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Dipodascus tetrasporeus sp. nov., an ascosporogenous yeast isolated from deep-sea sediments in the Japan Trench

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Dipodascus tetrasporeus sp. nov. is described as a novel yeast species in the family Dipodascaceae to accommodate an isolate recovered from sediments collected on the deep-sea floor in the north-western Pacific Ocean. In the clade comprising the genera *Dipodascus*, *Galactomyces* and *Geotrichum*, this is the only species that forms asci that bear four ascospores. The ascospore is surrounded by an irregular exosporium wall, similar to what is observed in the genus *Galactomyces*, but they are released by rupture, which is characteristic of *Dipodascus* and not *Galactomyces*. *D. tetrasporeus* is remarkably divergent (>10 % difference) in its D1/D2 26S rDNA sequence from any other known species. Although maximum-likelihood analysis of combined 18S rDNA and D1/D2 26S rDNA sequences cannot elucidate a reliable position for this species, it was placed among *Geotrichum carabidarum*, *Geotrichum cucujoidarum*, *Geotrichum fermentans* and *Geotrichum histeridarum*, which also have morphological and physiological affinity with the species. The species is homothallic. The type strain of *Dipodascus tetrasporeus* sp. nov. is strain SY-277^T (=NBRC 103136^T =CBS 10071^T).

According to Kurtzman & Fell (1998), ascomycetous yeast-like fungi characterized by the presence of arthroconidia were assigned to the genus *Geotrichum* Link: Fries, with their teleomorphic state in the genera *Dipodascus* and *Galactomyces* (de Hoog *et al.*, 1998a, b, c). Recently, these taxa were subdivided into two distinct groups. Group 1 includes the genera *Dipodascus* and *Galactomyces*, with *Geotrichum* anamorphs, and group 2 consists of *Magnusiomyces*, with *Saprochaete* anamorphs (de Hoog & Smith, 2004). The two groups are well separated phylogenetically, based on 18S rDNA sequences and 18S rRNA secondary structure (Ueda-Nishimura & Mikata, 2000), 26S rDNA sequences (Kurtzman & Robnett, 1998), internal transcribed spacer (ITS) and 5.8S rDNA sequences (de Hoog & Smith, 2004) and morphological characteristics (de Hoog *et al.*, 1986). Species of the teleomorphic genus *Dipodascus* are characterized by multispored asci containing eight or more ascospores, whereas species of *Galactomyces* and *Magnusiomyces* contain one (or rarely two) and four ascospores, respectively (de Hoog & Smith, 2004). At present, the genus *Dipodascus* includes six species, namely *Dipodascus aggregatus*, *D. albidus*, *D. armillariae*, *D. australiensis*, *D. geniculatus* and *D. macrosporus*.

During a survey of cold-seep microbial communities of the deep-sea floor along the landward slope of the northern Japan Trench, we isolated some yeasts from sediment samples. The Japan Trench arose from the subduction of the Pacific plate under the North American plate and is well known as the site of the deepest chemosynthesis-based communities, originating from cold methane seeps (Fujikura *et al.*, 1999). One yeast isolate possessed characters typical of the genus *Geotrichum* Link: Fries, whereas the others were identified as *Aureobasidium pullulans*, *Candida pseudolambica* and *Rhodotorula mucilaginosa*, which have often been found in deep-sea environments around the north-western Pacific Ocean (Nagahama, 2006). On the basis of 18S rDNA and 26S rDNA D1/D2 region sequences, as well as morphological

Abbreviations: ITS, internal transcribed spacer; ML, maximum likelihood.

The GenBank/EMBL/DDBJ accession number for the region covering the 18S rDNA and the 26S rDNA D1/D2 domain of *D. tetrasporeus* SY-277^T is AB300502.

and physiological features, strain SY-277^T is described as a member of a novel species, which we name *Dipodascus tetrasporeus* sp. nov.

Isolation and physiological characterization

Strain SY-277^T was isolated from sediment collected from a site about 27 km offshore at a depth of 1763 m in the Japan Trench (39° 19.2359′ N 142° 52.5384′ E) on 14 June 2002. Sampling was performed by means of a sampling system that prevents contamination by open water, as described previously (Nagahama *et al.*, 2001a). At the sampling site, some deep-sea zoobenthos such as sea anemones, sponges, starfishes and some kinds of fishes such as eels and chimaeriformes were found. In particular, many ophiuroids were observed to be spread out over the neighbourhood of the sampling sites.

