

## **Preliminary Studies on the Association between Zooplankton and the Stramenopilan Fungi, Aplanochytrids**

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### **ABSTRACT**

The relationship of the marine heterokont stramenopilan protists, the aplanochytrids with the zooplankton was studied in coastal waters. The aplanochytrids were fed to the zooplankton specimens and observed for grazing by two different approaches, 1) using fluorescently-labeled prey approach and 2) using internal transcribed spacer (ITS) based molecular probe and *in situ* hybridization approach. The aplanochytrid cells were detected in the guts as well as faecal pellets of the zooplankton, thus serving as prey to them. Aplanochytrids were consistently isolated from zooplankton specimens. The isolates did not produce a wide array of enzymes implicating that they may not play a major role in degradation of zooplankton exoskeleton. They were found to produce only protease considerably and sometimes lipase too. The Amplified rDNA Restriction Analysis (ARDRA) showed similar patterns suggesting that most of the isolates might be same strains of *Aplanochytrium* spp. The existence of aplanochytrids with the zooplankton in marine waters points towards their probable association either as predator-prey or as commensalistic rather than saprophytic type of association.

**Key Words.** Labyrinthulomycetes, protists, mesozooplankton grazing, fluorescently-labeled prey, molecular probe, amplified rDNA restriction analysis

## INTRODUCTION

Aplanochytrids are unicellular eukaryotic organisms belonging to Class Labyrinthulomycetes of the Kingdom Stramenopila (1). The aplanochytrids comprise a single genus *Aplanochytrium* with different species that share characteristics with each other (2, 3). Aplanochytrids were previously isolated from a variety of habitats like seawater, green algae, seagrasses etc (see 2). *Aplanochytrium haliotidis* causes pathogenic infection in abalone *Halotis kamtschatkana* (4). Recently molecular probes were designed for direct detection of these organisms in the oceanic environment (5) as the fluorescence microscopy technique (6) used for direct detection of Labyrinthulomycetes cannot distinguish the aplanochytrids against their 2 sister groups i.e., the thraustochytrids and the labyrinthulids. The thraustochytrids occasionally occur in high numbers in the water column and form a major portion of the microbial biomass of the seas. With regards to organic carbon contribution, an average cell is equivalent to almost 1000 bacterial cells and hence they periodically contribute higher carbon biomass than the bacterial biomass alone to the total microbial carbon (7). The aplanochytrids may also form important components of microbial population, since they are morphologically similar to thraustochytrids and are indistinguishable from thraustochytrids owing to their spherical shape as against spindle-shaped labyrinthulids. Hence the reports that mention thraustochytrid biomass (see 8) in nature may in fact be the thraustochytrid plus the aplanochytrid population.

The aplanochytrids were consistently isolated from the zooplankton of oceanic waters and their presence within their bodies was documented (5). How these 2 groups are associated with each other in the coastal environment is not yet known. Two hypotheses could be put forward. Firstly, since they are present in the water column, the aplanochytrid cells could be grazed upon by the zooplankton there. In other words, aplanochytrids might serve as a food source to the zooplankton, an aspect never studied before. Secondly, by producing different extracellular enzymes like thraustochytrids (3, 9-10) they might play a saprophytic role in decomposing the cadavers of zooplankton. With the objective of investigating the association of aplanochytrids with the zooplankton in coastal waters, the present study was carried out. Aplanochytrids were isolated from coastal zooplankton and characterized at physiological and molecular level. These were also fed to the coastal zooplankton to check the grazing pattern.

## MATERIALS AND METHODS

**Grazing studies:** The grazing of mesozooplankton on aplanochytrids was studied by 2 approaches; 1) using fluorescently-labeled prey cells and 2) using molecular probes.

*Fluorescently-labeled prey approach:* This was based on the 'fluorescently labeled bacteria' short term uptake technique of Sherr & Sherr (11). Mesozooplankton were sampled from the Dona Paula Bay using a zooplankton net of 200 µm mesh size (Hydrobios, Germany). Five to ten live specimens of mesozooplankton

