

Association of the stramenopilan protists, the aplanochytrids, with zooplankton of the equatorial Indian Ocean.

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ABSTRACT

Thraustochytrids and aplanochytrids, belonging to the Labyrinthulomycetes of the Kingdom Stramenopila have been frequently reported to occur as parasites or symbionts in a number of coastal invertebrates. Given the fact that these protists are widespread in coastal and oceanic waters, it is possible that they are also associated with pelagic zooplankton. This study examines their occurrence in zooplankton of the equatorial Indian Ocean waters. A total of 171 of 2100 individual specimens of zooplankton yielded Labyrinthulomycetes in culture, the colony morphology and/or life cycles of which corresponded to aplanochytrids. SSU ribosomal DNA sequence analysis of eight of the isolates placed them in a distinct clade among aplanochytrids, but closest to *Aplanochytrium yorkensis*/*A. kerguelensis*. The eight isolates were further segregated into two clusters, corresponding to isolates obtained from two different seasons. Biotinylated probes for two isolates of *A.kerguelensis* from the two different seasons were developed based on the Internal Transcribed Spacer (ITS) sequences. *In situ* hybridization (ISH) of zooplankton using the probes with streptavidin-alkaline phosphatase showed that aplanochytrid cells fed to copepods were grazed and could subsequently be detected in the animals. ISH on natural samples of zooplankton yielded a positive, but diffuse reaction in copepods, while cells resembling aplanochytrids were detected within chaetognaths. ISH using streptavidin-peroxidase conjugate lent further support for their presence within chaetognaths. This study suggests the predominant occurrence of the aplanochytrid *Aplanochytrium kerguelensis* in association with mesozooplankton of the equatorial Indian Ocean, particularly with chaetognaths. Further studies are suggested to understand if genetically distinct populations of *Aplanochytrium kerguelensis* are associated with chaetognaths in oceanic waters and if such an association is parasitic, mutualistic or commensalistic.

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INTRODUCTION

Stramenopilan protists belonging to the Labyrinthulomycetes, a group comprised of the osmoheterotrophic, unicellular thraustochytrids, aplanochytrids and labyrinthulids are extremely common in coastal and oceanic marine environments (Leander & Porter 2001, Raghukumar 2002). Labyrinthulids are mostly coastal. Thraustochytrids are ubiquitous and abundant in a variety of habitats in coastal and oceanic waters (Kimura et al. 1999, Damare et al. 2008). Aplanochytrids have been reported from both coastal and oceanic habitats, but their ecology is poorly known. Thraustochytrids produce high amounts of the ω -3 polyunsaturated fatty acid, docosahexaenoic acid (Fan et al. 2007, Raghukumar 2008). Since crustacean zooplankton require these fatty acids for their growth and reproduction but possess a limited or no ability to synthesize them (Kanazawa et al. 1979a, b, Lubzens et al. 1985, Nichols & Nichols 2008), Labyrinthulomycetes present in the water column might play an important role in the marine food web by serving as sources of these fatty acids to the zooplankton (Fell & Newell 1998, Kimura et al. 1999, Raghukumar 2002).

Thraustochytrids and aplanochytrids are known to be associated with invertebrates in a variety of ways. Raghukumar & Raghukumar (1999) observed thraustochytrids in the faecal pellets of salps. They were found to be endolithic in mollusk shell fragments especially mussel shells and clam shells (Porter & Lingle 1992) and were isolated from oyster tissue (Pekins 1973, Alderman 1976) and surface mucus of the hermatypic coral *Fungia granulosa* (Harel et al. 2008). *Labyrinthuloides haliotidis*, now known as *Aplanochytrium haliotidis*, was reported to cause pathogenic infection in the abalone *Haliotis kamtschatkana* (Bower 1987, Bower et al. 1989). Thraustochytrids were found to be the most common contaminants while developing cell lines and cultures of a number of invertebrates like sponges, cnidarians, crustaceans, mollusks, echinoderms and tunicates indicating some kind of an association (Rinkevich 1999, Frank et al. 1994, Rinkevich et al. 1998, Rinkevich & Rabinowitz 1997, Hsu et al. 1995).

Given the frequent association of Labyrinthulomycetes with invertebrates, it is important to know which of such associations are commensalistic, mutualistic or parasitic in order to understand their role in food web dynamics. In order to do this, it is necessary to isolate these organisms from particular invertebrate hosts and to locate them within their host tissues. For example, Raghukumar (1988) detected *Ulkenia visurgensis* in coelenteron and hydranth of a hydroid in tidal pools using immunofluorescence, an observation that strongly suggests a mutualistic association between the

invertebrate and the thraustochytrid. Oligonucleotide probes targeted against the small-subunit ribosomal DNA, the 16S rRNA gene in case of bacteria and the 18S rRNA gene in case of eukaryotes have emerged as powerful tools to detect microorganisms in their natural environment in recent years. The Internal Transcribed Spacer (ITS) portion of the rRNA gene is highly variable between species and often even strains and can be used to design probes to detect at species level.

There are few reports on the associations of Labyrinthulomycetes with zooplankton in the oceanic environment. In the present study we have attempted to study the associations of Labyrinthulomycetes with zooplankton of the equatorial Indian Ocean by isolating them from these invertebrates and characterizing them taxonomically. In addition, we have attempted *in situ* detection of aplanochytrid cells in zooplankton, with the help of oligonucleotide probes, directed against their ITS sequences.

MATERIALS AND METHODS

Isolation of Labyrinthulomycetes from zooplankton: Mesozooplankton were collected during two cruises to equatorial Indian Ocean on board ORV Sagar Kanya (SK 196 during September 2003 and SK 212 during October 2004) using Multiple Plankton Net (MPN, MultiNet Type Midi with 200 μ m mesh nets, Hydrobios, Germany). The location of the sampling stations is given in Fig. 1. Specimens were collected from different depth ranges, ranging from the surface to 1000 m. Zooplankton specimens were rinsed several times with sterile seawater and plated on Modified Vishniac (MV) Agar medium (Porter 1990) supplemented with 400,000 U procaine penicillin, 0.075 g streptomycin (Trade name Ambystin-S) and 1 % fetal bovine serum.

Isolation of DNA from aplanochytrid cultures: Isolation of whole genomic DNA of the isolates was carried out by a modification of phenol-chloroform:isoamyl alcohol method (Sambrook et al. 1989). Cell pellets obtained after growing the isolates for 5-7 days in MV broth in shake cultures were washed with sterile seawater in a microcentrifuge tube and subjected to three cycles of freezing and thawing using liquid nitrogen. Lysis buffer (2 % Triton X100, 1 % sodium dodecyl sulphate, 100 mM sodium chloride, 10 mM Tris hydrochloride pH 8.0, 1 mM EDTA) was added, followed by 1:1 phenol and chloroform : isoamyl alcohol. Sterile glass beads of 150-350 μ m size (Sigma) were added and vortexed till the entire contents of the tube turned viscous in nature. The tubes were stored at -20^o C for better precipitation. The pellets were collected by centrifugation at 12000 rpm for 10 minutes under cold conditions, washed once with cold 70 % ethanol and dried

using a centrifugal vacuum concentrator (Biotron Ecospin 314, S. No. Ec-050310). The dried DNA pellets were dissolved in sterile TE buffer (10 mM Tris-Cl, pH 8.0; 1mM EDTA, pH 8.0) and checked for purity by electrophoresis using 0.8 % agarose gel. DNA concentration was assessed spectrophotometrically by determining the OD₂₆₀/OD₂₈₀ ratio. The DNA was stored at -20°C until it was amplified by polymerase chain reaction (PCR). All PCR amplifications were carried out with final concentrations of 7.5 pM primer each, 100 µM dNTP mix, 1X PCR buffer containing 1.5 mM MgCl₂, and 1U Taq polymerase in BioRad Thermal Cycler (S. No. AL100938).

18S rDNA identification of the isolates and phylogenetic analysis: Two external primers designed by Honda et al. (1999) (18S001 and 18S13) and two universal internal primers (NS3 and NS4; White et al. 1990) were used to amplify the 18S rDNA regions of the isolates. PCR conditions were: denaturation at 94°C/4 min, followed by 30 cycles of denaturation 94° C/50s, annealing at 59.5° C/50 s for set I [18S001 (fwd) and NS4 (rev)] or 62.5° C/50 s for set II [NS3 (fwd) and 18S13 (rev)] and extension at 72° C/1 min, followed by a final extension step of 72° C/10 min. The amplified PCR products were purified using Montage PCR Clean Up Kit (Millipore) and sequenced. The 18S rDNA sequences obtained were deposited in the GenBank (Table 1). Phylogenetic trees based on the 18S rDNA sequences were generated using the Neighbour-Joining (NJ), Minimum-Evolution (ME) and the Maximum-Parsimony (MP) methods using MEGA version 4. The evolutionary distances in NJ and ME trees were computed using the Maximum Composite Likelihood method. The MP tree was obtained using the Close-Neighbor-Interchange algorithm. Sources of the other sequences, rooted to stramenopiles were obtained from NCBI database.

