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Kluyveromyces nonfermentans sp. nov., a new yeast species isolated from the deep sea

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Eleven strains of a new species of the genus *Kluyveromyces*, characterized as having evanescent asci and Q-6 as the major ubiquinone, were isolated from sediments, a clam and a crab collected at depths of 1000–2000 m in Suruga Bay and Sagami Bay, Japan. A phylogenetic tree based on small-subunit (18S) rRNA gene sequences placed these isolates into a cluster of *Kluyveromyces*. DNA complementarity and phylogenetic trees of internal transcribed spacer (ITS) regions and 5·8S rRNA genes showed that the isolates are closely related to *Kluyveromyces aestuarii*, but that these two species are genetically distinct. The isolates are described as *Kluyveromyces nonfermentans* sp. nov. Because this species lacks the fermentative ability considered to be an important criterion for the genus *Kluyveromyces*, the definition of the genus has been emended. The type strain of *K. nonfermentans* is strain SY-33^T (= JCM 10232^T).

Keywords: *Kluyveromyces nonfermentans* sp. nov., 18S rDNA, 5·8S rDNA, internal transcribed spacer, deep-sea isolates

INTRODUCTION

In the past, yeasts in marine environments have been investigated by many microbiologists and some of these have been recognized as new species (e.g. Rodrigues de Miranda & Norkrans, 1968; Fell & Statzell, 1971). Difficulties involved in collecting muds or organisms from deep-sea environments have limited most research to the shallower areas of the sea (Fell, 1976). We have isolated a number of micro-organisms from deep-sea samples obtained by the manned submersibles '*Shinkai* 2000' and '*Shinkai* 6500' and the unmanned submersible '*Kaiko*' (Kato *et al.*, 1996; Takami *et al.*, 1997). Many yeasts were present among these microbes, including strains tolerant of organic solvents (Fukumaki *et al.*, 1994). We examined more than 100 yeast strains isolated from sediments and benthic organisms obtained from deep-sea environments and found 11 strains of a previously unknown species of the genus *Kluyveromyces*, described in this paper as *Kluyveromyces nonfermentans* sp. nov.

Abbreviation: ITS, internal transcribed spacer.

The DDBJ accession numbers for the 18S rDNA sequences reported in this paper are AB011507–AB011510, AB011512–AB011521 and AB012264.

METHODS

Isolation. Yeasts were isolated from frozen deep-sea samples on YM agar (Difco) dissolved in artificial seawater (3% NaCl, 0·07% KCl, 1·08% $MgCl_2.6H_2O$, 0·54% $MgSO_4.7H_2O$, 0·1% $CaCl_2.2H_2O$) supplemented with 0·01% chloramphenicol and 0·002% streptomycin. The agar plates were incubated at a low temperature (5–10 °C) for the first 2 weeks and then at 20 °C for 1 month. All strains were grown at 25 °C in YM broth (Difco) or on YM agar (Difco) for purification and cultivation. The strains used in this study are listed in Table 1.

Physiological and biochemical characteristics. Strains were characterized morphologically and physiologically by standard methods with some modifications (van der Walt & Yarrow, 1984). Assimilation of nitrogen compounds was examined on solid media using a starved inoculum (Nakase & Suzuki, 1986). The vitamin requirements were investigated according to the methods of Komagata & Nakase (1967). Ubiquinones were extracted by the method of Yamada & Kondo (1973) with slight modifications and analysed by HPLC (Tamaoka *et al.*, 1983).

Nucleic acid analyses. DNA extraction and purification for DNA base composition and DNA reassociation analyses were performed by the procedure of Hamamoto & Nakase (1995) with slight modifications. DNA base composition was determined by the HPLC method of Tamaoka & Komagata (1984). DNA–DNA hybridization experiments

