

Chapter 2.

Aplanochytrids from Zooplankton of Equatorial Indian Ocean

2.1 Introduction

Most of the known species belonging to the Labyrinthulomycetes so far, including thraustochytrids, labyrinthulids and aplanochytrids have been discovered and taxonomically described from coastal marine environments. The labyrinthulid genus, *Labyrinthula*, has been known as a parasite of seagrass for more than 80 years now (Young III, 1943; Short *et al.*, 1987; Muehlstein *et al.*, 1988) and many species of this genus are regularly found in seagrasses or in decaying mangrove leaves (Bigelow *et al.*, 2005; Steele *et al.*, 2005). The first thraustochytrid, *Thraustochytrium proliferum* was described by Sparrow (1936) from marine algae in the vicinity of Woods Hole (Atlantic coastal waters). Many other species of *Thraustochytrium* and the other genera of thraustochytrids, namely *Japonochytrium*, *Schizochytrium* and *Ulkenia* were subsequently described from coastal regions (see Raghukumar, 2002). These include littoral waters bounding Woods Hole, Massachusetts (Goldstein, 1963a; lida *et al.*, 1996), New Haven, Connecticut (Goldstein, 1963b; Goldstein & Belsky, 1964), hypersaline waters of Great Salt Lake, USA (Amon, 1978), North Sea (Gaertner & Raghukumar, 1980; Gaertner, 1982), seawater from mangrove area of Yap Islands in west Pacific Ocean (Honda *et al.*, 1998) and mangrove waters of Chorao Islands in India (Bongiorni *et al.*, 2005c). The genus *Labyrinthuloides* (Watson and Raper) Perkins (now

synonymous with *Aplanochytrium* Bahnweg and Sparrow) was described from coastal waters of York River, Virginia (Perkins, 1973). Several other species of *Aplanochytrium* were subsequently described in association with marine algae (see Raghukumar, 2002).

In contrast to coastal waters, the diversity of Labyrinthulomycetes in oceanic waters and sediments is poorly known. The first member of Labyrinthulomycetes to be described from oceanic waters was *Aplanochytrium kerguelensis* from the Antarctic waters by Bahnweg and Sparrow (1972). These authors also described several new species of *Thraustochytrium* from these waters.

Attempts to culture and identify Labyrinthulomycetes have also been mostly confined to a variety of substrates in coastal ecosystems (Table 1).

Table 1. Examples of studies to culture Labyrinthulomycetes from various substrates from all over the world.

Organisms	Substrate	Association	Area of isolation	References
Thraustochytrids	Algae	Saprotrophic	Maine; Southsea Castle, Hampshire; Dona Paula Bay & Zuari river, Goa	Booth & Miller, 1968; Miller & Jones, 1983; Sathe-Pathak <i>et al.</i> , 1993; Raghukumar <i>et al.</i> , 1992; Raghukumar, 1988
Thraustochytrids, Labyrinthulids	Mangrove leaves or detritus	Saprotrophic	Orda, Goa; Okinawa Prefecture, Japan; sub-tropical mangroves (25-27°N); Coastal area of Takehara in Seto Inland Sea, Japan; Futial National Nature Reserve, Shenzhen, China; Hong Kong; Indonesia, Palombina & Poetto Beach, Goro Lagoon, Ancona Port (Italy)	Raghukumar, 1988; Perveen <i>et al.</i> , 2006; Yokochi <i>et al.</i> , 2001; Bowles <i>et al.</i> , 1999; Kumon <i>et al.</i> , 2006; Wong <i>et al.</i> , 2005; Fan <i>et al.</i> , 2002 a,b; Fan <i>et al.</i> , 2007; Kamlangdee & Fan, 2003; Unagul <i>et al.</i> , 2005; Bongiorno <i>et al.</i> , 2005c
Thraustochytrids, Aplanochytrids	Seagrasses	Saprotrophic	Florida, Puerto Rico; Eastern Canadian coastal sites from Nova Scotia, Newfoundland, Labrador	Leander <i>et al.</i> , 2004; Burja <i>et al.</i> , 2006
Labyrinthulids	Seagrasses	Parasitic	US, west coast of Europe; Japan	Short <i>et al.</i> , 1987; Muehlstein <i>et al.</i> , 1988; Ralph & Short, 2002; Short <i>et al.</i> , 1993; Bigelow <i>et al.</i> , 2005
Thraustochytrids, Aplanochytrids	Sediments	Saprotrophic	59-61°N, 50-51°N; Fladenground area of North Sea; Eastern	Bowles <i>et al.</i> , 1999; Raghukumar, 1980; Burja <i>et al.</i> ,

			Canadian coastal sites from Nova Scotia, Prince Edward Island, New Brunswick, Newfoundland, Labrador; South coast of Madeira, Portugal; Indonesia, Palombina & Poetto Beach, Goro Lagoon, Ancona Port (Italy)	2006; Jakobsen <i>et al.</i> , 2007; Bongiorno <i>et al.</i> , 2005b
Thraustochytrids, Labyrinthulids, Aplanochytrids	Invertebrates	Saprotrophic, Endolithic	Coasts of Maine, Georgia, Jamaica; York river, Virginia; Northern Arabian Sea; Estuarine region in southern Portugal	Porter & Lingle, 1992; Perkins, 1973; Raghukumar & Raghukumar, 1999; Azevedo & Corral, 1997; Naganuma <i>et al.</i> , 1996 (Kimura <i>et al.</i> , 1999)
Thraustochytrids, Labyrinthulids, Aplanochytrids	Invertebrates	Parasitic	Eastsound & Bellingham Bay, Washington; Buckingham Canal; Canada; India; Vancouver Island; Gulf of St. Lawrence; Northern Adriatic Sea;	McLean & Porter, 1982; Loganathan <i>et al.</i> , 1988; Jones & O'Dor, 1983; Anderson <i>et al.</i> , 2003; Polglase, 1980; Raghukumar, 1987a & b; Bower, 1987; Bower <i>et al.</i> , 1989; Whyte <i>et al.</i> , 1994; Schärer <i>et al.</i> , 2007
Thraustochytrids	Coral and coral mucous	Saprotrophic	Gulf of Eilat, Israel; India	Harel <i>et al.</i> , 2008; Raghukumar & Balasubramanian, 1991
Thraustochytrids	Cell lines of coral, tunicates, sponges, mollusks, grass prawn	Contaminant	Israel, Japan	Frank <i>et al.</i> , 1994; Rinkevich, 1999; Rinkevich & Rabinowitz, 1997; Rinkevich <i>et al.</i> , 1998; Hsu <i>et al.</i> , 1995; Kawamura & Fujiwara, 1995

Thraustochytrids and labyrinthulids have frequently been isolated from decaying mangrove leaves. Numerous parasitic, as well as non-parasitic associations with invertebrates have been reported. Among the important parasitic associations are the QPX- quahog parasite unknown in the clam *Mercenaria mercenaria*, the yellow spot disease in the skin of the nudibranch *Tritonia diomedea* and skin ulcers in the octopus *Eledone cirrhosa* (Polglasse, 1980; McLean & Porter, 1982; Whyte *et al.*, 1994; Smolowitz & Leavitt, 1997; Anderson *et al.*, 2003). *Schizochytrium* sp. was found to infect gills of squid *Illex illecebrosus* and cause fin rot and ulcerative lesions in estuarine fishes *Lates calcerifer* and *Etroplus suratensis* (Loganathan *et al.*, 1988; Jones & O'Dor, 1983). *Labyrinthuloides haliotidis*, now known as *Aplanochytrium haliotidis*, causes a pathogenic infection in abalone *Haliotis kamtschatkana* (Bower, 1987; Bower *et al.*, 1989).

Non-parasitic, saprophytic associations of thraustochytrids include their dense presence in faecal pellets of the salp *Pegea confoederata*, the occurrence of *Ulkenia visurgensis* in coelenteron and hydranth of a hydroid and their presence in gut contents and faecal material of the sea urchin *Lytechinus variegates* (Raghukumar & Raghukumar, 1999; Wagener-Merner *et al.*, 1980). When present in corals and coral mucus, they may provide nutritional sources to corals helping them survive during

bleaching events (Raghukumar & Balasubramanian, 1991; Harel *et al.*, 2008). Particularly interesting are thraustochytrids that are common contaminants in cell cultures of sponges, cnidarians, crustaceans, molluscs, echinoderms and tunicates (Rinkevich, 1999). These examples suggest a definite role for saprophytic thraustochytrids in association with these invertebrates.

Members of Labyrinthulomycetes have not often been brought into culture and identified from the oceanic environment. An exception is the isolation and description of *Aplanochytrium kerguelensis* and four *Thraustochytrium* species (Bahnweg & Sparrow, 1972 & 1974), from Antarctic waters. Therefore, we have no clear picture of their association with invertebrates in the oceanic environment, unlike what we know for coastal habitats. The dominant invertebrates in the oceanic water column are crustacean mesozooplankton.

The following objectives were formulated in light of the above.

- (1) To investigate the occurrence of Labyrinthulomycetes in zooplankton of the equatorial Indian Ocean water by isolating them in culture.
- (2) To taxonomically identify isolates of Labyrinthulomycetes, from zooplankton by studying their life cycle and morphology.

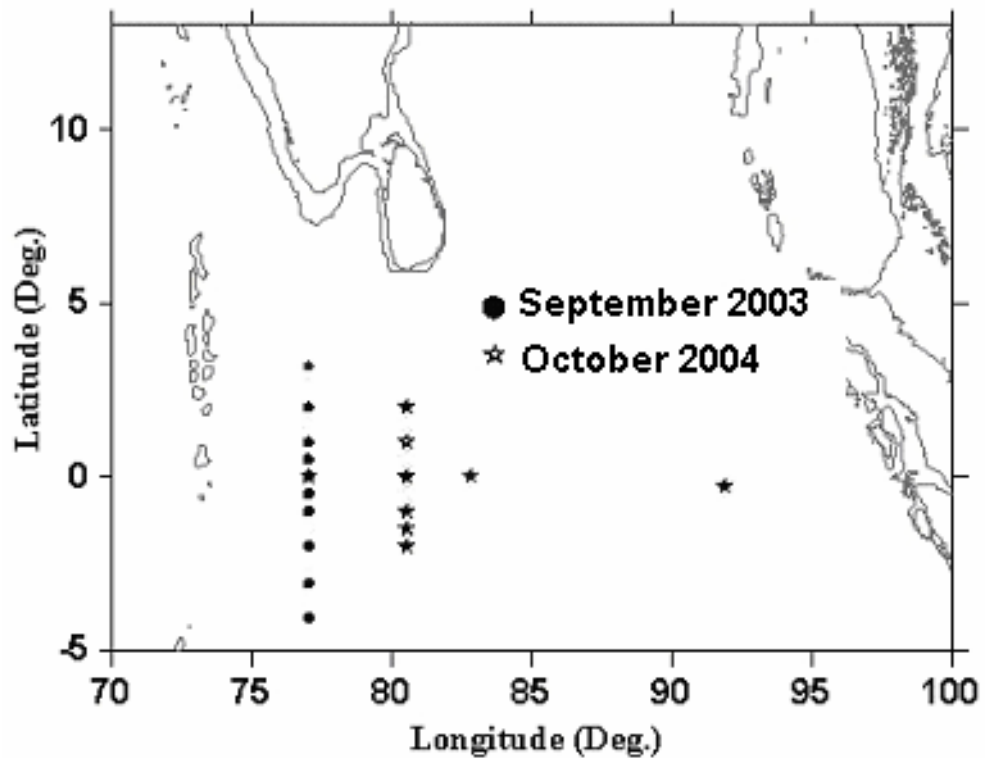
(3) To study a few physiological characteristics of the Labyrinthulomycetes isolated from zooplankton by studying their carbon and nitrogen requirements.

(4) To analyze the morphological and physiological groupings of the Labyrinthulomycetes isolated from zooplankton by carrying out Cluster analysis.

2.2 Materials and Methods.

2.2.1. Isolation of Labyrinthulomycetes: Labyrinthulomycetes were isolated from mesozooplankton from the equatorial Indian Ocean on board ORV Sagar Kanya during Cruise # SK 196 in September 2003 and Cruise # SK 212 in October 2004. Zooplankton were collected using a Multiple Plankton Net (MPN, MultiNet Type Midi with 200 μ m mesh nets, Hydrobios, Germany) from different depths ranging from surface to 1000 m . A total of 76 samples were collected from 10 stations in September 2003 and 9 stations in October 2004 (Fig. 1).

Fig. 1 ORV Sagar Kanya station locations in the equatorial Indian Ocean during cruises SK 196 (September 2003) and SK 212 (October 2004).



Zooplankton specimens were thoroughly washed in sterile seawater and individual animals were plated on to Modified Vishniac (MV) Agar plates (Porter, 1990) with the following composition (ingredients added as w/v).

- Dextrose: 1.0 %
- Peptone: 0,15 %
- Yeast extract: 0.01 %
- Liver infusion broth: 0.001 %;
- Agar: 0.8 %
- Seawater: 100 mL

- Fetal bovine serum: 1 % (Added separately to the autoclaved medium)
- Procaine penicillin: 40000 (added separately to the autoclaved medium)
- Streptomycin (Ambistryn): 0.075 g (Added separately to the autoclaved medium).