PCR amplification of ITS rDNA and sequencing: The ITS region of ribosomal DNA of 7 isolates was amplified using universal ITS primers (ITS1 and ITS4) designed by White et al. (1990). The reaction conditions were 94°C/4 min, followed by 30 cycles of 94° C/50 s, 57° C/45 s and 72° C/1 min, with a final elongation step of 72° C/7 min. The amplified PCR products were purified using Montage PCR Clean Up Kit (Millipore) and sequenced. Purified PCR products for which sequencing was unsatisfactory were cloned using TOPO Cloning Kit (Invitrogen, USA) and the plasmid from the transformants was extracted by Alkaline Lysis method (Birnboim & Doly 1979) and sequenced. The sequences obtained were deposited in the GenBank (Table 1).

Design of oligonucleotide probes: A total of 14 ITS sequences were used for the alignment. This consisted of 7 isolates of aplanochytrids obtained from zooplankton of equatorial Indian Ocean in

the present study and 7 of other organisms belonging to Kingdom Straminipila, downloaded from NCBI database. Amongst the Labyrinthulomycetes, ITS sequences of only Labyrinthulid quahog parasite QPX and *Thrausochytrium aureum* are available in the NCBI database out of which the sequences with accession numbers DQ641197, FJ533161 and FJ533163 were used in the present study. Four sequences of other organisms belonging to Kingdom Straminipila were downloaded for comparison. These 4 sequences showed 70-80 % similarity with aplanochytrid ITS sequences of the present study but with only 7-24 % of query coverage. These sequences belonged to *Sellaphora* AJ544676, *Hyaloperonospora parasitica* EU049281, *Peronospora tribulina* AY531414 and *Nitzschia* AY574377.

Multiple alignment of all sequences was carried out by ClustalW. The sequences which showed consensus regions only among the aplanochytrids and minimal or nil similarity with other organisms were chosen for designing probes for the aplanochytrids. These consensus sequences were analysed using Vector NTI 10 software (Invitrogen, USA). Based on the analysis, a total of four probes, consisting of one forward and one reverse primer for two isolates, S1961 and S2124 were designed (Table 2). Primer oligonucleotides were obtained from Sigma Genosys, Bangalore. All primers were biotinylated at the 5' end.

Confirmation of probe specificity: PCR amplification of the DNA of 8 aplanochytrid isolates obtained from zooplankton of equatorial Indian Ocean as well as three thraustochytrid isolates (PRA-147 (ATCC), Z7 and DP7) present in the culture collection of the National Institute of Oceanography, Goa, India was carried out using the designed set of primers i.e. S61F1-S61R1 and 138F1-138R1 respectively to check the specificity of the primers designed towards the two aplanochytrids. The annealing temperature conditions were optimized for both the primer sets by carrying out a gradient PCR in Peltier Thermal Cycler (MJ Research, PTC-200, S. No. AL083846). The temperature gradient ranging from 45° to 65° C was checked. The optimal conditions for the two primer sets were 94° C/ 4 min, followed by 30 cycles of denaturation at 94° C/40s, annealing at 56° C/50s for the former primer set and 60° C/50s for the latter and extension at 72° C/ 1min, with a final elongation step of 72° C/ 6 min.

The specificity was further confirmed by Southern Hybridization of the amplified bands with the respective probes using the Southern Hybridization Kit (Bangalore Genei, India). Hybridization and post-hybridization reactions were carried out on the membrane as well as on the gel to check if all the bands had blotted over the membrane as per the manufacturer's instructions. The bands were hybridized

with the probe S61F1 for isolates S1961, S1963 and S19610 and the probe 138F1 for isolates S2122, S2124, S2128 and S2129.

Standardization of pretreatment conditions for *In situ* hybridization (ISH) of whole aplanochytrid cells: Permeabilization conditions were optimized using cells of isolates S1961 and S19610. Five day old cells of the isolates grown in MV medium were harvested and suspended in phosphate-buffered saline containing formalin up to 2 % concentration and stored under refrigeration till further use. Four different permeabilization techniques were tried. (1) Cells were incubated overnight with Triton X100, in order to reduce hydrophobic interactions with the use of nonionic surfactant (Thimm & Tebbe 2003). (2) Cells were subjected to repeated freeze and thaw in liquid nitrogen to facilitate disruption of cells through ice crystal formation (Biegala et al. 2003). (3) Cells were incubated for 5 minutes with DTT, to reduce disulphide bonds of membrane proteins (Moter & Göbel 2000). Cells permeabilized after these three treatments were then smeared on glass slides coated with double-sided adhesive tape (La Cono & Urzi 2003) and also on Polyprep slides (Sigma) (Smolina et al. 2007) and dehydrated in an ethanol series. (4) Cells fixed with formalin were treated with 1 M DTT for 5 minutes, followed by washing with distilled water and final suspension in 1 M sorbitol. The cells were transferred in a cuvette and given an electric pulse of 2500 V for ~5 milliseconds in Gene Pulser Xcell™ (BioRad, S.No. 617BR105105) as per the protocol mentioned for eukaryotic cells in the manual of Gene Pulser Xcell™.

Standardization of *in situ* hybridization (ISH): ISH was standardized with whole aplanochytrid cells using the Southern Hybridization Kit (Bangalore Genei), following the manufacturer's instructions. The probes S61F1 and S61R1 were used separately at a final concentration of 5 pmol/μl. Two different probe conjugates were used for incubation of 20-45 mins, namely streptavidin-alkaline phosphatase (ALP) (Sigma, India) and streptavidin-peroxidase conjugates (Sigma, India). The cells were then incubated in respective substrate solutions till they took up the colour of the reaction. The substrate used for ALP was 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma, India) which produced blue colour in the cells while the substrate used for peroxidase was 3,3'-diaminobenzidine (DAB) tetrahydrochloride hydrate (Sigma, India) giving purple to dark red colouration to the cells. The incubation time of cells in the substrate varied from 3 to 12 hours for ALP and peroxidase respectively.

Application of probes to field samples:

(i) *Aggregates from the equatorial Indian Ocean:* Aggregates from the water samples of the equatorial Indian Ocean collected along with the mesozooplankton using multiple plankton net were subjected to different pretreatments, namely electroporation and air-drying over Polyrep slides prior to ISH with streptavidin-alkaline phosphatase conjugate.

(ii) *Control experiment using zooplankton fed with aplanochytrids:* The efficiency of standardized methodology of permeabilization and hybridization using aplanochytrid cells was examined on zooplankton specimens. Mesozooplankton were sampled from the Dona Paula Bay using a zooplankton net of mesh size 200 μm . Approximately 7-8 live specimens of mesozooplankton were kept in 5 separate beakers each under aeration in laboratory and starved for 6 hours. Cells of the aplanochytrid S1961 were fed to each experimental beaker. Cells were added up to twice the concentration of *Labyrinthulomycetes* found in natural waters of Dona Paula Bay ($232 \times 10^3 \text{ cells L}^{-1}$) as estimated independently using epifluorescence microscopy. A beaker each was sampled at 4, 6, 10, 15 and 24 hours by fixing the entire contents with 2 % formaldehyde. The specimens from each beaker were transferred to microcentrifuge tubes. Permeabilization of the entire zooplankton specimens presumably containing the aplanochytrid cells was then carried out as follows. The specimens were treated with 1M DTT for 5 mins in order to reduce disulphide bonds in membrane proteins. They were then washed twice with distilled water and once with 1 M sorbitol and finally suspended in 1 M sorbitol which acts as osmotic stabilizer. The entire specimens were then transferred to a cuvette and given an electric pulse of 2500 V for ~5 milliseconds as given above. The specimens were then subjected to ISH under the same conditions as mentioned before for the whole aplanochytrid cells using streptavidin-ALP conjugate.

(iii) *ISH for aplanochytrids in natural zooplankton population from equatorial Indian Ocean:* Zooplankton samples collected using the multiple plankton net during the two cruises on board ORV Sagar Kanya SK 212 (October 2004) and SK 228 (September 2006) from the water column of the equatorial Indian Ocean were preserved in formalin at 2 % concentration in refrigerator. ISH was carried out using S61F1 and 138F1 probes individually. Permeabilization was achieved through electroporation as mentioned earlier and hybridization was carried out using the streptavidin-ALP conjugate. After incubation with substrate solution for 1-2 hours, the specimens were transferred to distilled water and then on to a glass slide and observed using bright field microscopy (Olympus BX60).

Specimens with blue coloration were noted as positive. Photographs were captured using Olympus Digital DP70 camera.

ISH for aplanochytrids in chaetognaths, following in situ PCR: Specimens of chaetognaths from the Indian Ocean collected and fixed as above were washed with phosphate-buffered saline (PBS) and either dried under vacuum using centrifugal vacuum concentrator (Biotron Ecospin 314, S. No. Ec-050310) or suspended in 1M sorbitol and electroporated before carrying out *in situ* PCR. A total of 50 μ l of PCR buffer was added to each specimen in the PCR tube. *In situ* PCR using the two different sets of primers, i.e., S61F1-S61R1 and 138F1-138R1 was carried out separately, using BioRad Thermal Cycler (S. No. AL100938). PCR cycles consisted of 30 cycles of denaturation at 94° C for 40 s, primer annealing (56° C for S61F1-R1 primer set or 60° C for the 138F1-R1 primer set) for 50 s, and extension at 72° C for 60 s. The specimens were then subjected to ISH following the methods for natural zooplankton as given above and using streptavidin-ALP conjugate.