Table 1. Strains used in this study

Species	Strain	Isolate no.	Source	Locality	Accession no.
K. nonfermentans	JCM 10226	SY-05	Sediment at depth of 1200–1977 m	Suruga or Sagami Bay	–
	JCM 10227	SY-19	Sediment at depth of 1200–1977 m	Suruga or Sagami Bay	AB011507
	JCM 10228	SY-20	Sediment at depth of 1200–1977 m	Suruga or Sagami Bay	–
	JCM 10229	SY-26	Sediment at depth of 1200–1977 m	Suruga or Sagami Bay	–
	JCM 10230	SY-28	Sediment at depth of 1200–1977 m	Suruga or Sagami Bay	AB011508
	JCM 10231	SY-29	Sediment at depth of 1200–1977 m	Suruga or Sagami Bay	AB011509
	JCM 10232^T	SY-33^T	Sediment at depth of 1200–1977 m	Suruga or Sagami Bay	AB012264
	JCM 10233	SY-54	Sediment at depth of 1143 m	Suruga Bay	–
	JCM 10234	SY-56	Crab at depth of 1182 m	Suruga Bay	AB011510
	JCM 10235	SY-63	*Calyptogena* sp. at depth of 1156 m	Suruga Bay	–
	JCM 10236	SY-64	*Calyptogena* sp. at depth of 1156 m	Suruga Bay	AB011512
K. aestuarii	IFO 10597^T	–	CBS 4438^T	–	AB011513
K. dobzhanskii	IFO 10603^T	–	CBS 2104^T	–	AB011514
K. lactis	IFO 1090^T	–	NCYC 416^T	–	AB011515
	JCM 6846	–	NCYC 1548	–	AB011516
	JCM 9563	–	IFO 0648	–	AB011517
K. marxianus	IFO 10005^T	–	CBS 712^T	–	AB011518
	JCM 1630	–	IAM 12237	–	AB011519
	JCM 2188	–	IAM 12830	–	AB011520
K. wickerhamii	IFO 1675^T	–	CBS 2745^T	–	AB011521

were performed using DNA-fixed microplates, by the procedures described by Ezaki *et al.* (1989).

Phylogenetic analysis. DNA extraction for PCR was performed as described below. One loop of yeast culture was suspended in extraction buffer (200 mM Tris/HCl, pH 8·5, 250 mM NaCl, 25 mM EDTA, 0·5% SDS) and ground using a pellet mixer (Tref). The nucleic acids to be used as a template for PCR were extracted by phenol/chloroform treatment and propan-2-ol precipitation. The primers used for amplification and sequencing of 18S rRNA, 5·8S rRNA and the internal transcribed spacer (ITS) region were those described by White *et al.* (1990). The PCR products were purified by means of Suprec TM-02 (Takara) and sequenced using a LI-COR DNA sequencer model 4000L. All sequences were aligned using CLUSTAL W 1.75 (Thompson *et al.*, 1994) and were adjusted manually. Positions where one or more species contained a length mutation and where the sequences were unalignable were not included in the distance analysis. The evolutionary distances were calculated using the PHYLIP 3.57c program DNADIST (Felsenstein, 1995) with Kimura's two-parameter model and the trees were constructed in NEIGHBOR by the neighbour-joining method (Saitou & Nei, 1987). The robustness of branches in the tree was evaluated by performing a bootstrap analysis (Felsenstein, 1985) with 1000 replicates.

The sequences determined were deposited in the DDBJ database under the accession numbers shown in Table 1.

RESULTS AND DISCUSSION

Eleven strains isolated from sediments, a giant white clam (*Calyptogena* sp.) and an unidentified crab collected at depths of 1000–2000 m in Suruga Bay or Sagami Bay, Japan, were identified as species of the genus *Kluyveromyces* on the basis of formation of evanescent asci (Fig. 1) and having Q-6 as the major ubiquinone. The morphological, physiological and biochemical characteristics of these 11 isolates were nearly identical but, on the basis of heterogeneity in assimilation of three carbon compounds, cellobiose, salicin and ribitol, four sub-groups were recognized (SY-05, SY-20, SY-26, SY-28 and SY-29; SY-54, SY-56, SY-63 and SY-64; SY-19; and SY-33^T).