In addition to zooplankton specimens, attempts were made to isolate thraustochytrids and aplanochytrids from faecal pellets of zooplankton during October 2004. Faecal pellets were collected using the method modified from the one used by [Raghukumar & Raghukumar \(1999\)](#). Freshly collected zooplankton specimens were placed in 500 ml beakers containing 0.22 μm filter-sterilized seawater in such a way that they were suspended over a 100 μm mesh bolting silk. After 1 hour the animals were removed and the material that had passed through the mesh and collected at the bottom of the beakers were plated on MV medium. The plates were incubated for 7 – 14 days till colonies of thraustochytrids or aplanochytrids appeared. Colonies of these organisms were recognized by their general morphology. They resemble yeast colonies. However, unlike the shiny texture of yeasts, colonies of thraustochytrids and aplanochytrids possess rough textures. In addition, cells of thraustochytrids vary largely in size, ranging from 2 – 20 μm . The identity of the colonies was further confirmed by examining the colonies under the lower power of a microscope. Bacteria-free colonies were then sub-

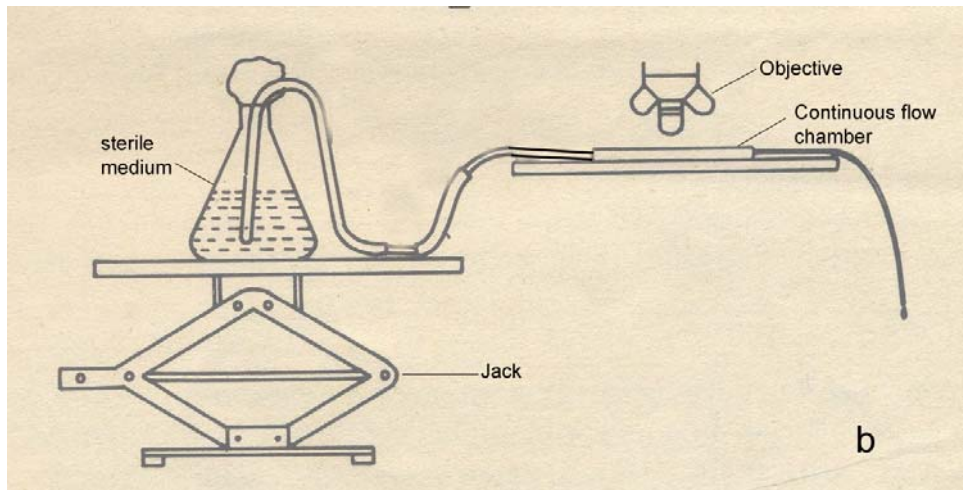
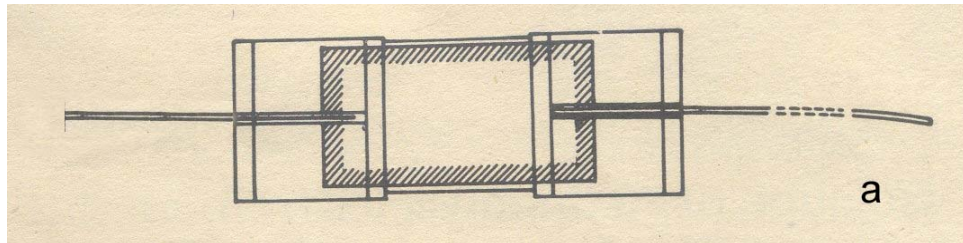
cultured on to sterile MV agar plates without antibiotics. In addition, they were also sub-cultured onto sterile seawater containing autoclaved *Artemia* larvae, obtained using standard methods of hatching *Artemia* cysts in seawater (Bahnweg & Sparrow, 1974). Growth on MV plates and *Artemia* larvae was observed after about three days of inoculation. Cultures were maintained by routine sub-culturing on MV agar tubes containing 0.4 % agar, instead of 0.8 % agar as in the isolation plates.

2.2.2. Morphology and Life Cycle: Colony and cell morphology of all the isolates was examined by streaking cultures on MV agar plates. A cover slip was gently placed over seven day old colonies which were then examined microscopically using a Carl Zeiss 'Axioskop' 2 plus microscope. Colony characteristics described by Leander *et al.* (2004) were used to distinguish the isolates.

Life cycle of 14 isolates was examined using a modified continuous flow chamber described by Raghukumar (1987). Basically, this chamber consists of a small piece of glass slide (3 x 2.3 cm) fixed on an aluminium slide containing groove of the same size as that of the glass piece (Fig. 2). A small drop of inoculum is placed in the centre of the slide. A coverslip of 24 mm² is placed on a firm base and silicon vacuum grease is applied on all edges, using a syringe. The coverslip is lowered on the slide and gently

pressed at the edges to make the grease spread thinly and evenly to seal the inoculum drop in the middle. At the two ends of the glass slide a silicon tubing (ID 0.8 mm, OD 2 mm) is fixed for the supply of nutrients or seawater. One end of the tubing is inserted into sterile seawater or liquid medium and the other end serves as an outlet for the same medium to ensure supply of fresh medium and removal of used medium. The flow of the water or medium is gentle to ensure that the inoculum is not washed off. The flow is regulated by adjusting the height of the jack on which the flask containing the liquid medium is rested. This device allows continuous microscopic observation and photomicrographic recording of cells for a period of 2 to 3 days. Life cycle and development of the isolates were photographed using a Zeiss AxioCam digital camera.

Fig. 2. Diagrammatic representation of continuous flow chamber (a) and assembly of the chamber for observation under microscope (b). (From Raghukumar, 1987).

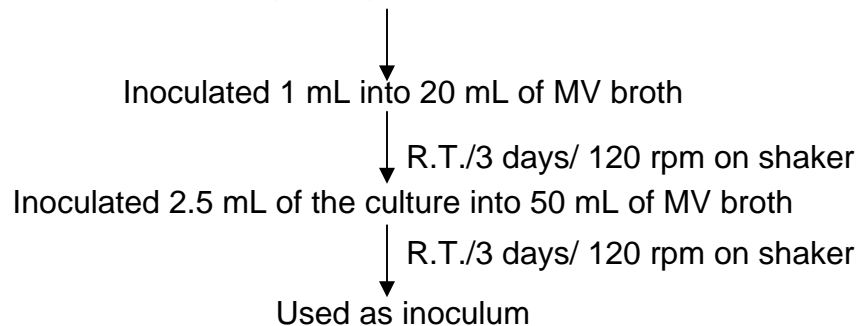


2.2.3. Physiological characterization: Fourteen isolates were characterized in terms of their carbon and nitrogen requirement, as well as the production of extracellular, hydrolytic enzymes.

2.2.3.1. Carbon requirements: Inoculum for experiments on carbon requirements was prepared as given in Figure 3. Experimental flasks contained 20 ml of MV medium using different sugars, in place of glucose. The sugars were autoclaved separately and added at a final concentration of 1 % to the MV medium excluding glucose. The inoculum volume corresponded to 5 % (1 ml).

Fig. 3 Flow diagram of preparation of inoculum of aplanochytrid isolates for the experiment on carbon utilization

Three day old culture growing in seawater/ pine pollen medium



A total of 16 sugars as follows were tried, based on [Bahnweg \(1979b\)](#).

- Hexoses: Glucose, Galactose, Rhamnose, Fructose
- Pentoses: Ribose, Xylose, Arabinose
- Disaccharides: Lactose, Sucrose, Maltose, Cellobiose, Trehalose, Mellibiose
- Trisaccharides: Raffinose
- Polysaccharides: Xylan, Starch

Growth was checked after 3 days of inoculation by measuring the increase in dry weight.

2.2.3.2. Nitrogen requirements: Fourteen isolates were examined.

Since many thraustochytrids and aplanochytrids do not use inorganic nitrogen, 16 amino acids were tested as follows, based on [Bahnweg \(1979a\)](#).

- Aliphatic: Glycine, Alanine, Leucine
- Aromatic: Tryptophan, Phenylalanine
- Acidic: Glutamic acid
- Basic: Histidine, Lysine, Arginine
- Amide: Asparagine
- Hydroxyl: Serine, Threonine
- Cyclic: Proline
- Sulphur-containing: Cysteine, Cystine
- Others: Ornithine

Growth of different nitrogen sources was compared in terms of colony diameter, since biomass differences in a liquid medium were not clear-cut.

The following medium was used for these experiments (all values w/v).

- Dextrose: 1.0 %.
- KH₂PO₄: 0.025 %.
- Agar: 0.6 %
- Seawater: 100 ml
- Fetal bovine serum (added after autoclaving the medium).
- Individual amino acids as above (filter-sterilized and added after autoclaving the medium): 1.0 %.
- Mixture of 0.005 % riboflavin and 0.005 % cyanocobalamin: 0.1 %
- Procaine penicillin: 20000 U
- Streptomycin (Trade name Ambystrin-S): 0.0375 g.

The serum, amino acids, vitamins, penicillin and streptomycin were added sterile after autoclaving the rest of the medium.

The cultures were streaked on the agar plates and the colony diameter was measured after 5 days of growth at R.T.

2.2.3.3. Production of extracellular enzymes: Qualitative assays were carried out on 14 isolates for the production of protease, lipase, amylase and chitinase. All isolates were inoculated from a 3 day old cultures growing in seawater/ pine pollen medium into MV agar plates supplemented with 1 % w/v of various substrates for the enzymes, as follows and the enzyme activity was examined after growth for 5 days.

- Protease ([Molitoris, 2000](#)): 1 % skimmed milk (Trade name, Sagar, India). Presence and extent of the zone of clearance around the colony indicated enzyme activity.
- Lipase ([Molitoris, 2000](#)): 1 % Tween 80 with 0.1 g L⁻¹ calcium chloride hydrous. Production of white precipitate indicates enzyme activity.
- Amylase ([Molitoris, 2000](#)): 1 % Starch. Blue colouration of the medium after the addition of Lugol (iodine) solution indicates absence of amylase activity and a colourless halo surrounding a colony indicates its presence.

- Chitinase: Chitin (Hi Media) was dissolved in 50 % sulphuric acid and precipitated with cold distilled water by diluting it 15 fold. The precipitate was washed with distilled water till the pH was close to 7.0. The precipitate was collected by centrifugation and weighed (Austin, 1988). The required amount was then autoclaved separately prior to adding to the autoclaved medium. Presence and extent of the zone of clearance around the colony indicated enzyme activity.

2.2.3.4. Cluster analysis: Cluster analysis using unweighted pair group average method in Statistica v 5.0 software was carried out between all the isolates to analyse the similarities between them. The characteristics used for the analysis are presented in Table 2. A total of 27 each of morphological as well as physiological characters were used for the analysis amounting to a total of 54 characters. Each character used for the analysis was designated plus or minus based on its presence or absence in that particular isolate. Likewise, characteristics of all the isolates were described as plus or minus. Phenograms were plotted based on the analysis in Statistica v 5.0 software.

Table 2. Characters used for cluster analysis of all the 14 isolates from equatorial Indian Ocean.

No.	Morphological characters	Physiological characters (the best C or N source)
1	Colony with agar penetration	Glucose
2	Colony with rays of continuous band of cells	Galactose
3	Colony with rays of disjoint patches of cells	Rhamnose
4	Colony with rays sprawling from centre outwards	Fructose
5	Colony with rays sprawling from periphery of colony	Ribose
6	Colony with clumps	Xylose
7	Young cell 3-6 μm	Arabinose
8	Young cell 6-8 μm	Cellobiose
9	Young cell 8-10 μm	Trehalose
10	Mature cell <10 μm	Lactose
11	Mature cell 10-20 μm	Maltose
12	Mature cell 20< μm	Sucrose
13	Sporangium 10-18 μm	Mellibiose
14	Sporangium 18-26 μm	Raffinose
15	Sporangium 26-34 μm	Xylan
16	Spore 3-4 μm	Starch
17	Spore 4-5 μm	Proline
18	Spore 5-6 μm	Glutamic acid
19	Spore 6-7 μm	Asparagine
20	Circular spores	Lysine
21	Cuniform spores	Arginine
22	Circular and cuniform spores	Glycine
23	Ellipsoidal spores	Alanine
24	Oval spores	Leucine
25	Intact cell wall during spore release	Serine
26	Disintegrating cell wall during spore release	Threonine
27	Presence of amoeboid cells	Ornithine

2.3. Results

2.3.1. Isolation of Thraustochytrids and Aplanochytrids

A total of 2100 zooplankton specimens, collected from 76 multiple plankton net samples and obtained from 19 stations during two cruises were plated onto MV medium for isolating thraustochytrids and aplanochytrids (Table 3). Out of this, a total of 171 specimens, corresponding to around 8 % were positive for the protists.

Out of the total 171 colonies obtained, a total of 14 isolates were subcultured and maintained. Five of these were isolated during September 2003 and the remaining 9 during October 2004 (Table 4). Three further isolates were obtained from the plates on which the filtrate containing fecal pellets was plated.

Table 3. Details of CTD stations where Multiple Plankton Net operations were carried out and sampling depths. Depth ranges given in bold indicate samples from which aplanochytrids were recovered in culture.

Sampling period	Time (hr)	Latitude	Longitude	Depths (m)
September 2003 (Cruise SK 196)	0750	3° N	77° E	500- 300, 300- 200, 200- 40, 40-0
	0230	02° N	77° E	500- 300, 300- 200, 200- 40, 40-0
	1100	01° N	77° E	500- 300, 300- 200, 200- 40 , 40-0
	1900	0.5° N	77° E	500- 300, 300- 200, 200- 70, 70-0
	0730	0° Eq	77° E	500- 300, 300- 200, 200- 40, 40-0
	0115	0.5° S	77° E	500- 300, 300- 200, 200- 30, 30-0
	0350	1° S	77° E	500- 300, 300- 200, 200- 40, 40-0
	0115	2° S	77° E	500- 300, 300- 200, 200- 30, 30-0
	0600	3° S	77° E	500- 300, 300- 200, 200- 40 , 40-0
	1700	4° S	77° E	500- 300, 300- 200, 200- 30, 30-0
October 2004 (Cruise SK 212)	2130	0° Eq	77° E	500- 300, 300- 200, 200- 60, 60-0
	0020	2° N	80.5° E	500- 300, 300- 200, 200- 20, 20-0
	2330	1° N	80.5° E	500- 300 , 300- 200, 200- 30 , 30-0
	1430	0° Eq	80.5° E	1000-500 , 500- 200, 200- 38, 38- 0
	1600	0° Eq	83° E	1000-500, 500- 300, 300- 30, 30- 0
	0830	1° S	80.5° E	1000-500, 500- 200, 200- 60, 60- 0
	1340	1.5° S	80.5° E	500- 300, 300- 200, 200- 60, 60-0
	2215	2° S	80.5° E	500- 300 , 300- 200, 200- 60, 60-0
	1400	0° Eq	93° E	1000-500, 500- 300, 300- 30, 30- 0

Table 4. Depth and location in the equatorial Indian Ocean from which isolates were obtained.