ISH of chaetognath specimens with streptavidin-peroxidase conjugate: Chaetognath specimens were subjected to ISH using streptavidin-peroxidase conjugate. In addition to skimmed milk powder, bovine serum albumin (BSA) was used as an alternate blocking agent at concentration similar to that of skimmed milk powder used for all the earlier experiments. The effect of BSA as blocking agent was checked in the presence and absence of 0.05% Tween 20.

RESULTS

Isolation of Labyrinthulomycetes from zooplankton of equatorial Indian Ocean: A total of 2100 zooplankton specimens, collected from 76 multiple plankton net samples and obtained from 19 stations during two cruises were plated on to MV medium for isolating thraustochytrids and aplanochytrids (Table 3). Out of this, a total of 171 specimens, corresponding to 8.14 % were positive for the protists. Labyrinthulomycetes were isolated from all the depths, ranging from 0 to 1000 m.

Out of the total 171 colonies obtained, 8 isolates that survived after repeated subculturing were maintained for further studies. Five of these were isolated during September 2003 and the remaining 9 during October 2004. Eight of these isolates were further characterized based on their 18S rDNA sequences.

Molecular identification of aplanochytrids and phylogenetic analysis: The 18S rDNA sequences of the 8 isolates from equatorial Indian Ocean ranged in size from 1353 to 1731 bp (Table 1). BLAST analysis of these sequences showed 98 to 100 % similarity to *Aplanochytrium kerguelense* and *A. stocchinoi*.

Phylogenetic analysis carried out using the three clustering algorithms, Neighbour-Joining, Minimum-Evolution and Maximum-Parsimony methods resulted in phylogenetic trees with similar topologies. A representative of the three phylogenetic trees (NJ tree) is presented in Fig. 2. Three distinct clades of the Labyrinthulomycetes were noticed. The first one comprised the thraustochytrids *Sicyodochytrium minutum*, *Botryochytrium radiatum*, *Parietichytrium sarkarianum*, *Thraustochytrium striatum*, *Schizochytrium aggregatum*, and *Ulkenia visurgensis* which formed a separate clade with a high bootstrap value of 100 % from the rest of the Labyrinthulomycetes. The other members of the Labyrinthulomycetes formed two clades, the first comprising the labyrinthulid *Labyrinthula* sp. and the second comprising the aplanochytrids supported by 64-69 % bootstrap values. All eight isolates obtained from oceanic zooplankton in the present study grouped with *Aplanochytrium yorkensis* and *A. kerguelensis* and separated from the rest of the aplanochytrids. Isolate S1962 was the closest to *A. yorkensis* and *Aplanochytrium* 367045 in all three trees. Isolates obtained during SK 212 cruise in October 2004 formed a clade distinct from the aplanochytrids obtained during SK 196 cruise (bootstrap value of 38 % in NJ and ME trees) in the topology.

Alignment of ITS sequences and designing of probes: The ITS rDNA PCR products of the 7 isolates studied ranged in size from 447 to 572 bp (Table 1). Since the 18S rDNA phylogenetic trees suggested that the aplanochytrids from the Indian Ocean were distributed as two distinct clusters, the probes were designed against two isolates, one from each cluster. Probe design analysis resulted in choosing S1961 from the SK196 cruise and S2124 from the SK212 cruise as the best possible candidates for the probe. Two sets of primers were designed against each of the two isolates. (Table 2).

Confirmation of probe specificity: PCR amplification with the designed set of primers showed that primer set S61F1-S61R1 amplified DNA of all the isolates obtained from the September 2003 cruise, namely S1961, S1963 and S19610. However, they did not amplify the DNA of the isolates obtained from the October 2004 cruise (Fig. 3). Similarly primer set 138F1-138R1 amplified DNA of S2122, S2124, S2128 and S2129, which had been isolated during October 2004 cruise (Fig. 4) but did not amplify the DNA of the isolates obtained from the September 2003 cruise.

Southern hybridization carried out using S61F1 probe on the amplified product of the isolates S1961, S1963 and S19610 for the first primer set and using 138F1 probe on the amplified product of the isolates S2122, S2124, S2128 and S2129 for the second primer set using streptavidin-ALP conjugate produced blue bands on the membrane with BCIP/NBT thus showing clear positive signals (Fig. 5).

Standardization of pretreatment conditions for *in situ* hybridization (ISH) of whole aplanochytrid cells: The four different permeabilization protocols tried prior to *in situ* hybridization of aplanochytrid cells using streptavidin-ALP conjugate showed positive results to varying degrees depending on the protocol used. Cells smeared on slides were not easily retained and were mostly washed off. Secondly, not all cells pretreated in this way showed the positive blue colour after reaction with streptavidin-conjugated alkaline phosphatase and substrate (Fig. 6a). Electroporation of cells to allow permeabilization provided more consistent results. All the cells treated in this way were stained blue but with different intensities (Fig. 6b). Thus, young cells stained dark as compared to older ones. Therefore electroporation was used for permeabilization before ISH for the other reactions that followed.

Electroporation of formalin-fixed cells of aplanochytrid S1961 followed by *in situ* hybridization with streptavidin-peroxidase conjugate consistently produced dark red stained cells after incubation with DAB hydrochloride hydrate, thus displaying positive results (Fig. 7). The positive reaction was seen after 3 hours of incubation with the substrate irrespective of the time of incubation with the conjugate.

***In situ* hybridization of field samples using probes:**

(i) *Aggregates from the water column:* The pretreatments of electroporation as well as air-drying revealed the presence of aplanochytrid probe-positive cells attached to the aggregates (Fig. 8). These cells were globose and measured 5 to 20 μm in diameter. The cells were generally present scattered in the aggregates.

(ii) *Zooplankton samples fed with aplanochytrids:* Zooplankton specimens that were fed with cells of the aplanochytrid S1961 for different time intervals were permeabilized using electroporation and subjected to ISH. Aplanochytrid cells that reacted to ISH were detected in the hind gut of several of the zooplankton specimens towards the end of 4 hours of feeding (Fig. 9). No aplanochytrid cells were detected in the specimens after 6 hours and more of feeding. However, blue colouration owing to reaction to alkaline phosphatase used in the ISH was noticed in different parts of the body of zooplankton, such as the appendages and metasome (Fig. 10). The colour intensity decreased after 15

hours of feeding. No reaction to ISH and, therefore, no development of blue colour that is characteristic of the alkaline phosphatase- BCIP/NBT test was detected in the tissue of the control zooplankton specimens that were not fed with aplanochytrids.

(iii) *Natural zooplankton population from equatorial Indian Ocean:* Specimens from both the cruises (October 2004 and September 2006) showed positive reaction towards probes developed for both the isolates S1961 and S2124 using the streptavidin-ALP conjugate (Tables 4 and 5). The use of S61F1 probe yielded positive reactions from 1.4 - 27.6 % zooplankton specimens sampled during October 2004 cruise, while the use of 138F1 probe yielded 0 – 34.9 % positive reactions. ISH-positive specimens of zooplankton were found in all the depths at all nine stations sampled during this cruise. On the contrary, only 2 of the 6 samples collected from the 3 stations were positive to the S61F1 probe and only 3 of the 6 samples to the 138F1 probe during the September 2006 cruise. Up to 12.5 % of the zooplankton specimens were positive to the S61F1 probe during the cruise, while 0 – 9.3 % were positive to the 138F1 probe at the other depths (Table 5). No distinct differential response to the two probes was noticed either between the cruises or the depths. Although positive reactions were seen to hybridization, no distinct aplanochytrid cells were detected in the specimens. The reaction was often generalized and found in various parts of the zooplankton.

While generally only a few copepods were positive for hybridization with the two probes using streptavidin-ALP, all the chaetognath specimens were positive (Figs. 11). Several globose, scattered cells that reacted positively to ISH were observed within the body of the chaetognaths. Positive reactions were mostly concentrated surrounding the gut and also inside the mouth parts. In one instance, an aggregate-like structure in the vicinity of the mouth part of the chaetognath reacted positively to ISH (Fig. 11a).

In situ PCR of chaetognath specimens revealed fewer ISH-positive cells within the tissue of chaetognaths compared to the procedure where only electroporation was used prior to ISH. However, the application of *in situ* PCR resulted in cells which stained much brighter with streptavidin-ALP (Fig. 12). Cells that reacted positively to *in situ* PCR-ISH were mostly ellipsoidal to irregular in shape. Such aplanochytrid-positive cells were present lining the gut.

ISH of chaetognath specimen using streptavidin-peroxidase conjugate: In order to eliminate the possibility of false positive reaction and as a step towards confirmation of probe efficiency, samples were subjected to ISH using streptavidin-peroxidase conjugate. BSA in the presence of Tween20 was

more efficient than skimmed milk powder as a blocking agent, only 10 out of the total 32 specimens of chaetognaths (31.3 %) showing a diffuse and generalized positive reaction to ISH unlike that seen with the streptavidin-ALP conjugate (100 % positive staining). Aplanochytrid cells that stained positively to DAB hydrochloride hydrate were observed in several parts of chaetognath specimens like in the head region, tail region and surrounding the gut (Fig. 13).

