

ORIGINAL PAPER

Thraustochytrium gaertnerium sp. nov.: a New Thraustochytrid Stramenopilan Protist from Mangroves of Goa, India

Lucia Bongiorno^{a,1}, Ruchi Jain^a, Seshagiri Raghukumar^{a,2,3}, and Ramesh Kumar Aggarwal^b

^aNational Institute of Oceanography, Dona Paula, Goa — 403 004, India

^bCentre for Cellular and Molecular Biology, Uppal Road, Hyderabad — 500 007, India

Submitted December 22, 2004; Accepted May 19, 2005
Monitoring Editor: Michael Melkonian

Thraustochytrids are ubiquitous, chemo-organotrophic, marine stramenopilan protists belonging to the class Labyrinthulomycetes. Their taxonomy is largely based on life cycle development stages. We describe here a new species of thraustochytrid isolated from mangroves of Goa, India. The organism is characterized by large zoosporangia with two distinct development cycles. In one, typical thalli with ectoplasmic net elements mature into zoosporangia that divide to form heterokont biflagellate zoospores, leaving behind a proliferation body. In the second type, the thalli develop into amoeboid cells, reminiscent of the genus *Ulkenia* Gaertner. Unlike *Ulkenia*, however, the ‘amoebae’ do not immediately produce zoospores, but round up prior to division into zoospores. The two types of development occur simultaneously in single cell-derived in-vitro cultures. Molecular characterization of the new isolate involving 18S rRNA gene typing and comparative phylogenetic analysis further establish it to be a new and distinct thraustochytrid species with *Schizochytrium aggregatum* Goldstein and Belsky and *Thraustochytrium kinnei* Gaertner as the closest forms. We have named this new species as *Thraustochytrium gaertnerium*, deriving its species name in honour of Dr Alwin Gaertner, a pioneer in the studies of taxonomy and ecology of thraustochytrids.

© 2005 Elsevier GmbH. All rights reserved.

Key Words: *Thraustochytrium gaertnerium* sp. nov.; thraustochytrids; Straminipila; mangroves; India.

Introduction

Thraustochytrids are a widespread group of marine fungi, belonging to the Kingdom Chromista (Cavalier-Smith 1993; Cavalier-Smith et al. 1994)

or Straminipila (Dick 2001; Raghukumar 2002). Typically chemoorganotrophic and morphologically resembling chytridiaceous and oomycetan fungi, they were originally placed among the phycomycetous fungi (Sparrow 1960) or the Kingdom Fungi (Eumycota of Ainsworth et al. 1973), prior to their transfer to the Straminipila. Thraustochytrids differ from members of the Kingdom Fungi in several ways. Their cell walls are multilamellate, made up of dictyosome-derived circular

¹Department of Marine Science, Polytechnic University of Marche, Via Breccia Bianache, 60131, Ancona, Italy

²Corresponding author;

e-mail raghu@darya.nio.org (S. Raghukumar)

³Present address: 313, Vainguinnim Valley, Dona Paula, Goa 403 004, India

scales consisting of sulphated polysaccharides, instead of chitin microfibrils (Chamberlain 1980; Chamberlain and Moss 1988; Darley et al. 1973). Their 'rhizoids', unlike those of chytrids, are extensions of the plasma membrane, associated with the organelle 'sagenogenetosome' or 'bothrosome' and are termed ectoplasmic net elements or EN (Moss 1985; Perkins 1973; Porter 1990). Presence of zoospores with heterokont flagella possessing tripartite hair, mitochondria with tubular cristae and 18S rRNA gene sequences have shown that thraustochytrids, together with oomycetes are 'straminipilan fungi', of the Kingdom Straminipila, related to heterokont algae such as the chrysophytes and diatoms (Cavalier-Smith 1993). Thraustochytrids constitute one of the 3 groups of Labyrinthulomycetes, the others being the labyrinthulids and the aplanochytrids (Leander and Porter 2001). They are broadly characterized by: (1) single cells that are not interconnected; (2) presence of ectoplasmic net elements (except in the genus *Althornia*); and (3) reproduction by zoospores. Five genera of thraustochytrids have been described to date, of which *Schizochytrium* Goldstein and Belsky alone is characterized by repeated binary divisions of the vegetative cells (Dick 2001; Goldstein and Belsky 1964). *Althornia* Jones and Alderman is characterized by an absence of the EN (Jones and Alderman 1971). *Ulkenia* Gaertner (Gaertner 1977) is distinguished by the transformation of the mature vegetative cell into an amoeboid cell prior to division into zoospores. *Japonochytrium* Kobayishi and Ookubo possesses an apophysis-like structure not found in the other genera (Sparrow 1960). Cytoplasmic contents of the mature cells of *Thraustochytrium* Sparrow directly divide into biflagellate zoospores (Sparrow 1960). There are 13 species described for this genus that are distinguished from each other by the presence or absence of proliferation bodies, size and shape of cells and the mode of division into zoospores (Dick 2001). We describe here a new species of the genus *Thraustochytrium* isolated from mangrove waters of Goa, India and present morphological and molecular evidence to support its novel species status.

Results and Discussion

Diagnosis

Thraustochytrium gaertnerium R. Jain, S. Raghukumar, L. Bongiorno, et R.K. Aggarwal, sp. nov