Phylogenetic analysis

We determined the 18S rRNA gene sequences of five isolates, SY-19, SY-28, SY-29, SY-33^T and SY-56, to establish their taxonomic placement within the genus *Kluyveromyces*, which is recognized as a relatively large genus consisting of 15 species. The five sequences were completely identical and were aligned with the published sequences of 33 ascomycetous yeasts, all of which contained Q-6 as the major ubiquinone, with the exception of *Holleya sinecauda*, which has Q-9, and *Debaryomyces hansenii* var. *hansenii* as an outgroup. A phylogenetic tree constructed by the neighbour-joining method (Saitou & Nei, 1987) on the basis of the 1636 positions alignable in all species is shown in Fig. 2. Our tree topology was consistent with the upper part of that previously reported (Cai *et al.*, 1996), except for some differences in branching with low confidence values of bootstrapping, consistent with the knowledge that the genus *Kluyveromyces* is a polyphyletic group (Cai *et al.*, 1996). The five isolates, SY-19, SY-28, SY-29, SY-33^T and SY-56, were precisely placed in cluster 2 described by Cai *et al.* (1996), comprising *Kluyveromyces aestuarii*, *Kluyveromyces dobzhanskii*, *Kluyveromyces lactis*, *Kluyveromyces marxianus* and

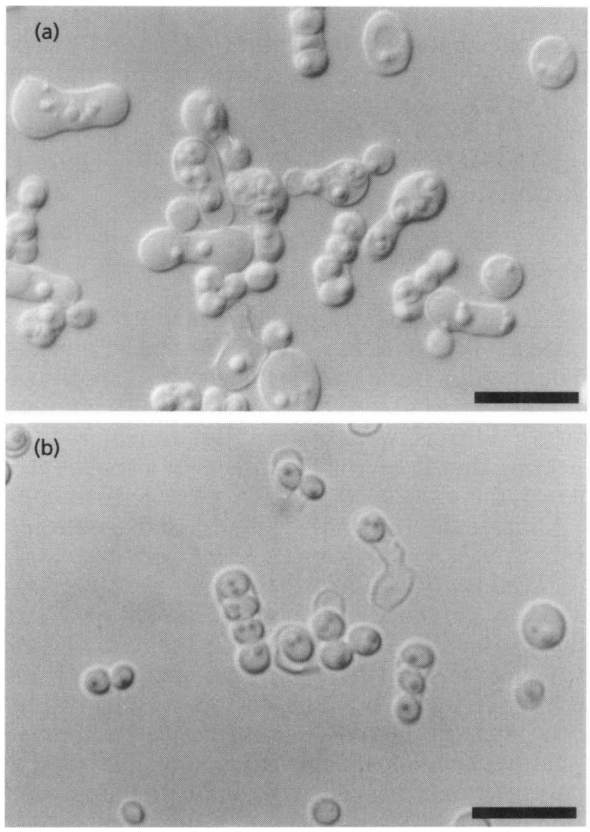

Fig. 1. Photomicrographs of *K. nonfermentans* SY-33^T (= JCM 10232^T) on YM agar. (a) Vegetative cells and asci after 3 d at 25 °C. (b) Disrupted asci after 3 weeks at 25 °C. Bars, 10 μm.

