

## **Chapter 7.**

# **Summary and Conclusions**

Thraustochytrids, aplanochytrids and labyrinthulids belong to the Class Labyrinthulomycetes of the Kingdom Straminipila or Chromista, which also includes diatoms, brown algae, oomycetan fungi and a variety of photosynthetic and non-photosynthetic flagellates. The Labyrinthulomycetes are heterotrophic and obligately marine. They have been found to be ubiquitous in the marine environment, occurring in a variety of habitats in both coastal and oceanic environments. A few recent studies have found thraustochytrids to be an important component of the microbial biomass in the water column. They have also been shown to be associated with a number of invertebrates in both parasitic and symbiotic modes. Owing to the production of omega-3 polyunsaturated fatty acids they also have wide ecological implications and biotechnological applications. The Labyrinthulomycetes are emerging to be an important group in the marine food web dynamics. Yet, their role in the marine environment has been poorly elucidated. They have been widely studied in coastal environment but little is known about their abundance and distribution in the oceanic environment.

The four major objectives of the present study were:

1. To isolate thraustochytrids and aplanochytrids from zooplankton in oceanic waters, to characterize them with respect to their life cycle in

order to identify them and to group them based on their carbon and nitrogen nutrition and production of extracellular enzymes.

2. To study the abundance of thraustochytrids and bacteria in oceanic water column to understand their relations.
3. To carry out molecular characterization of isolates obtained from zooplankton based on their 18S ribosomal RNA gene and ITS sequences
4. To design molecular probes for a few isolates to enable their detection in natural samples.

Most of this work was based on samples collected during three cruises on board ORV Sagar Kanya to the equatorial Indian Ocean during September 2003, October 2004, and September 2006. The following points summarize the work and conclusions.

1) Mesozooplankton collected from the equatorial Indian Ocean using a Multiple Plankton Net during the first two cruises were plated on culture media. A total of 171 specimens yielded colonies belonging to the Labyrinthulomycetes. Out of these, 14 isolates were purified and maintained for further studies. All isolates corresponded to the aplanochytrid *Aplanochytrium yorkensis* (Perkins) Leander and Porter based on morphology and life cycle. Colony morphology of all other cultures from mesozooplankton also resembled those of the species. The fourteen isolates showed numerous variations in terms of colony

morphology, life cycle and preferences to organic carbon and amino acids. Among the morphological characters, important variations include the colony margin, penetration of agar and production of amoebae. Only one isolate preferred glucose as the carbon source, all others utilizing pentoses and disaccharides more efficiently. All grew well on glutamate and proline. All isolates produced only protease among the 4 extracellular enzymes (protease, lipase, chitinase and amylase) tested. No thraustochytrids were isolated from zooplankton.

Members of thraustochytrids have often been reported from a variety of invertebrates in coastal environments. However, no studies have been carried out on their presence in zooplankton. *This study reports isolation and characterization of Labyrinthulomycetes from zooplankton for the first time.*

**2)** Molecular studies using 18S rDNA sequences of eight of the isolates confirmed morphological and life cycle observations that they belonged to the *Aplanochytrium yorkensis* group. The overall phylogenetic tree placed these isolates in the aplanochytrid group of the Labyrinthomycetes, distinct from thraustochytrids and labyrinthulids. Within this, they further corresponded to the *A. yorkensis* complex as suggested by [Leander et al. \(2004\)](#), being closest to that species.

*The present observations confirmed the predominance of aplanochytrids in association with mesozooplankton, which might have*

*been the result of a definite association of this group of organisms with mesozooplankton. It is also likely that aplanochytrids are the most important members of Labyrinthulomycetes in oceanic waters. Yet another possibility is the presence of uncultured thraustochytrids in the samples.*

**3)** Cluster analyses of the isolates based on morphological and physiological characters did not indicate any consistent grouping and did not provide weightage to any of the characters.