Yeast strains were characterized morphologically and physiologically using standard methods, with some modifications (Yarrow, 1998). Assimilation of nitrogen compounds was examined on solid media using a starved inoculum (Nakase & Suzuki, 1986). Vitamin requirements were investigated according to the method of Komagata & Nakase (1967). The DNA base composition was determined using the HPLC method of Tamaoka & Komagata (1984).

DNA sequencing and phylogenetic analysis

DNA extraction for PCR was performed using a QIAamp DNeasy Tissue kit (Qiagen) with some modifications (Nagahama *et al.*, 2001b) or a Microbial DNA Extraction kit (MOBIO) according to the manufacturer's instructions. The primers used for amplification and sequencing of the 18S rDNA, 5.8S rDNA and ITS regions were those described by White *et al.* (1990); the primers for the D1/D2 region of the 26S rDNA were those described by Fell *et al.* (2000).

Sequences were aligned using CLUSTAL W 1.81 (Thompson *et al.*, 1994) and the alignment was optimized manually. Positions where one or more species contained a length mutation and/or ambiguously aligned regions were excluded from subsequent phylogenetic analyses.

Nucleotide sequence phylogenies were derived using PAUP* 4.0b10 (Swofford, 1998). Maximum-likelihood (ML) analyses (Felsenstein, 1981) were performed using heuristic searches with random stepwise addition of 100 replicates and tree bisection–reconnection (TBR) rearrangements. The optimal model of nucleotide evolution for the ML analyses was determined using hierarchical likelihood ratio tests as implemented in MODELTEST 3.7 (Posada & Crandall, 1998). The model selected as the best fit for the combined dataset of 18S and 26S rDNA was TrN+I+G. For the bootstrap analyses (Felsenstein, 1985), 250 replicates were generated with five random additions and TBR. A posteriori probabilities were obtained by using Bayesian phylogenetic inference using the program MRBAYES 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) with the GTR+I+G model determined by using Mrmodeltest 2.2 (Nylander, 2004). Maximum-parsimony (MP) trees were obtained by 100 random addition heuristic search replicates using PAUP and 1000 bootstrap replicates were performed employing five random addition heuristic searches.

Species assignment and evolutionary position of SY-277^T among species related to genus *Geotrichum*

We sequenced a region comprising the 18S rDNA, ITS1, 5.8S rDNA, ITS2 and D1/D2 of the 26S rDNA for strain SY-277^T. Each sequence was aligned with those of species in the genus *Geotrichum* Link: Fries and related species from public databases (Table 1). Because alignments of 18S rDNA and D1/D2 were partly ambiguous owing to many length mutations, we edited the ambiguous regions manually and then removed unalignable sites from the 18S rDNA and D1/D2 sequences. In the end, we used 1326 18S rDNA and 319 D1/D2 nucleotide sites for the following analyses. Reliable alignments could not be obtained for the ITS/5.8S rDNA region, in part due to the paucity of available ITS1 sequences for species of *Geotrichum* (de Hoog & Smith, 2004).

We constructed phylogenetic trees from combined 18S rDNA and D1/D2 26S rDNA sequences using *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* as outgroups (Fig. 1). Species of *Geotrichum* formed two clades corresponding to groups 1 and 2 proposed by Ueda-Nishimura & Mikata (2000). Whereas group 2 was well supported statistically, group 1 and internal branches near the common node were not. These behaved as sister groups in this study, as well as in previous studies (Suh & Blackwell, 2006; Ueda-Nishimura & Mikata, 2000; Kurtzman & Robnett, 1998), whereas they were reported to be phylogenetically divergent in a broader context (de Hoog & Smith, 2004). Subclades containing three species of *Galactomyces* and the three *Dipodascus* species *D. albidus*, *D. australiensis* and *D. geniculatus* were clearly distinguished from the other species of group 1.