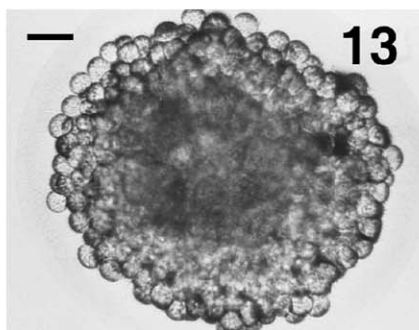
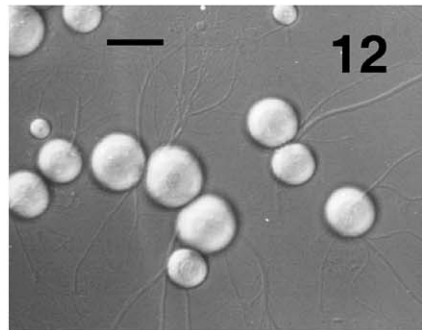
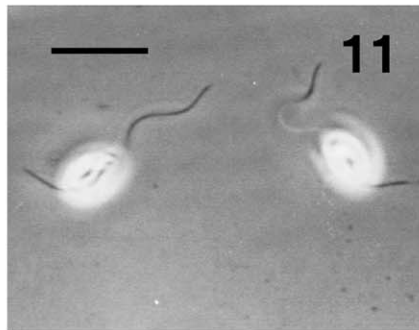
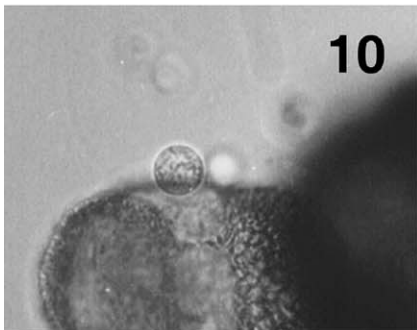
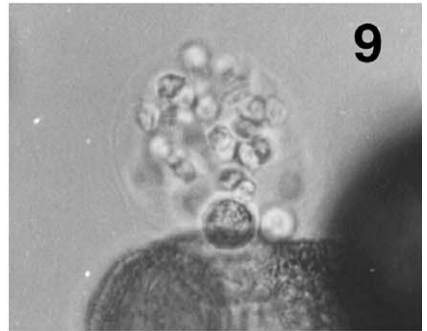
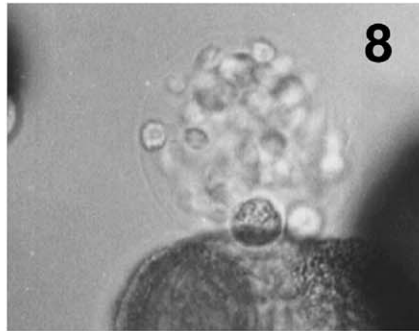
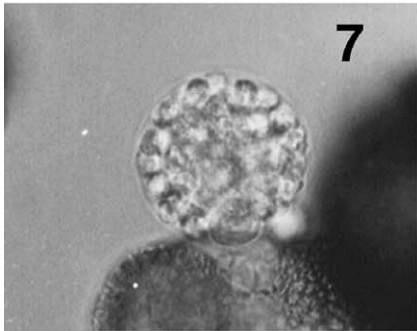
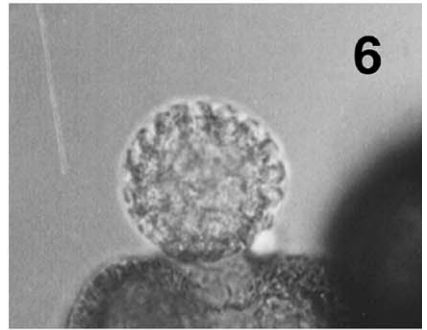
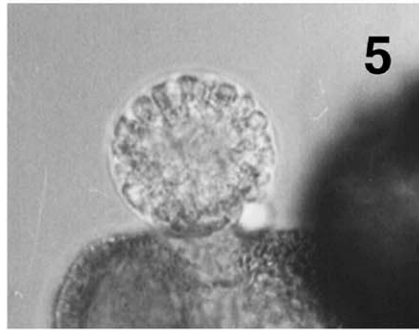
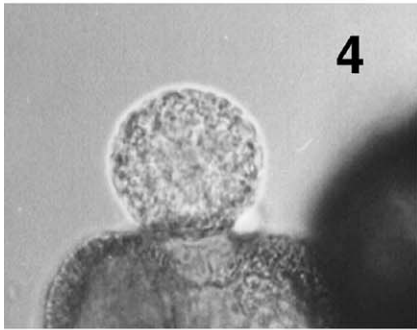
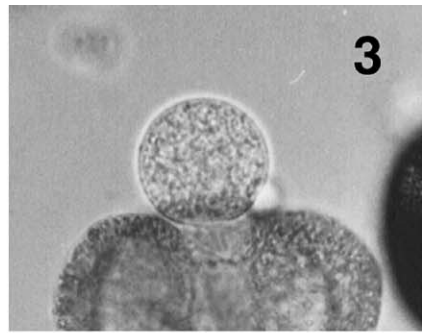
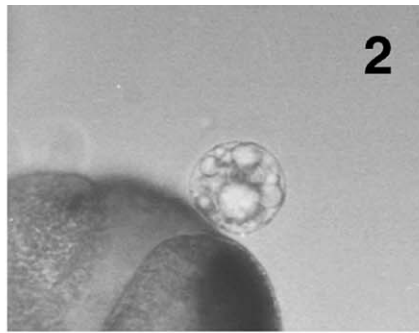
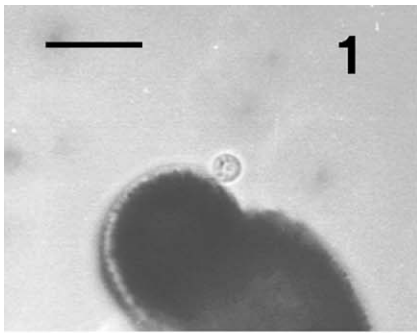
Thalli novelli 4–5 µm diametro sunt. Maturi thalli globosi, subglobosi, ectoplasmaticis reticulates partibus formati sunt. Thalli in aqua marina et pino polline colturis 15–30 µm diametro sunt; in MV medio nutritivo 17.5–50 µm diametro sunt. Fetura duabus rationibus zoosporarum procreationis est. Prima ratione maturi thalli zoosporangium directo fiunt; zoosporangia eucarpica, directo dividitur in 10–70 zoosporas. Zoosporae initio dismissae sunt ex rima in cellulae pariete, quae igitur plane extinguitus. Unum feturae corpus, 5–10 µm diametro, denique manet. Altera ratione maturi thalli amebiformes fiunt. Amebae 25–40 µm longae, 12.5–27.5 µm latae sunt, cum ectoplasma vitreo, vagina similes et endoplasma granoso, moto tardissimo. Amebae se congregant ad formandam zoosporangiam, ut supra dicitur. Nullum corpus feturae manet. Zoosporae 5.5–7.3 µm longa, 3–4.5 µm latae sunt.