were kept in 3 separate beakers each, containing 500 mL of 0.22 µm filtered seawater from the same site, under aeration in laboratory for 24 hours. Fluorescently-labeled aplanochytrid cells were added to each beaker at a concentration similar to the concentration of Labyrinthulomycetes in natural seawater at Dona Paula Bay during the sampling time [ $120 \times 10^3$  cells L<sup>-1</sup> as enumerated by Acriflavine Direct Detection (AfDD) technique according to Raghukumar & Schaumann (6)]. The fluorescently-labeled aplanochytrid cells were prepared by first harvesting the cells from the Modified Vishniac's broth after 5 days of growth. The cells were then washed with sterile 0.22 µm-filtered aged seawater and suspended in 100 mL of the same seawater, then stained for 2 hours at 60° C with 0.2 mg mL<sup>-1</sup> final concentration of the vital stain DTAF (5-(4,6-dichlorotriazin-2-yl) aminofluorescein; Sigma) prepared by dissolving it in 0.05 M Na<sub>2</sub>HPO<sub>4</sub>-NaCl solution, adjusted to pH 9.0. Later the cells were centrifuged and washed thrice with sterile 0.22 µm filtered seawater. The fluorescently-labeled cells were enumerated by filtering an aliquot over 0.45 µm black polycarbonate filter paper (Millipore) and observed under epifluorescence microscope. Cells were maintained at -20° C or lyophilized and refrigerated till further use.

During the grazing experiment each beaker was sampled at 6, 12 and finally 24 hours by withdrawing 25 mL of water and fixing with formalin (3 % final concentration) to enumerate fluorescent cells microscopically and follow their disappearance due to grazing. A control beaker containing 0.22 µm filtered seawater from the same sampling site with the same number of fluorescently-labeled aplanochytrids as that in experimental beakers was maintained without any zooplankton specimens to follow the disappearance of fluorescence of the prey cells with time.

Faecal pellets of zooplankton were collected with Pasteur pipette by observing under zoom stereo microscope (Olympus, SZH 10-141) at the end of 24 hours. These were checked for the presence of fluorescently-labeled aplanochytrids under blue excitation light using BX60 Olympus epifluorescence microscope (using 1000 X optical zoom) equipped with 100 W Hg lamp. A single factor ANOVA was conducted that examined the effect of time on decrease in the number of fluorescent cells in experimental as well as control beakers. The dependent variable was time and independent variables were the number of fluorescent cells in experimental and control beakers.

*Molecular probe-based approach:* Internal transcribed spacer (ITS) - based probe, S61F1, was used for this study. This probe was designed against the aplanochytrid isolate S1961 (isolated previously from oceanic zooplankton) for the detection of the aplanochytrids in natural samples (5). Live 7-8 specimens of mesozooplankton collected from Dona Paula Bay were kept in 5 separate beakers each under aeration in the laboratory. They were starved for a period of 6 hours. Cells of the aplanochytrid S1961 were then added in each experimental beaker. Cells were added up to twice the concentration found in natural waters of Dona Paula Bay as estimated independently by AfDD ( $232 \times 10^3$  cells L<sup>-1</sup>). A control beaker in which aplanochytrid cells were not added to the zooplankton was also maintained. The entire contents of each beaker were fixed with 3 % formalin at

4, 6, 10, 15 and 24 hours to preserve the specimens and to check if the aplanochytrids had been grazed and were present in their guts. The specimens from each beaker were transferred to microcentrifuge tubes. Permeabilization of the specimens followed by *in situ* hybridization (ISH) was carried out according to Damare & Raghukumar (5). ISH was carried out with the S61F1 probe at a concentration of 5 pM/μL using the Southern Hybridization Kit (Bangalore Genei, India), following the manufacturer's instructions. Streptavidin conjugated to alkaline phosphatase (ALP) (Sigma) was used as conjugate and 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma, India) were used as substrate solution.

**Isolation of Labyrinthulomycetes protists from zooplankton:** Labyrinthulomycetes were isolated from mesozooplankton from the Dona Paula Bay, Goa (India) during October 2011. Zooplankton were collected using a 200 μm mesh size plankton net from surface to approximately 5 m depth during high tide. Zooplankton specimens were thoroughly washed in sterile seawater and individual animals were plated on to Modified Vishniac (MV) Agar supplemented with antibiotics (500 mg each of ampicillin and cloxacillin) and 0.01% fetal bovine serum (2). The plates were incubated for 7 – 14 days till colonies of Labyrinthulomycetes protists appeared. The identity of the colonies was confirmed by examining the colonies under the low power of a microscope. Bacteria-free colonies were then sub-cultured on to sterile MV agar plates without antibiotics and were routinely sub-cultured hence forth.

**Physiological characterization of isolates:** The isolates were tested for the production of various extracellular enzymes like protease, lipase, amylase, chitinase, xylanase, pectinase, cellulase and laccase. This was done by incorporating respective substrates in the MV agar plates at final concentration of 0.1-1%. The substrates used were skimmed milk (1%) for protease, Tween 80 (1%) and calcium chloride (0.001%) for lipase, starch (1%) for amylase, chitin (1%) for chitinase, xylan (oatspelt, 1%) for xylanase, pectin (1%) for pectinase, carboxymethyl cellulose (0.1%) for cellulase and guaiacol (0.1%) for laccase. The positive result was indicated by a zone of clearance in case of protease, precipitation around the colony in case of lipase, zone of clearance against dark blue background after overlaying with Lugol's Iodine in case of amylase, zone of clearance after overlaying with 0.2% solution of Congo red dye in NaCl in case of chitinase, 0.05% Congo red dye solution in case of xylanase, pectinase and cellulase and red colouration around the colonies in case of laccase (12).