DISCUSSION

All fourteen isolates cultured from zooplankton of the Indian Ocean had earlier been identified on the basis of their life cycle and other morphological characteristics as belonging to *Aplanochytrium yorkensis* (Perkins) Leander and Porter (Damare & Raghukumar 2006). SSU ribosomal DNA sequences of eight of these isolates confirmed this identification. Thus, phylogenetic trees showed that these eight isolates were closest to *Aplanochytrium yorkensis* of the 'A. yorkensis complex' within the aplanochytrids, the other cluster being the '*Aplanochytrium minuta* complex' (Leander & Porter 2004). All isolates, therefore, corresponded to aplanochytrids, one of the three lineages of the Labyrinthulomycetes (Leander & Porter 2001). None belonged to thraustochytrids or labyrinthulids. The NJ and ME trees were fundamentally consistent with the trees reported by Honda et al. (1999) wherein labyrinthulids grouped with aplanochytrids distinctly separated from thraustochytrids. *Aplanochytrium yorkensis* is now considered synonymous to *A. kerguelensis* Bahnweg and Sparrow (1972) and is, therefore, referred to by that name in the rest of the discussion. Only 14 isolates that survived repeated subculturing of the total 171 isolates from zooplankton were earlier studied for their morphology and life cycle (Damare and Raghukumar, 2006) and eight of these that survived further subculturing were identified as that species based on 18S rDNA sequences. Such a small selection from the total may be suspected to have resulted in a bias. However, these were isolates from random depths and stations. Further, the colony morphologies of all 171 isolates corresponded to those of typical aplanochytrids described by Leander et al. (2004) and Damare & Raghukumar (2006). *Aplanochytrium kerguelensis* was originally reported from sub-Antarctic waters while *A. yorkensis* was described from oyster mantle, water samples, sediment and detritus from York river in Virginia (Bahnweg & Sparrow 1972, Perkins 1973). Thus, *Aplanochytrium kerguelensis* appeared to be the dominant species associated with the equatorial Indian Ocean zooplankton.

We believe that the predominance of this single species, *A. kerguelensis*, in the zooplankton of the equatorial Indian Ocean waters is significant.

(1) It is likely that aplanochytrids predominate in oceanic waters, compared to thraustochytrids and labyrinthulids. Raghukumar (1985) reported *Labyrinthuloides yorkensis* (*A. kerguelensis*) as the only species isolated from water samples of the Arabian Sea.

(2) All isolations in the present study were made from zooplankton. Aplanochytrids may have a definite association with zooplankton, compared to thraustochytrids. Zooplankton-associated *A. kerguelensis* of the equatorial Indian Ocean might represent a genetically distinct population of the species. Thus, seven of the eight isolates clustered apart from *A. yorkensis*, although this was the closest species in the phylogenetic trees.

(3) Although standard media for thraustochytrids and aplanochytrids were used to plate zooplankton, it is possible that only *A. kerguelensis* grew out of these and that oceanic thraustochytrids were not amenable to cultivation using these media. Indeed, culturable numbers of thraustochytrids in natural water samples appear to be far lower than actual numbers. Raghukumar et al. (2001) 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).

In order to further understand the association of aplanochytrids with zooplankton, ITS-based probes were successfully developed and tested against the protists in natural samples. There have been few similar attempts earlier. Lyons et al. (2005) generated two 18S rRNA targeting probes against the hard clam *Mercenaria mercenaria* parasite ‘Quahog Parasite Unknown’ or QPX. They used these probes to detect the presence of the parasite in the marine aggregates. Takao et al. (2007) have also designed an 18S rRNA targeted probe against thraustochytrids, especially *Aurantiochytrium*, formerly *Schizochytrium*. Though they have optimized the FISH protocol using this probe, they have not used it for detection in natural samples. Molecular probes can be designed using fluorescent labels such as FITC and rhodamine or biotinylated probes against streptavidin-conjugated enzymes such as alkaline phosphatase (ALP) or horseradish peroxidase (HRP). Fluorescent *in situ* hybridization (FISH) probes were avoided in this study because zooplankton, on which the probes were intended to be used, have an autofluorescent chitinous exoskeleton that could cause severe interference in detecting the required organism. Therefore, it was decided to use a bright-field microscopy based biotinylated probe which can react with chromogenic substrate against enzymes to give a coloured reaction. Two streptavidin-

conjugated enzymes, namely alkaline phosphatase (ALP) and peroxidase were used. Both probes tested positive against cultured cells, as well as cells in the aggregates collected from natural water samples.

There are two possible ways by which aplanochytrids can occur in association with the zooplankton. They may occur as passive inhabitants in the guts of the animals that had fed upon their cells present in the water column, or occur as a regular symbiotic component in the bodies of the zooplankton. ISH on coastal zooplankton fed with aplanochytrid cells clearly showed the presence of the protistan cells in the guts of the animals. None of the control specimens (not fed with the aplanochytrids) were positive. Many copepods showed a blue colouration in their hind guts 4 hours after the cells of the aplanochytrid were added (Fig. 9), thus confirming that the cells were eaten and had passed into the hind gut and that the probes were effective. ISH carried out on chaetognaths from the equatorial Indian Ocean often yielded positive reaction in the foregut part of the organism, suggesting the presence of the aplanochytrids in that region, probably through feeding (Figs. 11 and 12). The importance of Labyrinthulomycetes (thraustochytrids) in the marine food web as sources of the omega-3 polyunsaturated fatty acid (PUFA), docosahexaenoic acid (DHA) to zooplankton has been stressed by many (Fan & Newell 1998, Kimura et al. 1999, Raghukumar 2002). Although there have been several reports of zooplankton feeding on Labyrinthulomycetes in laboratory as well as in aquaculture (Brown 2002, Alonzo et al. 2005, Bergé & Barnathan 2005), there are few reports of actual feeding of zooplankton on these protists in nature. The present study provides strong evidence towards this. There are no detailed reports of PUFAs in aplanochytrids (Raghukumar 2008). If these too are rich in DHA, they would then play an important source of the PUFA to zooplankton.

The possibility that aplanochytrids might live within the body parts of these zooplankton, probably in a mutualistic association is supported by the observation of clusters of globose cells located within different parts of the body of many chaetognaths, using both the streptavidin-alkaline phosphatase as well as streptavidin-horseradish peroxidase conjugates. *In situ* PCR on chaetognaths followed by ISH using streptavidin-alkaline phosphatase provided better evidence of the presence of aplanochytrids within the animals. Distinct, angular to amoeboid cells within the tissues of the chaetognaths (Fig. 12), typical of *A. yorkensis* on a nutrient agar medium were observed. This was also seen during ISH using streptavidin-peroxidase wherein, clumps of cells were noticed in the tail region of a few specimens (Fig. 13). Rinkevich & Rabinowitz (1993) have suggested the occurrence of thraustochytrid cells circulating in the circulatory system of the tunicate *Botryllus schlosseri* and have found them in various body parts of tunicates, corals, sponges. These protists were reported as major

contaminants in cell lines of these animals (Rinkevich & Rabinowitz 1997, Rinkevich 1999). However, the detection of aplanochytrids only in a few of the chaetognath specimens casts doubt on a mutualistic association. The possibility of cells growing below the chitinous exoskeletonous moult of the specimens (Fig. 13) cannot also be ruled out.

Although the above observations were highly suggestive of the presence of *A. kerguelensis* in chaetognaths, they were confounded by the possibility of false positives. Care was taken to inhibit endogenous ALP activity by pre-treatment with dithiothreitol (DTT), a strong reducing agent. In addition, the specimens had been fixed in formalin, which destroys endogenous alkaline phosphatase activity (Bussolati & Leonardo 2008). Despite these precautions, the streptavidin-alkaline phosphatase conjugate appeared to yield false positives wherein the colour reaction appeared in a diffused manner in many parts of the body of the zooplankton (Fig. 10). On the one hand this could have resulted from aplanochytrids that were attached to the exterior of various body parts. The diffused colouring and the absence of distinct cells of the aplanochytrid might have also resulted from the destruction of the aplanochytrid cells during processing. However, it is also likely that the reaction was due to the alkaline phosphatase present in the body tissues of zooplankton. Adults as well as juvenile copepods and branchipods are known to produce alkaline phosphatase often associated with cell surfaces (Lespilette et al. 2007, Cembella et al. 1984). Zooplankton secrete this enzyme in the environment (Jamet & Boge 1998, Gambin et al. 1999, Boge et al. 2006). In view of the above, we also used streptavidin-peroxidase conjugate for ISH. This resulted in much less generalized and diffuse staining of the chaetognaths. Among all the mesozooplankton, chaetognaths were more positive to ISH with alkaline phosphatase as compared to copepods. ISH using streptavidin-peroxidase conjugate on chaetognaths too showed positive reaction but to lesser extent than that seen with alkaline phosphatase system.

The eight species of zooplankton-associated aplanochytrids appeared to be comprised of two seasonally differing populations. The SSU ribosomal sequences showed that cultures obtained during SK 212, S2122, S2124, S2125, S2128 and S2129 clustered distinctly apart from the isolates S1961 and S19610, isolated during SK 196 (Fig. 2). The ITS probes developed for the isolate S2124 was positive for all the others isolated during the same period, namely S2122, S2125, S2128 and S2129 (Fig. 4). Likewise, the probe developed for S1961 was positive for all cultures isolated during SK 196 (Fig. 3). However, these observations need to be confirmed by future studies since they were inconsistent with the ISH studies, where specimens of copepods, as well as chaetognaths collected from any one cruise were positive to both probes.