*Although cluster analysis suggested that *Aplanochytrium yorkensis* in zooplankton of the equatorial Indian Ocean exists as populations with a high variability and mixing, phylogenetic trees based on 18S as well as ITS rRNA gene sequences grouped the isolates of the October 2004 cruise distinctly as a separate clade within the *A. yorkensis* complex, suggesting that these probably represented a new taxon. Use of other taxonomic characters, such as fatty acid and carotenoid profiles as has been recently used for thraustochytrids will help in the elucidation of the taxonomy of aplanochytrids. Different populations of *A. yorkensis* or different species of the *A. yorkensis* complex might be associated with mesozooplankton in different seasons or locations.*

**4)** In order to shed light on the association of aplanochytrids with zooplankton, attempts were made to locate them on or within zooplankton

using molecular probes. Based on ITS sequences of 5 isolates obtained in the present study, four probes consisting of one forward and one reverse primer each for two isolates were designed. The oligonucleotide probes varied in length from 19 to 20 nucleotides. These probes were cross-checked for their effectiveness with the particular isolates by PCR, southern hybridization and *in situ* hybridization (ISH). The latter two were carried out with biotinylated probes, using streptavidin-conjugated alkaline phosphatase and colour detection using a chromogenic substrate for the enzyme, followed by bright field microscopy. Lack of cross-reactions with other isolates was confirmed using the same techniques. Cells of aplanochytrids fed to copepods could be detected using ISH. Aggregates collected from the water column of the equatorial Indian Ocean revealed cells of the aplanochytrid upon ISH. Likewise, the gut portions of chaetognaths reacted positively to ISH carried out. Cells resembling aplanochytrids were also detected within the tissues of chaetognaths using ISH. Copepod samples from the equatorial Indian Ocean water column reacted positively to ISH using the probes. However, no definite cells were detected in these and the reaction was diffuse, being present on various parts of the body of the copepods. A similar reaction was also often noticed with chaetognaths.

*The present study confirms the presence of aplanochytrids in marine aggregates of the water column. It further suggests that aplanochytrids were consumed by zooplankton and probably were also*

*present within their body tissues, as has been suspected in the case of tunicates and other coral reef invertebrates. Further studies are required to confirm these findings.*

5) An insight into the role of thraustochytrids and aplanochytrids in the oceanic water column can be gained by studying their abundance and distribution, as well as their relation to bacteria. Enumeration based on epifluorescence microscopy using the acriflavine direct detection method showed that they were present from below detection levels to as high as  $766.6 \times 10^3$  cells L<sup>-1</sup> at times, comparable to values obtained from studies in the Arabian Sea and the Japan Sea. They often showed distinct distribution profiles in the water column, generally being higher in the mixed layer than below it. However, their numbers were also often substantial below 200 m of the water column. Their contribution of carbon to total microbial (thraustochytrid + bacterial) biomass was very high (99.4 %, 15.8 µg C L<sup>-1</sup>) at certain locations. Thraustochytrids were positively related to bacteria during October 2004, but not during the other cruises.

Thraustochytrids were patchier in their distribution compared to bacteria, probably because they were more particle- or aggregate-dependent for their growth than the latter. Thraustochytrids grow well on particulate organic matter by virtue of their ectoplasmic net elements, which help them to attach to such substrates and obtain nutrition from them through the production of various degradative enzymes.

Transparent Extracellular Particles (TEPs) form the matrix of aggregates. Therefore, the relationship between thraustochytrids, bacteria and TEPs in the water column was studied during two cruises, in October 2004 and September 2006. Thraustochytrids displayed a significantly positive correlation with TEPs in the water column at some, but not at all locations during October 2004. Their numbers often peaked about 20 m above peak TEPs concentration. This might be due to the release of DOC from the sinking particles that left a plume supporting growth of thraustochytrids.