Alternatively to check for chitinase production, the MV Agar was incorporated with Congo red dye solution to a concentration of 0.05 %, autoclaved and sterile chitin solution was added to a final concentration of 1%. Another set of plates were made by incorporating 0.05 % concentration of Rhodamine B dye instead of Congo red in the MV Agar and observed for a zone of clearance after streaking, indicating a positive reaction (13). The 5 % stock solution of chitin for the above purpose was prepared by dissolving 5 g of chitin in 100 ml of 50 % sulfuric acid using the magnetic stirrer, precipitated with cold distilled water by diluting it 15-fold. The precipitate was washed with distilled water till the pH was adjusted to 7 and autoclaved separately.

### **Molecular characterization of isolates:**

*DNA isolation and PCR:* Isolates were grown in large volumes of MV broth for 5 days and their biomass was extracted by centrifugation. The cell pellets were stored at -20° C until DNA was extracted from them. DNA was isolated using ZR Fungal/Bacterial DNA MiniPrep™ kit (Cat. No. D6005, Zymo Research). The quality of DNA was checked by running agarose gel electrophoresis and documented using a multi-image light cabinet, AlphaImager2200 (Alpha Innotech Corporation) and processed using AlphaImager2200 software. Molecular identification was done by selectively amplifying the 18S rDNA region employing a PCR, purifying the PCR products, obtaining the sequences and performing a database search in order to find the closest neighbors. 18S rDNA PCR was performed using two universal primers (NS3 and NS4) and two primers described by [Honda et al. \(14\)](#) (18S001 and 18S13) to amplify full length 18S rDNA. The first set of primers was 18S001-NS4 and the second set of primers was NS3-18S13. Appropriate amount of DNA was added to 50 µl PCR reaction solution containing 1X Taq assay buffer with MgCl<sub>2</sub>, 5 mM dNTP solution, 0.5 U of Taq DNA polymerase and 10 pM of each primer in DNase RNase free water. PCR amplifications were performed in a thermal cycler (Eppendorf vapoprotect) with the following cycling program: initial denaturation for 5 min at 95°C, 35 amplification cycles of 50 sec at 95° C, 50 sec at 59° C for primer set I and 62° C for primer set II and 1 min at 72° C, with a 10 min extended elongation step. PCR products were visualized along with a 1kb ladder on 1 % agarose gel incorporated with ethidium bromide dye. The PCR products were purified using AxyPrep™ PCR Cleanup Kit supplied by Axygen Biosciences (Cat. No. AP-PCR-250). The quality of the purified PCR products was also checked by electrophoresis and the purified PCR products were stored at -20°C till further use.

*Molecular differentiation amongst the isolates:* Amplified rDNA Restriction Analysis (ARDRA) using the amplified 18S rDNA region was carried out. A restriction digestion reaction was performed with the enzymes XbaI, SacI-HF and EcoRI-HF. The enzymes were selected based on the 18S rDNA sequences from NCBI database. A 10 µL reaction mixture containing 1X concentration of NE Buffer 4, 100 µg/ml concentration of BSA, 0.01X concentration of each XbaI, SacI-HF and EcoRI-HF, 5 µl of the purified PCR product in DNase RNase free water was made. The restriction digestion was performed in a thermal cycler (Eppendorf) at 37° C for 2 hours followed by the enzyme inactivation by incubating them at 65° C for 20 min. The products were visualized on a DNA chip (Kit No.5067-1504) using BioAnalyzer 2100 (Agilent Technologies, Germany) as per the protocol of the manufacturer. Apart from the 29 isolates, 2 aplanochytrid isolates (S1961 and S19615) obtained from oceanic zooplankton and 9 more isolates obtained from detritus and sediments of mangrove regions (Chorao and Divar Islands) and Ribandar salt pan were used for comparison. These were isolate CLF1, DPZF4, DiAJ8, CSF8, RAMr46, RAMr14, DPZMr14 and DPSgMr15.

*Sequencing and phylogenetic tree construction:* The purified PCR products were sequenced using a 3130xl Genetic Analyzer (ABI Sequencer 2200), Applied Biosystems by capillary electrophoresis. The sequences

were analyzed using the DNA Baser sequence assembly software (trial version 3.2.5.124) and Basic Local Alignment Search Tool (BLAST) provided by NCBI. The phylogenetic tree was constructed using the software MEGA 4.0.1. Firstly the sequences obtained after sequencing and their respective neighboring sequences obtained as a result of BLAST analysis were aligned using CLUSTALW tool inbuilt within the software. The aligned sequences were then resized to consider only the aligned region. Later construction of the phylogenetic tree using Neighbor-Joining (NJ) method was done by fixing the bootstrapping value at 1000.