Strain SY-277^T was placed among *Geotrichum carabidarum*, *Geotrichum cucujoidarum*, *Geotrichum fermentans* and *Geotrichum histeridarum*, but the grouping of strain SY-277^T with *Geotrichum* species was weakly supported by bootstrap resampling. In addition, the shortest pairwise distances calculated between the sequences of strain SY-277^T and the nine closest species were to *D. macrosporus* and *Geotrichum klebahnii*, which are not sister taxa of strain SY-277^T in Fig. 1. Strain SY-277^T was closest to *D. macrosporus* in 18S rDNA sequence (97.5 %) and to *G. klebahnii* in 26S rDNA sequence (89.7 %). Differences of three to six substitutions in the D1/D2 region are often interpreted as representing the critical boundary between conspecificity and non-conspecificity. In the present case, a difference in excess of 10 % between strain SY-277^T and

Table 1. Accession numbers of 18S and 26S rDNA sequences used in this study

Species	18S rDNA		26S rDNA	
	Strain	Accession no.	Strain	Accession no.
Dipodascus aggregatus	IFO 10816^T	AB000645	NRRL Y-17564^T	U40120
Dipodascus albidus	IFO 1984	AB000642	NRRL Y-12859^T	U40081
Dipodascus armillariae	IFO 10804	AB000639	NRRL Y-17580^T	U40093
Dipodascus australiensis	IFO 10805^T	AB000643	NRRL Y-17565^T	U40100
Dipodascus geniculatus	IFO 10806^T	AB000644	NRRL Y-17628^T	U40130
Dipodascus macrosporus	IFO 10807^T	AB000640	NRRL Y-17586^T	U40121
Dipodascus tetrasporeus sp. nov.	SY-277^T	AB300502	SY-277^T	AB300502
Galactomyces citri-aurantii	IFO 10822	AB000665	NRRL Y-17913^T	U84233
Galactomyces geotrichum	IFO 9541^T	AB000647	NRRL Y-17569^T	U40118
Galactomyces reessii	IFO 10823^T	AB000646	NRRL Y-17566^T	U40111
Geotrichum carabidarum	NRRL Y-27727^T	AY520162	NRRL Y-27727^T	AY520292
Geotrichum cucujoidarum	NRRL Y-27731^T	AY520175	NRRL Y-27731^T	AY520305
Geotrichum fermentans	IFO 1199^T	AB000651	NRRL Y-17567^T	U40117
Geotrichum histeridarum	NRRL Y-27729^T	AY520227	NRRL Y-27729^T	AY520357
Geotrichum klebahnii	IFO 10826^T	AB000641	NRRL Y-17568^T	U40114
Magnusiomyces capitatus	IFO 10820	AB000650	NRRL Y-17686^T	U40084
Magnusiomyces ingens	JCM 9471^T	AB018130	NRRL Y-17630^T	U40127
Magnusiomyces magnusii	IFO 10808	AB000653	NRRL Y-17563	U40097
Magnusiomyces ovetensis	IFO 1201	AB000657	NRRL Y-17574^T	U40116
Magnusiomyces spicifer	IFO 10809^T	AB000649	NRRL Y-17578^T	U40115
Magnusiomyces tetrasperma	IFO 10810^T	AB000654	NRRL Y-7288^T	U40086
Saprochaete fragrans	IFO 10825^T	AB000656	NRRL Y-17571^T	U40119
Arxula adeninivorans	IFO 10858^T	AB000659	NRRL Y-1769	U40094
Arxula terrestris	IFO 10828^T	AB000663	NRRL Y-17704^T	U40103
Candida allociferrii	IFO 10193	AB000658	IFO 10194^T	AB041003
Stephanoascus farinosus	IFO 10873^T	AB000660	NRRL Y-17593^T	U40132
Stephanoascus smithiae	IFO 10879^T	AB000661	NRRL Y-17849^T	U76531
Schizosaccharomyces pombe	NRRL Y-12796^T	AY046272	NRRL Y-12796^T	AY048171
Saccharomyces cerevisiae	Unknown	M27607	NRRL Y-12632^T	AY048154

any of its nearest relatives provides strong evidence that the strain represents an independent species, *D. tetrasporeus*. However, the phylogenetic placement of the novel species was unclear in this study because the relationship between it and neighbouring *Dipodascus* species is not statistically robust. Multigene analyses (Kurtzman & Robnett, 2003) may be useful in resolving this matter in future.

