

Chapter 3.

Molecular characterization of Aplanochytrids from the equatorial Indian Ocean

3.1 Introduction

Conventional taxonomy has relied on phenotypic characters such as morphology and life cycle in the case of higher organisms and biochemical characteristics in the case of microorganisms. Molecular methods have provided some of the most powerful tools for identification and phylogenetic determination of organisms in recent years. Gene sequences permit the construction of phylogenetic trees that reflect the evolutionary history of the species in a given clade and thus provide a natural taxonomy ([Edwards-Ingram *et al.*, 2004](#)).

Molecular taxonomy using genetic information in the form of DNA sequences as a universal character set for identification of organisms has several advantages.

- Molecular taxonomy is important especially to those groups in which distinctive phenotypical features are difficult to observe or compare.
- The use of appropriate genes for phylogenetic study also helps in inferring evolutionary relationships.
- Molecular taxonomy avoids subjectivity that often characterizes the use of morphology.
- Molecular taxonomy and evolution requires the use of a gene sequence which can be called as a molecular chronometer, as defined by Woese in 1970. These are molecular sequences having no phenotypic expression and which can undergo changes over time that become fixed in the organisms. Such changes can be used to

measure time in relative sense and therefore these sequences can be used for constructing genealogies. However, defining a single general marker gene for the taxonomic classification of an organism on all taxonomic levels, such as species, genus, phylum and Kingdom, is challenging. Thus, a fast-evolving marker may be needed for classification on the species level, but it may be too divergent for reconstructing the phylogeny at a higher level ([Schultz *et al.*, 2005](#)). Thus not all molecular chronometers are equally useful for reconstructing genealogies.

The most useful molecular chronometers actually are ribosomal RNA molecules ([Woese, 1987](#)).

- rRNAs are large molecules and hence contain considerable information and their size also makes them less erratic chronometers than smaller molecules.
- They are present in multiple copies within a cell.
- They don't seem to be subjected to lateral gene transfer.
- They are easy to isolate in large quantities.
- They are highly conserved and contain regions that evolve at different rates and are useful at the level of species to higher phylogenetic levels.

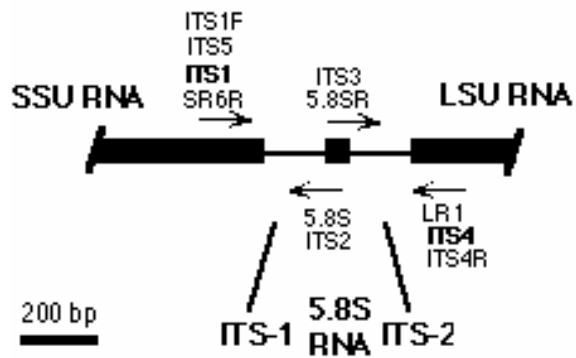
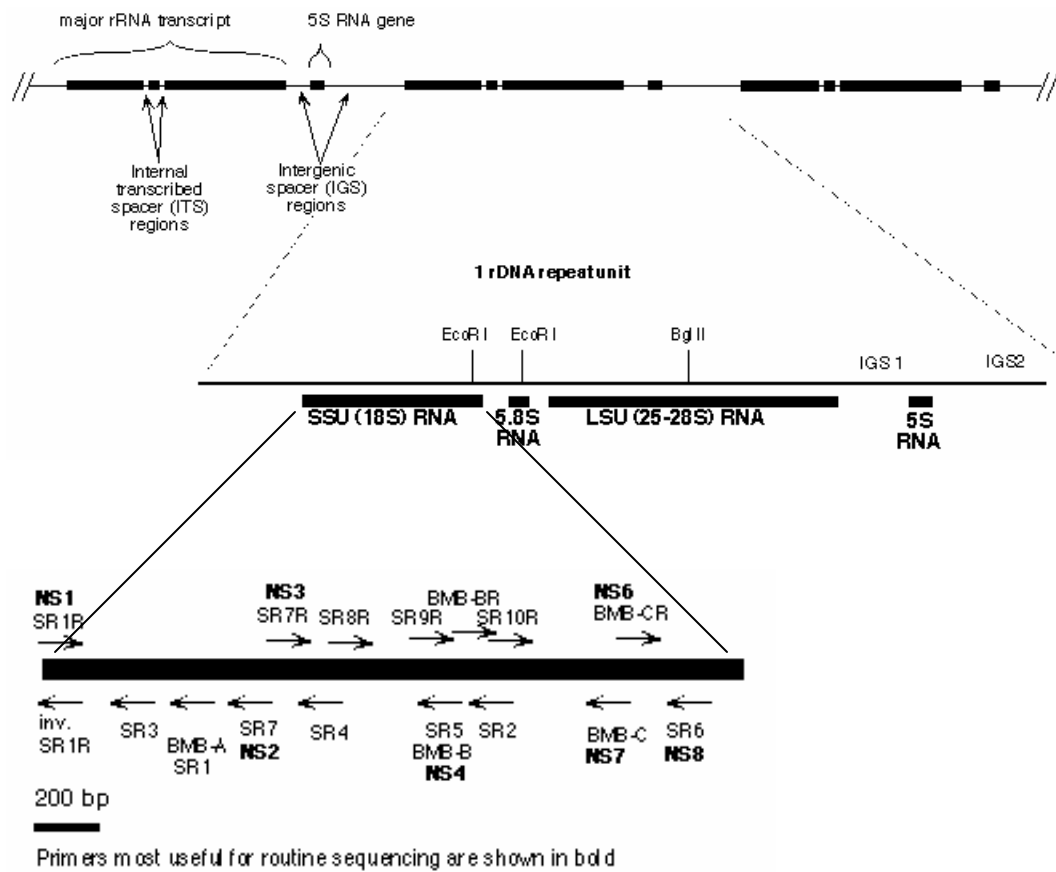
Ribosomal DNA sequences encoding the 16S for prokaryotes and 18S for eukaryotes have found increasing use in inferring

relationships among and, especially, within the major taxonomic groupings.

The rDNA or the nuclear-encoded ribosomal RNA genes exist as a multiple-copy gene family comprised of highly similar DNA sequences (typically from 8-12 kb each) arranged in a head-to-toe manner (Fig. 1). Each repeat unit has coding regions for one major transcript (containing the primary rRNAs for a single ribosome), punctuated by one or more intergenic spacer (IGS) regions. In some groups, each repeat also has a separately transcribed coding region for 5S RNA whose position and direction of transcription may vary among groups. The ITS regions are stretches of DNA between the 18S, 5.8S and 28S rRNA genes. The ITS region, a part of rDNA cistron, is highly conserved intraspecifically but variable between different species. Hence it is well suited marker for low level phylogenetic analyses ([Schultz *et al.*, 2006](#)). Thus using different markers like ITS for low level and 18S or 28S rDNA for high level classification helps in taxonomic studies.