*The present study has shown that thraustochytrids and aplanochytrids are common inhabitants of the water column of oceanic waters, as they are also in coastal waters. Their high densities, occasionally corresponding to biomass values more than that of bacteria indicates that they might play an important role in the water column. The varying relations between bacteria and thraustochytrids suggests that the factors that govern the growth and populations of the two groups may vary. Likewise, the varying relations of thraustochytrids to TEPs might have been caused by the varying chemistry of TEPs, formed from different processes.*

**6)** Experiments using roller table bottles to generate TEPs from natural seawater were carried out to understand the reasons behind the variability in relationships between thraustochytrids and TEPS. Various

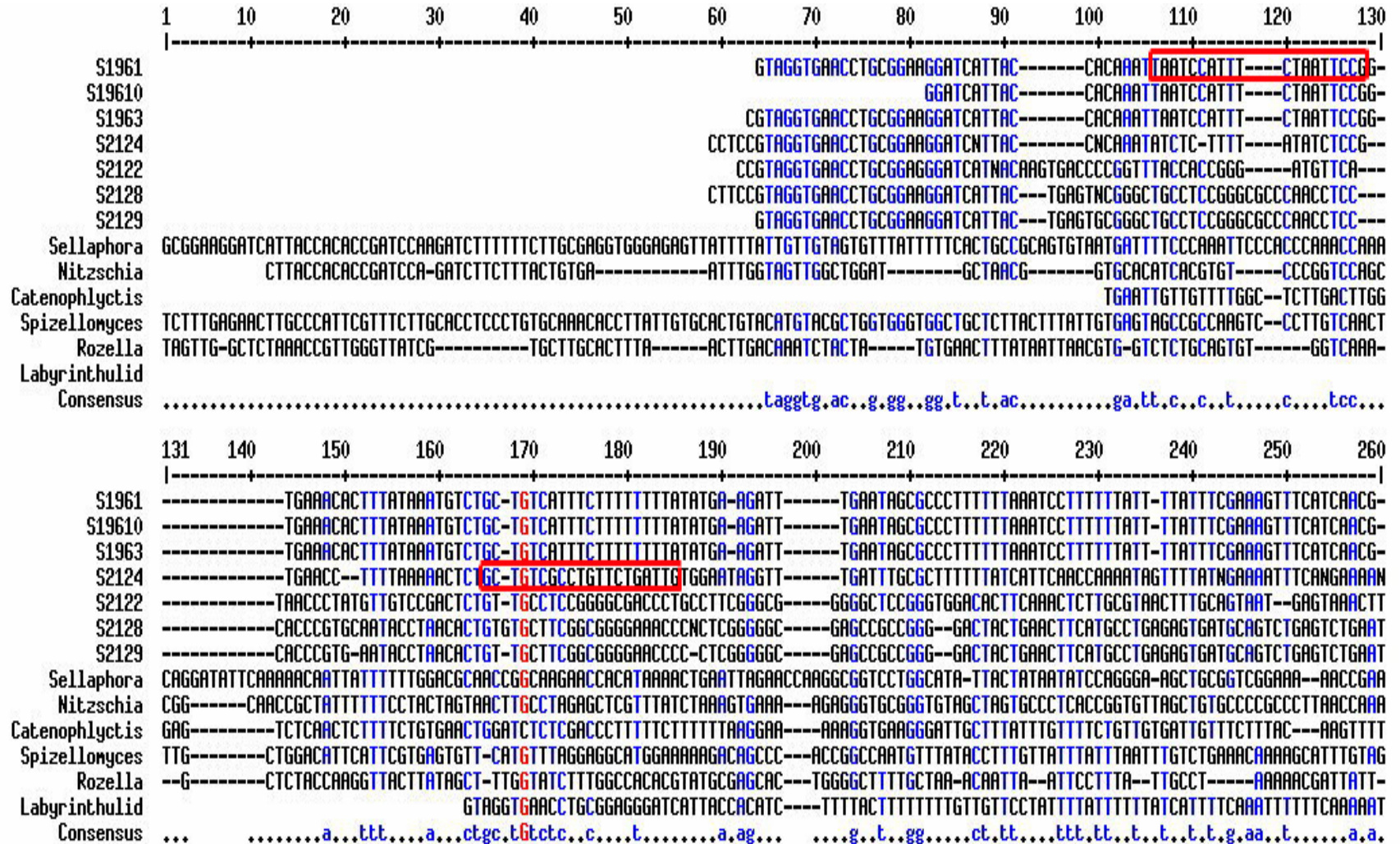


experiments were carried out using oceanic and coastal waters filtered through 200 or 20  $\mu\text{m}$ , such that the waters contained phytoplankton, microzooplankton, thraustochytrids and bacteria, or in the presence of the above consortia, but without phytoplankton. The carbohydrate chemistry of the TEPs was assessed using three different lectins.