Morphological and physiological characteristics

Since de Hoog & Smith (2004) assigned four-spored species of *Dipodascus* to the genus *Magnusiomyces* in accordance with their molecular divergence, the genus *Dipodascus* consists only of species having asci that contain between eight and more than 100 ascospores (de Hoog & Smith, 2004). Group 1, which comprises species of the genera *Dipodascus* and *Galactomyces* as well as their anamorphs in the genus *Geotrichum*, was more divergent than group 2 in terms of morphological characteristics. Our phylogenetic analysis places *D. tetrasporeus* in group 1. The novel species is of special interest in view of the unique combination of characters that it possesses. Reproduction involves the formation of arthroconidia (Fig. 2a) as well as occasional buds or blastoconidia (Fig. 2b), as observed also in *Geotrichum fermentans* and *Geotrichum cucujoidarum* (Table 2). The three blastoconidiogenous species form a separate subclade (Fig. 1). *D. tetrasporeus* forms asci that contain four spores, a unique character within group 1 species, although it is widespread among ascosporic yeasts including the group 2 (*Magnusiomyces*) species. Here, the ascospores were globose or subglobose and were surrounded by an irregular exosporium wall (Fig 2b, c, f), typical of those observed in species of *Galactomyces*, which is phylogenetically divergent from *D. tetrasporeus* (Fig. 1). Moreover, the ascospores of the novel species have a gelatinous coating (Fig 2c, f) similar to that observed in species of *Dipodascus* and *Magnusiomyces* and, as observed in those species, the asci of *D. tetrasporeus* release the ascospores through rupture of persistent walls (Fig 2b, e).

D. tetrasporeus is also unusual from the physiological standpoint in its fermentation ability, which is rare among *Dipodascus* species (de Hoog *et al.*, 1998a; de Hoog & Smith, 2004). In group 1 species, this ability is found in *Geotrichum* species such as *Geotrichum carabidarum*, *Geotrichum fermentans*, *Geotrichum histeridarum* and *Geotrichum klebahnii* and some species of *Galactomyces*.

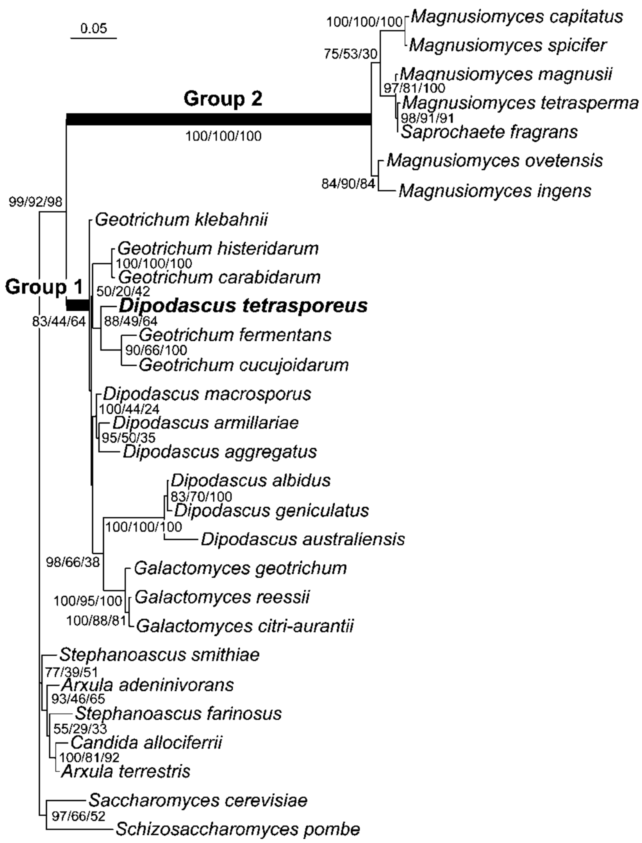

Fig. 1. Phylogenetic relationships between *Dipodascus tetrasporeus* sp. nov. SY-277^T and related species, based on nucleotide sequences of the 18S rDNA and the D1/D2 region of the 26S rDNA. Details of strain names and sequence accession numbers are given in Table 1. The ML tree was constructed as described in the text. Numbers are confidence values for nodes supported by 50 % or more (values represent posterior probabilities in a Bayesian analysis/bootstraps for maximum-likelihood analysis with 250 replicates/bootstraps for maximum-parsimony analysis with 1000 replicates). Groups 1 and 2 are as defined by Ueda-Nishimura & Mikata (2000). Bar, 0.05 substitutions per site.