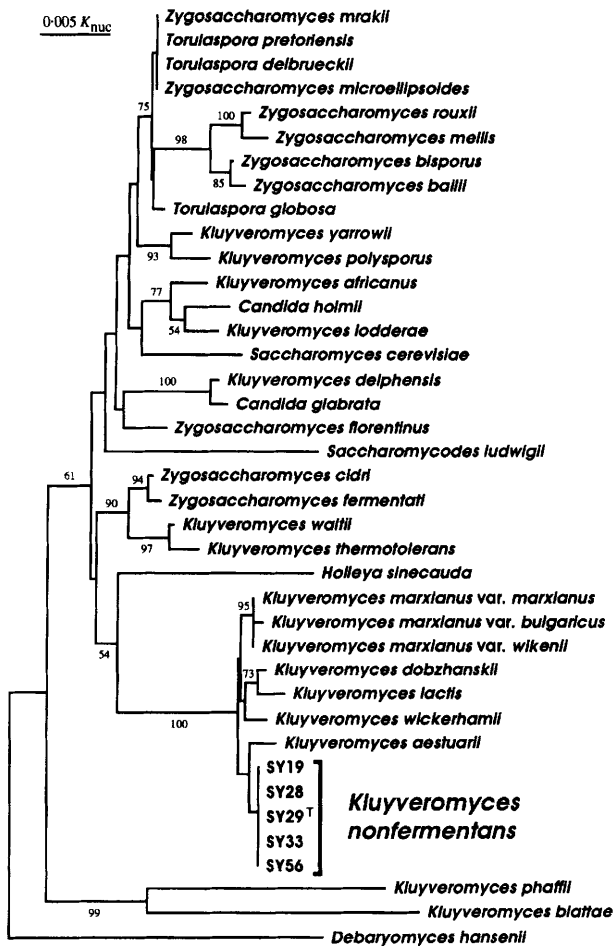

Fig. 2. Phylogenetic positions of five *K. nonfermentans* isolates, inferred from 18S rRNA gene sequences. The tree was constructed as described in the text. Numbers by nodes are bootstrap frequencies (values <50% not shown) derived from 1000 replicates. The accession numbers of reference sequences used in this tree are: *Z. mrakii*, X90757; *T. pretoriensis*, X84638; *T. delbrueckii*, X53496; *Z. microellipsoides*, X90756; *Z. rouxii*, X90758; *Z. mellis*, X90755; *Z. bisporus*, X91084; *Z. bailii*, X91083; *T. globosa*, X84639; *K. yarrowii*, X89528; *K. polysporus*, X83825; *K. africanus*, X89519; *C. holmii*, X78601; *K. lodderae*, X83824; *S. cerevisiae*, M27607; *K. delphensis*, X83823; *C. glabrata*, X51831; *Z. florentinus*, X91086; *S. ludwigii*, X69843; *Z. cidri*, X91085; *Z. fermentati*, X77930; *K. waltii*, X89527; *K. thermotolerans*, X89526; *H. sinecauda*, U53443; *K. marxianus* var. *marxianus*, X89523; *K. marxianus* var. *bulgaricus*, X89524; *K. marxianus* var. *wikenii*, X89522; *K. dobzhanskii*, X83822; *K. lactis*, X51830; *K. wickerhamii*, X83826; *K. aestuarii*, X89520; *K. phaffii*, X89525; *K. blattae*, X89521; and *D. hansenii*, X58053.

Kluyveromyces wickerhamii, closer to *K. aestuarii* than to the other species in this cluster. Cluster 2, including the five isolates, was clearly distinguished from the others with a significant level (100%) of bootstrap confidence, in agreement with the previous report (Cai *et al.*, 1996).

To clarify the relationships within cluster 2 further, we determined and analysed the sequences of the ITS region and the 5·8S rRNA gene of 15 strains, i.e. the type strains of *K. aestuarii*, *K. dobzhanskii* and *K. wickerhamii*, three strains of *K. marxianus*, three strains of *K. lactis* and six isolates, SY-19, SY-28, SY-29, SY-33^T, SY-56 and SY-64, selected from the four subgroups recognized on the basis of the results of assimilation tests. Because the ITS region displays a high substitution rate relative to the 18S and 26S rRNA genes, it has been found to be useful in resolving relationships between close taxonomic relatives (Berbee *et al.*, 1995; Waalwijk *et al.*, 1996; James *et al.*, 1996; Oda *et al.*, 1997). Phylogenetic trees based on ITS1–5·8S rDNA–ITS2, ITS1 and ITS2 are shown in Figs 3(a), (b) and (c), respectively. No intraspecific variation in ITS sequences was evident among the six isolates or among three strains of *K. marxianus*, in contrast to three strains of *K. lactis*. All trees successfully placed the 15 strains examined here into each species, but differences in tree topologies were evident due to ambiguity with respect to the branching point of *K. dobzhanskii*. The SY strains were placed closer to *K. aestuarii* in these trees, in agreement with the placement observed in the 18S-based tree (Fig. 2). Evolutionary distances between species determined on the basis of ITS1 were longer than those obtained on the basis of ITS2, whereas a remarkably long branch between the *K. aestuarii*–SY strains cluster and the other cluster was obtained on the basis of ITS2. Also, two of four