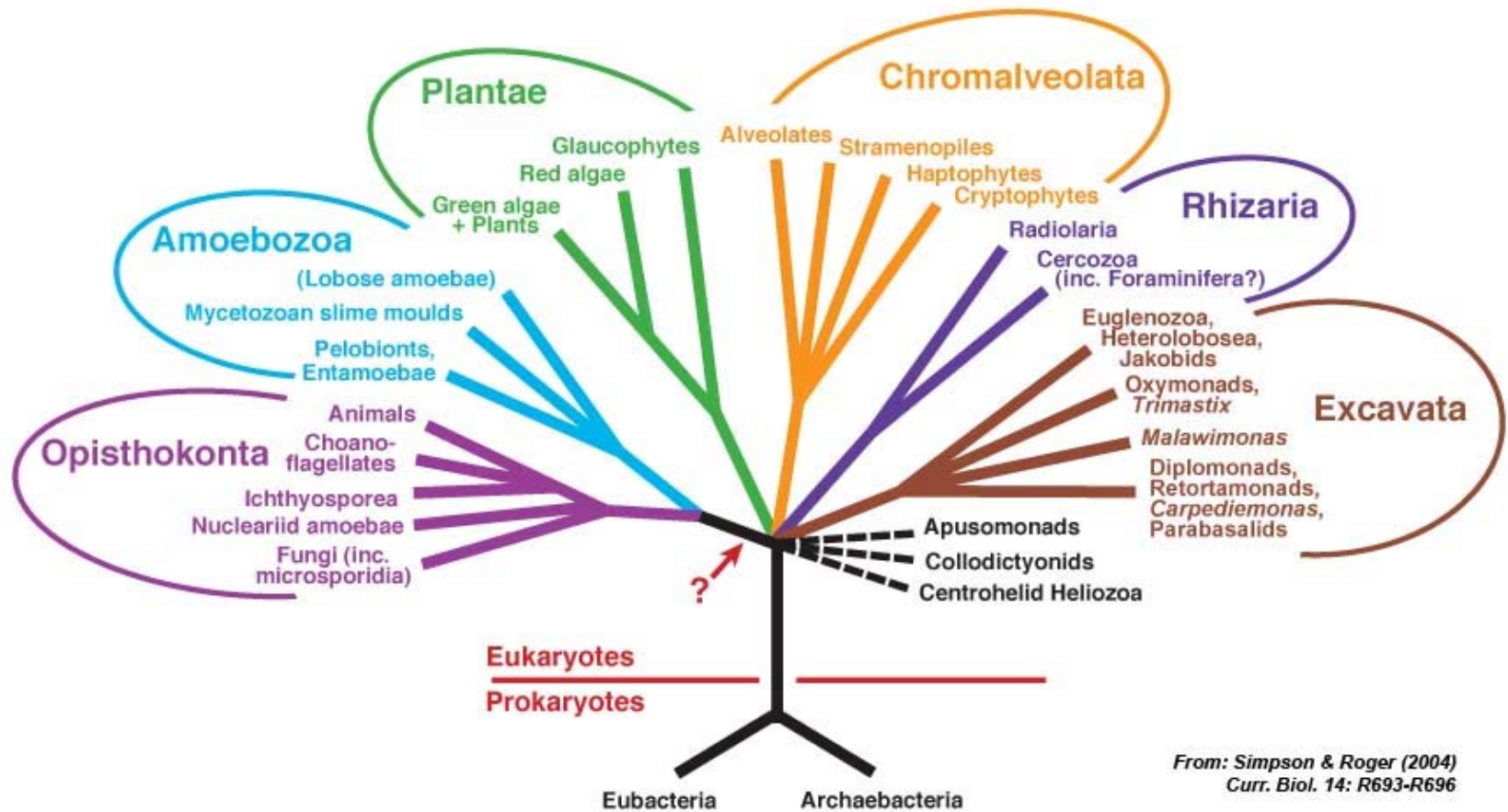
Molecular taxonomy has aided in recognizing fundamental domains in the phylogeny of living organisms and classifying them into more natural groups. A diagrammatic tree depicting the organization of most eukaryotes into six major groups, showing their relationship with the Bacteria and Archaea is shown in Fig. 2 ([Simpson & Roger, 2004](#)).

Fig. 1. Schematic view of ribosomal DNA repeat unit (From <http://www.biology.duke.edu/fungi/mycolab/primers.htm>)



Primers for routine sequencing are shown in bold

Fig. 2. Diagrammatic representation of various groups of organisms in tree of life.



The small subunit (SSU) 18S rDNA sequences and ITS sequences are now widely applied to study the taxonomy and phylogeny of both prokaryotes and eukaryotes, both in the terrestrial and marine environment. SSU sequences have also been used to study the diversity of organisms in a given environment, without resorting to isolating or culturing them. While such studies have been widely used to analyse environmental prokaryotic diversity, it is only since about a decade that molecular tools have been used to study cultured and uncultured marine microbial eukaryotic diversity. These studies have shown that picoeukaryotes (less than 1 μm in size) are fundamental components of marine ecosystem ([Massana *et al.*, 2004](#); [Moreira & Lopez-Garcia, 2002](#)). Deep-sea sequences from up to (2000 m depth) have shown the presence of thraustochytrids and labyrinthulids in such samples ([Moreira & Lopez-Garcia, 2002](#)). [van Hannon *et al.* \(1999\)](#) described the eukaryotic diversity in a detritus-derived experimental system using molecular methods. rRNA-targeted eukaryotic specific probes can be used for fluorescent in situ hybridization to enumerate small eukaryotic cells in marine samples ([Lim *et al.*, 1996](#)). Ribosomal DNA sequences are increasingly used for inferring evolutionary relationships amongst non-cultivated protists too, e.g. Radizoa ([Moreira *et al.*, 2007](#)).

The importance of 18S r DNA sequences in understanding the taxonomy of thraustochytrids, aplanochytrids and labyrinthulids was highlighted by the work of [Honda *et al.* \(1999\)](#), who made a detailed study on numerous isolates of these organisms. These authors showed that the exclusive use of morphology and life cycle for classifying the Labyrinthulomycetes could be inadequate or even misleading. This study was also instrumental in revising the taxonomy of the thraustochytrid genus *Schizochytrium*, which was reorganized into 3 new genera ([Yokoyama & Honda, 2007](#)). [Bongiorni *et al.*, \(2005c\)](#) used the SSU sequences to describe a new species of *Thraustochytrium*. [Leander *et al.*, \(2004\)](#) made a detailed taxonomic and phylogenetic revision of the aplanochytrids based on the 18S rDNA sequences.