In particular, *Geotrichum fermentans*, *Geotrichum klebahnii* and *D. tetrasporeus* were similar in their ability to ferment both glucose and galactose. Furthermore, *Geotrichum klebahnii* is physiologically similar to *D. tetrasporeus* in being able to grow on vitamin-free medium and in not utilizing cellobiose as a carbon source. *D. tetrasporeus* can be differentiated from other species of group 1 by the absence of growth on D-mannitol and D-glucitol (Table 2), which are otherwise utilized by group 2 species. The novel species produces a fruity odour when growing in 2 % malt extract (ME) medium or 4 % malt extract/0.5 % yeast extract (MEYE) medium, as reported for *Geotrichum fragrans* and *Galactomyces geotrichum* (de Hoog *et al.*, 1998b, c).

Owing to the lack of phylogenetic robustness among group 1 species, it may be difficult to speculate on the evolutionarily origin of the morphological traits of *D. tetrasporeus*. Distinctive characters such as the four-spored asci and the *Galactomyces*-type ascospores are shared with the more phylogenetically divergent species. We presume that it might be evidence that the novel species retains character states that are ancestral for group 1, although convergent appearance cannot be ruled out.

The description of *D. tetrasporeus* calls for the emendation of the description of the genus *Dipodascus*, although further changes may be required as a more definitive phylogenetic placement of the species becomes possible. The availability of only a single isolate from a deep-sea sediment cannot be viewed as evidence that this is the true habitat of the species, although the ability to grow at very low temperatures (<4 °C) could be regarded as a relevant property. Psychrotolerance was common among the other yeast species isolated from same sampling area (*A. pullulans*, *C. pseudolambica* and *R. mucilaginosa*) around the north-western Pacific Ocean (Nagahama, 2006). *A. pullulans* and *R. mucilaginosa* have also been reported from PCR-based or culture-based studies in deep-sea hydrothermal vents (Edgcomb *et al.*, 2002; Gadanho & Sampaio, 2005; López-García *et al.*, 2007). Among relatives of *D. tetrasporeus*, three *Geotrichum* species, *Geotrichum carabidarum*, *Geotrichum cucujoidarum* and *Geotrichum histeridarum*, were discovered in association with beetle guts (Suh & Blackwell, 2006). Although we did not collect benthic invertebrates at the sampling site visited on this cruise, it may be that *D. tetrasporeus* is associated with them.

Genus *Dipodascus* de Lagerheim emend. Nagahama et Abdel-Wahab

Asci hyaline, subspherical to broadly ellipsoidal, cylindrical to tubular or acicular, containing four to more than 100 ascospores. Ascospores globose to ellipsoidal or cylindrical, hyaline, smooth-walled, each with an even, gelatinous coat, occasionally with an irregular exosporium similar to those found in species of *Galactomyces*. This description is a partial revision of that of de Hoog & Smith (2004).

Latin diagnosis of *Dipodascus tetrasporeus* Nagahama et Abdel-Wahab sp. nov.

Cultura in agaro malti post dies 10 (20 °C) *plana*, *sicca*, *capillata*, *candida*, *centralia pulveracea et intumescens. Hyphae ad* 3–5 μm *latis*, *apicibus rotundatis*, *in arthroconidia cylindrica fragmentata* (2.5–4.0 × 5.0–36.0 μm). *Gametangia prope septa hypharum utrinque vel ex hyphis separatus. Asci sphaeroidei vel late ellipsoidei*, 5–10 × 6–14 μm, 1–4 *ascosporas continentes. Ascosporae late ellipsoideae*, 3.0–5.0 μm *diametro*, *exosporio irregulariter inflato. Glucosum et galactosum fermentantur. Sucrosum*, *maltosum*, *lactosum*, *raffinosum*, *melibiosum non fermentantur. Glucosum*, *galactosum*, L-*sorbosum*, D-*xylosum*, D-*arabinosum* (*exiguum*), D-*ribosum* (*exiguum*), *ethanolum*, *glycerolum*,