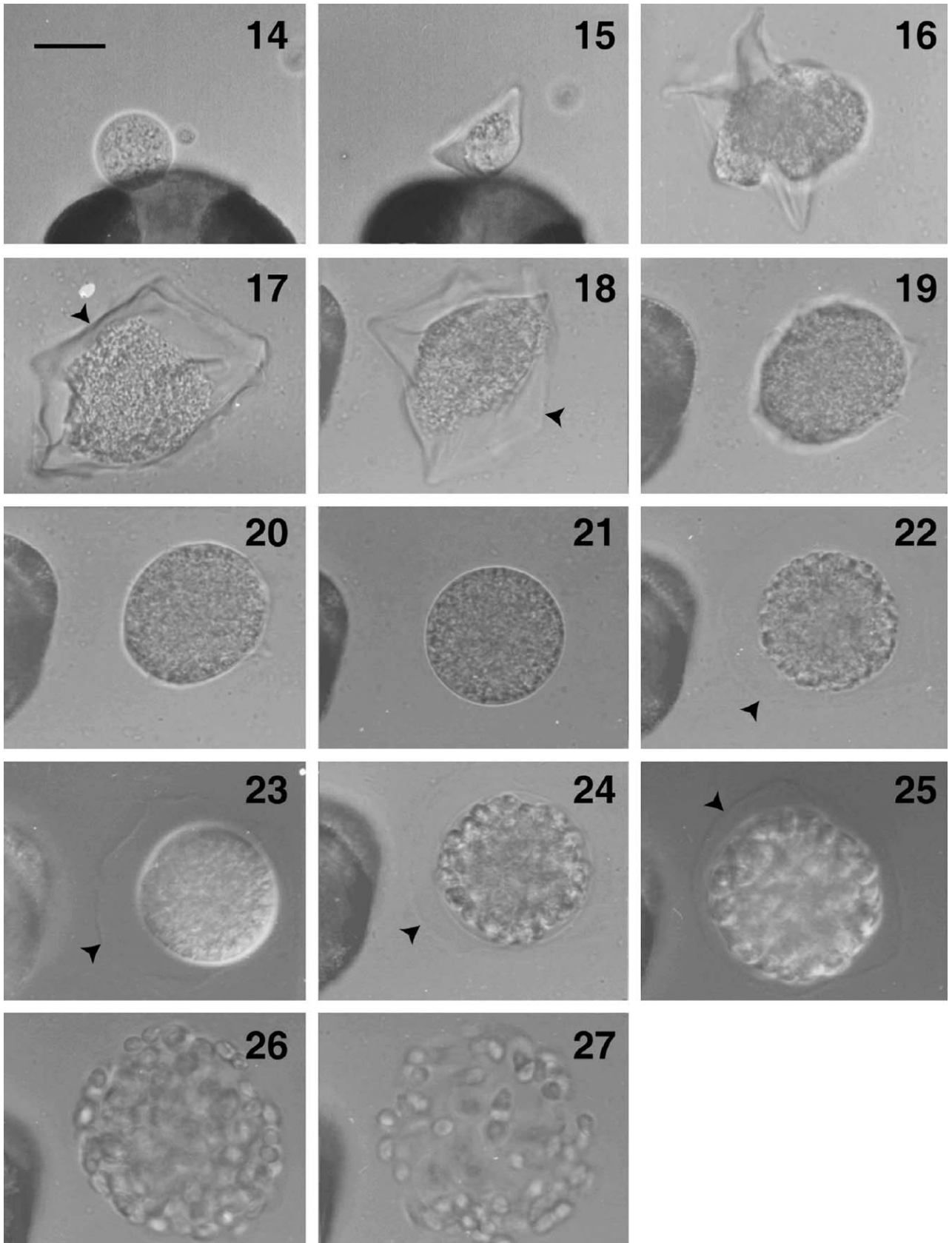
Etymology: The specific epithet 'gaertnerium' is in honour of Dr Alwin Gaertner, a pioneer in the studies of ecology and taxonomy of thraustochytrids.

Holotype: Figures 1–26, isolated from waters of Chorao mangroves, Goa, India, Lat. 15°25'N; Long. 73°95'E. The axenic culture is deposited at ATCC, accession no. ATCC PRA-148 and also at NIO, Goa, accession no. NIOS-6

Young thalli 4–5 µm in diameter; mature thalli globose, sub-globose and with ectoplasmic net elements. Sporangia in seawater/pine pollen cultures 15–30 µm in diameter; in MV medium 17.5–50 µm in diameter. Reproduction occurring by 2 different methods of zoospore formation. Mature thalli in the first type directly becoming zoosporangia; zoosporangia eucarpic, dividing directly into 10–75 zoospores. Zoospores released initially by a rupture in the cell wall, the latter disintegrating totally afterwards. A single proliferation body, 5–10 µm in diameter left

Figures 1–13. *Thraustochytrium gaertnerium* sp. nov. **1–10:** Type I development. **1.** Freshly encysted zoospore on pine pollen. **2.** Young thallus with prominent globules. **3.** Mature thallus with granular contents. **4.** Young zoosporangium showing initiation of zoospore cleavage. **5.** Centripetal cleavage of zoospore initials from zoosporangium. **6.** Mature zoosporangium with zoospores. **7.** Mature zoosporangium with zoospores. The proliferation body is clearly delimited. **8–9.** Liberation of zoospores. Note the persistent zoosporangial wall. **10.** Proliferation body left after zoospore discharge. Bar in Figure 1 is common to Figures 1–10 and represents 20 µm. **11.** Biflagellate zoospores. Bar represents 5 µm. **12.** Cells with ectoplasmic net elements. Bar represents 20 µm. **13.** Colony on MV agar medium. Bar represents 50 µm.





behind. Mature thalli in the second type becoming amoeboid. Amoebae 25–40 μm in length and 12.5–27.5 μm wide, with a hyaline, sheath-like ectoplasm and a granular endoplasm, exhibiting very slow movements. Amoebae rounding up to form zoosporangia as above. No proliferation body remains. Zoospores measuring 5.5–7.3 μm in length and 3–4.5 μm wide.

Life cycle and Taxonomy

Young vegetative cells resulting from freshly encysted zoospores measured around 4–5 μm in diameter (Fig. 1). In pine pollen cultures, such cells developed into mature thalli in about 24–25 h. During the course of development (after 18 h), young thalli of 10–15 μm diameter were filled with prominent droplets (Fig. 2). Two different types of development were observed. In Type I, the young thallus developed directly into a mature thallus. At the onset of maturity, prior to zoospore formation, the contents of the mature thallus, the zoosporangium, became granular in appearance (Fig. 3). The zoosporangium was characterized by a thin cell wall and measured 15–30 μm in diameter. The first sign of zoospore formation was the appearance of an undulate outer margin of the zoosporangial contents (Fig. 4). Cleavage of zoospores then took place rapidly and outlines of the zoospores became visible (Figs 5, 6). The proliferation body was clearly visible at this stage (Fig. 7). Prior to their liberation, the zoospores showed rapid movements inside the zoosporangium for a very short period. Zoospores were initially released through a tear in the cell wall (Fig. 8). As zoospore release progressed, most of the cell wall disintegrated totally (Figs 9, 10). A large proliferation body, 5–10 μm in diameter was left, following the release of the zoospores (Fig. 10). The zoosporangia produced 10–75 zoospores. Zoospores were bean-shaped and possessed two flagella (Fig. 11). The flagella were apically attached, the long anterior and the shorter posterior located one below the other. The zoospores measured 3–4.5 \times 5.5–7 μm in size, with a length-to-width ratio of 1.4:1 to 2:1. The duration,

starting from the time of zoospore initiation till the release of all the zoospores was approximately 60 min. Amoebae and zoospores were also observed in the agar medium. Prominent, branched ectoplasmic net elements were produced both in pine pollen culture and MV agar medium (Fig. 12). Colonies of the thraustochytrid in MV agar were circular to subcircular and contained large, globose, hyaline cells (Fig. 13).