## RESULTS

**Grazing studies: *Fluorescently-labeled prey approach:*** It was seen that the number of fluorescently-labeled aplanochytrid cells in the experimental beakers decreased from an initial number of 115 to 32 cells mL<sup>-1</sup>. In the control beaker wherein there were no zooplankton specimens, the number of fluorescent cells dropped to 66 cells mL<sup>-1</sup> within 24 hours. This indicates that there was a natural loss of fluorescence with time. An average of the numbers is plotted against time in hours (Fig. 1). Statistical analysis showed that the F value (29.3) was more than the F<sub>crit</sub> value (3.4) by significant P value (1.7E-07), thus rejecting the null hypothesis and showing that the means of experimental and control conditions were not identical (Table 1). Faecal pellets collected from the bottom of the beakers with the help of Pasteur pipettes also revealed fluorescent cells (Fig. 2).

*Molecular probe based approach:* Aplanochytrid cells that reacted positively to ISH were detected in the hind gut of several of the zooplankton towards the end of 4 hours of feeding (Fig. 3a). No aplanochytrid cells were detected in the specimens after 6 hours and more of feeding. However, blue colouration owing to reaction of alkaline phosphatase with BCIP/NBT used in the ISH was noticed in different parts of the zooplankton body (Fig. 3b-d). 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.

**Isolation and physiological characterization studies:** Altogether 29 isolates were obtained after plating of zooplankton specimens on MV agar. Out of the 29 isolates, 24 isolates produced protease, 2 isolates (DPZO 1.7 and 3.1) produced lipase and only one isolate (DPZO 1.19) produced amylase. None of the isolates produced chitinase, pectinase, cellulase, xylanase and laccase.

**Molecular characterization studies:** The 18S rDNA region that was amplified using primers NS3 and 18S13 was digested by the restriction enzymes (Fig. 4) whereas the region amplified using 18S001 and NS4 was not completely digested by them. The ARDRA displayed similar pattern of 4 bands for almost all the isolates except isolates DPZO 1.3, 1.11, 2.6, 2.9 and 4.7. The restriction pattern of the 2 oceanic aplanochytrids was different from the coastal ones. BLAST analysis of 18S rDNA region of all the isolates showed that except for the isolate DPZO 1.19, all the other isolates belonged to the genus *Aplanochytrium*. Isolate 1.19 showed high similarity with

a thraustochytrid. The nine other isolates that were used for comparison in ARDRA were also found to be thraustochytrids. CLF1 and DPZMr14 were *Schizochytrium*, RAMr14 was *Botryochytrium*, CSF8 was *Ulkenia* and the rest were *Thraustochytrium* spp. Some undigested PCR product was left over in the wells of the isolates DPZO 2.10, 4.7, 8.2 and 8.4, which could also be seen along with the restricted bands. The phylogenetic tree constructed using Mega 4.0.1 based on NJ Method is shown in Fig. 5 along with the respective bootstrap values.

## DISCUSSION

The present work was carried out to check the grazing behavior of coastal zooplankton on aplanochytrids as well as to study their existence with the zooplankton by means of culturing and then to examine their saprophytic nature. Grazing studies revealed that zooplankton fed on the aplanochytrids. The number of fluorescently-labeled aplanochytrids decreased in time in both the experimental as well as control conditions. Statistically both the conditions were significantly different from each other indicating that the reduction in cell numbers in experimental conditions was on account of grazing and not by chance or natural decay in fluorescence. Some of the fluorescent aplanochytrids were seen in the faecal pellets, which might have passed there passively through the guts of zooplankton without getting digested. Raghukumar & Raghukumar (15) also detected thraustochytrids in faecal pellets of salps by AfDD staining. The consumption of aplanochytrids by zooplankton implies towards the supply of essential nutrients to them. Probably these also produce polyunsaturated fatty acids (PUFAs) similar to thraustochytrids. PUFAs are essential nutrients for the growth and reproduction of zooplankton but cannot be synthesized *de novo*. Almost 98 % of the lipids in the zooplankton body are obtained through dietary intake (16). When thraustochytrid-fed zooplankton like rotifers and crustaceans served as feed to fish in aquaculture, it was observed that zooplankton incorporated fatty acids from thraustochytrids in their body (17-19). Heterotrophic protists are responsible for trophic upgrading wherein they improve poor algal quality for subsequent use by higher trophic organisms (20). Copepods generally have high clearance rates on heterotrophic protists and may even prefer them over algae (see 21).