*Thraustochytrids appeared to be inhibited in the presence of phytoplankton. Thraustochytrid populations increased when phytoplankton were excluded, or when only diatom polysaccharides were introduced in seawater. These studies again showed varying relationships between thraustochytrids, bacteria and TEPs. Thraustochytrids and bacteria did not appear to compete against each other in most cases. Aggregate formation seemed to be enhanced in water samples supplemented with antibiotics and favoured growth of not only thraustochytrids but also fungi.*

# Appendix

Appendix 1- Multiple alignment file of ITS sequences of 7 aplanochytrid isolates of the present study and 6 other organisms belonging to Kingdom Straminipila. The regions of probe design are marked as red boxes.





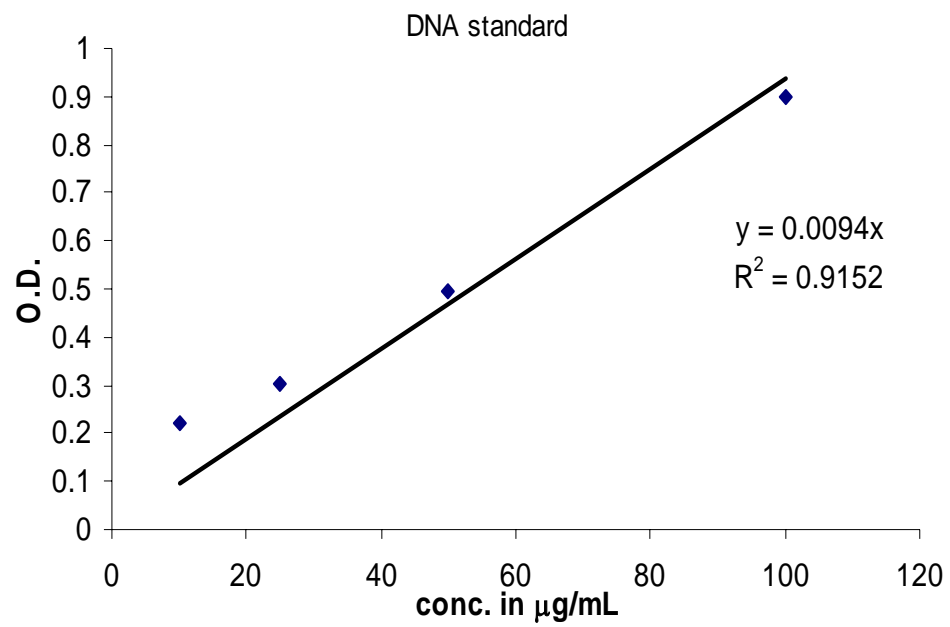
	261	270	280	290	300	310	320	330	340	350	360	370	380	390
	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----													
S1961	---AACATTGAATAACTTTTAGCGATGGATGCTAGGTTCTCGCAACGATGAAGAACGCAGCGAATGCGATACGTAATGCGAATTGCAGAA-TCCGTGAATCATCAAAATTTTGACGCACATTGCAC													
S19610	---AACATTGAATAACTTTTAGCGATGGATGCTAGGTTCTCGCAACGATGAAGAACGCAGCGAATGCGATACGTAATGCGAATTGCAGAA-TCCGTGAATCATCAAAATTTTGACGCACATTGCAC													
S1963	---AACATTGAATAACTTTTAGCGATGGATGCTAGGTTCTCGCAACGATGAAGAACGCAGCGAATGCGATACGTAATGCGAATTGCAGAA-TCCGTGAATCATCAAAATTTTGACGCACATTGCAC													
S2124	GTAAATTTTTTATAACTTTTANCGANGGANGTCTAGGTTCTCGCAACGATGAANAACNCANCGAANGCGATCCGTAAGGCGAATNGCAGAA-NCCGNGAANCATCAAAATTTTGACGCACATTGCAC													
S2122	AATTAATAAATAAACTTTTACACAGGATCTCTGGTTCTGGCATCGATGAAGAACGCAGCGAATGCGATAGTAATGTGAATTGCAGAATTCAGTGAATCATCGATCTTTGACGCACATTGCAC													