In addition to the above mode of zoospore formation, an amoeboid mode (Type II) was seen simultaneously in cultures. Some of the mature thalli, instead of developing into zoosporangia, began to assume an amoeboid shape (Figs 13, 14). This transformation into amoebae was rapid and was complete in less than 1 minute. Such 'amoebae' ranged from 25 to 40 μm in length and 12.5–27.5 μm in width. They were characterized by a very prominent, hyaline and sheath-like ectoplasm, while the rest of the cell was densely granular (Fig. 15–17). The amoebae were capable of rapid transformation of shape and moved very slowly (Figs 15–18). The amoeboid stage lasted about 15 min. We did not detect any bacterivory by such amoebae in cultures contaminated with bacteria. Subsequently, the amoebae rapidly rounded up into cells that had the same dimensions as normal zoosporangia (Figs 18–20). Rounded up protoplasts were smaller than the amoebae, presumably because the latter were flat. At this stage the presence of the cell wall that had earlier surrounded the cell could be discerned (Figs 21–24). These cells, the zoosporangia of 'Type II' divided into zoospores in a manner similar to those of 'Type I' (Figs 21–25). No proliferation bodies were detected in the Type II zoosporangia at the end of zoospore division (Figs 26, 27). The number of zoospores produced by these was similar to those of Type I and no differences were noticed in the zoospore shape. Numerous amoeboid cells were seen at the bottom of the Petri plate in seawater/pine pollen cultures of about 4–7 days. During the early stages of establishing axenic cultures, numerous cigar-shaped, limax amoebae were regularly observed in the cultures. These were not subsequently noticed in seawater/

Figures 14–27. *Thraustochytrium gaertnerium* sp. nov. Type II Developmental stages. **14.** Mature zoosporangium. **15.** Transformation of mature cell into an amoeboid cell. **16–18.** Rapid changes in shape of the amoeboid cell. Arrow in Figs 17 and 18 indicate the ectoplasm. **19–20.** Rounding up of the amoeboid cell. **21.** Initiation of zoospore cleavage of the rounded up amoeboid cell. **22–24.** Mature zoosporangium. Note the presence of a loose cell wall. **25–27.** Zoospore release. Figs 16–27 are from the same cell. Arrows in Figs 22–25 indicate the remnants of a cell wall. Bar in Figure 14 represents 20 μm and is common to Figs 14–27.

pine pollen cultures. However, in one instance, when a culture grown on MV broth was transferred to the continuous flow chamber, as mentioned under 'Methods', numerous normal, globose cells transformed into such limaciform amoebae within 3 h. Such amoebae measured 22–26 µm in length and 3.6–5.5 µm in width and also rounded up later to form Type II zoosporangia. Motile zoospores were observed in cultures up to 1 month old. Limaciform amoebae are not unusual among thraustochytrids. Honda et al. (1998) have described very similar amoebae in the life cycle of *Schizochytrium limacinum*. Such amoebae also form part of the life cycle of *Ulkenia amoeboidea* (Raghukumar 1980).

The behaviour of the thraustochytrid in a rich, organic medium, the MV broth, did not differ from that in seawater/pine pollen cultures. Type I zoosporangia, amoebae and Type II zoosporangia were produced also in MV broth. However, the zoosporangia were much larger and measured 17.5–50 µm in diameter. Zoospore numbers reached values of 20–80 per sporangium.

Phylogenetic analyses based on the 18S rRNA gene sequence variation, irrespective of the clustering algorithm (NJ and ML), robustly establish the protist species analysed and described in this study, to be a new member of the Labyrinthulomycetes, genetically closest but significantly distinct from all other reference thraustochytrids compared in the analysis (Figs 28, 29; Table 2). The overall topologies of different phenetic trees obtained in the study were similar to each other except for minor differences in the branch lengths. Further, the phylogenetic analysis revealed three broad lineages of the compared taxa, as has been observed earlier by others, namely the labyrinthulids, aplanochytrids and the thraustochytrids (Honda et al. 1998; Leander and Porter 2001). The phylogenetic tree shows that within the lineage comprising the thraustochytrids, species of the 3 major genera, *Thraustochytrium*, *Ulkenia* and *Schizochytrium* are interspersed among each other. A similar observation has been made by Honda et al. (1998). This suggests that the major morphological characters on which the thraustochytrid taxonomy is based are homoplasious. In other words, binary division, the amoeboid stage, or division within zoosporangia might have evolved independently several times within the thraustochytrids. This makes the classification of thraustochytrid species difficult, when based solely on morphological characters. In the present case, the species morphologically resembles the genus *Thraustochytrium* Sparrow in its Type I

development and *Ulkenia* Gaertner in its Type II development. In *Thraustochytrium*, the contents of the zoosporangium divide into zoospores while still enclosed within the rigid cell wall. Zoospores are liberated by a tear in the wall or its partial or total disintegration. Gaertner (1977) erected the genus *Ulkenia* to include species in which the cytoplasmic contents are released as a naked protoplast either because of a total disappearance of the cell wall or through the escape of the protoplast through an opening in the cell wall. The protoplast then undergoes amoeboid movements, prior to division into zoospores. The amoeboid movement of the protoplast in the Type II development of the present species resembles that in *Ulkenia*. However, the protoplast subsequently rounded up prior to division into zoospores and did not undergo divisions while the amoeboid stage persisted, as in the genus *Ulkenia* (Gaertner 1977). Besides, the cell wall of the zoosporangium appeared to have persisted throughout, becoming evident when the 'amoebae' rounded up (Figs 21–24). Molecular phylogenetic analyses provided a clearer picture of the taxonomic position of this species. In all the phenetic trees *Thraustochytrium gaertnerium* appears as a distinct new member of the thraustochytrid group with *T. kinnei* as the closest known species of the Genus *Thraustochytrium*. However, the sister taxon (and thus the closest relative) to *T. gaertnerium* is a group of four taxa comprising *S. aggregatum* and 3 other thraustochytrids whose accession nos. are given in Table 2. The present species is being described as a new species of *Thraustochytrium*, based both on the 18S rRNA gene sequences and the Type I development.