The grazing studies thus demonstrated that aplanochytrids act as potential prey to the zooplankton and in turn serve as important links in the microbial loop of the food web in marine waters. In order to substantiate the above, isolation and characterization of Labyrinthulomycetes protists from the zooplankton was carried out. Identification based on molecular taxonomy revealed that most of the isolates obtained in the present study from the coastal zooplankton belonged to the genus *Aplanochytrium* similar to the observation made with oceanic zooplankton. This proposes a strong association between the zooplankton and aplanochytrids. They may probably exist as commensals as proposed by Damare & Raghukumar (5) or as saprophytes.

The aplanochytrid isolates obtained in the present study showed an overall tendency for the production of protease enzyme. Almost 83 % of the isolates produced this enzyme. None of them produced any of the other enzymes studied except for isolate DPZO 1.7 which produced lipase and not protease, DPZO 3.1 which produced

lipase as well as protease and DPZO 1.19 which produced protease and amylase. Taoka et al. (22) observed that amylase was produced only by *Thraustochytrium* genus and not by *Schizochytrium* and *Aurantiochytrium* genera in their study. In the present study we observed that aplanochytrids did not produce amylase while isolate 1.19, which showed close similarity with *Thraustochytridae* sp. strain C41 produced this enzyme. None of them produced chitinase and it was cross-checked by growing them on chitin-supplemented MV plates containing different dyes like Congo Red and Rhodamine B (13). Damare & Raghukumar (3) also observed that aplanochytrids isolated from zooplankton were unable to produce chitinase and they produced only protease. On the other hand, a variety of enzymes were produced by thraustochytrids isolated from crustaceans, one of them being chitin-degrading enzyme (9). Although the thraustochytrids were not identified up to generic level in their study by Bongiorno et al. (9), it could be that the thraustochytrids probably have a higher tendency for degrading refractory organic material than the aplanochytrids. Hence a wide array of enzymes was produced by them which were not seen in the case of aplanochytrids. It could be implicated that these aplanochytrids did not play a direct role in degradation of zooplankton exoskeleton due to their inability to produce chitinase, instead could probably degrade other proteinaceous organic matter.

ARDRA analysis was carried out to differentiate the aplanochytrid isolates at their molecular level. ARDRA has been extensively carried out on bacterial communities and a few have been reported for fungi too (23-24). Stoeck & Epstein (25) have reported eukaryotic lineages, belonging to fungi and Straminipila by ARDRA in oxygen-depleted marine environment. Of the 29 isolates isolated from Dona Paula Bay, there were a few isolates producing banding patterns that differed from the rest. These isolates, namely DPZO 1.11, 1.3, 2.10, 4.7, 8.2 and 8.4, could probably be different strains of the same *Aplanochytrium* spp.. The ARDRA results correlate to some extent with the enzyme profiles. Probably the isolates that produced only protease and displayed similar band pattern could be the same strain of *Aplanochytrium*. These isolates seemed to be different from the oceanic aplanochytrids as seen by their different ARDRA patterns.

In conclusion, the aplanochytrids play an unassailable role with the zooplankton in marine environment. The existence of aplanochytrids with the zooplankton entails that these protists could be commensals, but their exact nature is not known. They would also fall quarry to the zooplankton but why would zooplankton consume aplanochytrids have to be determined by studying the fatty acids from the aplanochytrids that could be incorporated in their bodies. These aspects open up new directions for future investigation.

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TABLES

**Table 1** Summary of the results of ANOVA analysis examining the effect of time on decrease in the number of fluorescently-labeled aplanochytrid cells in experimental as well as control beakers.

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	32008.07	2	16004.03	29.32614	1.7E-07	3.354131
Within Groups	14734.6	27	545.7259			
Total	46742.67	29				

## FIGURE LEGENDS

Fig. 1 Change in abundance of fluorescently-labeled aplanochytrid cells with respect to time in the presence of zooplankton from Dona Paula Bay.