The present study demonstrates the predominant occurrence of the aplanochytrid *Aplanochytrium kerguelensis* in association with mesozooplankton of the equatorial Indian Ocean. It might be expected that if such an association occurred, the percentage frequency of isolation of aplanochytrids would have been higher than the observed 8 % specimens yielding colonies of aplanochytrids. However, such an association might be related to the zooplankton taxon. No attempts were made to separate distinct zooplankton taxa prior to plating on culture media. ISH studies suggested a strong relation between aplanochytrids and chaetognaths. Further studies using electron microscopy and other molecular probes will throw light on possible mutualistic relationships of aplanochytrids with chaetognaths and the role of aplanochytrids in the diet of oceanic mesozooplankton.

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Table 1. Details of 18S and ITS rDNA sequences of the aplanochytrids isolated from zooplankton of the equatorial Indian Ocean.

| rDNA type | Isolate | Period of isolation | Size of sequence | Gene bank accession No. |
|-------------|---------|---------------------|------------------|-------------------------|
| 18S rDNA | S1961 | Sep 2003 | 1682 bp | EU851167 |
| | S1962 | Sep 2003 | 1353 bp | EU851174 |
| | S19610 | Sep 2003 | 1675 bp | EU851168 |
| | S2122 | Oct 2004 | 1644 bp | EU851172 |
| | S2124 | Oct 2004 | 1683 bp | EU851169 |
| | S2125 | Oct 2004 | 1682 bp | EU851170 |
| | S2128 | Oct 2004 | 1731 bp | EU851173 |
| | S2129 | Oct 2004 | 1681 bp | EU851171 |
| ITS rDNA | S1961 | Sep 2003 | 556 bp | EU872090 |
| | S1963 | Sep 2003 | 503 bp | EU872091 |
| | S19610 | Sep 2003 | 503 bp | EU872092 |
| | S2122 | Oct 2004 | 552 bp | - |
| | S2124 | Oct 2004 | 447 bp | EU872093 |
| | S2128 | Oct 2004 | 572 bp | - |
| | S2129 | Oct 2004 | 562 bp | - |

Table 2. Sequences of probes designed for two isolates of *Aplanochytrium kerguelense* from zooplankton of the equatorial Indian Ocean. Probes were designed by alignment of 7 isolates from the present study with 7 other organisms from the Kingdom Stramenopila.

| Isolate No. | Probe | Length | Sequence | Type |
|-------------|-------|--------|-------------------------|---------|
| S1961 | S61F1 | 19 | 5'-TAATCCATTTCTAATTCCG | Forward |
| | S61R1 | 19 | 5'-TACATAAAAACCCAAAATC | Reverse |
| S2124 | 138F1 | 20 | 5'-GCTGTCGCCTGTTCTGATTG | Forward |
| | 138R1 | 20 | 5'-ACATACTTGCGTACATATTG | Reverse |

Table 3. Sampling locations in the equatorial Indian Ocean and depth ranges from where zooplankton samples were collected for isolation of Labyrinthulomycetes. Depth ranges in bold indicate samples from which the protists were recovered in culture.

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

Table 4. Response of individual zooplankton specimens collected during October 2004 from the equatorial Indian Ocean to ISH using probes designed against two isolates of *Aplanochytrium kerguelensis*.

| Station | Depth | S61F1 probe | | | 138F1 probe | | |
|-------------|----------|-----------------|----------------|-------------|-----------------|----------------|-------------|
| | | Total specimens | Total positive | % positive | Total specimens | Total positive | % positive |
| 1°N 80.5°E | 30-200 | 63 | 3 | 4.8 | 43 | 15 | 34.9 |
| 0°Eq 80.5°E | 38-200 | 63 | 4 | 6.3 | 135 | 4 | 9.3 |
| 0°Eq 80.5°E | 200-500 | 59 | 5 | 8.5 | 54 | 6 | 14.0 |
| 0°Eq 83°E | 500-1000 | 40 | 3 | 7.5 | 64 | 0 | 0.0 |
| 0°Eq 83°E | 300-500 | 22 | 3 | 13.6 | 29 | 5 | 11.6 |
| 0°Eq 83°E | 0-30 | 58 | 16 | 27.6 | 77 | 7 | 16.3 |
| 1°S 80.5°E | 500-1000 | 73 | 1 | 1.4 | 68 | 0 | 0.0 |
| 1°S 80.5°E | 200-500 | 61 | 3 | 4.9 | 52 | 7 | 16.3 |
| 0°Eq 77°E | 60-200 | 50 | 2 | 4.0 | 56 | 2 | 4.7 |

Table 5. Response of individual zooplankton specimens collected during September 2006 from the equatorial Indian Ocean to ISH using probes designed against two isolates of *Aplanochytrium kerguelensis*.

| Station | Depth | S61F1 probe | | | 138F1 probe | | |
|-----------|---------|-----------------|----------------|-------------|-----------------|----------------|------------|
| | | Total specimens | Total positive | % positive | Total specimens | Total positive | % positive |
| 0°Eq 77°E | 200-500 | 17 | 0 | 0.0 | 23 | 3 | 7.0 |
| 0°Eq 77°E | 0-200 | 17 | 0 | 0.0 | 31 | 1 | 2.3 |
| 0°Eq 83°E | 200-500 | 21 | 0 | 0.0 | 36 | 0 | 0.0 |
| 0°Eq 83°E | 0-200 | 9 | 1 | 11.1 | 9 | 0 | 0.0 |
| 0°Eq 93°E | 200-500 | 7 | 0 | 0.0 | 5 | 0 | 0.0 |
| 0°Eq 93°E | 0-200 | 24 | 3 | 12.5 | 24 | 4 | 9.3 |

Legends to figures

- Fig. 1.** Area in the equatorial Indian Ocean where zooplankton were collected by deploying multiple plankton net during cruises SK 196 (September 2003) and SK 212 (October 2004).
- Fig. 2.** Neighbour-Joining (NJ) tree of 18S rDNA of aplanochytrids of the present study (bold letters) with *Bacillaria paxillifer* (Accession no. M87325) and *Ochromonas danica* (Accession no. M32704 J02950) as outgroups.
- Fig. 3.** Agarose gel electrophoresis of PCR-amplified products using S61F1-S61R1 primer set of aplanochytrids from the equatorial Indian Ocean (Lanes 1: S1962, 3: S2122, 5: S19610, 6: S2124, 7: S2129, 8: S2128, 9: S1963 and 11: S1961) and thraustochytrids from NIO culture collection. (Lanes 2-Z7, 4-CW1 and 10-DP7). Lane 12: negative control and Lane 13: 1 kb DNA ladder. Bands produced by PCR amplification are shown by arrows.
- Fig. 4.** Agarose gel electrophoresis of PCR-amplified products using 138F1-138R1 primer set of aplanochytrids from the equatorial Indian Ocean (Lanes 1: S1962, 3: S2122, 5: S19610, 6: S1961, 7: S2129, 8: S2128, 9: S1963 and 11: S2124) and thraustochytrids from NIO culture collection (Lanes 2-Z7, 4-CW1 and 10-DP7). Lane 12: negative control and Lane 13: 1 kb DNA ladder. Bands produced by PCR amplification are shown by arrows.
- Fig. 5.** Southern hybridization of S1961 amplified product using S61F1-S61R1 primer set as seen stepwise (i) amplified PCR product of approximately 350 bp in length in agarose gel electrophoresis (Lane 1-1 kb DNA ladder, 2- S1961 PCR product), (ii) portion of the gel containing the band cut prior to electroblotting and (iii) band blotted on nylon membrane visualized by hybridization reaction.
- Fig. 6.** The aplanochytrid S1961 pretreated by different methods like (a) overnight incubation in Triton X100 and (b) electroporation showing positive reaction for *in situ* hybridization using biotinylated probes and streptavidin-conjugated alkaline phosphatase. Bar represents 10 μm .
- Fig. 7.** The aplanochytrid S1961 showing a positive reaction for *in situ* hybridization using biotinylated probes and streptavidin-conjugated peroxidase. Bar represents 10 μm .

- Fig. 8.** *In situ* hybridization of aggregates from the equatorial Indian Ocean using biotinylated ITS-probes developed against the aplanochytrid S1961 and S2124 and streptavidin-alkaline phosphatase conjugate. Bar represents 10 μm .
- Fig. 9.** A specimen of zooplankton fed with the aplanochytrid S1961 showing positive reaction in their guts for *in situ* hybridization using probes for the aplanochytrid isolate and with streptavidin-alkaline phosphatase conjugate. Fig. b is high power photograph of a. Bar represents 100 μm in a and 50 μm in b.
- Fig. 10.** Blue reaction in the appendage (a) and metasome (b) of the zooplankton body to the alkaline phosphatase- BCIP- NBT following *in situ* hybridization using probes for the aplanochytrid S1961. Bar represents 50 μm .
- Fig. 11.** *In situ* hybridization of chaetognaths from equatorial Indian Ocean using biotinylated ITS-based probes developed against the aplanochytrid S1961 and S2124; (a) an aggregate-like structure in the vicinity of the mouth part of the chaetognath reacted positively to ISH, (b) contents inside the mouth parts of a specimen showing positive reaction, (c) several positively stained cells seen scattered within the chaetognath body, and also (d) surrounding the gut. Bar represents 100 μm in a and b and 10 μm in c and d.
- Fig. 12.** *In situ* hybridization of chaetognaths from the equatorial Indian Ocean following *in situ* PCR, using biotinylated ITS-probes developed against the aplanochytrid S2124. Bar represents 10 μm .
- Fig. 13.** *In situ* hybridization of chaetognaths from equatorial Indian Ocean using biotinylated ITS-based probes developed against the aplanochytrid S1961 and S2124 and streptavidin-peroxidase conjugate. Positively stained cells are seen in the (a) head region, (b) and (c) tail region, and (d) the region surrounding the gut but towards periphery. Bar represents 10 μm in a, b and d and 100 μm in c.