Date	Latitude	Longitude	Depth (m)	Isolate No.
05 Sep.2003	02° 59.5' N	77° 01.4' E	300- 200	S1961
05 Sep.2003	02° 59.5' N	77° 01.4' E	200- 40	S1962
05 Sep.2003	02° 59.5' N	77° 01.4' E	40- 0	S1963
06 Sep.2003	02° 00.1'N	77° 00.3' E	40- 0	S19610
07 Sep.2003	01° 00.2' N	77° 00.0' E	200- 40	S19615
28 Oct.2004	00° 59.893' S	80° 30.317' E	1000- 500	S2121
28 Oct.2004	00° 59.893' S	80° 30.317' E	60- 0	S2122
29 Oct.2004	01° 28.917' S	80° 30.629' E	60- 0 (faecal pellets)	S2123, S2124
30 Oct.2004	01° 55.932' S	80° 39.926' E	200- 60	S2125, S2126, S2127
30 Oct.2004	01° 55.932' S	80° 39.926' E	500- 300	S2128
06 Nov.2004	00° 00.539' S	92° 58.552' E	30- 0 (faecal pellets)	S2129

2.3.2. Morphology and Life Cycle

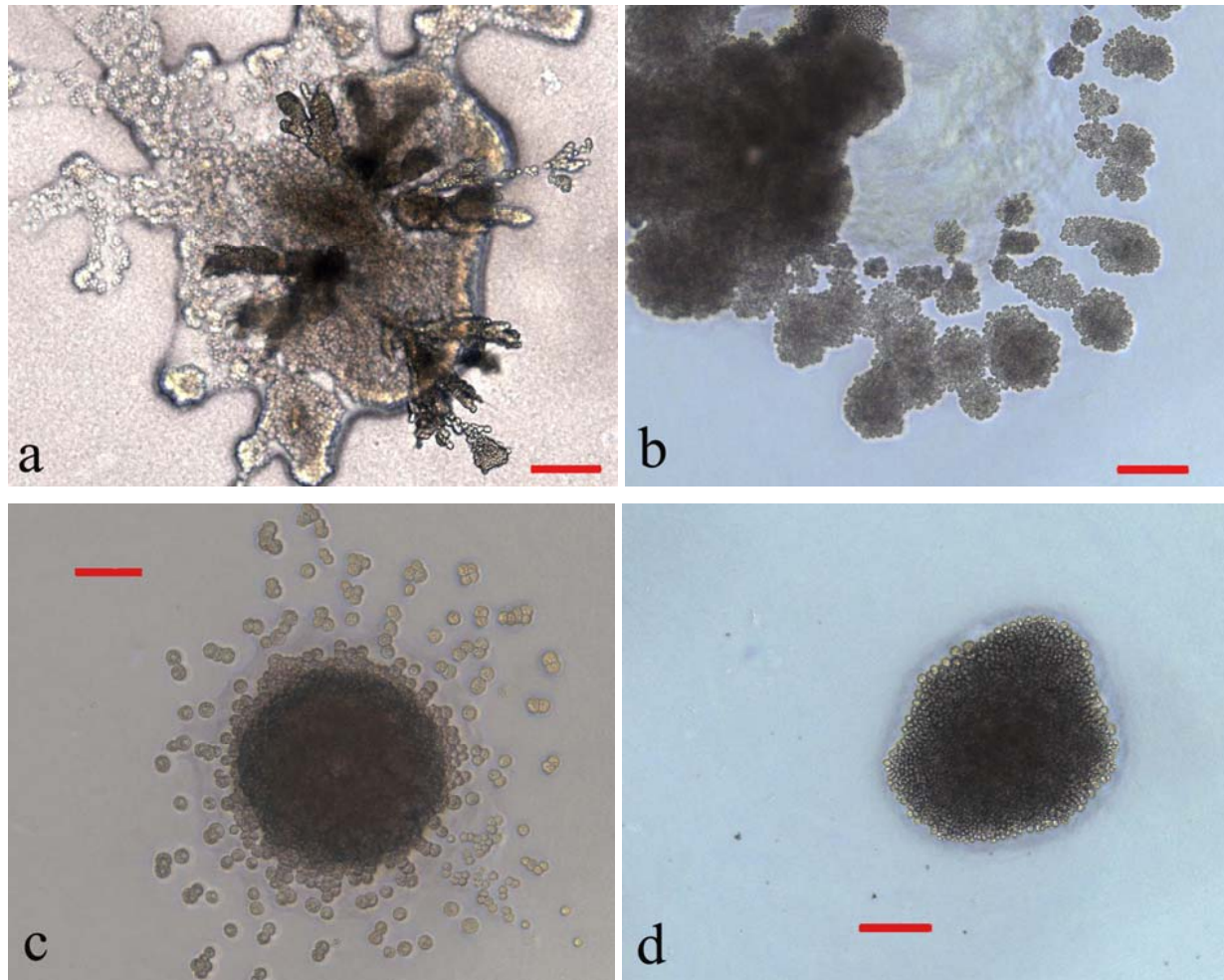
Colony morphology of the isolates could be broadly classified into two categories (Fig. 3). In the first type, cells of the organism penetrated the agar and grew within (Fig. 3a), while such behaviour was absent in the other category (3b-d). These two groups could be further subdivided into two subgroups each, namely those in which the colonies produced distinct

broad rays of continuous band or patches of cells (Fig. 3a,b,c) and those in which the rays were absent (Fig. 3d). The characteristics of individual isolates in terms of colony morphology are presented in Table 5.

Table 5. Colony characteristics of the 14 isolates from Indian Ocean.

Colony Type			Isolate No.
Agar penetration present	Colonies forming broad rays sprawling from the center outwards		S1961, S1963, S19615
	Colonies forming clumps of cells on the agar surface	Radial pattern, wavy margin	S1962
	Colonies forming clumps of cells on the agar surface along with distinct rays	Radial pattern, wavy margin	S19610
		Radial pattern absent	-
No agar penetration	Colonies forming broad rays sprawling from the center outwards		S2123, S2125
	Colonies forming clumps of cells on the agar surface	Radial pattern, wavy margin	S2126
		Radial pattern absent	S2122, S2129
	Colonies forming clumps of cells on the agar surface along with distinct rays	Radial pattern	S2127
		Radial pattern absent	S2121, S2124, S2128

Fig. 3. Examples of different colony morphologies of various isolates from the equatorial Indian Ocean. Bar represents 100 μ m. (a) Colony of isolate S1961 producing rays of continuous band of cells with agar penetration; (b) colony of S2123 with same type of rays without agar penetration; (c) clump-like colony of S2127 producing rays of disjointed patches of cells from the edge of the colony; (d) clump-like colony of S1962.



The ectoplasmic net elements (EN) showed three kinds of morphology. Isolates S1962, S1963, S2123, S2125, S2127 and S2129 produced fine filaments of EN, that were less than 0.5 μm in width (Fig. 4a). EN elements of isolate S2125 had a very broad base of 3 μm or more (Fig. 4b). Isolates S1961, S19610, S19615, S2121, S2126 and S2128 also produced fine filaments, but the filaments often showed broad areas in between, that were 0.5 to 1 μm wide (Fig. 4c). Isolates S2122 and S2124 produced only broad filaments, that were 0.5 to 1 μm wide (Fig. 4d).

All 14 isolates showed a similar development and life cycle pattern, as follows (Fig 5). Motile spores, ranging in size from 3.1 to 7.0 μm settled down and grew into mature vegetative cells. These cells were globose to subglobose in size and ranged in size from 7.8 to 27.8 μm in diameter. Mature vegetative cells transformed into sporangia, whereby the cytoplasmic contents divided simultaneously into 12 to 32 spores. The spores were enclosed within the cell wall of the sporangium. Alternatively, the mature vegetative cell divided into a diad and then a tetrad and subsequently into a number of small cells, the spores, all enclosed within the sporangial wall. The spores were later released from the fully mature sporangium either by dissolution of its cell wall or through a tear or break at any point on the cell wall (Fig. 6). In the former case, the cell wall disappeared and the spores moved away from each other by gliding

Fig. 4. Examples of the different morphologies of the ectoplasmic net elements (EN) (arrows) of various isolates from the equatorial Indian Ocean. Bar represents 100 μ m. (a) Cells possessing very fine filaments of EN; (b) cells possessing EN with a broad base; (c) cells possessing fine EN filaments with broad areas in between; (d) cells with broad filaments of EN.

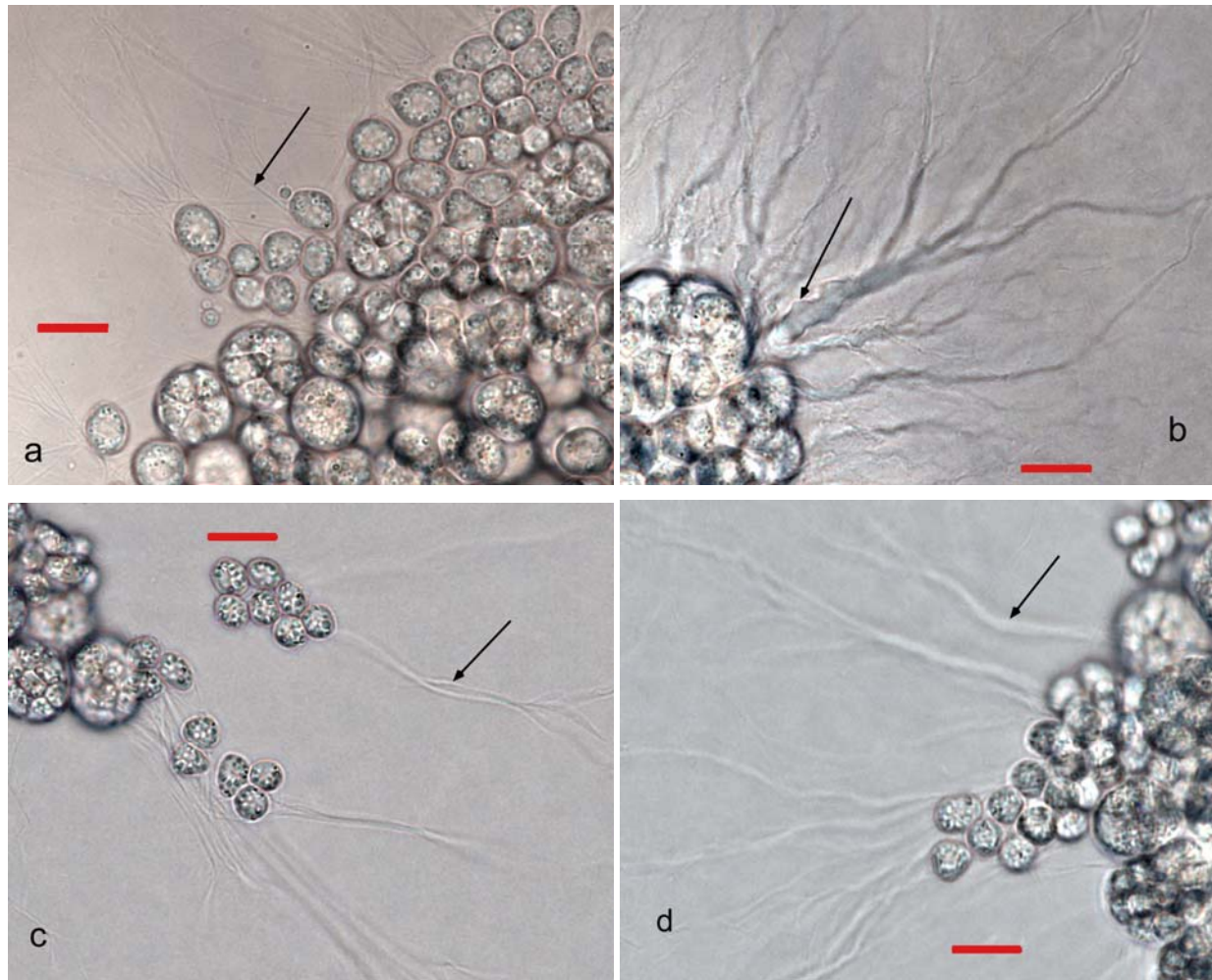
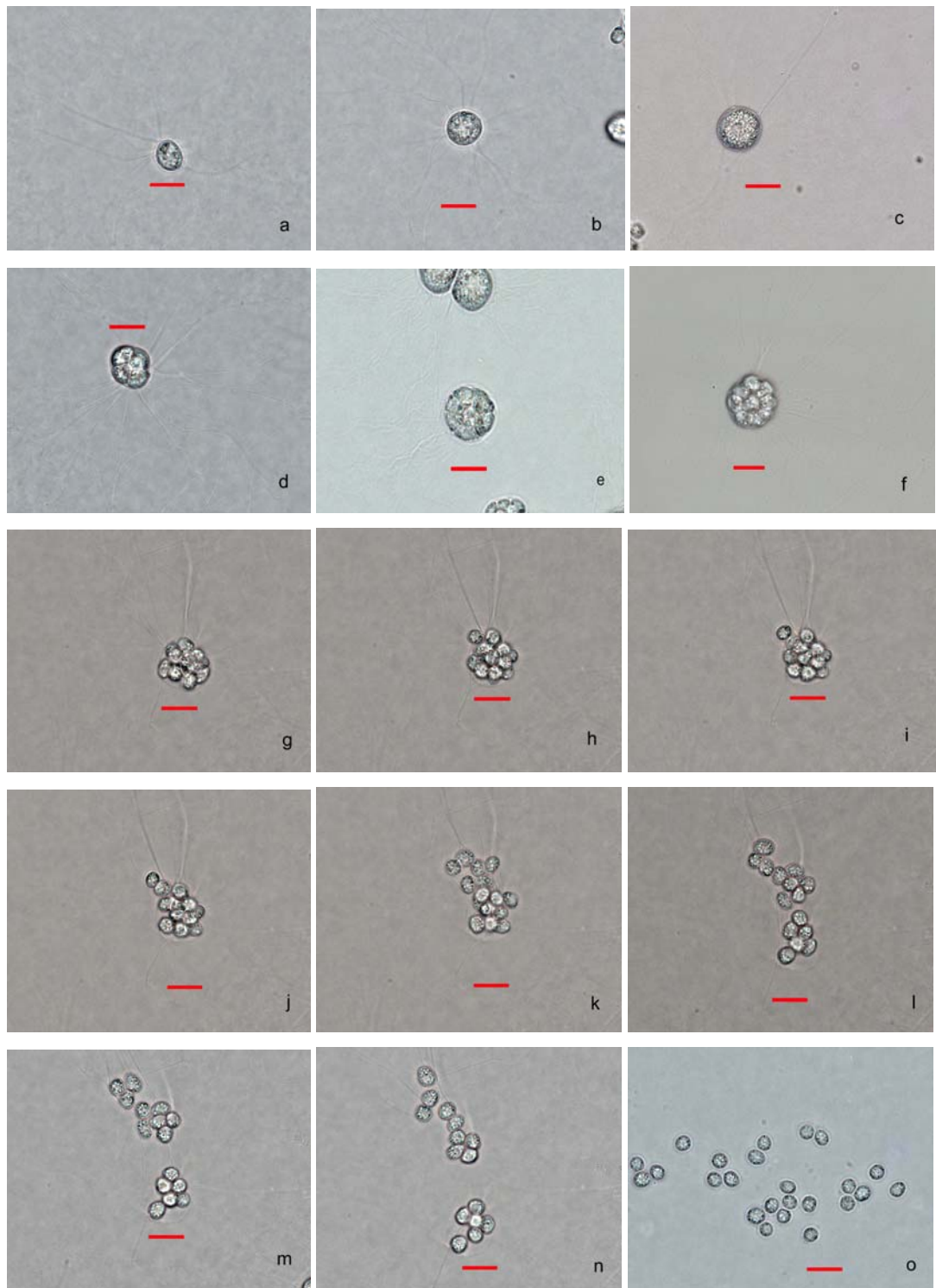