The genus *Thraustochytrium* Sparrow is characterized by monocentric, eucarpic, epi- and endobiontic thalli that produce ectoplasmic net elements. The entire contents or part of the contents of the mature thalli, the zoosporangia divide into zoospores. Zoospores are characteristically heterokont, with a longer apical, tinsel and shorter, posterior whiplash flagellum. One or more proliferation bodies may be produced. Zoospores are liberated by a tear in the wall or a partial or total disintegration of the cell wall of the zoosporangia. The proliferation bodies persist after zoospores are liberated. Morphologically, the present species resembles *Thraustochytrium aureum* Goldstein (Goldstein 1963a), *T. motivum* Goldstein (Goldstein 1963b), *T. kinnei* Gaertner (Gaertner 1967) and *T. benthicola* Raghukumar (Raghukumar 1988) in the production of a single proliferation body. The proliferation body in *T. motivum* and *T.*

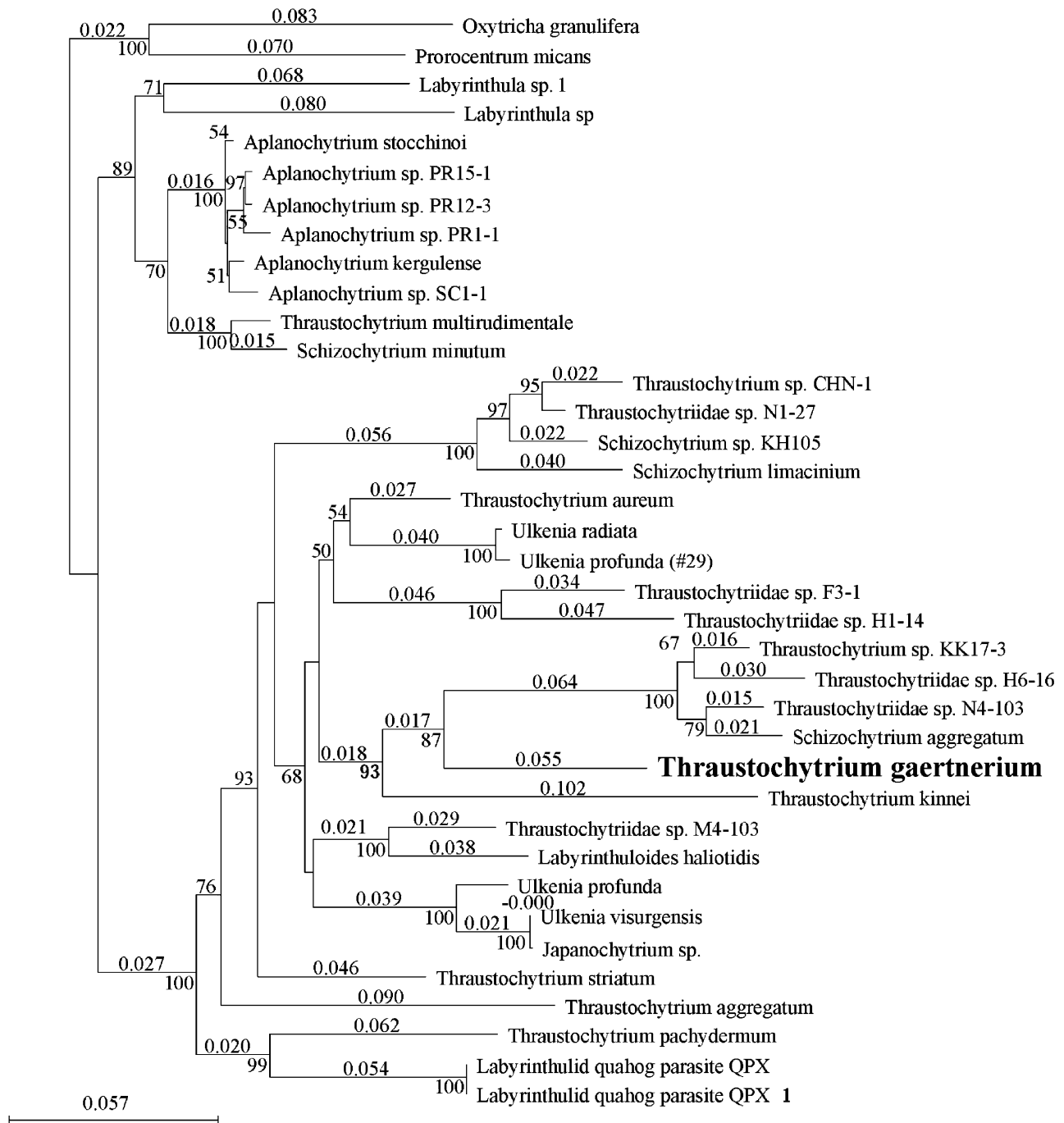


Figure 28. Phylogenetic analysis of the thraustochytrids, aplanochytrids and labyrinthulids based on the multiple alignments of 18S rDNA sequences. The Neighbour joining tree is constructed using Kimura 2-parameter. The numbers at each internal branching shows bootstrap values only for the nodes supported by more than 50%. (500 replicates). The numbers on the branches are the branch lengths. Bar represents 0.065 mutations per site.

kinnei is distinctly large and formed much before the zoospores are cleaved. The sporangia are pyriform to ovate as compared to the globose to

subglobose zoosporangia of *T. gaertnerium* and fewer zoospores are produced. *Thraustochytrium benthicola* produces irregularly shaped zoospor-