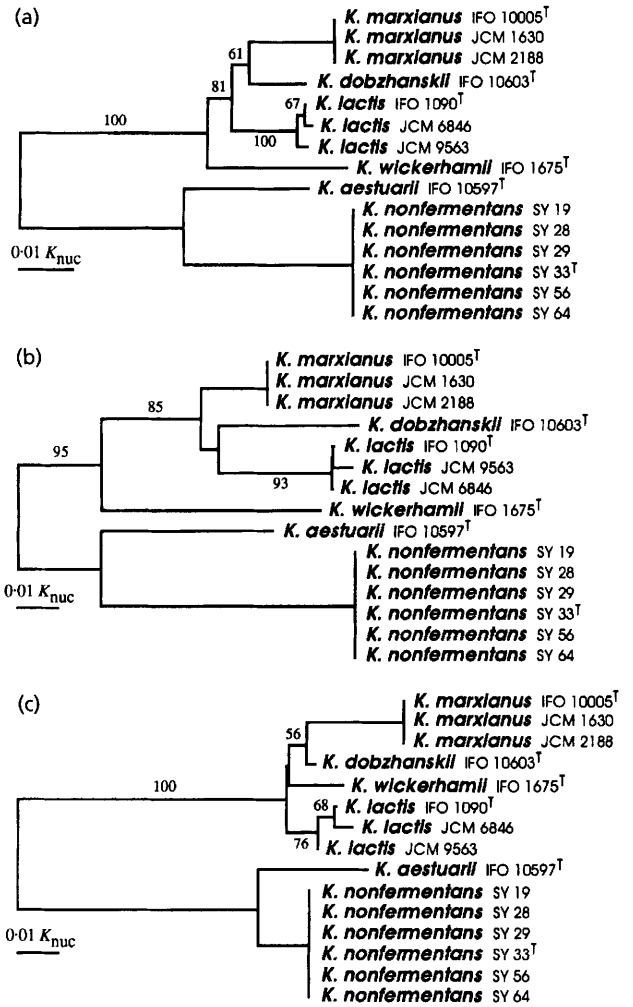

Fig. 3. Phylogenetic relationships among strains of *K. nonfermentans* and related species. The trees were constructed from the evolutionary distance data for ITS1–5·8S rDNA–ITS2 (a), ITS1 (b) and ITS2 (c), as described in the text. Numbers by nodes are bootstrap frequencies (values <50% not shown) derived from 1000 replicates.

substitutions in the 5·8S rRNA gene occurred within the branch that separated the *K. aestuarii*–SY strains cluster from the others. The remaining substitutions in the 5·8S rRNA gene were within the branches that led to both the SY strains and *K. aestuarii*. The variations in the 5·8S rRNA gene could be useful as signatures for differentiation of the three groups.

DNA–DNA reassociation

DNA–DNA reassociation experiments were performed at 40 °C among strains identified within cluster 2 in our phylogenetic analysis. The results are shown in Table 2. The relative DNA binding values among the four SY strains were within the range 91–107%, indicating that these were members of a single species, whereas the values of 22–52% obtained for DNA–DNA reassociation among SY-33^T and six strains of *K. marxianus*, *K. dobzhanskii*, *K. lactis*, *K. wickerhamii* and *K. aestuarii* confirmed that the SY strains were genetically unrelated to previously known species. The highest value of 52% obtained in the case of DNA–DNA reassociation between *K. aestuarii* IFO 10597 and SY-33^T was consistent with the results of phylogenetic analysis. The correspondence between the ITS-based trees and the values obtained in the DNA–DNA reassociation experiments may indicate the possibility of employing ITS sequences as a taxonomic tool for delineating species.

Genus *Kluyveromyces* van der Walt emend. Nagahama, Hamamoto, Nakase *et* Horikoshi

Asexual reproduction is by multilateral budding on a narrow base. Cells are ovoid, ellipsoid, cylindrical to elongate. Pseudomycelium may be formed. True hyphae are not produced. Conjugation may or may not precede ascus formation. The ascospores are smooth, reniform, bacilliform, ellipsoidal or spheroidal, tending to agglutinate after liberation. One to

Table 2. Levels of DNA complementarity among strains of *K. nonfermentans* and related species