As with most microorganisms, molecular characterization has become an important tool for taxonomic identifications of thraustochytrids, in addition to conventional methods. Although the fourteen isolates from zooplankton of the equatorial Indian Ocean were characterized in terms of their morphology and life cycle, it was essential also to confirm these in terms of their 18S rDNA sequences. Since all of them corresponded to a single species, *Aplanochytrium yorkensis*, it was considered appropriate to characterize them further in terms of their ITS sequences, which shed light on intra-species

differences more effectively than the 18S sequences. In the light of the above, the following objectives were defined.

- (1) To sequence and analyze the 18S rRNA gene of the isolates obtained from zooplankton of equatorial Indian Ocean in order to classify them to species level.
- (2) To sequence and analyze the ITS rDNA of the isolates in order to understand their intra-species relationships.

3.2 Materials and Methods

3.2.1 Genomic DNA isolation: Isolation of whole genomic DNA from the isolates obtained from equatorial Indian Ocean was carried out by a modification of phenol-chloroform:isoamyl alcohol method ([Sambrook et al., 1989](#)). The cell pellets obtained after growing the isolates for 5-7 days in MV broth, were washed with sterile seawater and subjected to repeated freeze-thaw cycles using liquid nitrogen. After 3 freeze-thaw cycles, lysis buffer was added into the tubes followed by equal amounts of phenol and chloroform:isoamyl alcohol. Sterile glass beads of 150-350 μm size (Sigma) were added and vortexed till the entire contents of the tube turned viscous in nature. The tubes were then centrifuged at 12000 rpm for 15 mins, the supernatant collected and treated with chloroform:isoamyl alcohol and centrifuged at 12000 rpm for 3 mins. The supernatant was collected

and DNA was precipitated with 2.5 times volume of cold ethanol. The tubes were stored at -20° C for better precipitation. They were then centrifuged at 12000 rpm for 10 min under cold conditions. The pellets formed were 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 and checked for purity by electrophoresis using 0.8 % agarose gel. The DNA was stored at -20° C until further analysis.

3.2.2 DNA amplification: 18S rDNA regions of the isolates were amplified by polymerase chain reaction with primers designed by [Honda *et al.* \(1999\)](#). Two universal internal primers (NS3 and NS4) were used (Table 1). Amplification was 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. The reaction conditions were standardized for each primer pair [set I- 18S001 (fwd) & NS4 (rev) and set II- NS3 (fwd) & 18S13 (rev)] by carrying out gradient PCR in Peltier Thermal Cycler (MJ Research, PTC-200, S. No. AL083846). The temperature range checked for annealing of both primer sets was 55-65° C. The conditions were denaturation at 94° C/4 min, 30 cycles of denaturation 94° C/50s, annealing 59.5° C/50 s for set I and 62.5° C/50 s for set II and

extension 72° C/1 min, and final extension step of 72° C/10 min in BioRad Thermal Cycler (S. No. AL100938).

ITS rDNA regions of the isolates were amplified similarly using ITS1 and ITS4 primers (White *et al.*, 1990). Amplification was carried out with final concentrations of 7.5 pM primer each, 100 µM dNTP mix, 1X PCR buffer containing 1.5mM MgCl₂, and 1U Taq polymerase. The reaction conditions that were standardized for this primer pair by carrying out gradient PCR as mentioned above were 94° C/4 min, 30 cycles of 94° C/50 s, 57° C/45 s, 72° C/1 min, and final elongation step of 72° C/7 min.

Table 1. List of primers used for 18S and ITS rDNA amplification in the present study.

Primers	Type	Sequence
18S001	Forward	AACCTGGTTGATCCTGCCAGTA
18S13	Reverse	CCTTGTTACGACTTCACCTTCCTCT
NS3	Forward	GCAAGTCTGGTGCCAGCAGCC
NS4	Reverse	CTTCCGTCAATTCCTTTAAG
ITS1	Forward	TCCGTAGGTGAACCTGCGG
ITS4	Reverse	TCCTCCGCTTATTGATATGC

3.2.3 PCR product purification and sequencing: The amplified PCR products were checked by electrophoresis on 1.2 % agarose gel. The PCR products were purified using Montage PCR Clean Up Kit (Millipore) and sent for sequencing. The purified PCR products of the isolates for which sequencing failed were cloned using TOPO

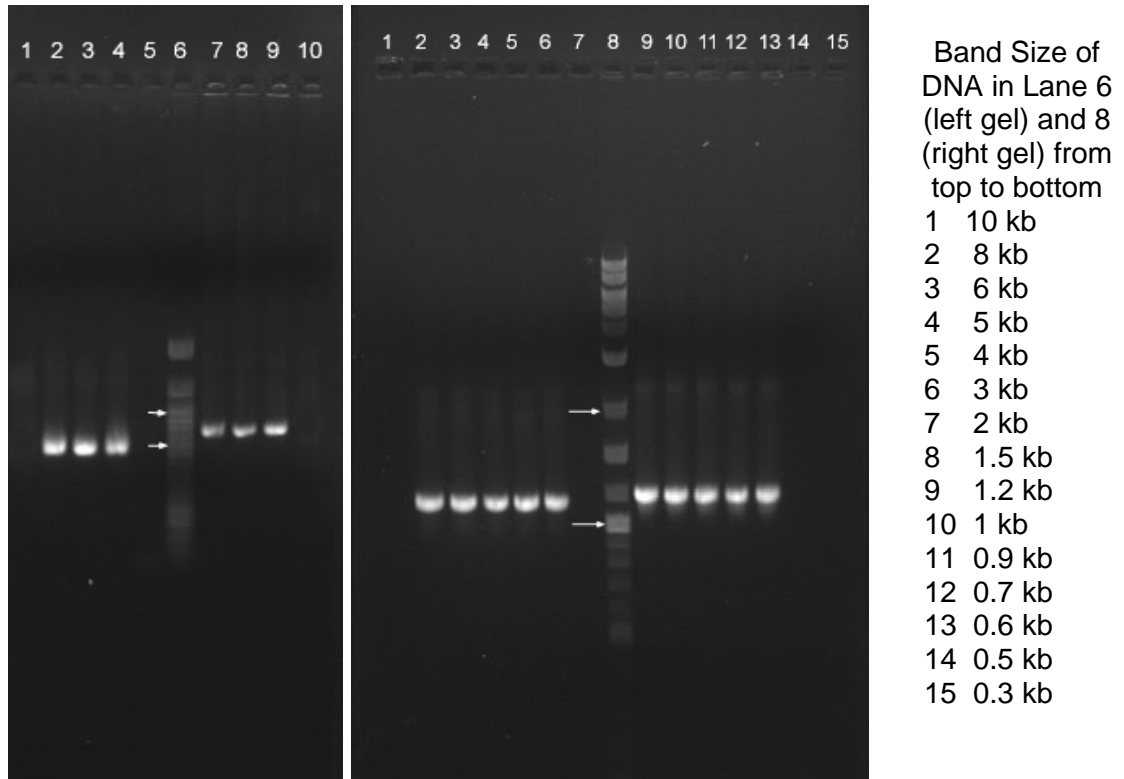
Cloning Kit (Invitrogen, USA) and the plasmid from the transformants was extracted by Alkaline Lysis method (Birnboim & Doly, 1979) and were sequenced by Microsynth AG (Switzerland) or Bangalore Genei (India).