S2128	ATAAATCAGTCAAACTTTTACCAATGGATCTCTGGTTCCGGCATCGATGAAGAACGCAGCGAATGCGATAGTAATGTGAATTGCAGAATTCAGTGAATCATCGATCTTTGACGCACATTGCAC													
S2129	ATAAATCAGTCAAACTTTTACCAATGGATCTCTGGTTCCGGCATCGATGAAGAACGCAGCGAATGCGATAGTAATGTGAATTGCAGAATTCAGTGAATCATCGATCTTTGACGCACATTGCAC													
Sellaphora	---ATTGTAATACAACTTTTACGCGAGGATGCTAGGTTCCCAACGATGAAGAACGCAGCGAATGCGATACGTAATGCGAATTGCAGAACTTCGTGAATCATCAAGATTTTGACGCACATTGCAC													
Nitzschia	CCTAATTGACAAACAACTTTTACGCGGATGGATGCTAGGTTCCCAACGATGAAGAACGCAGCGAATGCGATACGTAATGCGAATTGCAGAACCTTCGTGAATCATCAAAATTTTGACGCACATTGCAC													
Catenophlyctis	CTTTAATGAAGACAACTTTTACCAATGGATCTCTGGTTCTGGCATCGATGAAGAACGCAGCGAATGCGATAGTAATGTGAATTGCAGATCC--GTGACTCATCGAATTTTGACGCACATTGCAC													
Spizellomyces	TAATATACAAAACAACTTTTGGCAACGGATCTCTGGTTCTCGCAACGATGAAGAACGCAGCGAATGCGATAGTAATGTGAATTGCAGAATTCAGTGAATCATCAATCTTTGACGCACATTGCAC													
Rozella	TATAATCACAAACAACTTTTACCAATGGATCTCTGGTTCCGCAACGATGAAGAACGCAGCGAATGCGATAGTAATGTGAATTGCAGATTCAGTGAATCATCGATCTTTGACGCATATTGCAC													
Labyrinthulid	TTAAACATTTTTATGACTTTTAGCGATGGATGCTAGGTTCTCGCAACGATGAAGAACGCAGCGAATGCGATACGTAATGCGAATTGCAGAATTCAGTGAATCATCAATCTTTGACGCACATTGCAC													
Consensus	...aA...a.a.A.aACTTTTAgCgAtGGATgTCTaGgtTctcgCAcGATGAAGAACGCAGCGAAaTGCGATAcGTAATGcGAATTGCAGaa,tccGTGAATCATCaAaacTTTGACGCACATTGCAC													
	391	400	410	420	430	440	450	460	470	480	490	500	510	520
	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----													
S1961	TTCTGGTTC--CCGGG--AAGTATTTCTGTTTGAGTGTCTGTCTCTCATCT---CACTGAATTTCAAGTGGATTTGGGTTTTATGTATGT--GTGCGCGCAGTGGCATGTTGACCTAAACCTGTTTG													
S19610	TTCTGGTTC--CCGGG--AAGTATTTCTGTTTGAGTGTCTGTCTCTCATCT---CACTGAATTTCAAGTGGATTTGGGTTTTATGTATGT--GTGCGCGCAGTGGCATGTTGACCTAAACCTGTTTG													