Species	Strain	Relative binding (%) to DNA from:									
		1	2	3	4	5	6	7	8	9	10
1. *K. nonfermentans*	SY-19	100	97	95	93	28	–	–	–	–	–
2. *K. nonfermentans*	SY-28	93	100	97	91	30	–	–	–	–	–
3. *K. nonfermentans*	SY-56	101	107	100	96	28	–	–	–	–	–
4. *K. nonfermentans*	SY-33^T	102	105	105	100	42	22	41	25	38	52
5. *K. marxianus*	JCM 1630	43	38	38	41	100	100	57	41	37	39
6. *K. marxianus*	IFO 10005^T	–	–	–	37	101	100	44	42	22	33
7. *K. dobzhanskii*	IFO 10603^T	–	–	–	32	57	48	100	53	41	41
8. *K. lactis*	IFO 1090^T	–	–	–	36	49	44	57	100	19	34
9. *K. wickerhamii*	IFO 1675^T	–	–	–	26	40	31	39	24	100	27
10. *K. aestuarii*	IFO 10597^T	–	–	–	46	38	32	34	36	21	100

Table 3. Physiological and biochemical characteristics that differentiate *K. nonfermentans* from related species

v, Variable; w, weak.

Characteristic	*K. nonfermentans*	*K. aestuarii*	*K. dobzhanskii*	*K. lactis*	*K. marxianus*	*K. wickerhamii*
Assimilation of:						
Saccharose	−	+	+	+	+	+
Glycerol	−	+	+	+	v	+
Lactic acid	−	+	+	+	+	+
Succinic acid	−	+	+	+	+	+
Fermentation of D-glucose	−	+	+	v	+	+
Growth in the absence of thiamin	−/w	+	+	+	+	+
DNA G+C content (mol%)	35·8–37·4	39·5–39·9	42·6	39·9–40·7	40·8–41·5	40·0–42·4

four or, in some species, many ascospores are formed per ascus. Fermentation is usually present. Nitrate is not assimilated. Diazonium blue B reaction is negative. This description is based on that of Lachance (1998).

Latin diagnosis of *Kluyveromyces nonfermentans* sp. nov.

In medio liquido post dies 3 *cellulae vegetativae spheroideae vel ellipsoideae* (2·0–6·5 × 2·0–7·5 μm), *singlae vel binae. Post unum mensem sedimentum formatur. Cultura in agaro* YM, *infimo-convexa, glabra vel verruculosa, pauro hebia, cremea vel glauco-cremea, butyrosa et margine glabra, undulata vel lobiforma. Hyphae et pseudohyphae non formantur. Post dies* 3 *in agaro* YM *aut malti* (5%), *asci formantur, evanescentes post dies* 14. *Asci conjugati, habentes* 1–4, *sporos spheroides. Fermentatio nulla. Galactosum, cellobiosum* (*varium*), *lactosum* (*vel exiguum*), *ethanolum,* D-*mannitolum et salicinum* (*varium*) *assimilantur, at non* L-*sorbosum, saccharosum, maltosum, trehalosum, melibiosum, raffinosum, melezitosum, inulinum, amylum solubile,* D-*xylosum,* L-*arabinosum,* D-*arabinosum,* D-*ribosum,* L-*rhamnosum, glycerolum, erythritolum, ribitolum* (*varium*), *galactitolum,* D-*glucitolum, methyl* α-D-*glucosidum, glucono-δ-lactonum, acidum* 2-*keto-gluconicum, acidum* 5-*ketogluconicum, acidum* DL-*lacticum* (*aut exiguum*), *acidum succinicum, acidum citricum, inositolum, acidum* D-*glucuronicum nec acidum* D-*galacturonicum. Ethylaminum, lysinum et cadaverinum assimilantur at non kalium nitricum nec natrium nitricum. Ad crescentiam biotinum, niacinum et thiaminum necessarium sunt.* G+C *acidi deoxyribo-nucleati* 35·8–37·4 mol%. *Ubiquinonum majus* Q-6. *Typus stirps* SY-33 *ex luta,* Suruga Bay, Japan, *isolata est. In collectionibus culturarum quas* Japan Collection of Microorganisms, Wako, Saitama *sustentant, no.* JCM 10232 *deposita est.*

Description of *Kluyveromyces nonfermentans* sp. nov.

Kluyveromyces nonfermentans (non.fer.men'tans. L. adj. *nonfermentans* not causing fermentation).