Fig. 5. Representative development and life cycle pattern based on different isolates of *Aplanochytrium yorkensis* isolated from the equatorial Indian Ocean. Bar represents 10 μ m.



motion over the ectoplasmic net elements. In the latter case, the spores were released through one or two points of break in the cell wall. The spores when released from the sporangium moved away from the same by gliding over the ectoplasmic net elements. The spores were cuniform, circular, oval or ellipsoidal in shape (Fig. 7). The isolates were identified as *Aplanochytrium yorkensis* (Perkins) Leander and Porter (syn.: *Labyrinthuloides yorkensis* Perkins (1973), based on their developmental cycle and presence of a large eccentric vacuole in the cell that was obvious in case of larger cells (Fig. 8). Amoeboid cells were produced occasionally. These either divided into non-motile vegetative cells (Fig. 9) or motile spores (Fig. 10).

None of the 14 isolates corresponded to thraustochytrids. The morphology of all colonies that grew out of zooplankton also resembled those of *Aplanochytrium* as described above. Although all the isolates corresponded to *Aplanochytrium yorkensis*, they differed widely in cell shape and size, the number, shape and size of spores formed, the mode of liberation of the spores and the presence or absence of amoeboid cells. These differences are tabulated in Table 6.

Fig. 6. Release of spores by dissolution of cell wall (a,b,c) and break at two points (d) and one point in cell wall (e,f). Bar represents 10 μ m.

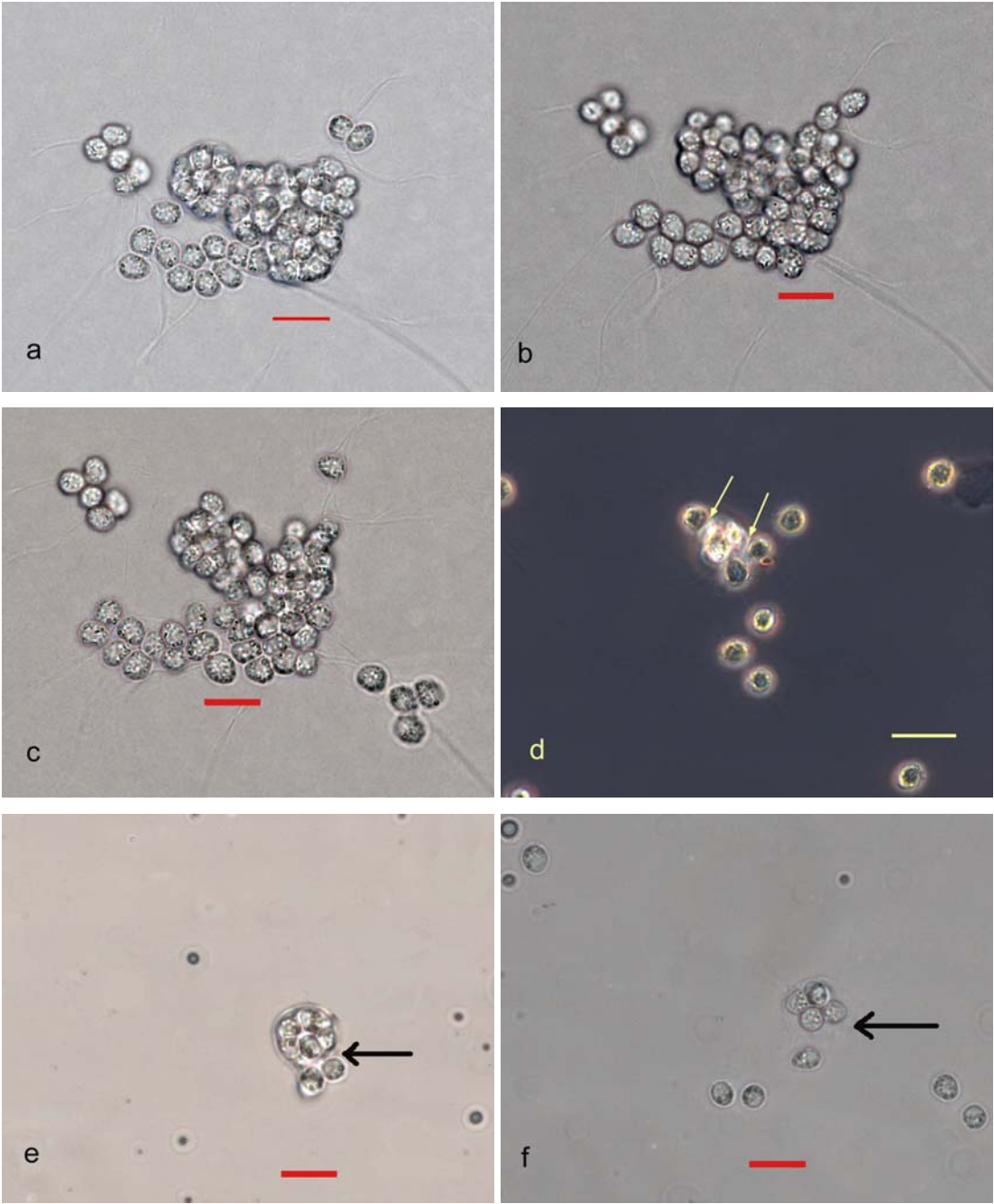


Fig. 7. Various spore shapes (a) cuniform, (b) circular, (c) ellipsoidal (marked by arrow) and (d) oval (marked by arrow) (Bar represents 10 μ m).

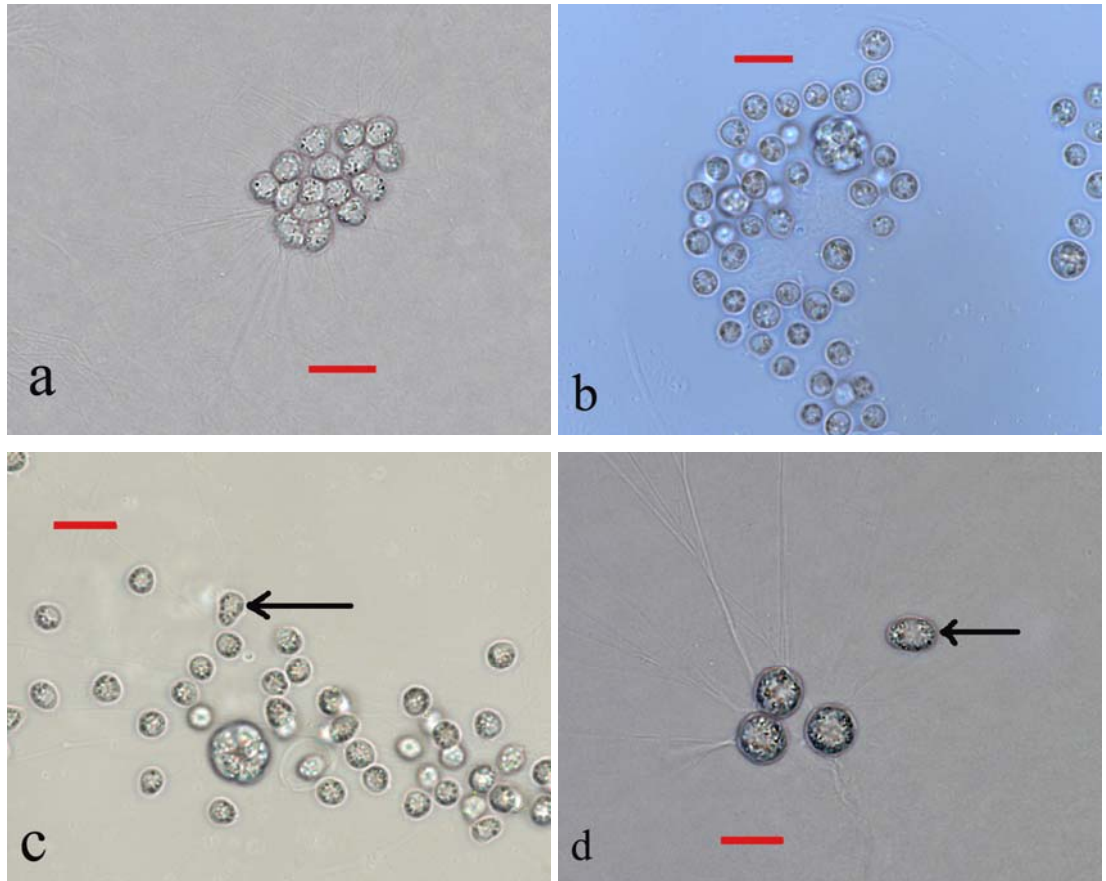


Fig. 8. Various developmental stages of aplanochytrid isolate S2127 displaying large eccentric vacuoles inside the cells (Bar represents 20 μm).

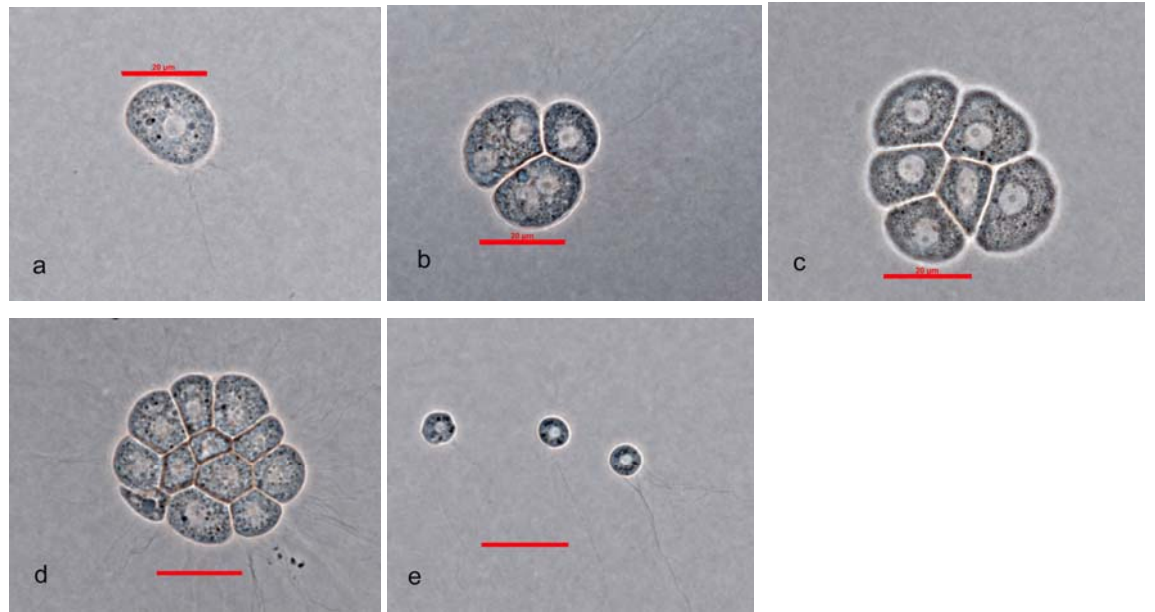


Fig. 9. Fragmentation of amoeboid cells into vegetative cells. (Bar represents 10 μm in a-e, 5 μm in f)

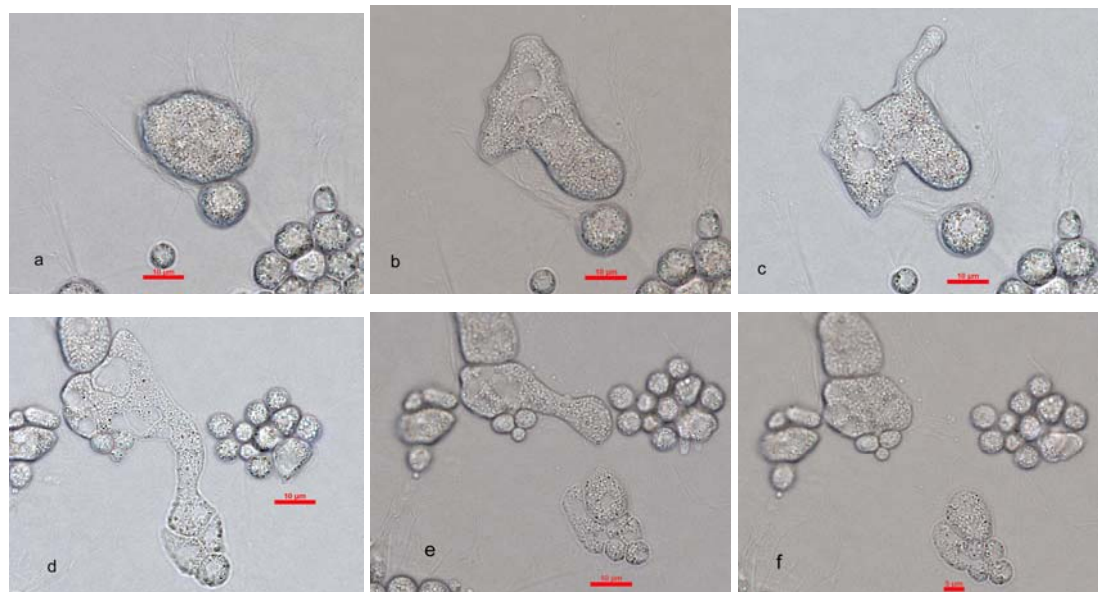


Fig. 10. Successive binary fission of amoeboid cells into spores.
(Bar represents 10 μm in a-c, e, g-l and 5 μm in d and f)