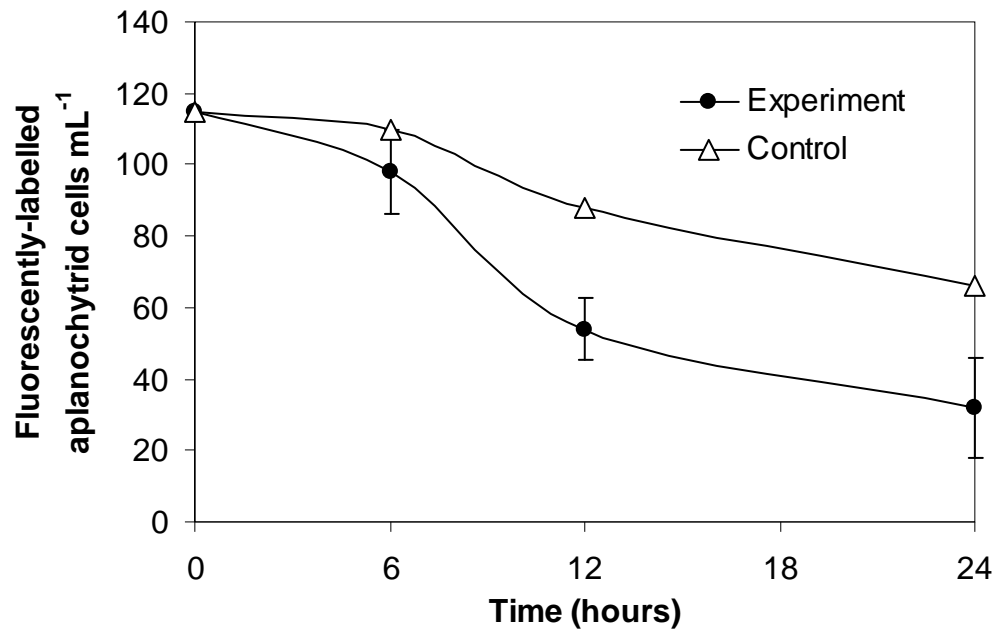
Fig. 2 Fluorescently-labeled aplanochytrid cell (marked by arrow) inside a faecal pellet of a mesozooplankton specimen under (a) blue light and (b) bright light. Scale bar = 50  $\mu\text{m}$ .

Fig. 3 Reaction of BCIP-NBT substrates to streptavidin-conjugated alkaline phosphatase bound to biotinylated S61F1 probe during *in situ* hybridization on mesozooplankton fed with aplanochytrid culture #S1961 for different time intervals; (a) after 4 hours, (b) after 6 hours, (c) after 10 hours, (d) after 15 hours. Scale bar = 50  $\mu\text{m}$  for Fig. a, c and d and scale bar = 100  $\mu\text{m}$  for Fig. 4b.

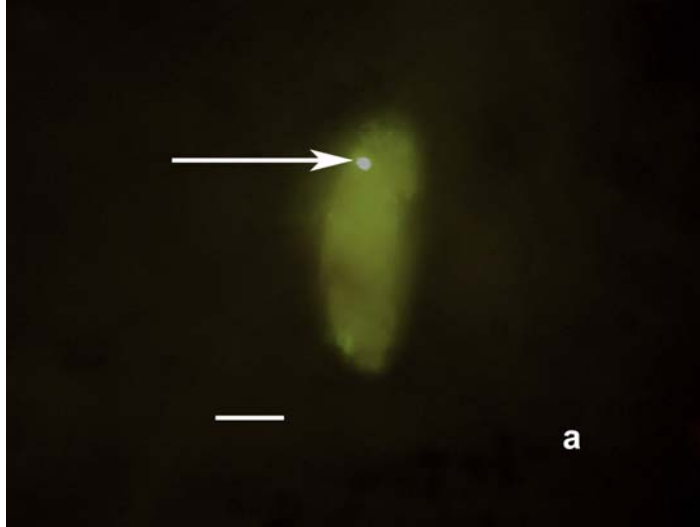
Fig. 4 ARDRA pattern as displayed in BioAnalyzer 2100 after restriction digestion of 18S rDNA amplified with NS3 and 18S13 primers.

Fig. 5 Neighbour Joining Tree of the aplanochytrids from present study along with other thraustochytrid isolates used for comparison and standard sequences from NCBI database using MEGA4.0.1. Some sequences which were too divergent were not considered for the tree construction.