After 3 d at 25 °C in YM broth (Difco), the cells are spheroidal to ellipsoidal (2·0–6·5 × 2·0–7·5 μm) and occur singly or in parent–bud pairs. A sediment is formed after 1 month. After 1 month at 25 °C on YM agar, streak cultures are low-convex, smooth or verrucose, somewhat dull, cream to greyish-cream and butyrous. The margin is smooth, undulating to lobiform. Branching hyphae or pseudohyphae are not formed in Dalmau plate cultures on cornmeal agar (Difco). Asci, usually formed with conjugation, are evanescent and form one to four spheroidal ascospores. Sporulation is abundant on 5% malt extract (Difco) or YM agar after 2–3 d at 25 °C. Fermentative ability is negative. The following carbon compounds are assimilated: D-glucose, galactose, cellobiose (variable), lactose (or weak), ethanol, D-mannitol and salicin (variable). No growth occurs on L-sorbose, saccharose, maltose, trehalose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, glycerol, erythritol, ribitol (variable), galactitol, D-glucitol, methyl α-D-glucoside, glucono-δ-lactone, 2-ketogluconic acid, 5-ketogluconic acid, lactic acid (or weak), succinic acid, citric acid, inositol, D-glucuronic acid or D-galacturonic acid. The following nitrogen compounds are assimilated: ethylamine, lysine and cadaverine. No growth occurs on sodium nitrate or sodium nitrite. The vitamins required are biotin (no growth or weak), niacin and thiamin (no growth or weak). Growth at 37 °C is variable. No growth occurs on 50% glucose–yeast extract agar. Growth occurs on YM agar containing 10% NaCl. No growth occurs in the presence of 100 p.p.m. cycloheximide. Starch-like substances are not produced. Gelatin liquefaction is negative. Splitting of fat is negative. The diazonium blue B reaction is negative. Urease activity is negative. The major ubiquinone is Q-6. The G+C content of the nuclear DNA is 35·8–37·4 mol%. The type strain of *Kluyveromyces nonfermentans*, strain SY-33^T, was isolated from deep-sea mud in Suruga Bay or Sagami Bay, Japan. This strain has been deposited in the Japan Collection of Microorganisms (JCM), the Institute of Physical and Chemical Research (RIKEN), Wako, Saitama, Japan, as strain JCM 10232^T. The other 10

strains examined, SY-05, SY-19, SY-20, SY-26, SY-28, SY-29, SY-54, SY-56, SY-63 and SY-64, are also deposited in the JCM, as JCM 10226, JCM 10227, JCM 10228, JCM 10229, JCM 10230, JCM 10231, JCM 10233, JCM 10234, JCM 10235 and JCM 10236, respectively.

Additional information

The name *Kluyveromyces nonfermentans* sp. nov. is proposed for the 11 non-fermenting strains described here in consideration of their remarkable lack of fermentative ability in comparison with other *Kluyveromyces* species. With the description of *K. nonfermentans* sp. nov., the genus *Kluyveromyces* has been emended as given above.

K. nonfermentans, a new species from deep-sea environments, can be distinguished from the other cluster-2 species, *K. marxianus*, *K. dobzhanskii*, *K. lactis*, *K. wickerhamii* and *K. aestuarii*, on the basis of the lack of assimilation of sucrose, lactic acid and succinic acid as a sole carbon compound and lack of fermentation of D-glucose (Table 3). The G + C content of the DNA of *K. nonfermentans*, within the range 35·8–37·4 mol %, is relatively low compared with that of the other cluster-2 species (Table 3), but this may be due to the use of different protocols for determination of DNA base composition.

K. aestuarii, a species phylogenetically related to *K. nonfermentans*, appears to be associated with saline water, mud and some invertebrates in estuaries or other marine environments (Fell, 1961; Ahearn *et al.*, 1968; de Araujo *et al.*, 1995). The coincident occurrence of *K. nonfermentans* and *K. aestuarii*, which appears to be restricted to marine environments, supports the view that these two species may have shared a common ancestor in the aquatic environment. A characteristic discontinuity between *K. nonfermentans* and the other cluster-2 species, including *K. aestuarii*, has led to the hypothesis that *K. nonfermentans* may be originally derived from a *K. aestuarii*-like organism that evolved through adaptation to deeper regions of marine environments.

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