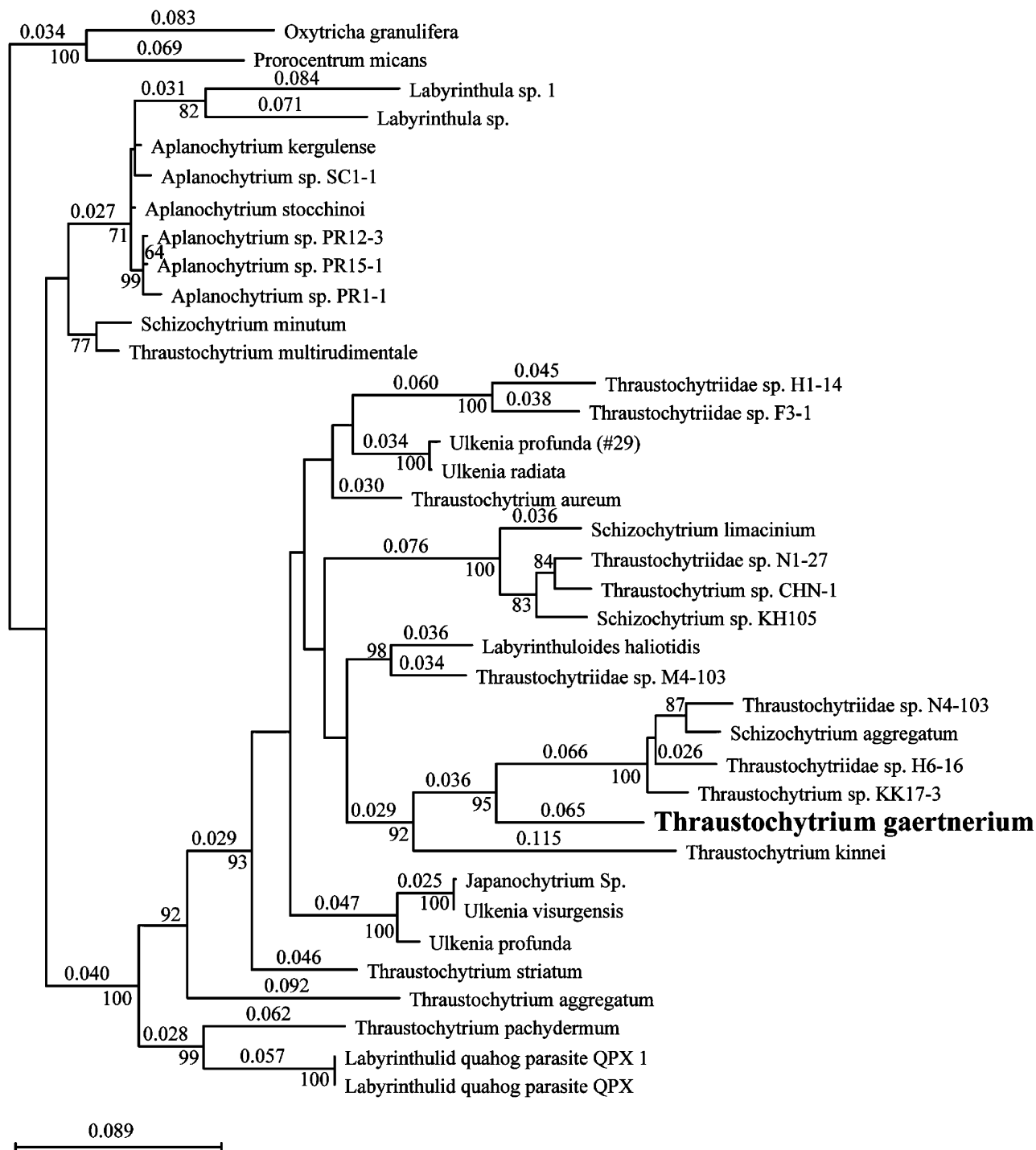


Figure 29. Phylogenetic analysis of the thraustochytrids, aplanochytrids and labyrinthulids bases on the multiple alignments of 18S rDNA sequences. The phylogram is the best maximum likelihood tree ($\ln(L) = -10234.017$) and distance is calculated using gamma corrected Kimura 2-parameter. The numbers at each internal branching shows bootstrap values only for the nodes supported by more than 50%. (500 replicates). The numbers on the branches are the branch lengths. Bar represents 0.089 mutations per site.

angia. *T. aureum* is distinct from the present species in that it produces smaller zoosporangia (8–17 µm) and contains fewer zoospores (less than 50). None of the above species has the special Type II development found in *T. gaertnerium*. *Schizochytrium aggregatum* which appears in a sister clade to *T. gaertnerium* is morphologically a distinct genus, characterized by repeated binary divisions of the vegetative cells. The following key distinguishes *T. gaertnerium* from other species of *Thraustochytrium* that produce one or more proliferation bodies.

seawater were poured in sterile 5 cm Petri dishes. A small amount of pine pollen, sterilized in an oven at 100 °C for 48 h was added as 'bait'. The plates were incubated at room temperature (approximately 28–30 °C) and were examined for growth of thraustochytrids after 3–7 days. The organism was isolated in axenic cultures by subculturing in seawater/pine pollen as above, but with an addition of 0.075% streptomycin and 0.036% penicillin. Absence of bacteria in this culture was confirmed microscopically and also by streaking the culture on Modified Vishniac's (MV) agar plates

1. Single proliferation body produced..... 2
1. More than one proliferation body produced..... 8
2. Proliferation body delineated prior to zoospore formation; prominent..... 3
2. Proliferation body delineated during zoospore formation; not prominent..... 6
3. Zoospores nonmotile at the time of discharge, lacking flagella; developing flagella subsequently....
..... *Thraustochytrium proliferum*
3. Zoospores motile even at the time of discharge, with fully formed flagella..... 4
4. Cell wall mostly persistent, or persistent as a small collar following zoospore discharge.....5
4. Cell wall totally disintegrating following zoospore discharge.....
..... *Thraustochytrium antarcticum*
5. Zoosporangia globose, subglobose or obpyriform, most of the cell wall persistent after zoospore liberation..... *Thraustochytrium motivum*
5. Zoosporangia obpyriform, most of the cell wall disintegrates during zoospore liberation, leaving just a collar around the zoosporangium..... *Thraustochytrium kinnei*
6. Zoosporangia smaller than 20 µm ; not more than 50 zoospores per zoosporangium; no amoeboid stages produced during life cycle..... 7
6. Zoosporangia often up to 30 µm diam; up to 75 zoospores produced; Amoeboid stages present during life cycle..... ***Thraustochytrium gaertnerium* sp. nov.**
7. Zoosporangia irregular in shape; zoospores ovoid; flagella apical and subapical.....
..... *Thraustochytrium benthicola*
7. Zoosporangia up to 17 µm in diam; less than 50 zoospores produced; flagella lateral.....
..... *Thraustochytrium aureum*
8. Number of proliferation bodies not more than 4, zoospores motile at discharge.....
..... *Thraustochytrium multirudimentale*
8. Number of proliferation bodies more than 4.....10
9. Number of proliferation bodies 5–50; zoospores not motile at the time of discharge.....
..... *Thraustochytrium rossii*
9. Number of proliferation bodies 3–10; zoospores motile at discharge.....
..... *Thraustochytrium kerguelensis*

Methods

The protist analysed and described in this study was isolated during April 1999 from waters collected from Choroa mangroves in Goa (Lat: 15°27'N; Long: 73°48'E). Samples were collected using sterile glass vials and brought to the laboratory within 1 h for isolation using the pine pollen baiting method (Gaertner 1968; Porter 1990). About 3 ml water sample and 2 ml of sterile

(Perkins 1972). Bacteria-free colonies were subcultured in seawater/pine pollen cultures. A single pine pollen grain with a thraustochytrid cell attached to it was picked up using a fine glass loop. Several single cell cultures were established from this initial culture. The cultures were maintained on MV agar butts as stab cultures. The type culture, on which the description and analyses are based is deposited at the American Type Culture Collection (ATCC), USA under the accession

Table 1. Primers used in this study.

Primer name	Primer sequence	Direction	Reference
18S001	5'-AACCTGGTTGATCCTGCCAGTA-3'	Forward	Honda et al. 1999
18S13	5'-CCTTGTTACGACTTCACCTTCCTCT-3'	Reverse	Honda et al. 1999
NS3	5'-GCAAGTCTGGTGCCAGCAGCC-3'	Reverse	White et al. 1990
NS4	5'-CTTCCGTCAATTCCTTTAAG-3'	Forward	White et al. 1990
M13F	5'-GTAAAACGACGGCCAGT-3'	Forward	
M13R	5'-AACAGCTATGACCATG-3'	Reverse	

number ATCC PRA-148. A subculture of this is also deposited in the culture collection of the National Institute of Oceanography, Goa, under Accession number NIOS-6.