Fig. 1

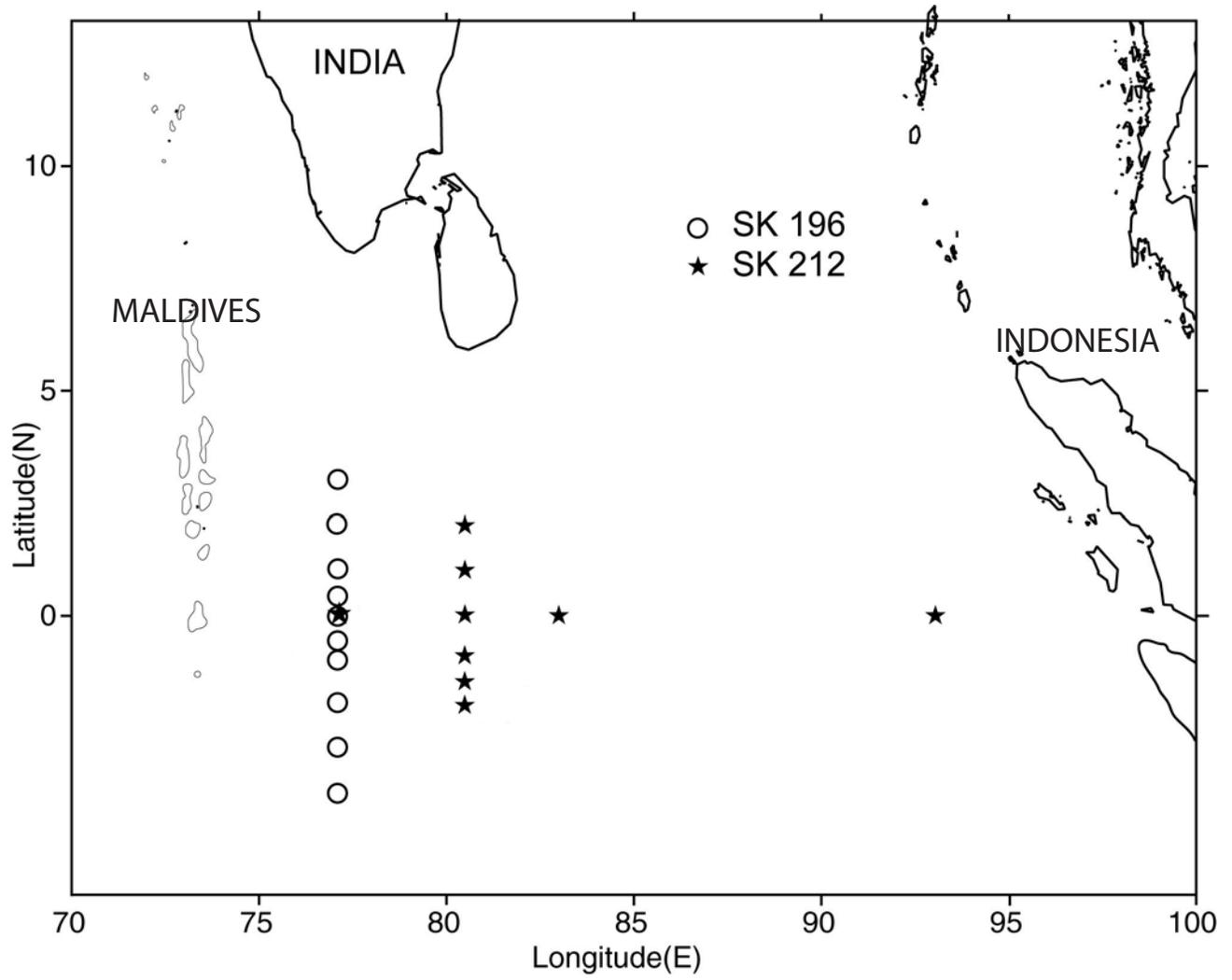


Fig. 2

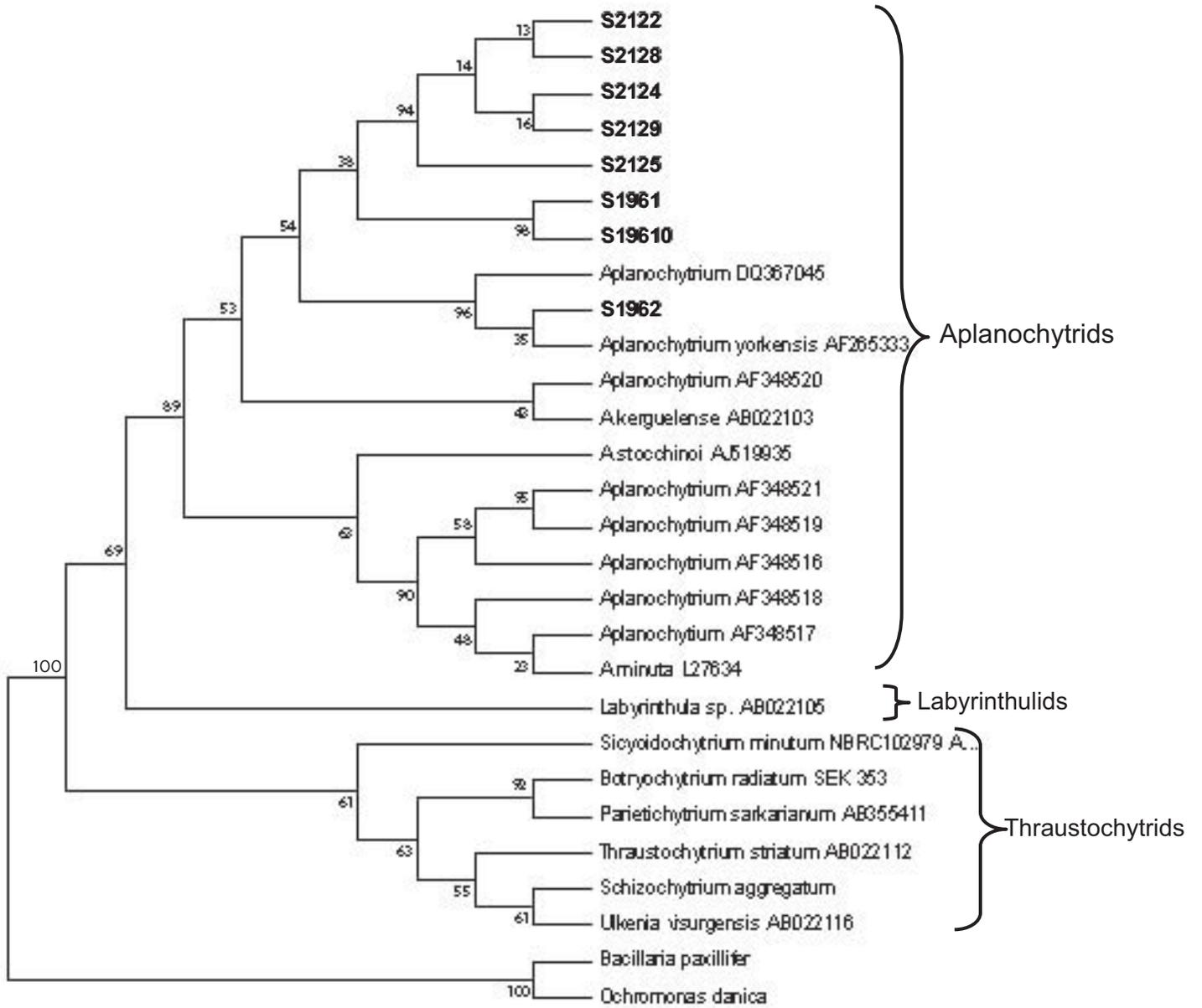


Fig.3

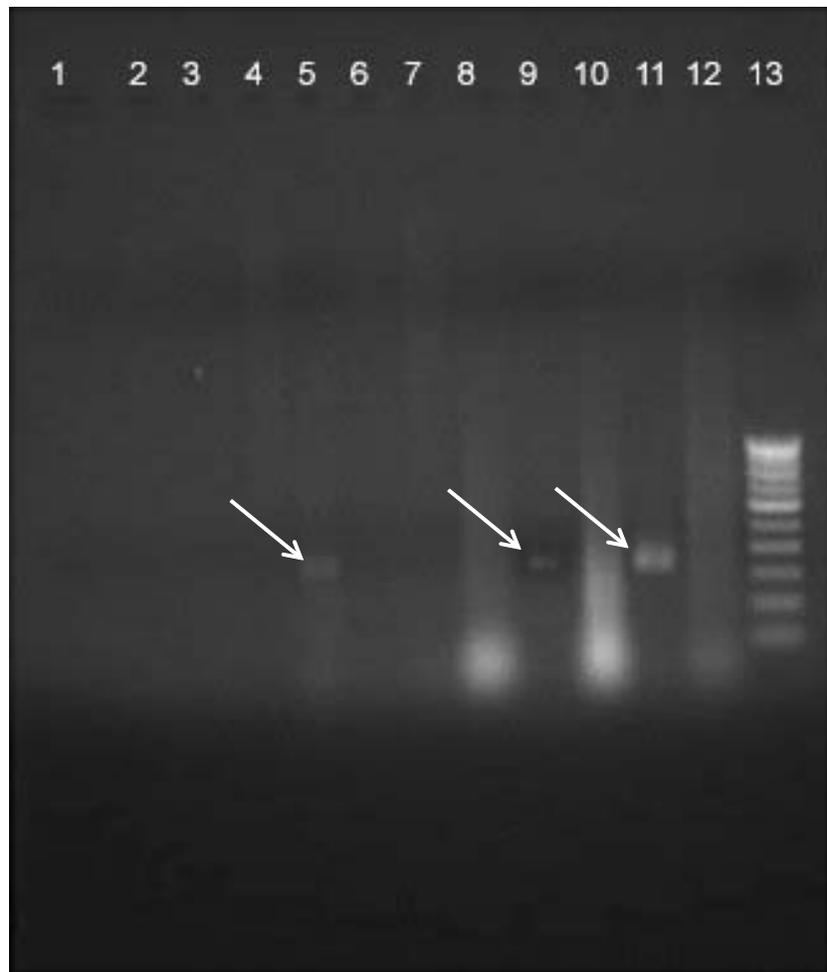


Fig. 4

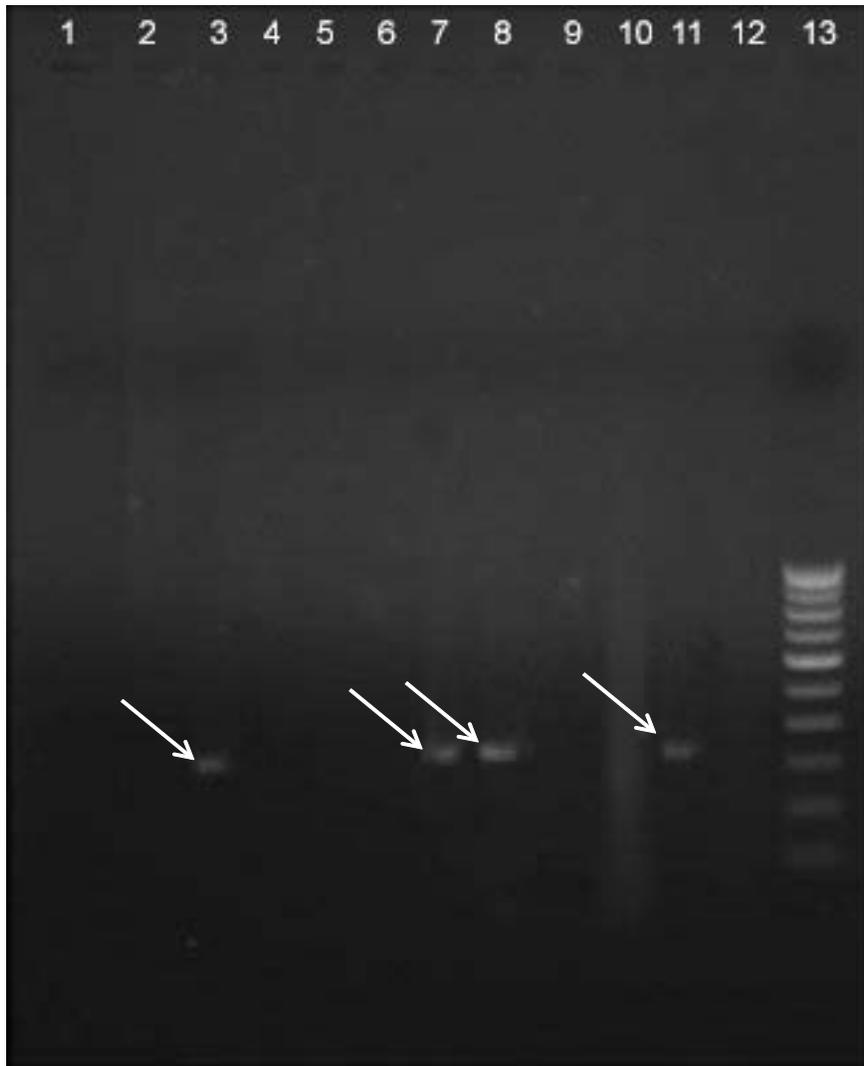


Fig.5