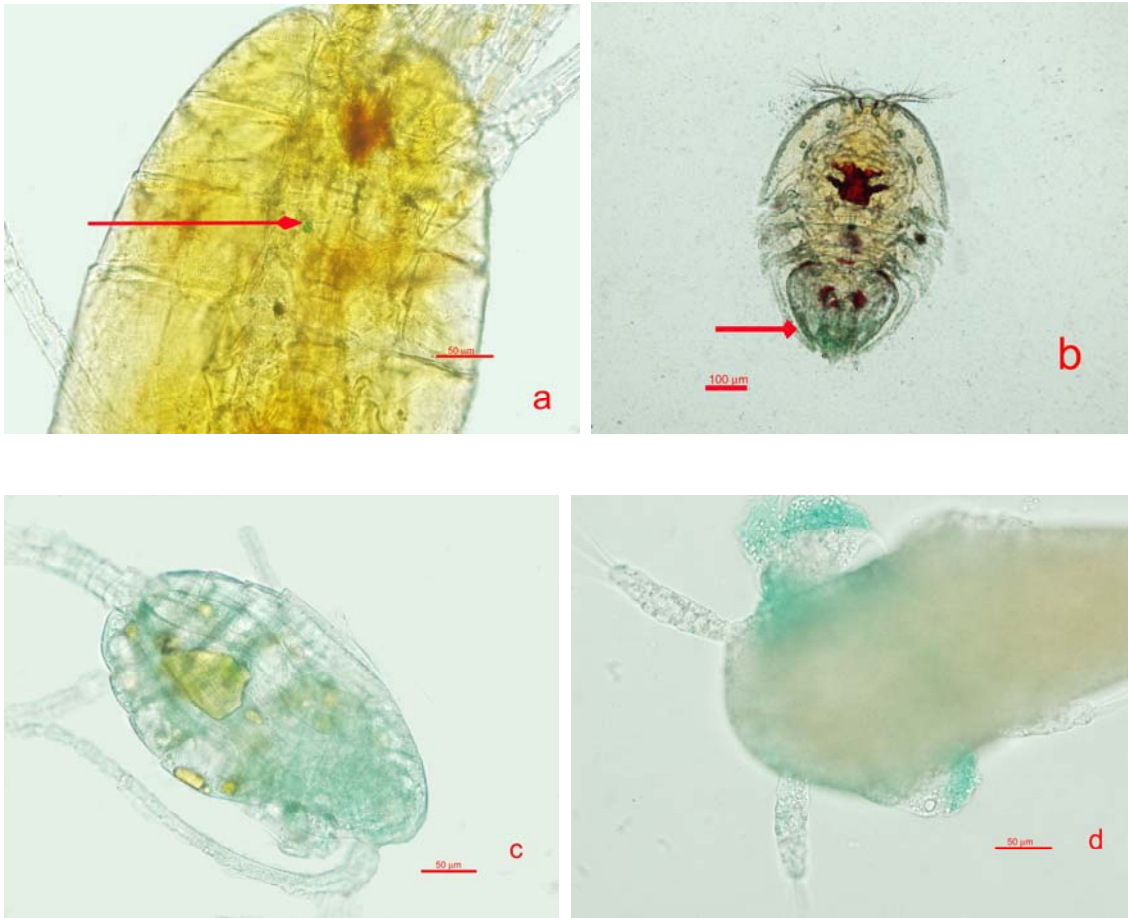
**Fig. 1**



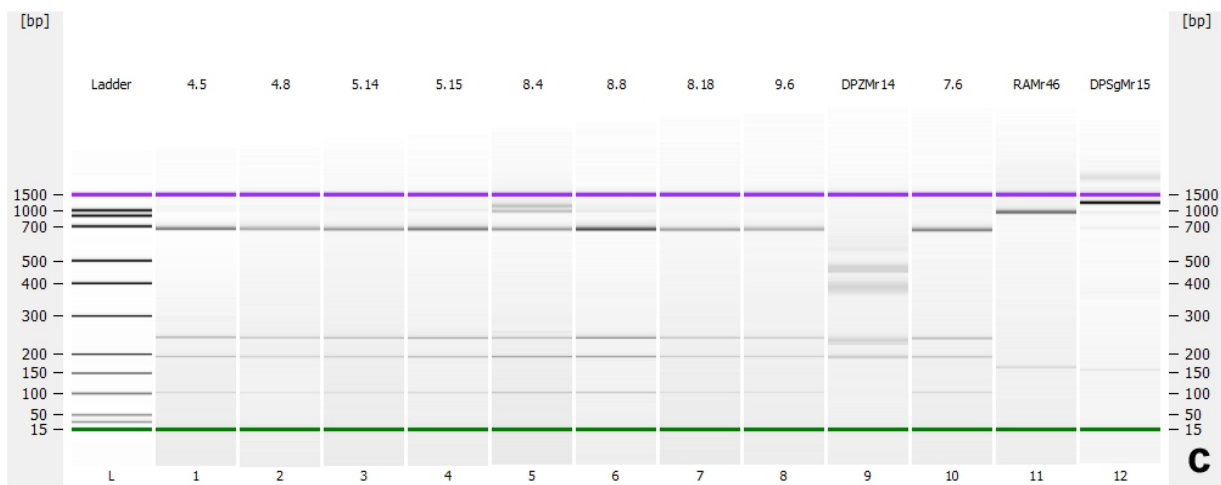
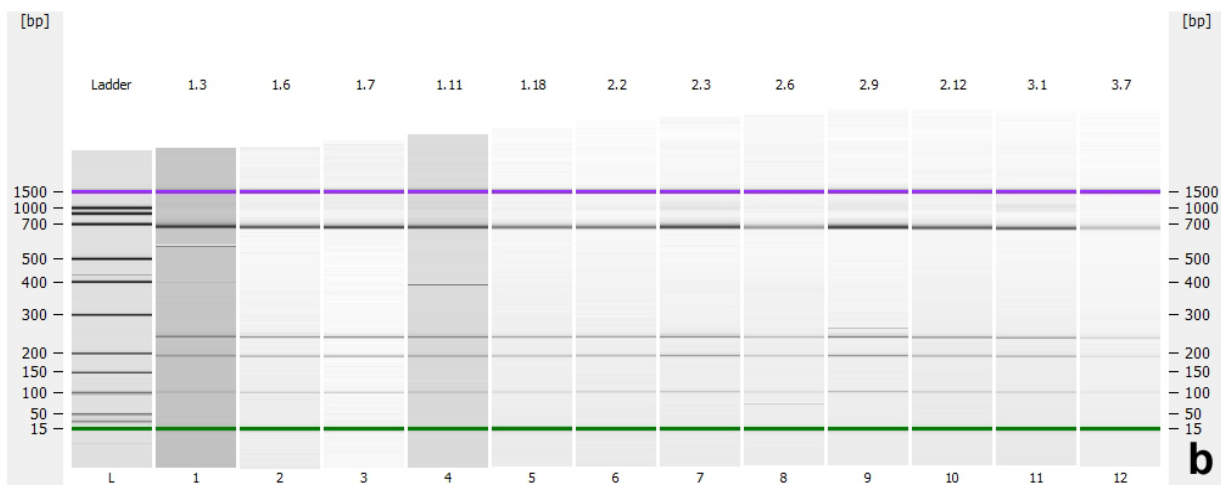