Development of the thraustochytrid was studied using sea water/pine pollen cultures or 2 days old cultures in MV broth. A modified design of a continuous flow micro-chamber as described by Raghukumar (1987) was used for observing the life cycle under a microscope for up to 3 days. All observations were made using an Olympus BX 60 microscope and photomicrography using Olympus PM-20 unit.

The 18S rRNA gene-based phylogenetic analysis was undertaken to ascertain the taxonomic affiliation of the thraustochytrid. Most of the methods used for the purpose were as detailed by Sambrook et al. (1989), except for the indicated modifications. Total genomic DNA was isolated according to the procedure of Sheriff et al. (1994). The concentration and purity of the DNA was checked on 0.8% agarose gel in 1% TAE buffer. The small subunit rRNA gene was amplified by polymerase chain reaction (PCR) on the DNA thermocycler PTC 200 (MJ Research), using the 18S rDNA specific terminal primers 18S001 and 18S13 (Table 1). The primers corresponded to the nucleotides 1–22 and 1,733–1,757 of the *Ochromonas danica* 18S rRNA gene (accession number: M32704, J02950) (Honda et al. 1999). Approximately 25 ng of genomic DNA was taken in 25 µl reaction volume containing 1 × PCR buffer, 200 µM each of dATP, dCTP, dGTP and dTTP, 5 pMoles of each primer, 1.5 mM MgCl₂, and 1.0 U *ampliTaq* Gold DNA polymerase (Applied Biosystems). Amplification conditions comprised: an initial denaturation step of 10 min at 94 °C followed by 35 cycles of 1 min denaturation at 94 °C, 1 min of annealing at 55 °C and 2 min of extension step at 72 °C and in the end a final extension step of 10 min at 72 °C. The PCR products were checked on 1.5% agarose gel in 1% TAE buffer before

further characterization by cloning and sequencing.

The PCR products were ligated into pCR[®]2.1 vector using the TA cloning kit (Invitrogen) according to the instructions of the manufacturer. The constructs were transformed into *Escherichia coli* DH5α competent cells by heat shock method. Recombinant plasmids were isolated by alkaline lysis method and inserts were amplified using M13 universal primers (Table 1). Amplified inserts were then sequenced for both the strands on an automated DNA sequencer ABI-Prism3700 using the fluorescence-based detection approach and dideoxy-termination sequencing chemistry as per the manufacturer's details (Applied Biosystems). For sequencing, two additional internal primers (Table 1) were used in conjunction with the M13 universal primers that were originally used to amplify the cloned inserts.

The 18S rRNA gene sequences were edited and assembled using Auto-assembler program (Applied Biosystems) and deposited in the NCBI nucleotide sequence databank (GenBank Accession No. AY705753). The final sequences were used to identify and retrieve 36 related homologous sequences of reference organisms (Table 2) available in the GenBank database (National Center for Biotechnology Information, USA: NCBI Home page <http://www.ncbi.nlm.nih.gov>), using the BLASTn program (Altschul et al. 1990). The 18S rDNA sequences obtained for the organism were aligned with corresponding sequences belonging to the 34 reference Labyrinthulomycetes (Table 2) using the programs Clustal-X (<http://www.igbmc.u-strasbg.fr/BioInfo/>) (Higgins and Sharp 1989) and GenDoc (<http://www.psc.edu/biomed/genedoc>) (Nicholas et al. 1997) and also checked manually for large gaps. The aligned sequences were flushed at the ends to avoid missing information for any compared reference entries. This resulted in a final alignment of 1600 bp, which was used for further compar-

Table 2. List of 18S rRNA gene sequences of related Labyrinthulomycota species used as reference taxa in this study for ascertaining the generic affiliation of the new isolate.

Organism	GenBank accession number
<i>Oxytricha granulifera</i>	X53486
<i>Prorocentrum micans</i>	M14649
<i>Aplanochytrium kerguelense</i>	AB 022103
<i>Japanochytrium</i> Sp.	AB022104
<i>Labyrinthuloides haliotidis</i>	U21338
<i>Labyrinthula</i> Sp.	AB022105
<i>Schizochytrium aggregatum</i>	AB022106
<i>Schizochytrium limacinium</i>	AB022107
<i>Schizochytrium minutum</i>	AB022108
<i>Thraustochytrium aggregatum</i>	AB022109
<i>Thraustochytrium aureum</i>	AB022110
<i>Thraustochytrium kinnei</i>	L34668
<i>Thraustochytrium multirudimentale</i>	AB022111
<i>Thraustochytrium striatum</i>	AB022112
<i>Thraustochytrium pachydermum</i>	AB022113
<i>Ulkenia profunda</i>	L34054
<i>Ulkenia profunda</i> (#29)	AB022114
<i>Ulkenia radiata</i>	AB022115
<i>Ulkenia visurgensis</i>	AB022116
Thraustochytriidae sp. N4-103	AB073309
Thraustochytriidae sp. N1-27	AB073308
Thraustochytriidae sp. H6-16	AB073306
Thraustochytriidae sp. H1-14	AB073305
Thraustochytriidae sp. F3-1	AB073304
<i>Thraustochytrium</i> sp. CHN-1	AB126669
<i>Aplanochytrium stocchinoi</i>	AJ519935
<i>Schizochytrium</i> sp. KH105	AB052555
<i>Thraustochytrium</i> sp. KK17-3	AB052556
Labyrinthulid quahog parasite QPX	AF261664
Labyrinthulid quahog parasite QPX	AF155209
<i>Aplanochytrium</i> sp. SC1-1	AF348520
<i>Aplanochytrium</i> sp. PR1-1	AF348516
<i>Aplanochytrium</i> sp. PR12-3	AF348517
<i>Aplanochytrium</i> sp. PR15-1	AF348518
<i>Labyrinthula</i> sp.	AF348522
Thraustochytriidae sp. M4-103	AB073307

isons. The aligned sequences were then used to derive corrected Kimura two-parameter distance (Kimura 1980) estimates and infer phylogenetic relationships using both distance based as well as direct character-state-based methods, namely, neighbour-joining (Saitou et al. 1987) and maximum likelihood, with analytical routines available in the software packages PHYLIP 3.5c (Felsenstein 1993) (<http://evolution.genetics.washington.edu/phylip.html>) and Phylo_win (Galtier et al. 1996). In phylogenetic analysis, the two alveolates, *Prorocentrum micans* (Dinoflagellata) and *Oxytricha granulifera* (Ciliophora) were selected as outgroups to root the phenetic trees as they often form sister groups with the straminopiles in the 18S rDNA eukaryotic tree. The robustness of the phenetic clustering was ascertained by 500 bootstrap resamplings (Felsenstein 1985).