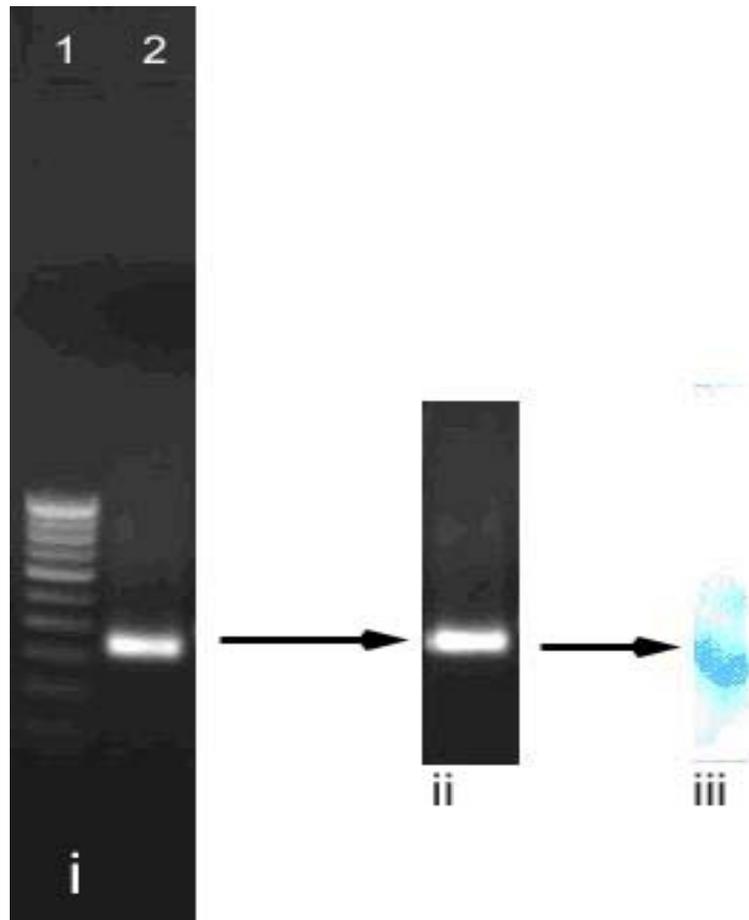


Fig.6

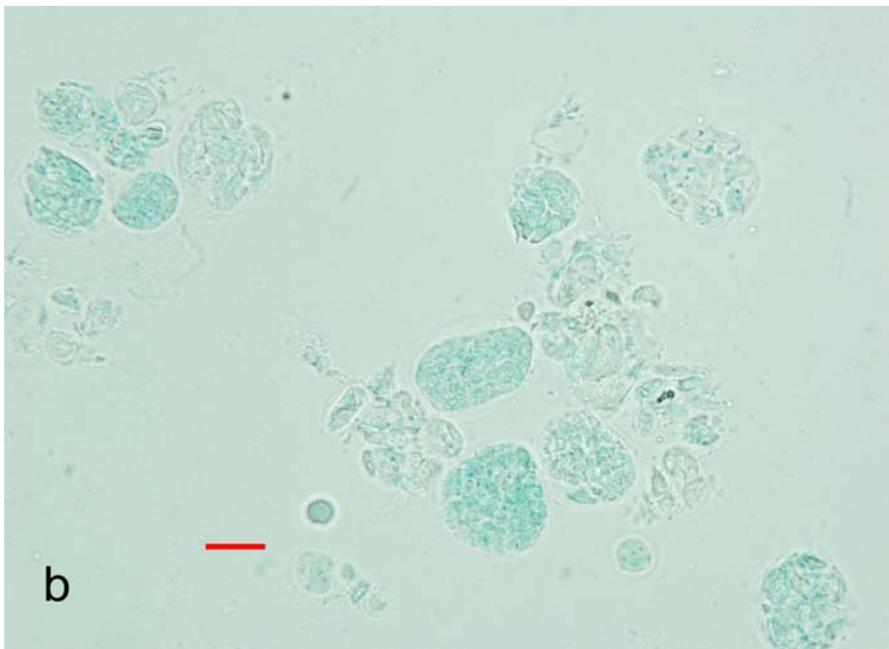


Fig.7

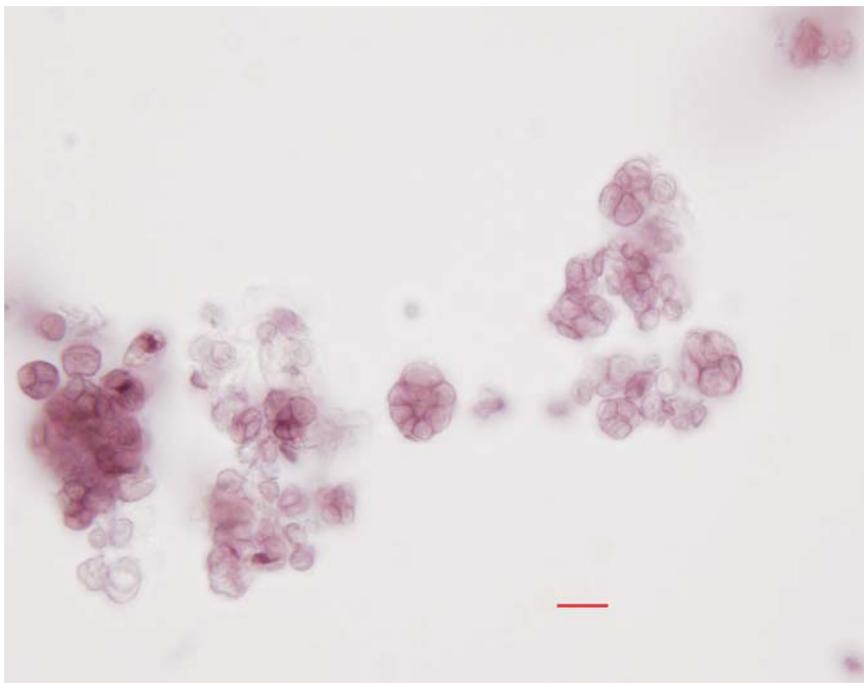


Fig.8

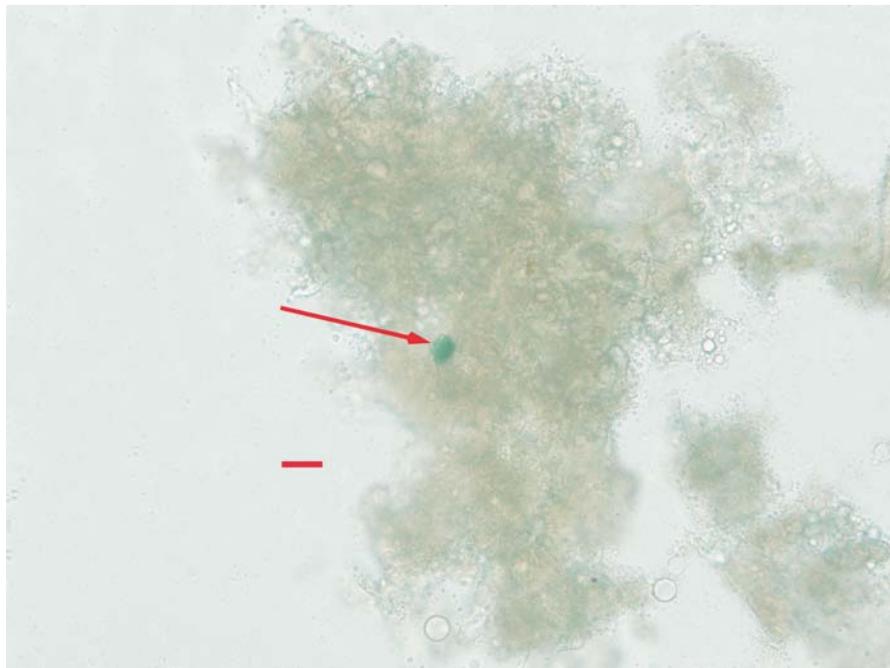


Fig. 9



Fig. 10