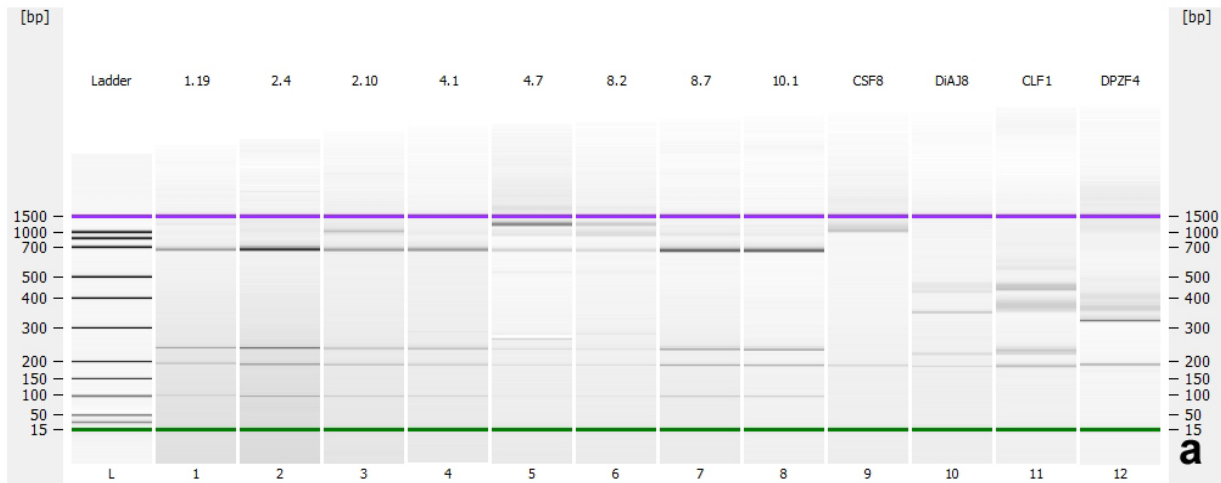
**Fig. 2**



**Fig. 3**

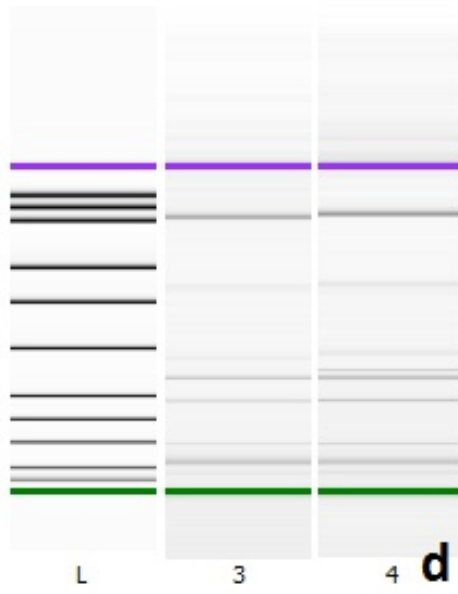


**Fig. 4**





Ladder S19615 S1961



**Fig. 5**