Acknowledgements

One of us (L.B.) is grateful to the Indian Council of Cultural Relations, New Delhi for the award of a Post-doctoral fellowship under the Indo-Italian Cultural Exchange Programme, during the tenure of which this work was carried out. We are also thankful to the Council of Scientific and Industrial Research, New Delhi and the Directors of NIO and CCMB for support. This is NIO contribution no. 4003

References

Ainsworth GC, Sparrow FK Jr., Sussman AS eds (1973) The Fungi. An Advanced Treatise, Vol. IV B. Academic Press, New York

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403–410

Cavalier-Smith T (1993) Kingdom Protozoa and its 18 phyla. *Microbiol Rev* **57**: 953–994

Cavalier-Smith T, Allsop M, Chao EE (1994) Thraustochytrids are chromists not fungi: signature sequences of Heterokonta. *Philos Trans R Soc Lond B* **346**: 387–397

Chamberlain AHL (1980) Cytochemical and ultrastructural studies on the cell walls of *Thraustochytrium* spp *Bot Mar* **23**: 669–677

Chamberlain AHL, Moss ST (1988) The thraustochytrids: a protist group with mixed affinities. *Biosystems (Amsterdam)* **21**: 341–349

Darley WM, Porter D, Fuller M (1973) Cell wall composition and synthesis via Golgi-directed scale formation in the marine eucaryote, *Schizochytrium aggregatum*, with a note on *Thraustochytrium* sp. *Arch Mikrobiol* **90**: 89–106

Dick MW (2001) Straminipilous Fungi. Kluwer Academic Publishers, The Netherlands

- Felsenstein J** (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791
- Felsenstein J** (1993) Documentation of PHYLIP (Phylogeny Inference Package) version 3.5c. University of Washington, Seattle, USA
- Gaertner A** (1967) Ökologische Untersuchungen an einem marinen Pilz aus der Umgebung von Helgoland. *Helgoländer wiss. Meeresuntersuchungen* **15**: 181–192
- Gaertner A** (1968) Eine Methode des quantitativen Nachweises niederer, mit Pollen köderbarer Pilze im Meerwasser und im Sediment. *Veröff Inst Meeresforsch Bremerh* **3**: 75–92
- Gaertner A** (1977) Revision of the Thraustochytriaceae (Lower marine fungi) I. *Ulkenia* nov. gen., with a description of three new species. *Veröff Inst Meeresforsch Bremerh* **16**: 139–157
- Galtier N, Gouy M, Gautier C** (1996) SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. *CABIOS* **12**: 543–548
- Goldstein S** (1963a) Morphological variation and nutrition of a new monocentric marine protist. *Arch Mikrobiol* **45**: 101–110
- Goldstein S** (1963b) Development and nutrition of a new species of *Thraustochytrium*. *Am J Bot* **50**: 799–811
- Goldstein S, Belsky M** (1964) Axenic culture of a marine phycocyanobacterium possessing an unusual type of asexual reproduction. *Am J Bot* **51**: 72–78
- Higgins DG, Sharp PM** (1989) Fast and sensitive multiple sequence alignments on a microcomputer. *CABIOS* **5**: 151–153
- Honda D, Yorochi T, Nakahara T, Erata M, Higashihara T** (1998) *Schizochytrium limacinum* sp. nov., a new thraustochytrid from a mangrove area in the west Pacific Ocean. *Mycol Res* **102**: 439–448
- Honda D, Yorochi T, Nakahara T, Raghukumar S, Nakagiri A, Schaumann K, Higashihara T** (1999) Molecular phylogeny of labyrinthulids and thraustochytrids based on the sequence of 18S ribosomal RNA gene. *J Eukaryot Microbiol* **46**: 637–647
- Jones EBG, Alderman DJ** (1971) *Althornia crouchii* gen. et sp. nov., a marine biflagellate fungus. *Nova Hedwigia* **21**: 381–389
- Kimura M** (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**: 111–120
- Leander C, Porter D** (2001) The Labyrinthulomycota is comprised of three distinct lineages. *Mycologia* **93**: 459–464
- Moss ST** (1985) An ultrastructural study of taxonomically significant characters of the Thraustochytriales and the Labyrinthulales. *Bot J Linn Soc* **91**: 329–357
- Nicholas KB, Nicholas HB Jr** (1997) GeneDoc: a tool for editing and annotating multiple sequence alignments [www.psc.edu/biomed/genedoc]
- Perkins FO** (1972) The ultrastructure of holdfasts, “rhizoids”, and “slime tracks” in thraustochytriacous fungi and *Labyrinthula* spp. *Arch Mikrobiol* **84**: 95–118
- Perkins FO** (1973) Observation on thraustochytriacous (Phycocyanobacteria) and labyrinthulids (Rhizopodiaceae) ectoplasmic nets on natural and artificial substrata—an electron microscope study. *Can J Bot* **51**: 485–491
- Porter D** (1990) Phylum Labyrinthulomycota. In Margulis L, Corliss JO, Melkonian M, Chapman DJ (eds) *Handbook of Protozoology*. Jones and Bartlett Publ., Boston, pp 388–398
- Raghukumar S** (1980) *Thraustochytrium benthicola* sp. nov.: a new marine protist from the North Sea. *Trans Br Mycol Soc* **90**: 273–278
- Raghukumar S** (1987) A device for continuous observation of microscopic aquatic organisms. *Indian J Mar Sci* **30**: 83–89
- Raghukumar S** (1988) *Schizochytrium mangrovei* sp. nov.: a thraustochytrid from mangroves in India. *Trans Br Mycol Soc* **90**: 627–631
- Raghukumar S** (2002) Ecology of the marine protists, the Labyrinthulomycetes (Thraustochytrids and Labyrinthulids). *Europ J Protistol* **38**: 127–145
- Saitou N, Nei M** (1987) The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**: 408–425
- Sambrook J, Fritsch EF, Maniatis T** (1989) *Molecular Cloning, A Laboratory Manual*, second ed. Cold Spring Harbor Press, New York
- Sheriff C, Whelan MJ, Arnold GM, Lafay JF, Brygoo I, Bailey JA** (1994) Ribosomal DNA sequence analysis reveals new species groupings in the genus *Colletotrichum*. *Exp Mycol* **18**: 121–128

Sparrow FK (1960) Aquatic Phycomycetes. The University of Michigan Press, Ann Arbor

White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and Direct Sequencing of Fungal

Ribosomal RNA Genes for Phylogenetics. In Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: A Guide to Methods and Application. Academic Press Inc., New York, pp 315–322

Available online at www.sciencedirect.com