S1963	TTCTGGTTC--CCGGG--AAGTATTTCTGTTTGAGTGTCTGTCTCTCATCT---CACTGAATTTCAAGTGGATTTGGGTTTTATGTATGT--GTGCGCGCAGTGGCATGTTGACCTAAACCTGTTTG													
S2124	TTCTGGTTNACCGAGCAAGTATTTCTGTTTGAGTGTCTGTCTCCCTCT---CACTGAATTTCAAGTGGACTTTGGGTTTT---CAAT--ATGTACGCAAGT---ATGTATACCTGAAAAAAGTT													
S2122	CCCCGTTATCCG--GGGGCATGCCTGTTCGAGCGCATTTCA--CCACNACAGCCTCGCTTG--GATTTGGCAGCAGCGGTCGGCCGGTGCCTCAATC--GACCGGNTGGGCTTC----TGTC--													
S2128	CCCCGTTATCCG--GGGGCATGCCTGTTCGAGCGCATTTCA--CCACNACAGCCTCGCTTG--GATTTGGCAGCAGCGGTCGGCCGGTGCCTCAATC--GACCGGNTGGGCTTC----TGTC--													
S2129	CCCCGTTATCCG--GGGGCATGCCTGTTCGAGCGCATTTCA--CCACNACAGCCTCGCTTG--GATTTGGCAGCAGCGGTCGGCCGGTGCCTCAATC--GACCGGNTGGGCTTC----TGTC--													
Sellaphora	TTCCGGGATTTTCCCGGAGCATGCTTGTCTGAGTGTCTGTGAACCC---C-A-CTCGGCGTTACGATGC---GTGACA--GCCGGAATAAAATATACTT--TGGCGTGCACACTACTAAACTAGT													
Nitzschia	TTTCGGGTTTTACCGGTAGCATGCTTGTCTGAGTGTCTGTGAACCC---C-A-CTCAGCGTCCGCTGCTCCCTTGTGGTGTACCGGTTGCTGAGCTTTGCTAGTGTGAGTTGCGTTATGCGAACCTCTG													
Catenophlyctis	TTGGTGGTATCCGCCA--AGTATGCTTGTTTGAGTGTCTGTGACTCTCTC--T-CTCTCAACTTTTTTTTTTTTTTTTTTNGCTTCGGTGAAGAAAGAAAGAAAAAAGT--TGTGGAGGATTTT													
Spizellomyces	TTCTGGTATCCG--GGGAGTATGCCGTTTCAGAACTATTAATCTCA--A-ATAAATTTGTTGTTGGATATGATAGTCTGGAGTTTCGGCCTCAGTC--TAG--TTGAAAT--GATTGTGCAAGC-													
Rozella	TTCTGGTATCCG--GGGAGTATGCTGTTTGAGTATCATATCTCTCTCA--CTATACTTGTA--TAGAAGAGGACTTGAAGCTCATTCTTACCAGGAGCTGCTTTAAT--TATGGGGCGTGA													
Labyrinthulid	TTTCGGGTGATCC--GGGAGCATTCCTGTTTGAGTGTGTTGTTT--TCTCACTTGTCTTTATA--GAGCAGCTTGTGGGCTGTTTCTGT--TTCTCTTTTGGGAATAGGTGGCCTTGAGATAGTAT													
Consensus	ttctcGGt,ttcCg,G,aGtATgccTGTtGAGtGTctgTtctctC.c.c.a.Ctctgc,T,t.,gttg...ttggg,tt...cg...gt.....cgc.agtgg,.tg.....ct.....t.t.													