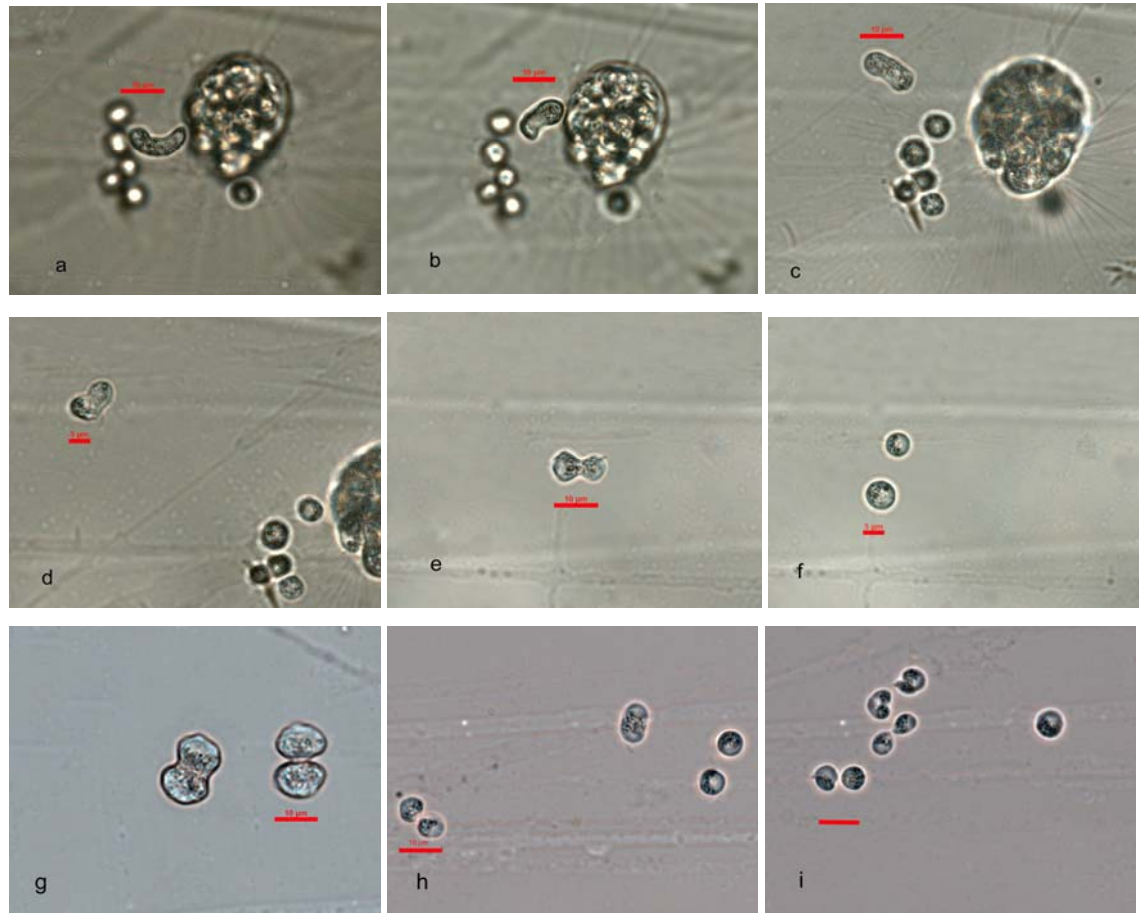


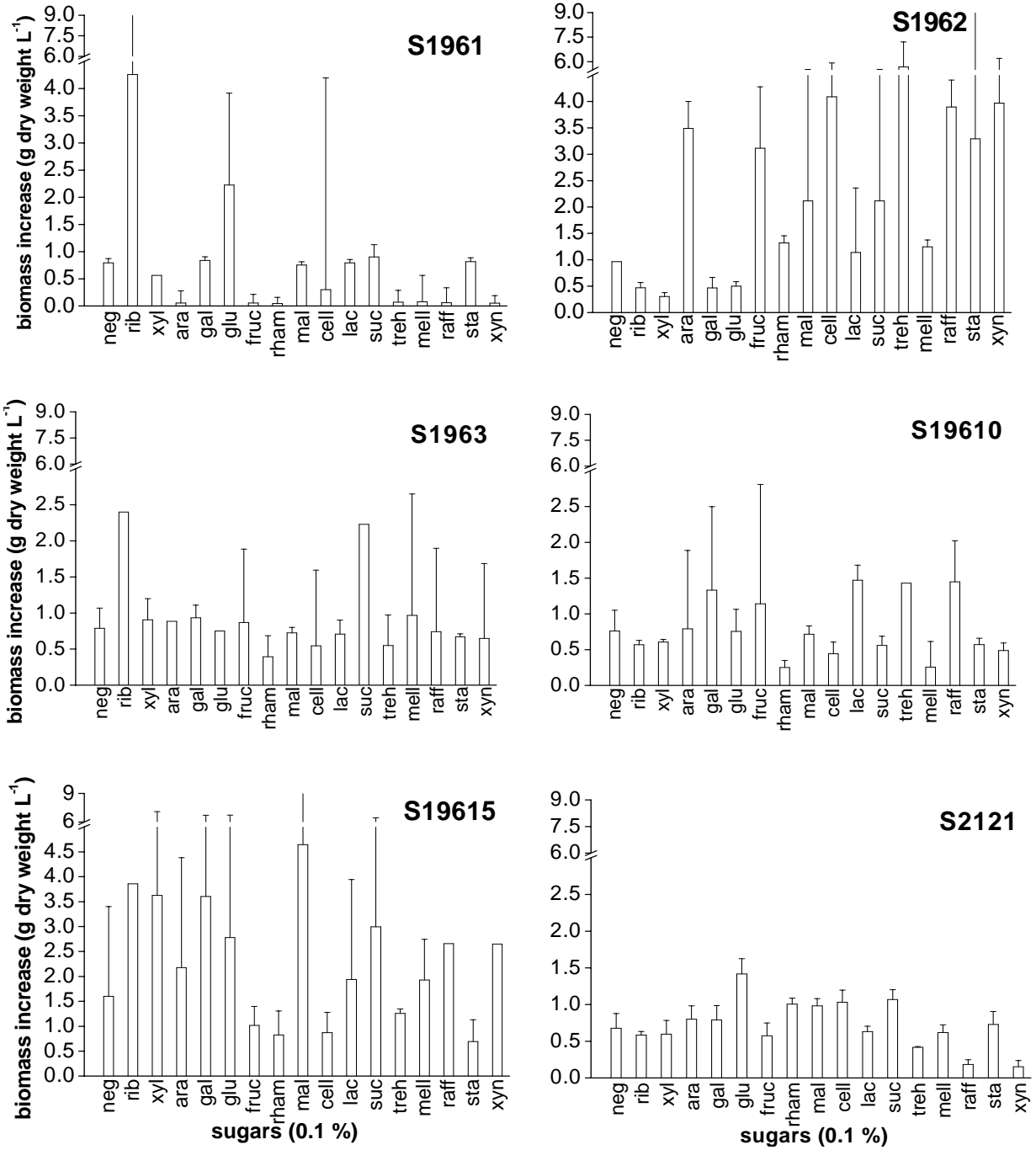
Table 6. Differences in cell morphology of the 14 isolates isolated from equatorial Indian Ocean

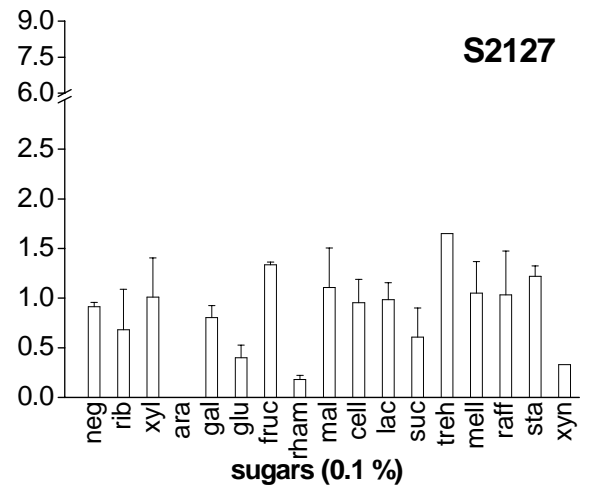
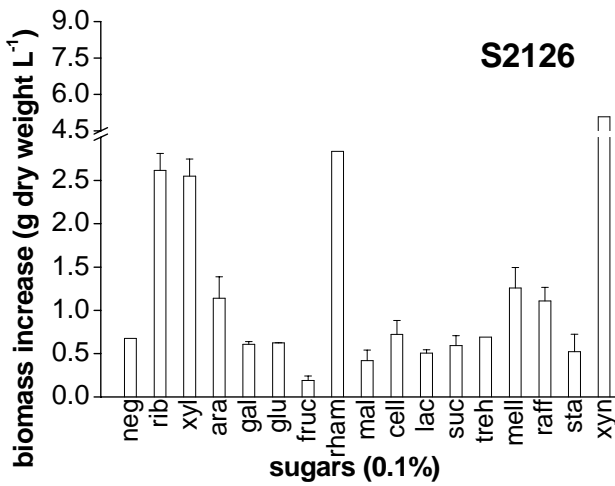
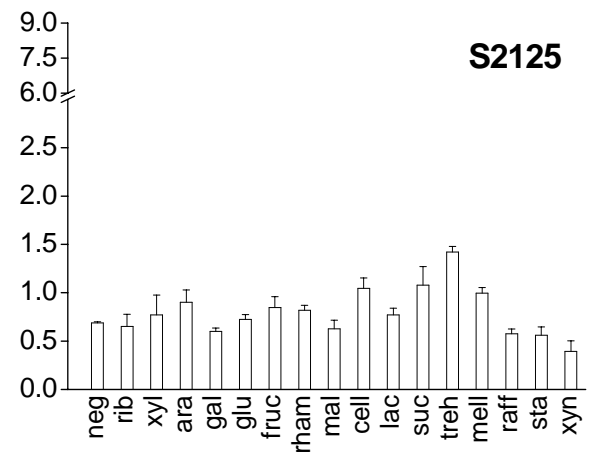
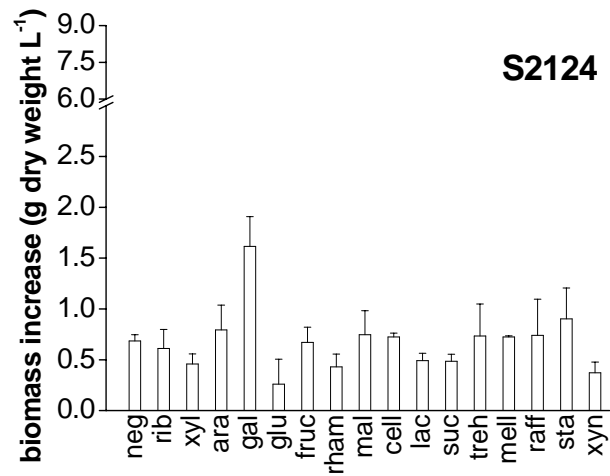
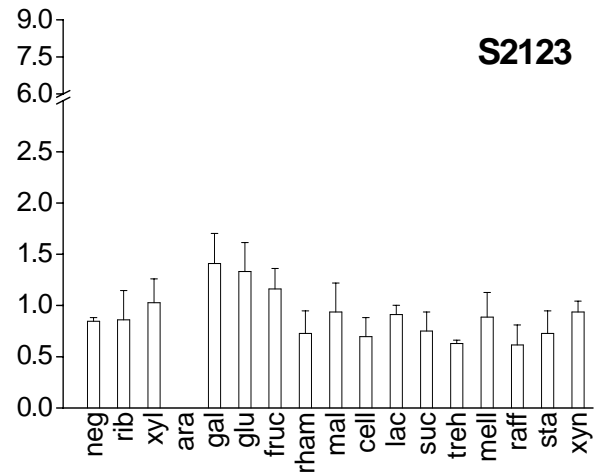
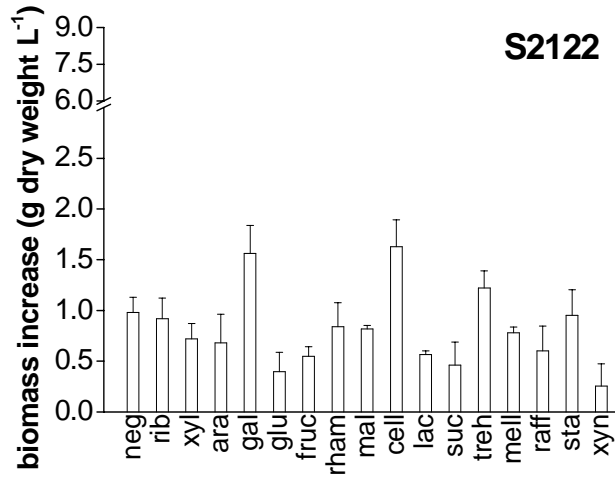
Isolate No.	Size (μm) of			No. of spores	Released spore shape	Presence of ectoplasmic net	Presence of intact cell wall	Presence of amoeboid cells
	Mature cell before formation of spores	Sporangium containing fully mature spores	Spore					
S1961	19.1- 27.8	19.4- 35.0	5.0- 5.8	23- 28	Cuniform	Fine with broad areas	disintegrating	Present sometimes
S1962	9.4- 13.5	13.8- 14.9	3.8- 4.6	-	Circular, cuniform	Fine filaments	-	Nil
S1963	10- 15.4	13.3- 16.9	3.1- 4.5	26- 32	Circular, cuniform, ellipsoidal	Fine filaments	Present (break at two points)	Nil
S19610	15.7- 20	20.0- 20.2	3.1- 5.8	17- 21	Circular	Fine with broad areas	disintegrating	Nil
S19615	10.6- 13.3	10.6- 17.6	3.3- 3.8	16- 26	Cuniform, ellipsoidal	Fine with broad areas	Present (break at one point or disintegrating)	Nil
S2121	10.7- 12.0	14.5-16.4	3.9- 5.0	15- 22	Cuniform	Fine with broad areas	disintegrating	Nil
S2122	7.8-13	13.3-17.5	3.8- 4.9	16- 18	Circular, cuniform	Broad	Present (break at one point)	Present
S2123	10.3- 15.7	12.5- 23.6	4.0- 5.7	16- 22	Circular, cuniform	Fine filaments	Present (break at one point)	Nil
S2124	12.5-15	13.3- 16.7	3.8- 4.9	12- 15	Circular, cuniform	Broad	Present (break at one point)	Present
S2125	9.4- 11.4	16.0- 22.5	4.4- 4.7	12- 13	Cuniform	Fine filaments broad base	Present (break at one point)	Present
S2126	13.5- 15.2	18.0- 27.6	4.8- 7.0	~ 28	Circular, oval	Fine with broad areas	disintegrating	Present
S2127	12.7- 27	18.3- 32	4.4- 6.5	-	Circular, cuniform	Fine filaments	disintegrating	Present
S2128	13.0- 15.2	15.0- 21.6	3.6- 5.0	21- 22	Circular, cuniform	Fine with broad areas	disintegrating	Nil
S2129	10.0- 12.5	13.8- 25	3.8- 5.1	12- 18	Cuniform	Fine filaments	Present (break at one point)	Nil