3.2.4 Phylogenetic analysis: Phylogenetic trees based on the 18S rDNA and ITS sequences were generated using the Neighbour-Joining (NJ), Minimum-Evolution (ME) and the Maximum-Parsimony (MP) methods. The analyses were performed by using MEGA version 4. Sources of the other sequences, rooted to Stramenopiles were obtained from NCBI database. For phylogenetic trees based on ITS sequences, only those sequences generated from this study were used, since no ITS sequences for aplanochytrids are available.

3.3 Results

The 18S rDNA of 8 cultures and ITS rDNA of 7 cultures isolated from zooplankton of the equatorial Indian Ocean water column were sequenced. Figs. I and II show the amplified products of 18S rDNA and ITS rDNA respectively as visualized on agarose gel. Partial sequences of 18S rDNA and ITS rDNA were obtained. The 18S rDNA sequences ranged in size from 1353 to 1731 bp and that of the ITS region ranged from 447 to 572 bp.

Fig. I. Agarose gel electrophoresis of PCR –amplified products of 18S rDNA of 7 isolates of *Aplanochytrium yorkensis* with both the primer sets. (Upper arrow marks band of size 2 kb and lower of 1 kb).



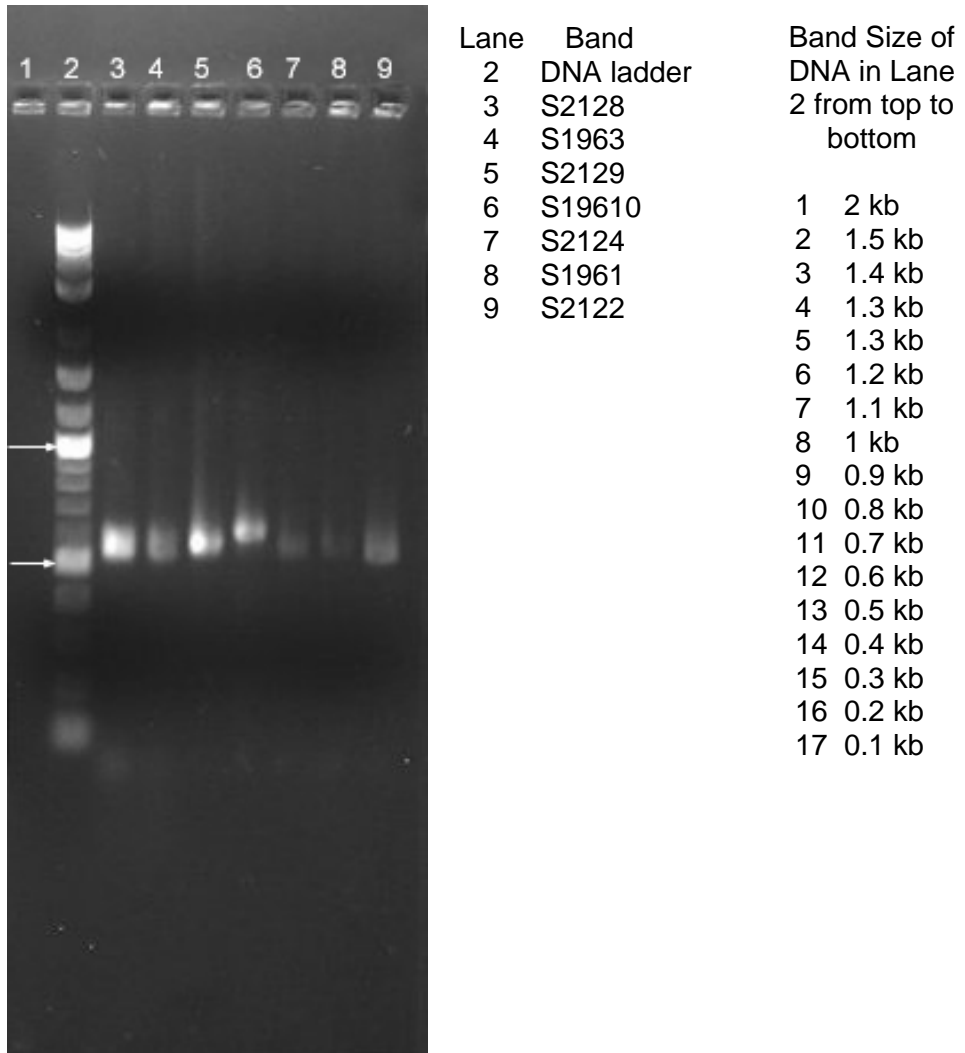
Band Size of DNA in Lane 6 (left gel) and 8 (right gel) from top to bottom

- 1 10 kb
- 2 8 kb
- 3 6 kb
- 4 5 kb
- 5 4 kb
- 6 3 kb
- 7 2 kb
- 8 1.5 kb
- 9 1.2 kb
- 10 1 kb
- 11 0.9 kb
- 12 0.7 kb
- 13 0.6 kb
- 14 0.5 kb
- 15 0.3 kb

Lane	Band
2	S1961 primer set I
3	S1962 primer set I
4	S19610 primer set I
5	Negative control
6	DNA ladder
7	S1961 primer set II
8	S1962 primer set II
9	S19610 primer set II
10	Negative control

Lane	Band
2	S2122 primer set I
3	S2124 primer set I
4	S2125 primer set I
5	S2128 primer set I
6	S2129 primer set I
7	Negative control
8	DNA ladder
9	S2122 primer set II
10	S2124 primer set II
11	S2125 primer set II
12	S2128 primer set II
13	S2129 primer set II
14	Negative control

Fig. II. Agarose gel electrophoresis of PCR-amplified products of ITS rDNA. (Upper arrow marks band of size 1 kb and lower of 0.5 kb)



BLAST analysis of the 18S rDNA sequences of the 8 isolates obtained from equatorial Indian Ocean showed 98 to 100 % similarity to *Aplanochytrium kerguelense* and *A. stocchinoi* (Table 2).

