbosum, maltosum, sucrosum, cellobiosum, trehalosum, lactosum, mellibiosum, raffinsum, melezitolum, inulinum, amylum, D-xylosum, L-arabiosum, D-arabiosum, D-ribosum, L-rhamnosum, ethanolum, erythritolum, ribitololum, galactitololum, D-mannitololum, D-glucitololum, α-methyl D-glucosidum, salicinum, acidum lacticum, acidum succinicum, acidum citricum, inositololum, 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 % glucoso. Augmentum in 34 °C. Diazonium caerulian 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 10929^T).

Description of *Tetrapisispora iriomotensis* sp. nov.

Tetrapisispora iriomotensis (i.ri.o.mo.ten'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 Dalmau 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-

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

Latin diagnosis of *Tetrapisispora nanseiensis* sp. nov.

In medio liquid cum glucoso, peptono et extracto levidinis post dies 2 ad 24 °C, cellulae ovoideae vel cylindricae, 1.0–3.0 × 3.0–6.0 µm, singulae aut binae, per gemmationem multilateralem reproducentes. Cultura in agar cum glucoso, peptono et extracto levidinis post unum mensem ad 24 °C, butyrosa, glabra, nitida et eburnea. Pseudomycelium nullum. Asci per transformationem cellularum vegetativarum diploidearum, 1–4 ascoporas continentes. Ascosporae ovoideae. Asci non rumpuntur.

Glucosum et galactosum fermentantur, at non L-sorbosum, maltosum, sucrosum, cellobiosum, trehalosum, lactosum, melibiosum, raffinsum nec D-xylosum. Glucosum, galactosum, glycerolum, D-glucono-1,5-lactonum et gluconicum assimilantur, at non L-sorbosum, maltosum, sucrosum, cellobiosum, trehalosum, lactosum, melibiosum, raffinsum, melezitosum, inulinum, amylum, D-xylosum, L-arabiosum, D-arabiosum, 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. Non crescit sine vitaminis. Exiguum crescere potest in 0.01% cycloheximido. Non crescere potest in 10% NaCl/5% glucoso. Augmentum in 34 °C. Diazonium caerulian 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 10899^T).

Description of *Tetrapisispora nanseiensis* sp. nov.

Tetrapisispora nanseiensis (nan.se.i.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. No 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 U96-107-4^T (= IFO 10899^T) 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 U96-107-4^T (= IFO 10899^T) 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 gemmationem multilateralem reproducentes. Cultura in agar cum glucoso, peptono et extracto levidinis post unum mensem ad 24 °C, butyrosa, glabra, nitida et eburnea. Pseudomycelium nullum. Asci per transformationem cellularum vegetativarum diploidearum, 1–4 ascoporas continentes. Ascosporae ovoideae vel reniformes. Asci non rumpuntur.

Glucosum et galactosum fermentantur, at non L-

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

Nitras kalicus, *ethylaminum*, *lysinum* nec *cadaverinum* non assimilantur. Non crescit sine vitaminis. Non crescere potest in 0.01% cycloheximido. Exiguum crescere potest in 10% NaCl/5% glucoso. Augmentum in 34 °C. Diazonium caeruleum B non respondens. G + C *acidi deoxyribonucleati* 31 mol% per HPLC. *Systema coenzymatis* Q-6 adest.

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

Description of *Tetrapisispora arboricola* sp. nov.

Tetrapisispora arboricola (ar.bo.ri'co.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 2.0–4.0 × 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. 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. 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, trehalose and glycerol. No growth occurs on *L*-sorbose, maltose, sucrose, cellobiose, 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, *D*-glucono-1,5-lactone, *DL*-lactate, succinate, citrate, inositol, gluconate, glucosamine or arbutin. The following nitrogen compounds are not assimilated: nitrate, ethylamine, lysine and cadaverine. No 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 31 mol% by HPLC. Ubiquinone system is Q-6.

Strains U97-412-1^T (= IFO 10925^T) and U97-412-3 (= IFO 10926), from flowers of *Elaeocarpus decipiens*, U97-436-2 (= IFO 10927), from a leaf of *Bruguiera 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 U97-412-1^T (= IFO 10925^T) 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: U96-107-4^T and Yk-305a2, and U97-412-1^T, U97-412-3, U97-436-2 and U97-437-2. The 18S rDNA sequences showed no more than one substitution between conspecific strains U96-107-4^T 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 U97-101-2^T (*T. iriomotensis*), asci ruptured soon after formation; in the other six strains (*T. nanseieis*, *T. arboricola*), asci 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, U97-101-2^T 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).

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 multispored 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. nanseiensis* and *T. arboricola*, asci of which were persistent, were not assigned to the genus *Fabospora*. The type species of genus *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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