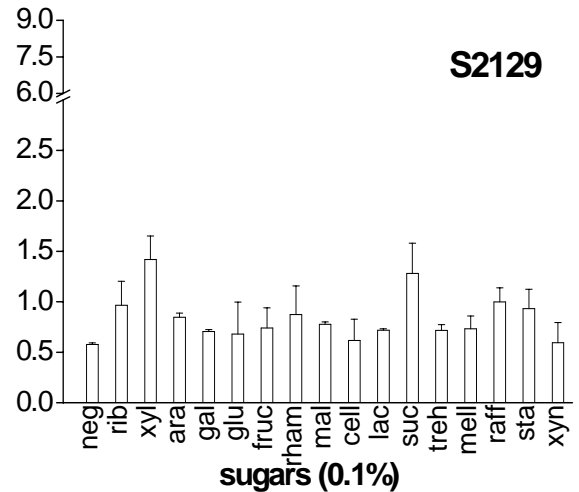
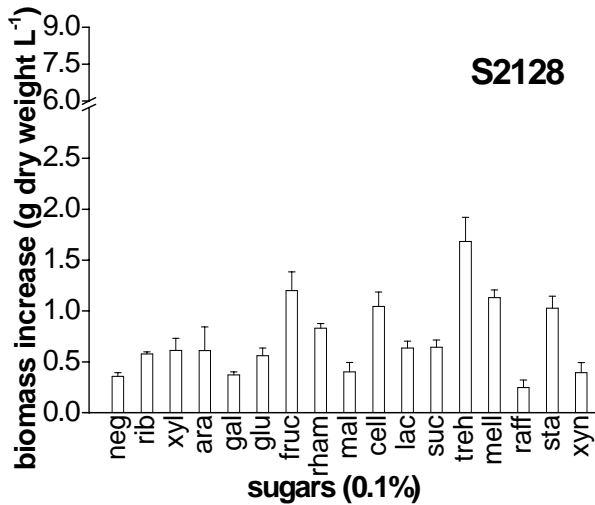
2.3.3 Physiological characterization

Most isolates utilized a broad range of sugars (Fig. 11). However, individual isolates varied in their preference to the different sugars. Three isolates, namely S1961, S1963 and S2129 grew best in the presence of the pentoses, ribose and xylose respectively. S2121, S2123 and S2124 utilized hexoses best. Of these, isolate S2121 preferred glucose, while S2123 and S2124 showed maximum growth in the presence of galactose. Eight isolates showed a preference for the disaccharides maltose, trehalose, rhamnose, cellobiose and lactose. Isolate S2122 also showed best growth in presence of cellobiose followed by galactose. Isolate S2126 exhibited best growth in the presence of the polysaccharide xylan followed by isolates S1962 and S19615.

Fig. 11. Growth of the 14 aplanochytrid isolates on various carbon sources. (Key: neg- negative control, rib- ribose, xyl- xylose, ara- arabinose, gal- galactose, glu- glucose, fruc- fructose, rham- rhamnose, mal- maltose, cell- cellobiose, lac- lactose, suc- sucrose, treh- trehalose, mell- mellibiose, raff- raffinose, sta- starch, xyn- xylan)

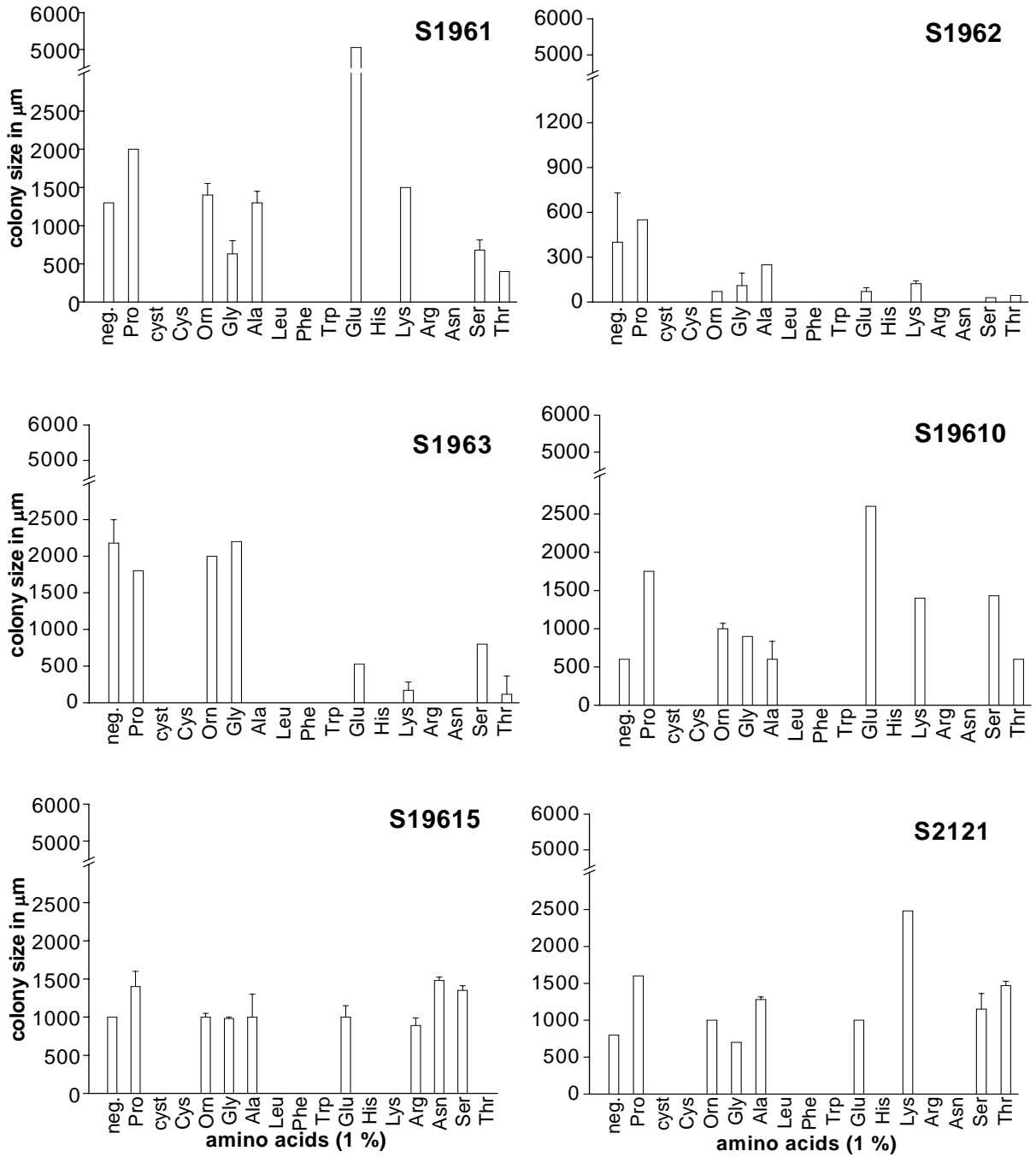


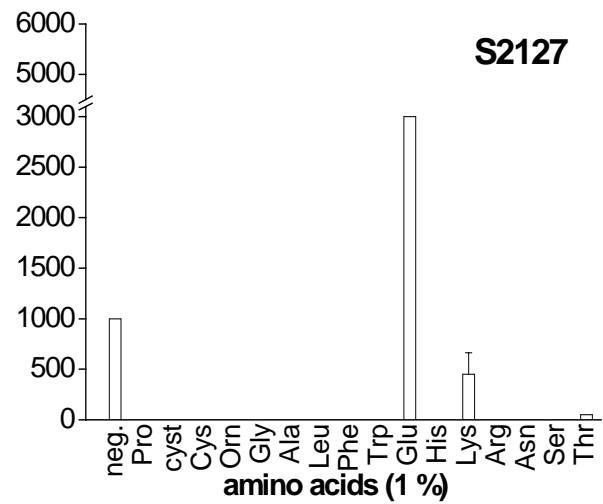
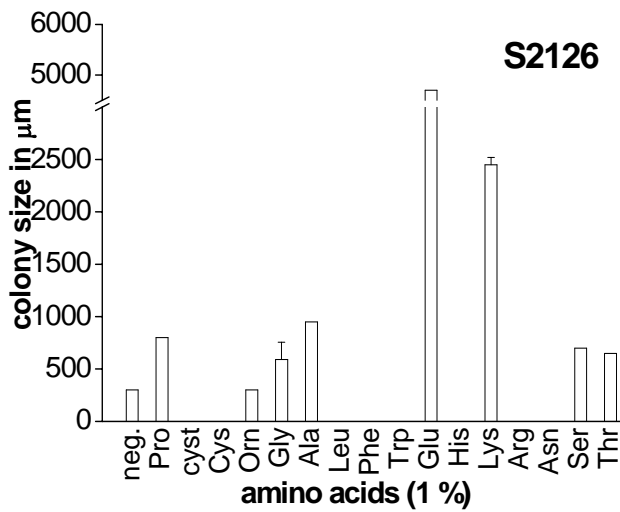
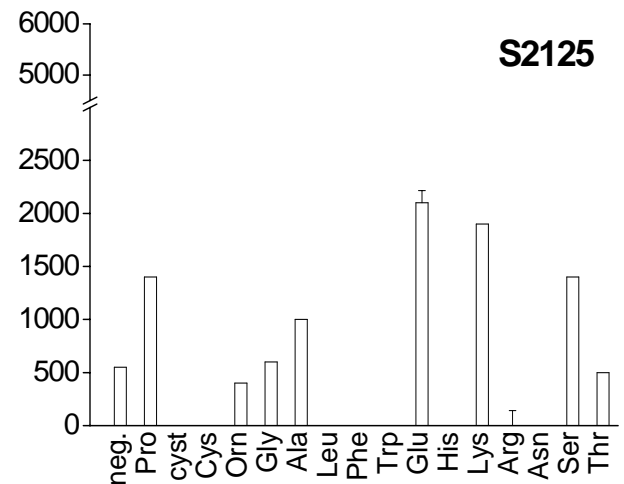
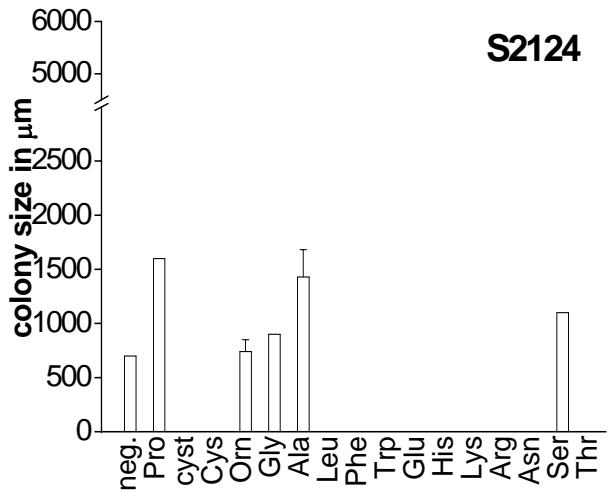
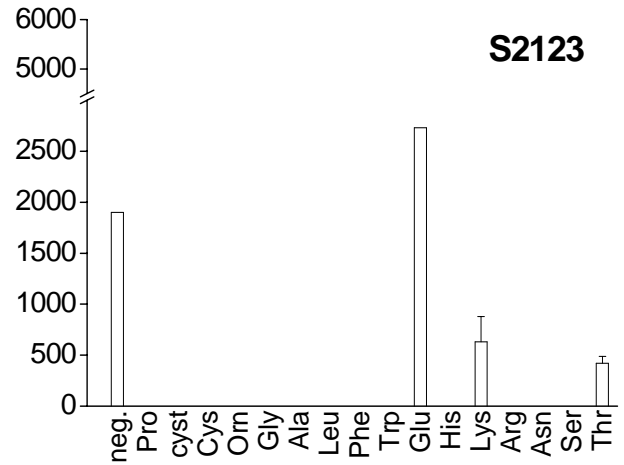
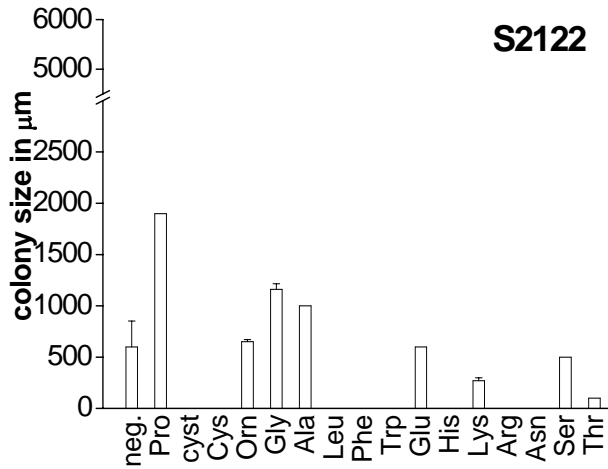