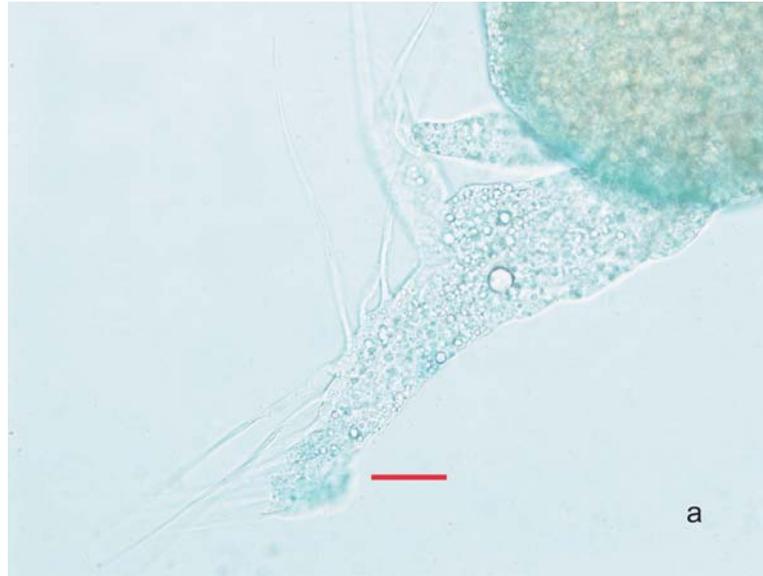


Fig. 11

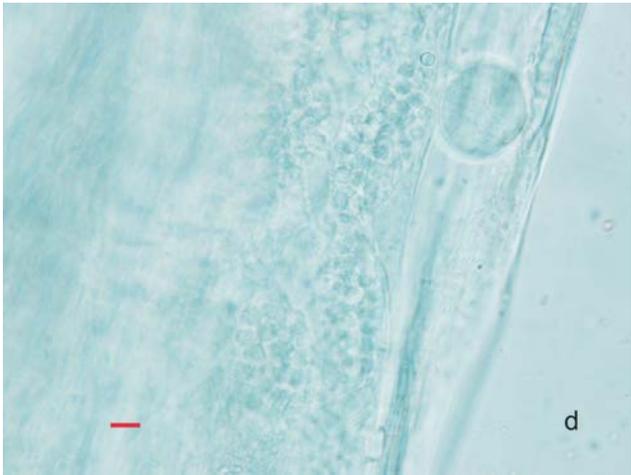
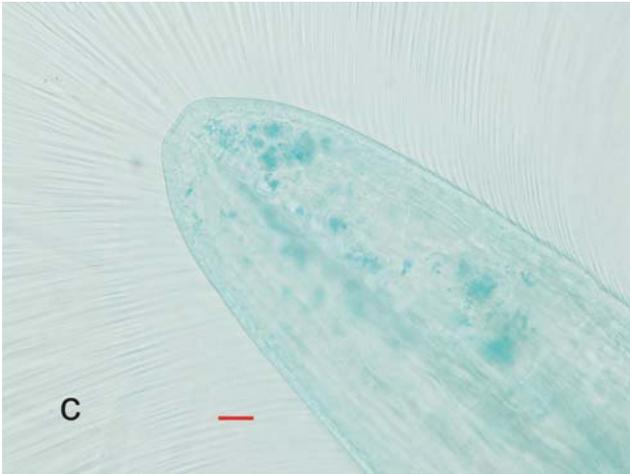
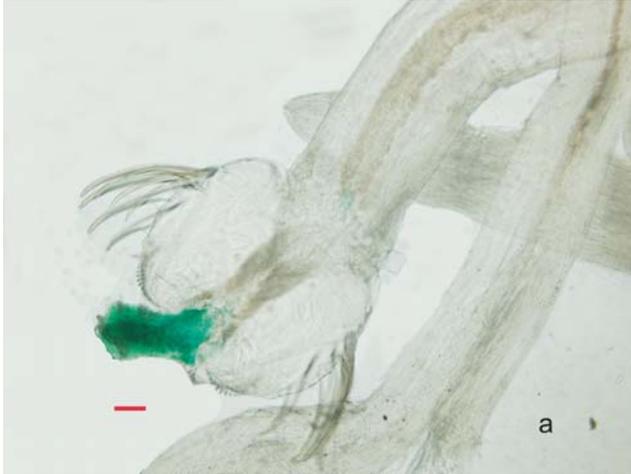


Fig. 12

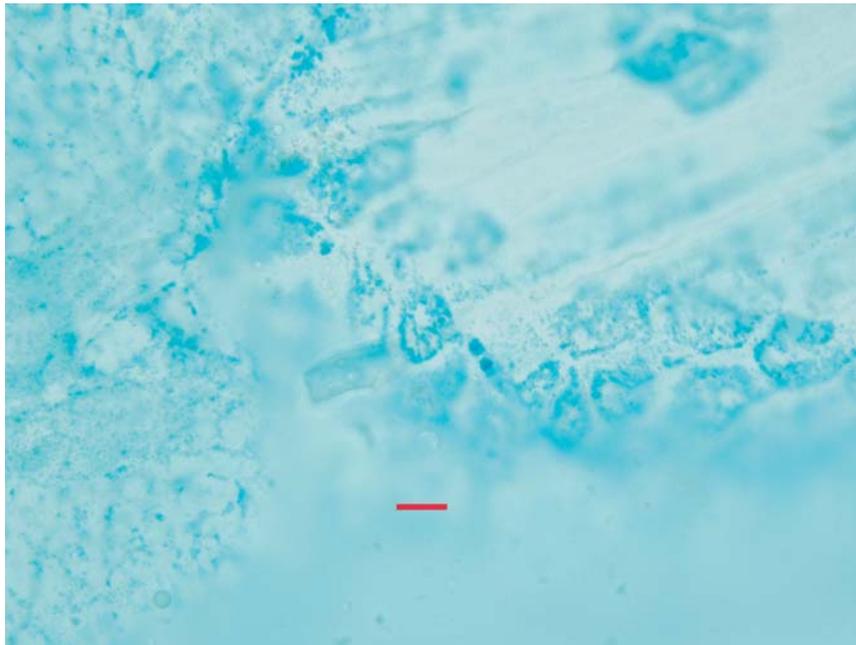


Fig. 13