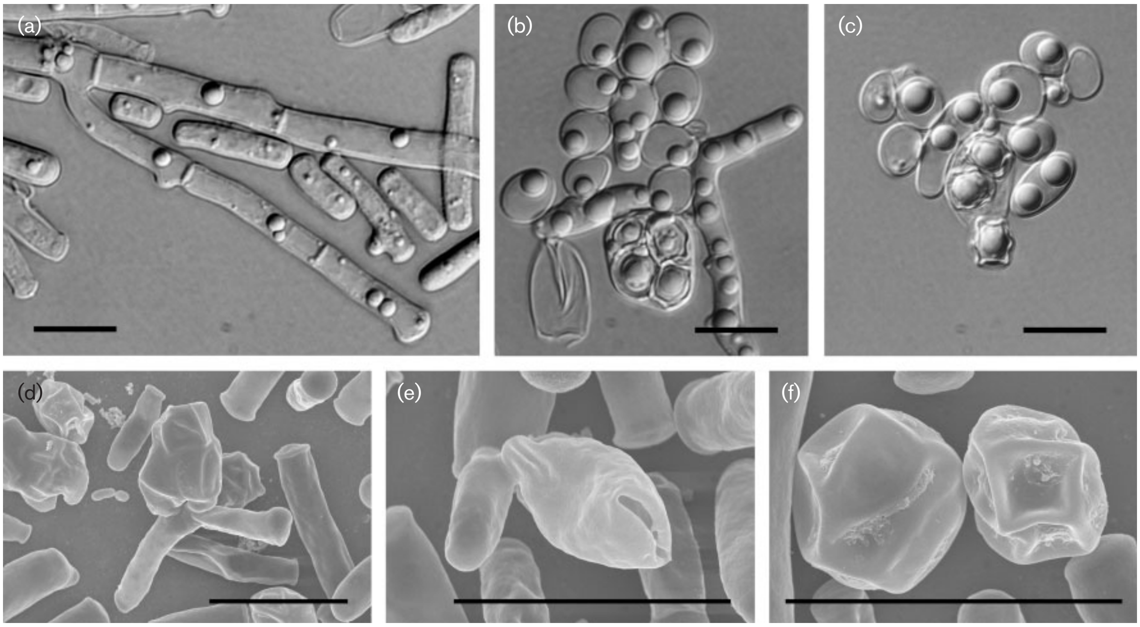

Fig. 2. (a–c) Photomicrographs of strain SY-277^T after 10 days. Bar, 10 µm. (a) Branching hyphae and cylindrical arthroconidia (on MEYE agar). (b) Blastoconidia, an ascus with four ascospores and an empty ascus after rupture (on ME agar). (c) Ascospores with an irregular exosporium and sheath, being released from asci (on MEYE agar). (d–f) Scanning electron micrographs of strain SY-277^T on MEYE agar after 1 month of culture. (d) Ascus on arthroconidia and ascospores; (e) ruptured hole of empty ascus; (f) ascospores surrounded by an exosporium with gelatinous substance.

glucono-β-lactonum, acidum DL-*lacticum, acidum succinicum et acidum* D-*galacturonicum assimilantur, at non sucrosum, maltosum, cellobiosum, trehalosum, lactosum, melibiosum, raffinosum, melezitosum, inulinum, amylum solubile,* L-*arabinosum,* L-*rhamnosum, erythritolum, ribitolum, galactitolum,* D-*mannitolum,* D-*glucitolum, methylum α-*D-*glucosidum, salicinum, acidum* 2-*ketogluconicum, acidum* 5-*ketogluconicum, inositolum, acidum citricum nec acidum* D-*glucuronicum. Ethylaminum, lysinum et cadaverinum assimilantur, at non kalium nitricum nec natrium nitrosum. Vitamina externa ad crescentiam non necessaria sunt.* G+C *acidi deoxyribonucleati* 40.7 mol% (*per* HPLC).