Table 2. Details of 18S rDNA sequences of 8 aplanochytrid isolates from the present study.

Isolate	Date	18S rDNA size	BLAST (% query coverage- % similarity)	Gene bank accession No.
S1961	Sep 2003	1682 bp	<i>A. stocchinoi</i> (99%) & <i>A. kerguelense</i> (100%)- 98%	EU851167
S1962	Sep 2003	1353 bp	<i>A. stocchinoi</i> (99%)- 98%	EU851174
S19610	Sep 2003	1675 bp	<i>A. stocchinoi</i> (99%) & <i>A. kerguelense</i> (100%)- 98%	EU851168
S2122	Oct 2004	1644 bp	<i>A. stocchinoi</i> & <i>A. kerguelense</i> (99%)- 98%	EU851172
S2124	Oct 2004	1683 bp	<i>A. stocchinoi</i> (99%) & <i>A. kerguelense</i> (100%)- 98%	EU851169
S2125	Oct 2004	1682 bp	<i>A. stocchinoi</i> (99%) & <i>A. kerguelense</i> (100%)- 98%	EU851170
S2128	Oct 2004	1731 bp	<i>A. stocchinoi</i> (99%)- 98% similarity & <i>A. kerguelense</i> (99%)- 97%	EU851173
S2129	Oct 2004	1681 bp	<i>A. stocchinoi</i> (99%) & <i>A. kerguelense</i> (100%)- 98%	EU851171

Phylogenetic analysis carried out using the three clustering algorithms, Neighbour-Joining, Minimum-Evolution and Maximum-Parsimony methods displayed similar topologies of the phenetic trees except for minor differences in the branch lengths (Figs. 3-5). Three distinct clades of the Labyrinthulomycetes were noticed. The first one comprised the labyrinthulid, *Labyrinthula* sp., which formed a separate clade with a high bootstrap value of 99 to 100 % from the rest of the Labyrinthulomycetes. The other members of the Labyrinthulomycetes formed two clades, the first comprising the two thraustochytrids, *Schizochytrium minutum* and *Thraustochytrium multirudimentale*, which formed a monophyletic group supported by comparatively high bootstrap value of 73 % in NJ tree, 88 % in ME tree and 64 % in MP tree, separated from the second clade comprising the aplanochytrids (Figs. 3-5). Within the *Aplanochytrium* clade, the Indian Ocean isolates and also *Aplanochytrium* DQ367045 formed a distinct clade separated from *A. kerguelensis*, with a bootstrap value of 62 % in NJ tree, 61 % in ME tree and 66 % in MP tree. The Indian Ocean isolates were themselves clustered in two distinct clades, those isolated during SK 212 cruise in October 2004 forming one clade (bootstrap value of 98, 97 and 78 % in NJ, ME and MP trees respectively), while those isolated during SK 196 cruise of September 2003 formed another distinct clade in all 3 trees (63, 72 and 56 % bootstrap values in NJ, ME and MP trees respectively).

ITS sequence data are not available for Labyrinthulomycetes in data banks, except for the labyrinthulid Quahog Parasite X. The ITS sequences of the 8 isolates (Appendix 1) showed 90 % homology to the Quahog Parasite X (Table 3). The ITS rDNA tree of seven isolates obtained from equatorial Indian Ocean is shown in Fig. 6. The topologies of the 3 trees were basically similar in that isolates S19610, S1963 and S 1961, isolated during September 2003 formed a separate clade. The branch length of each of these isolates was negligible. The closest relative of these was S2124, although the bootstrap length (100 %) distinctly separated this from the others. Except for the isolate S2124, the other isolates obtained during October 2004, i.e. S2122, S2128 and S2129 were clustered into one clade.

Table 3. Details of ITS rDNA sequences of 7 aplanochytrid isolates from the present study.

Isolate	Date	ITS rDNA size	BLAST (% query coverage- % similarity)	Gene bank accession No.
S1961	Sep 2003	556 bp	Labyrinthulid quahog parasite QPX isolate clone (28%)- 90% similarity	EU872090
S1963	Sep 2003	503 bp	Labyrinthulid quahog parasite QPX isolate clone (31%)- 90% similarity	EU872091
S19610	Sep 2003	503 bp	Labyrinthulid quahog parasite QPX isolate clone (31%)- 90% similarity	EU872092
S2122	Oct 2004	552 bp	<i>Cladosporium cladosporioides</i> (100%)- 98% similarity	-
S2124	Oct 2004	447 bp	<i>Hyaloperonospora tribulina</i> (24%) - 78% similarity	EU872093
S2128	Oct 2004	572 bp	Hypocreaceae clone (99%)- 98% similarity	-
S2129	Oct 2004	562 bp	Hypocreaceae clone (99%)- 98% similarity	-

Fig. 3. Neighbour-Joining (NJ) tree of 18S rDNA of aplanochytrids of the present study with *Prorocentrum micans* and *Oxytricha granulifera* as outgroups. (Key: AYC- *Aplanochytrium yorkensis* complex, AA- all aplanochytrids, AMC- *Aplanochytrium minuta* complex, T- thraustochytrids, L- labyrinthulids)