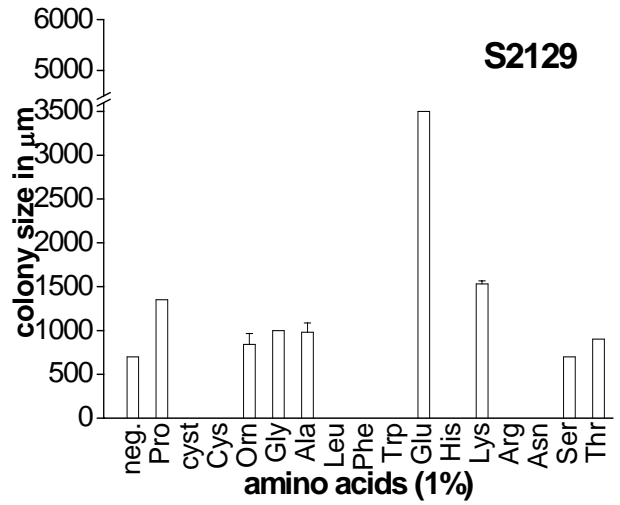
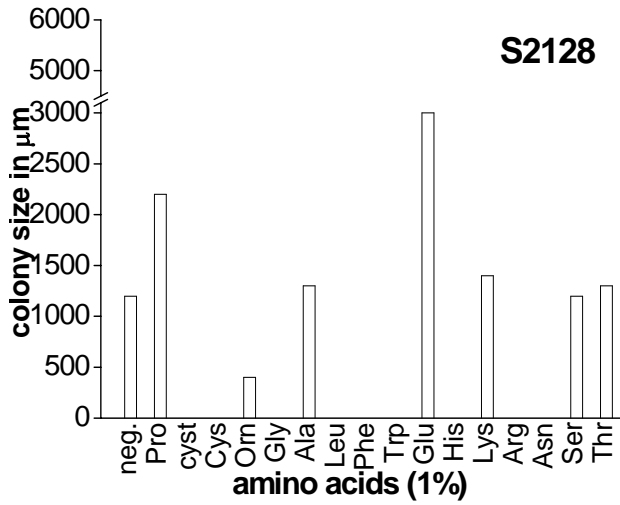


All isolates utilized glutamate. S1961 and S2126 isolates grew best in the presence of this amino acid, compared to others. All isolates except for S2123 and S2127 utilized proline and S2122 and S2124 preferred this amino acid as the nitrogen source to others. None of the isolates utilized the aromatic amino acids phenylalanine and tryptophan as well as others like cystine, cysteine, histidine and leucine.

Fig 12. Growth of the 14 isolates on various nitrogen sources. (Key: neg- negative control, Pro- proline, cyst- cystine, Cys- cysteine, Orn- ornithine, Gly- glycine, Ala- alanine, Ser- serine, Lys- lysine, Arg- arginine, Asn- asparagine, Thr- threonine, Glu- glutamic acid, His- histidine, Leu- leucine, Phe- phenylalanine and Trp- tryptophan)







Ten of the 14 isolates produced protease. None produced amylase, lipase or chitinase. Isolate S1963 showed the largest clearance zone on skimmed milk medium, while isolate S2123 showed the least (Table 7).

Table 7. Protease activity of 14 aplanochytrid isolates from equatorial Indian Ocean, as observed by the zone of clearance of milk protein.

Isolate no.	Zone of clearance of milk protein (mm)
S1961	16.5
S1962	nil
S1963	18.125
S19610	10.75
S19615	-
S2121	nil
S2122	nil
S2123	2
S2124	13.25
S2125	11.5
S2126	2.625
S2127	3.5
S2128	10.75
S2129	7.75

Cluster analysis was carried out using three different combinations of characters. (1) Morphological and life cycle characters; (2) physiological characters and (3) both morphological and physiological characters. Two distinct clusters were found in the phenogram done only with morphological characters (Fig. 13), one containing isolates with sporangia

> 25 μm size and the second containing those with < 25 μm . All other characters appeared in both clusters.

The phenogram generated using carbon and nitrogen requirements (Fig. 14) separated out S2121 in a cluster. This isolate preferred glucose as a carbon source and lysine and threonine as a nitrogen source. Within the second cluster, isolates S1961, S1963 and S19615, which utilized the pentose sugar ribose were clearly separated from the rest. Trehalose-utilizing isolates, S1962, S2125, S2127 and S2128 also stood out as a separate cluster.

The phenogram generated using a combination of both morphological as well as physiological characters placed a greater emphasis on the cell size (Fig. 15). Here again two distinct clusters were formed, one containing isolates with cell size up to 27.8 μm and sporangium size >25 μm and second containing isolates with smaller cell size up to 15.4 μm and smaller sporangium <25 μm . Rest all characters appeared to be scattered throughout the phenogram.

Fig. 13. Phenogram made by using 27 different morphological characters of fourteen isolates of *Aplanochytrium yorkensis*, isolated from the equatorial Indian Ocean.

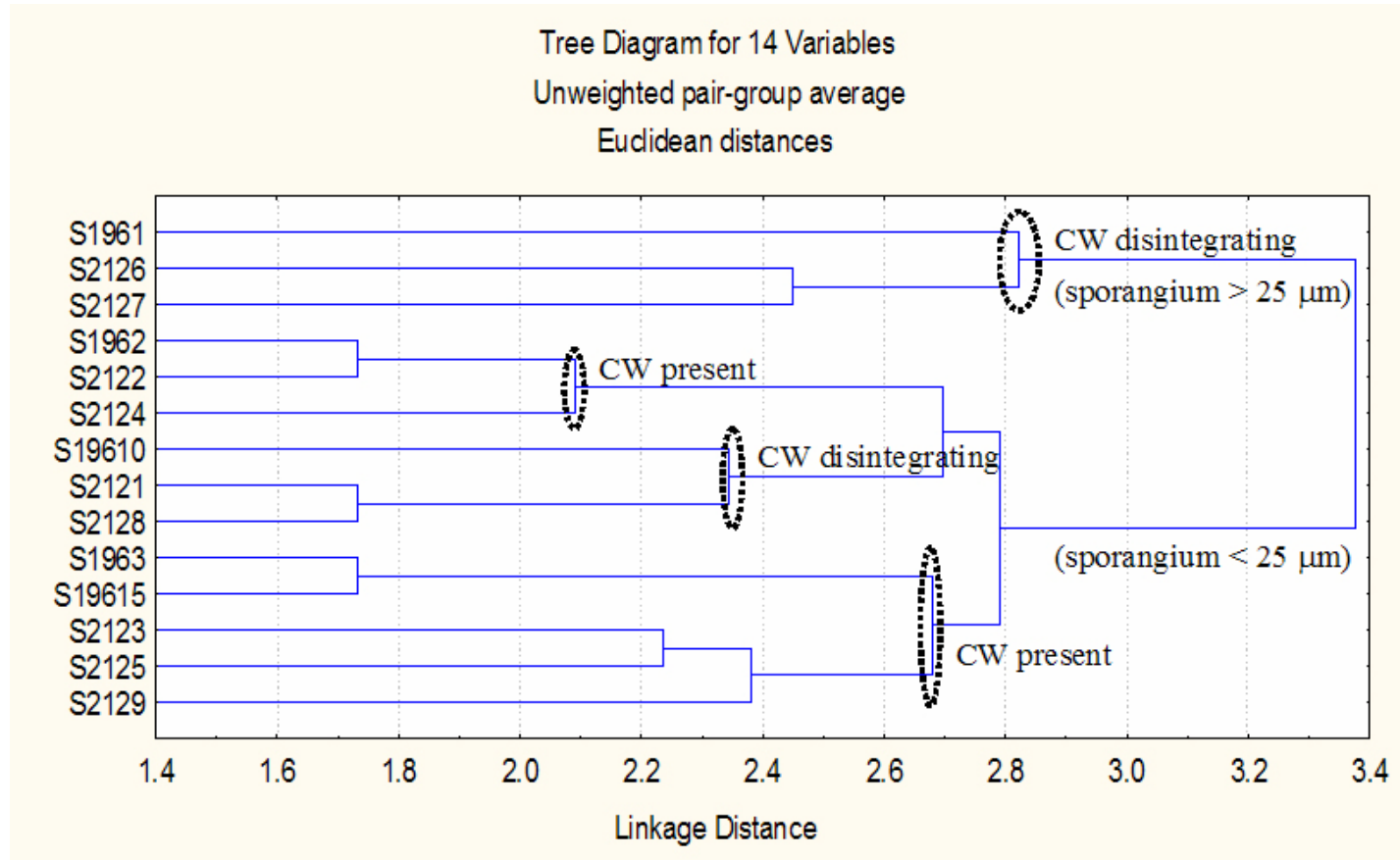


Fig. 14. Phenogram made by using 27 different physiological characters based on carbon and nitrogen nutrition of fourteen isolates of *Aplanochytrium yorkensis*, isolated from the equatorial Indian Ocean.

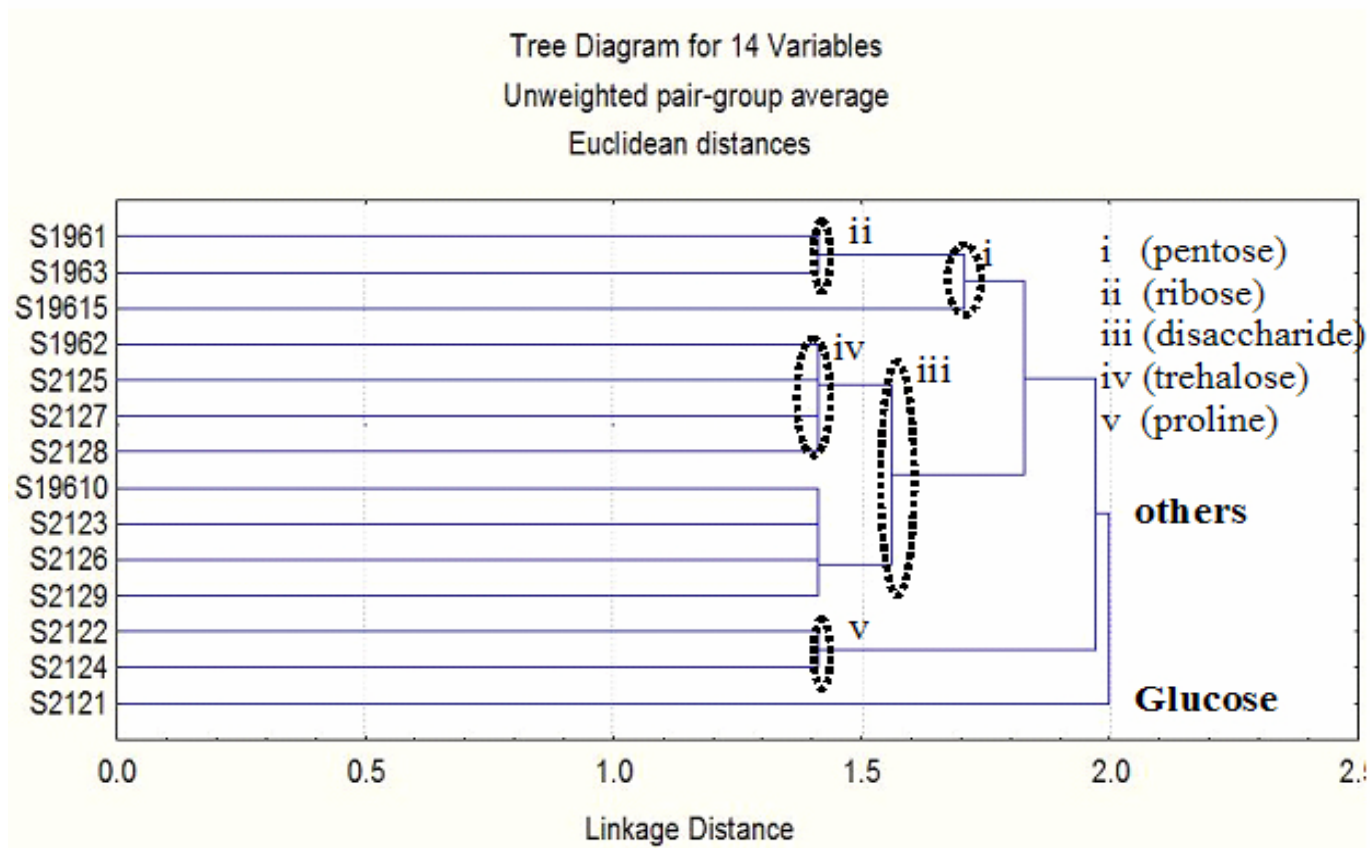
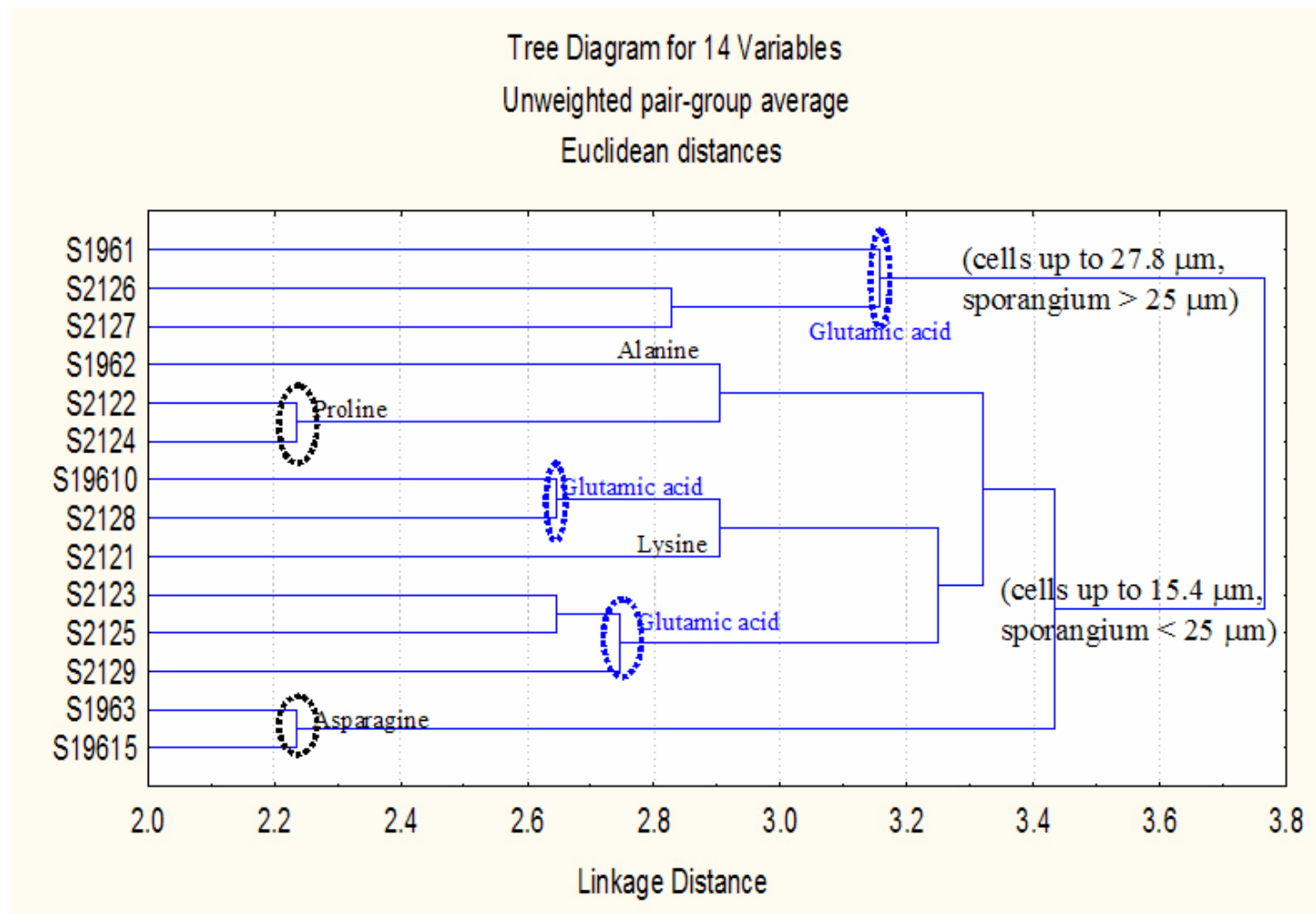