	521	530	540	550	560	570	580	590	600	610	620	630	640	650
	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----													
S1961	GTATGCTAAGCCTCACA AAAAC TTTGGATAATCTACTTTTTTCAAGAAA AAGGTG CAGGTG TTTTACCGCATAAA AACACACAT TACAATCA-GACCTCAATCAGGA AAGAAGACCCCTGAATTTAA													
S19610	GTATGCTAAGCCTCACA AAAAC TTTGGATAATCTACTTTTTTCAAGAAA AAGGTG CAGGTG TTTTACCGCATAAA AACACACAT TACAATCA-GACCTCAATCAGGAAA													
S1963	GTATGCTAAGCCTCACA AAAAC TTTGGATAATCTACTTTTTTCAAGAAA AAGGTG CAGGTG TTTTACCGCATAAA AACACACAT TACAAT													
S2124	ACAAGCTAACGATCATATCAA-TTTGGATAATTT													
S2122	-----CCT-AAGCGTTGTGGAA--CTATT--CGCTAAGGGTGACTA--CGGGAGAGCTACGCCGTAAAACAA CCCCAT--TTCTA-AGGTTGACCTCGGATCAGGTAGGGATACCCGCTGA ACTTAA													
S2128	-----CCTNGAGCGTATGGGGCT--TTGTCACCCGCTCGACTAGGGCCGGCCGGGCAGCCGACGTCTC-CAACCATTT--TTCTTCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGA ACTTAA													
S2129	-----CCTCGAGCGTATGGGGCT--TTGTCACCCGCTCGACTAGGGCCGGCCGGGCAGCCGACGTCTC-CAACCATTT--TTCTTCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGA ACTTAA													
Sellaphora	G---TGCTTGGTCGAATGCACG---TTAT-GGCGGACGCACTTGCATCA--TTGGCGCAGCCGTCCTCTGTAGAGAAATACTATTTTTTCAAGGAGAGTACA-----CAGTCGTCGCATA													
Nitzschia	GCTTAATTTGACCCACTGGGTT---TGCTAGGAGGCCGTTGGGATTCA CCCC GGTTG AAGGGCTACGTCACTAGCTCC-TCGTCTATCTCTGGTAGCAGTGTTCGGCCTTGTGCCGCTATTATTGG													
Catenophlyctis	GGTCTCTTTGCGCCAGCGA ACTGACTTTTGAGTATTTGCGGCATGCTTTT---TTTACTGAAGGGTTTACTGGCCTTGAAGTCTTAAAGC--GCAGCCTGGATTG--TTTGATGGCGCCTTGTGCCTG													
Spizellomyces	-----CGGCCCA--AGAGTT-----CGGTAGC---TTC--AACTGCAGCAGAT-TTATCTATTCTTT---TGAAGTTCCTTTGT---AACTATTGGACTGTGTTGCTGCCT-----CAA													
Rozella	GCTTAACACTGCC TAGTAGCTTACAGGCTTACCAGTGC---TAA CAAACAGCGTTTGCCTTATCATGCCATTCA GTGTGGTAGTAAAGC---AGGC GTTGATGGTAA TGTAGAGTTGACGAACGG													
Labyrinthulid	TTCACAAATATCTAGCAAT TATTAACTTTTTACAACATCAACCTCAATCAGGA AAGAGGACCCGCTGA ACTTAAGCATAT													
Consensus	g...c...g..cca...gaa.t...t.t.....t.g.....c.a...g....a...g.....tc.c.taac.....T...a.a....ga.....gg....g.....c.....													
	651	660	670	680	690	700	710	720	730	740	750	760	768	
	-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----+-----													
S1961	GCATATCAATAAGCGG													
S19610	GCATATCAATAAGCGG													
S1963	GCATATCAATAAGCGG													
S2124	GCATATCAATAAGCGG													
S2122	GCATATCAATAAGCGGAGG													
S2128	GCATATCAATAAGCGGAGG													
S2129	GCATATCAATAAGCGGA													
Sellaphora	AGTGCTCG--CAATTGTTGC---ATC--CTTTTTCAATTCCTGATCTCAGATCAAGC AAGAAGACCCGCCGAATTTAAG---CATATCAATAAGCGGAGGA													
Nitzschia	AGTTGCGGGACAGATGGGAGCTGAACGGCTAGACTGAAGCCAGGTGTGAGTTTGTARCGACTTCCCGAGCTAGTTGGTCTCAGACCCGCTAGTTGTTGATTCTGTTCCGGTCTCAG													
Catenophlyctis	AAAAATTAATTCACGTTGTGTCCTTTGGCTTTGAGGAGATTTGGTTTACTTGTGACGTTGAGCAAGTCGGATTGTTTTTGCCTAACAGCAAAAACACACTTTTTTCCATACT													
Spizellomyces	CTAGTACACAGTGTGTTTGTTCGAGAAGACA-AGCCTCT													
Rozella	TTAGCAAGCTGTCCTTTGGCTTTTAAAGTCAGAGTATGATTAAG													
Labyrinthulid	TTAGCAAGCTGTCCTTTGGCTTTTAAAGTCAGAGTATGATTAAG													
Consensus	..a.....g..g.....													

## Appendix 2. DNA Standard

DNA (HiMedia, Cat. RM 511) was dissolved in sterile distilled water to get the desired concentration and the absorbance was read at 260 nm.



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