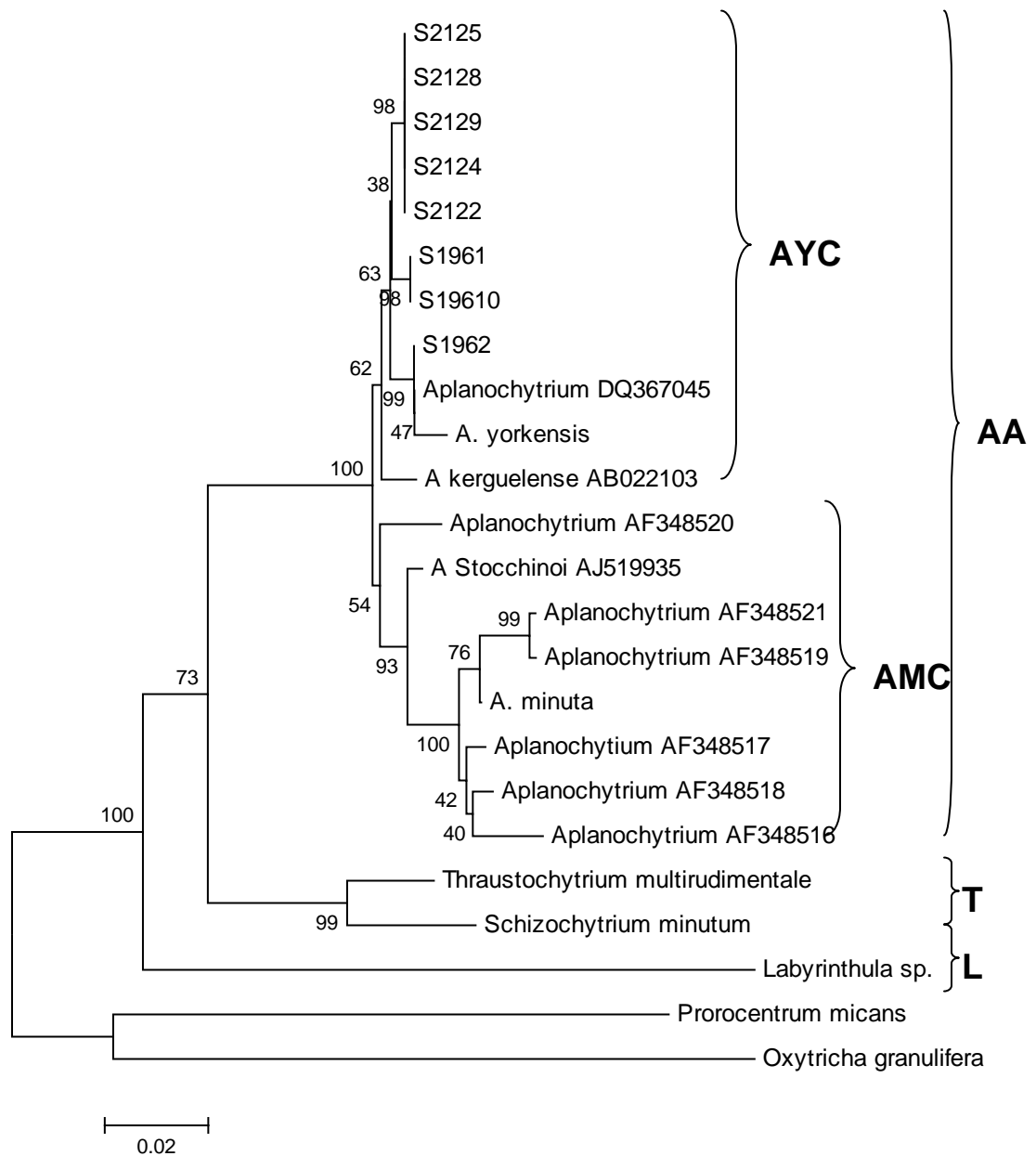


Fig. 4. Minimum-Evolution (ME) tree of 18S rDNA of aplanochytrids of the present study with *Prorocentrum micans* and *Oxytricha granulifera* as outgroups. (Key: AYC- *Aplanochytrium yorkensis* complex, AA- all aplanochytrids, AMC- *Aplanochytrium minuta* complex, T- thraustochytrids, L- labyrinthulids)

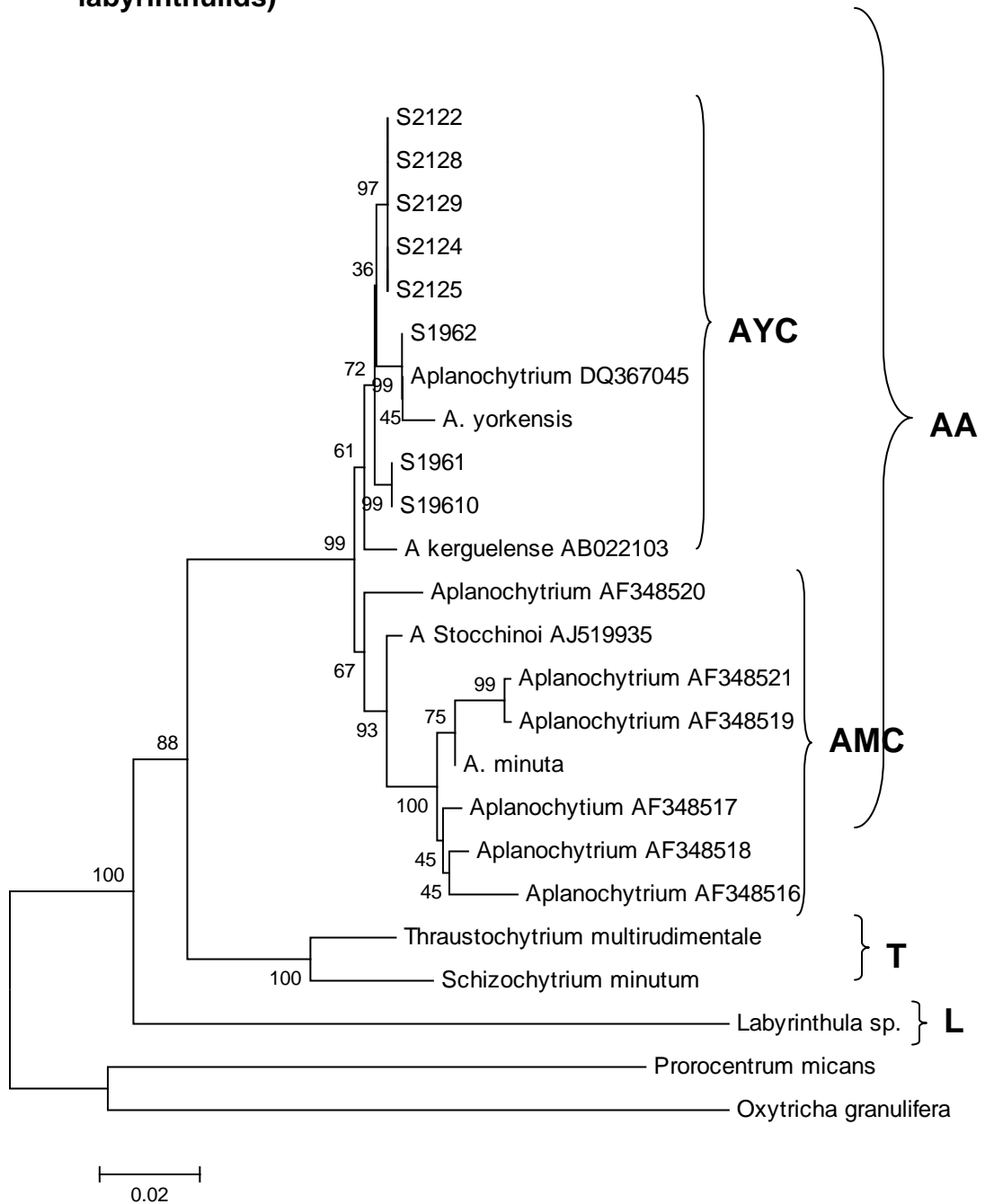


Fig. 5. Maximum-Parsimony (MP) tree of 18S rDNA of aplanochytrids of the present study with *Procentrum micans* and *Oxytricha granulifera* as outgroups. (Key: AYC- *Aplanochytrium yorkensis* complex, AA- all aplanochytrids, AMC- *Aplanochytrium minuta* complex, T- thraustochytrids, L- labyrinthulids)