Fig. 15. Phenogram made by using 27 morphological and 27 physiological characters of fourteen isolates of *Aplanochytrium yorkensis*, isolated from the equatorial Indian Ocean.



2.4 Discussion

Taxonomic identity of the organisms: All 14 isolates cultured from zooplankton and identified up to species level based on morphology and life cycle corresponded to *Labyrinthuloides yorkensis* Perkins, originally described from Virginia waters in 1973 (Perkins, 1973). *Labyrinthuloides yorkensis* was described with the following features.

- Uninucleate vegetative cells capable of gliding motility utilizing EN
- EN contain no cytoplasmic organelles, only membrane-bound cisternae
- Cells move independently and may reverse direction of movement
- EN do not enrobe cells
- Vegetative multiplication by binary fission or sporangial formation as a result of successive bipartition or progressive cleavage of the protoplast
- Zoospores, if formed, are biflagellate
- Plasmodia and amoebae may be formed

Except for the absence of biflagellate zoospores, the life cycle of the present isolates fully corresponded to *Labyrinthuloides yorkensis* with regards to the pattern of formation of gliding spores by either successive bipartition or progressive cleavage and formation of motile amoebae and plasmodia on agar surfaces as described by Perkins (1973).

While reviewing the aplanochytrids based on a number of morphological, as well as molecular characteristics, [Leander and Porter \(2000\)](#) concluded that the genus *Labyrinthuloides* was synonymous with *Aplanochytrium*. The type species of the latter genus, *Aplanochytrium kerguelensis* Bahnweg and Sparrow, was originally described from the Antarctic waters near Kerguelen Islands ([Bahnweg and Sparrow, 1972](#)). Since *Aplanochytrium* has precedence over *Labyrinthuloides*, all species described under the latter genus have now been placed under *Aplanochytrium*. The 14 isolates obtained in the present study, therefore, were identified as *Aplanochytrium yorkensis* (Perkins) Leander and Porter.

[Leander et al. \(2004\)](#) found that *A. yorkensis* and *A. kerguelensis* consistently grouped together in a clade based on 18S rDNA sequences and they named this as 'A. yorkensis complex'. *A. kerguelensis* is very similar to *A. yorkensis* in all respects, except for the fact that Bahnweg and Sparrow reported that the spores of *A. kerguelensis* were non-motile and crawled out of the sporangium. It is likely that the authors did not notice the spores gliding using the ectoplasmic net elements.

There are several possible reasons for the predominance of *Aplanochytrium yorkensis* in the present study.

1. *Aplanochytrium yorkensis* might be the most dominant species in oceanic waters. For example, [Raghukumar \(1985\)](#) attempted to culture Labyrinthulomycetes from oceanic waters of the Arabian Sea

and succeeded in isolating only *A. yorkensis*. No thraustochytrids could be isolated in his samples.

2. All isolations in this study were made from mesozooplankton. It is likely that aplanochytrids have a definite association with mesozooplankton. This needs to be verified by future studies dealing with isolation of thraustochytrids and aplanochytrids from zooplankton in different waters.
3. Thraustochytrids might have been associated with the mesozooplankton but might not have been amenable to culture using the medium adopted in this study. This is a likely possibility, since [Raghukumar *et al.* \(2002\)](#) observed that while up to 1313×10^3 cells of thraustochytrids were present per litre seawater of the Arabian Sea, culture methods yielded only 10.5×10^3 cells L⁻¹. being a mere 0.08 % of the total numbers. It is well known with bacteria that culturable numbers are far less than the actual numbers, a phenomenon termed as 'the plate count anomaly' ([Staley & Konopka, 1985](#)). Novel and unconventional methods and media for culturing thraustochytrids might shed more light on the diversity of thraustochytrids in the oceans.

Variations among the *Aplanochytrium yorkensis* isolates: Although all isolates in the present study corresponded to *Aplanochytrium yorkensis*,

they varied considerably from each other in a number of morphological characters. These were: colony characteristics, ectoplasmic net elements, mode of liberation of spores, presence or absence of amoebae, shape and size of the spores and organic carbon and nitrogen preferences (Tables 5-7, Figs. 3,4,6,7,9-12). Such variations among species of *Aplanochytrium* have been studied in detail by [Leander et al. \(2004\)](#). Based on colony morphology, these authors divided their *Aplanochytrium* isolates into 2 main categories, one forming distinct rays sprawling from the centre outwards and the second forming clumps without radial pattern. These two categories were observed also in the present study. However, the more important differentiation appeared to be the presence or absence of agar penetration, since this corresponded to isolates from different cruises. Thus, the isolates obtained from the first cruise in September 2003 produced colonies that penetrated the agar, while the isolates belonging to the second cruise in October 2004 did not do so.

Amoeboid cells were produced by a few isolates in the present study. [Perkins \(1973\)](#) found amoeboid cells in fresh cultures of *A. yorkensis*, but these were not seen to replicate. They rounded up and became sporangia after a brief period of motility. The amoeboid cells produced by the Indian Ocean isolates did not round up into vegetative cells. Instead, they either fragmented into vegetative cells which later formed sporangia or fragmented successively to form spores. The former

phenomenon is observed in *A. thasii* and *A. schizochytrids*. Amoebae are also known in many thraustochytrids (Gaertner, 1977; Raghukumar, 1982; Honda *et al.*, 1998; Bongiorni *et al.*, 2005c). However, the reason behind their production is not known. Interestingly in the present study, amoeboid cells were mostly produced by isolates that did not penetrate the agar. Out of the nine isolates which did not penetrate the agar, five produced amoebae. Only one isolate amongst the five which penetrated the agar, produced amoebae and that too occasionally. Owing to their osmoheterotrophic mode of nutrition, these organisms need to spread out over a large area to be able to attach to new substrates and absorb nutrition with the help of EN elements. This may be facilitated by the production of amoeboid cells.

Physiological characteristics: Thraustochytrids are known to produce a variety of degradative enzymes (Bongiorni *et al.*, 2005b). Aplanochytrids are also expected to play a role in degradation and mineralization of complex organic compounds in the marine environment, being heterotrophic like their sister group, the thraustochytrids (Raghukumar, 2002). Since physiological characters might throw light upon their ecological behaviour, the production of different degradative enzymes, as well as carbon and nitrogen requirements of aplanochytrids was studied.

Members of the Labyrinthulomycetes generally seem to be inefficient in degrading chitin, which is a structural part of the exoskeleton of zooplankton (Jeuniaux & Voss-Foucart, 1991). None of the Labyrinthulomycetes studied by Bahnweg (1979b) hydrolysed chitin. Likewise, none of the present isolates produced chitinase indicating that aplanochytrids may not play a significant role in degradation of zooplankton exoskeleton. They also did not produce amylase and lipase.

Members of Labyrinthulomycetes appear to be highly efficient in degrading protein. All the 23 isolates studied by Bahnweg (1979b) and 7 out of the 11 isolates studied by Bongiorno *et al.* (2005b) produced protease. Likewise, most of the isolates in the present study were capable of producing proteases. The production of protease as the sole extracellular degradative enzyme suggests that aplanochytrids might have an important role in the degradation of complex proteinaceous compounds of zooplankton cadavers.

Glucose is generally the favoured carbon source for Labyrinthulomycetes (Bahnweg, 1979b). Interestingly, the present isolates of *Aplanochytrium yorkensis* generally preferred pentoses and disaccharides to glucose. Yet, all these isolates were also capable of growing well with other carbon sources, thus being highly versatile in terms of their carbon nutrition.

In the case of amino acids, all the isolates displayed poor growth in liquid medium showing almost insignificant differences in dry weight of biomass. Therefore they were plated on solid media containing different amino acids and their response to different amino acids was measured in terms of their colony diameter. Amino acid requirements of the aplanochytrids in the present study confirmed to earlier results of Bahnweg (1979a) and others (Goldstein, 1963a,b,c; Goldstein & Belsky, 1964; Alderman & Jones, 1971; Sykes & Porter, 1973; Vishniac, 1955) on thraustochytrids and aplanochytrids, in that glutamate was the most favoured amino acid for most isolates and the second most preferred after proline, lysine and alanine for the others. In fact, glutamate can serve as an excellent carbon, as well as nitrogen source for thraustochytrids and aplanochytrids. Bahnweg (1979a) observed that *A. yorkensis* was able to grow also on leucine, isoleucine and glutamine along with glutamate and proline.

Microorganisms utilize glutamate for synthesis of glutamine, proline or aspartic acid (Cameron *et al.*, 1952; Smith, 1957). This might be true also of aplanochytrids. Since proline cannot replace glutamic acid for growth (Smith, 1957), there may be higher requirement for glutamic acid followed by proline as seen in the present study. However, Bahnweg (1979a) observed the opposite wherein growth in presence of proline was more than that in the presence of glutamic acid. Similar to his observation,

the isolate S2124 in the present study also showed maximum growth in the presence of proline. The high requirement for proline might be due to its ATP-synthesizing ability by driving mitochondrial oxidative phosphorylation (Shetty & Wahlqvist, 2004). None of the isolates in the present study showed any growth on histidine, arginine, asparagine, cystine, cysteine, leucine, phenylalanine and tryptophan.

Every individual of the fourteen *Aplanochytrium yorkensis* isolates differed in its sum total of physiological and morphological characteristics. However, cluster analyses trees made separately based either on morphology or physiology did not result in similar groupings of isolates (Figs. 13-14). Thus, for example, they could be separated into two distinct clades when physiological characters were used, the single isolate S2121 preferring glucose as carbon source separating from the rest at the first level. Among the other characters, those that preferred pentoses (S1961, S1963 and S19615) and those that preferred disaccharides (8 isolates) formed distinct clades at the third level (Fig. 14). Using morphological characters, they could be separated into two distinct clades, the ones with sporangia larger than 25 μm (S1961, S2126 and S2127) and those with smaller sporangia forming distinct clades at the first level. Since it was not clear whether the morphological or physiological characteristics were more significant, cluster analyses using both these characters together was carried out (Fig. 15). This yielded two clades, those with sporangia

above 25 μm and those that were smaller, at the first level. Strains that preferred asparagine as an amino acid were distinct from those that had other preferences at the second level. These results lead to the following conclusions.

1. All isolates belonged to a single species, *Aplanochytrium yorkensis*, but with high variability in morphological and physiological characteristics.
2. Individual isolates probably represent distinct clones.
3. The lack of grouping based on any of the characters was likely to have been the result of a high degree of mixing between the populations. The fact that all the isolates were obtained from zooplankton from the same area, namely the equatorial Indian Ocean (77° E, 80.5° E and 93° E), emphasizes this view.
4. The morphological and physiological variations observed may thus be inherent variations of a species and may not be reliable tools to distinguish species within the genus *Aplanochytrium*.
5. Such a mixing could have been the result of sexual reproduction. Although no sexual reproduction has been reported in the Labyrinthulomycetes so far, it cannot be ruled out. Perkins & Amon (1969) reported synaptonemal complexes of chromosomes in their electron microscopic studies on *Labyrinthula*, suggesting the presence of sexual reproduction in it (Porter, 1990).

6. Alternatively, it is possible that although morphologically the isolates corresponded to *A. yorkensis* complex, morphology and physiology alone might not be sufficient to resolve the taxonomy of these organisms. This is discussed further in Chapter 3.

2.5 Conclusions

No thraustochytrids were isolated from zooplankton. All isolates corresponded to the aplanochytrid *Aplanochytrium yorkensis*, suggesting either a predominance of this species in zooplankton or the presence of uncultured thraustochytrids in the samples. The fourteen isolates showed numerous variations in terms of colony morphology, life cycle and preferences to organic carbon and amino acids. Among the morphological characters, important variations include the colony margin, penetration of agar and production of amoebae. Only one isolate preferred glucose as the carbon source, all others utilizing pentoses and disaccharides more efficiently. All grew well on glutamate and proline. All isolates produced protease, while none produced lipase, amylase and chitinase. No consistent grouping of the isolates using cluster analyses was noticed using either morphological or physiological characters. It is suggested that *Aplanochytrium yorkensis* in zooplankton of the equatorial Indian Ocean exists in populations with a high variability and mixing.