Table 2. Differentiating characteristics of *D. tetrasporeus* sp. nov. and relatives

Species: 1, *D. tetrasporeus* (strain SY-277^T); 2, *Geotrichum klebahnii*; 3, *D. macrosporus*; 4, *D. armillariae*; 5, *Geotrichum carabidarum*; 6, *Geotrichum histeridarum*; 7, *Geotrichum fermentans*; 8, *Geotrichum cucujoidarum*. +, Positive; −, negative; d, delayed positive; W, weak; V, variable depending on the strain.

Characteristic	1	2	3	4	5	6	7	8
Pairwise sequence similarity with strain SY-277^T								
26S rDNA	(100)	89.7	88.7	87.9	86.1	86.1	81.3	79.5
18S rDNA	(100)	97.2	97.5	96.6	95.3	95.1	94.6	94.6
Fermentation of glucose	+	+	−	−	d	d/W	+	−
Fermentation of galactose	+	W/−	−	−	−	−	+	−
Growth in vitamin-free medium	+	+	+	+	−	−	+	+
Growth on sole carbon compounds								
Cellobiose	−	−	+	+	−	−	+	+
D-Mannitol	−	+	+	+	W/−	+	+	+
D-Glucitol	−	+	+	V	W	+/d	+	+
Number of ascospores per ascus	1–4	−	10–30	4–12	−	−	−	−
Presence of blastoconidia	+	−	−	−	−	−	+	+

Typus stirps SY-277^T *ex sedimentum, fossa Japana, Oceanus Pacificus, isolata est. In collectionibus culturarum quas* NITE Biological Resource Center, Kisarazu, Chiba *sustentant, no.* NBRC 103136^T (=CBS 10071^T) *deposita est.*

Description of *Dipodascus tetrasporeus* Nagahama et Abdel-Wahab sp. nov.

Dipodascus tetrasporeus (te.tra.spo′re.us. Gr. adj. *tetra* four; Gr. n. *spora* a seed and, in biology, a spore; N.L. masc. adj. *tetrasporeus* with four spores, representing the formation of four ascospores per ascus).

After 10 days on MEYE agar at 20 °C, colonies are 25–30 mm in diameter, white, flat, dry, centrally powdery and swelling, with finely hairy margins. Hyphae are 3–5 μm wide, with rounded apices, and with some basitonous branchings, with slight differentiation between main and lateral branches, branches soon disarticulating into cylindrical arthroconidia (2.5–4.0 × 5.0–36.0 μm). Abundant true mycelia and arthroconidia are formed. On ME agar medium, hyphae and arthroconidia produce clusters of globose to subglobose blastospores, 4.0–6.0 μm in diameter. Gametangia are located on opposite sides of septa or on separate hyphae. Asci are subspherical to broadly ellipsoidal, 5–10 μm wide and 6–14 μm long, and contain one to four ascospores. Ascospores are globose to subglobose 3.0–5.0 μm in diameter, with an irregular exosporium and gelatinous sheath. The species is homothallic. D-Glucose and galactose are fermented but sucrose, maltose, lactose, raffinose and melibiose are not. The following carbon compounds are assimilated: D-glucose, galactose, L-sorbose, D-xylose, D-arabinose (weak), D-ribose (weak), ethanol, glycerol, glucono-*β*-lactone, DL-lactic acid, succinic acid and D-galacturonic acid. No growth occurs on sucrose, maltose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, L-arabinose, L-rhamnose, erythritol, ribitol, galactitol, D-mannitol, D-glucitol, methyl *α*-D-glucoside, salicin, 2-ketogluconic acid, 5-ketogluconic acid, citric acid, inositol or D-glucuronic acid. The nitrogen compounds ethylamine, lysine and cadaverine are assimilated but potassium nitrate and sodium nitrite are not. Growth occurs at 27 °C and is weak at 30 °C but does not occur at all at 33 °C on MEYE agar. Growth occurs on vitamin-free medium. No growth occurs on 50 % glucose/yeast extract agar. Growth occurs in the presence of 100 p.p.m. cycloheximide. No growth occurs in the presence of 10 % sodium chloride. No starch-like substances are produced. The diazonium blue B reaction is negative. Urease activity is negative. The G+C content of the nuclear DNA is 40.7 mol% (by HPLC).

The type strain, strain SY-277^T (=NBRC 103136^T =CBS 10071^T), was isolated from sediments collected from the deep-sea floor in the Japan Trench, Pacific Ocean.

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