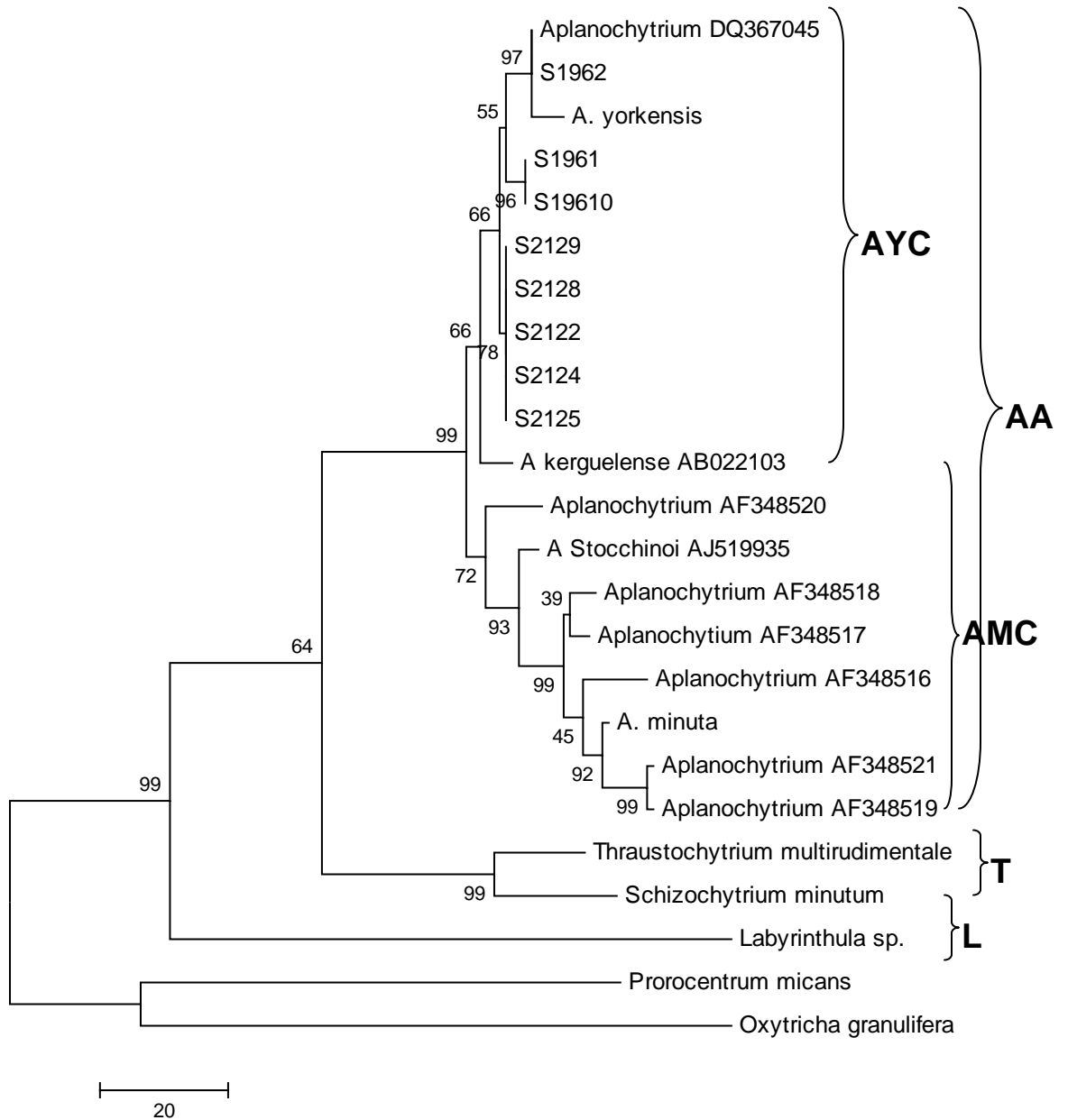
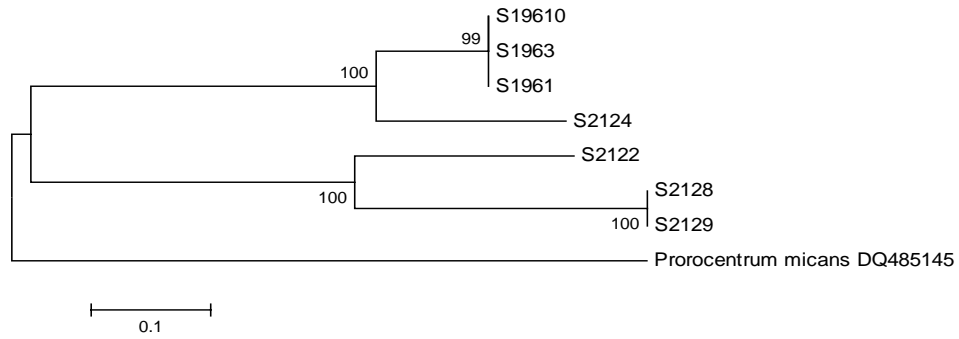
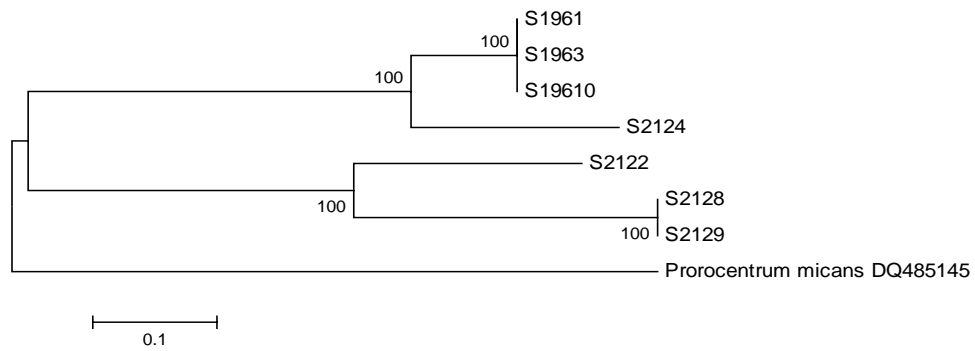


Fig. 6. Phylogenetic trees of ITS rDNA of aplanochytrids of the present study with *Prorocentrum micans* as outgroup.

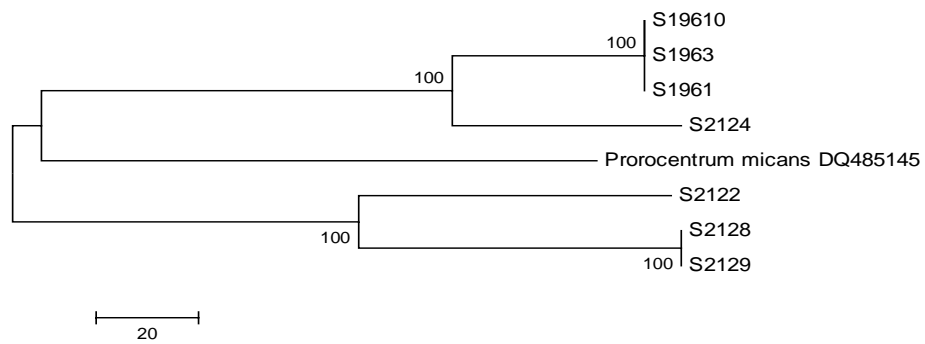
a- NJ tree



b- ME tree



c- MP tree



3.4 Discussion

Phylogenetic trees generated using the 18S rDNA sequences of the eight *Aplanochytrium* isolates, as well as other Labyrinthulomycetes confirmed the findings of [Honda *et al.* \(1999\)](#), [Leander & Porter \(2000\)](#) and [Bongiorni *et al.* \(2005c\)](#), that the Labyrinthulomycetes are comprised of three distinct phylogenetic groups, namely the thraustochytrids, aplanochytrids and labyrinthulids. The eight isolates of *Aplanochytrium yorkensis* of the present study, together with other species of *Aplanochytrium*, namely *A. kerguelensis*, *A. yorkensis*, *A. minuta*, *A. stocchinoi*, *A. schizochytrops*, *A. thaisii*, *A. saliens* and *A. haliotidis* formed a monophyletic group distinct from thraustochytrids and labyrinthulids.

Based on their morphological as well as molecular studies, [Leander *et al.* \(2004\)](#) divided the aplanochytrids into 2 groups or complexes. They found that *A. yorkensis* and *A. kerguelensis* were consistently placed together forming a clade and named it as '*Aplanochytrium yorkensis* complex'. The second clade, the '*Aplanochytrium minuta* complex' consists of the other 6 species. The eight isolates from zooplankton of the equatorial Indian Ocean fall under the '*Aplanochytrium yorkensis*'. Based on the morphology and life cycle (Chapter 2), as well as their 18S rDNA sequences, it is clear that the eight isolates in the present study belonged to the '*Aplanochytrium yorkensis*' complex.

In the 18S phylogenetic trees generated in the present study, *A. kerguelensis* (the *A. yorkensis* complex) was the earliest diverging aplanochytrid followed by the isolates of the present study, together with *A. yorkensis* and *Aplanochytrium* Dq367045. [Leander et al. \(2004\)](#) also concluded that *A. kerguelensis* diverged before the rest of the aplanochytrids. Although BLAST showed that the present isolates had a high degree of homology to *A. stochhinoi*, it is clear from the 18S phylogenetic trees that they were closer to *A. yorkensis*. The genus *Aplanochytrium* is characterized by the gliding of vegetative cells via ectoplasmic net (EN) and also the formation of motile spores that too glide using the EN. The several taxonomic problems for this genus have been pointed out by [Leander et al. \(2004\)](#).

The 8 isolates of the present study were closest to 'Aplanochytrium Dq367045', *Aplanochytrium yorkensis* and *A. kerguelensis* in all three phylogenetic trees and could be placed in the *A. yorkensis* complex based on their 18S rDNA sequences (Figs. 3-5). All the 8 isolates obtained in the present study formed a clade distinct from *A.kerguelensis*, with a bootstrap value of 63, 61 and 66 % in NJ, ME and MP trees respectively. These results suggest that the present isolates might constitute a new taxon, even if their life cycle and morphology corresponded to those of *A. yorkensis*. The studies of [Honda et al. \(1999\)](#) have shown that conventional morphological characteristics may not be sufficient to define

species of thraustochytrids. [Yokoyama & Honda \(2007\)](#) recently emended the genus *Schizochytrium* that was based on morphology and life cycle and instead divided it into three different genera, *Schizochytrium* sensu stricto, *Aurantiochytrium* and *Oblongichytrium* gen. nov. based on carotenoid and fatty acid profiles. It is likely that morphology and life cycle are also not sufficient to classify aplanochytrids. It will be interesting to examine the fatty acid and carotenoid profiles of isolates from equatorial Indian Ocean and other aplanochytrids to understand their taxonomy better. No detailed work on these aspects has been carried out so far.

The eight isolates from the Indian Ocean formed a separate clade both from *Aplanochytrium kerguelensis* and *A. yorkensis*. However, these isolates themselves were distinctly separated into those isolated during September 2003 (cruise # SK 196; isolates S1961, S19610, S1962) and those isolated during October 2004 (cruise # SK 212; isolates S2122, S2124, S2125, S2128 and S2129). The two groups showed a significant bootstrap value of 99 % in the 18S sequences. Trees based on ITS sequences confirmed the distinctiveness of the two groups, except for isolate S2124. Thus the two groups showed a 100 % bootstrap value using these sequences (Fig. 6). The ITS sequences clearly showed that the Indian Ocean isolates from the two seasons differed significantly, but not among themselves. This leads to the interesting question as to

whether genetically distinct populations appear in association with zooplankton during different seasons.

3.5 Conclusions: Phylogenetic trees based on the 18S rDNA sequences confirmed morphological observations that the eight isolates of the present study, obtained from zooplankton of the equatorial Indian Ocean were closest to *Aplanochytrium yorkensis*. The eight isolates could be classified as belonging to the '*Aplanochytrium yorkensis* complex' that comprises *A. yorkensis* and *A. kerguelensis* and distinct from the thraustochytrid and labyrinthulid complexes. Among the eight isolates, those obtained from each of the two cruises clustered together, indicating that distinct populations of the aplanochytrids could be present at different times. Among these five isolates from October 2004 cruise formed a distinct clade from the rest of the '*A. yorkensis* complex', thus raising the possibility of being new species. This grouping of the eight isolates was also confirmed by trees based on ITS sequences.