

# Molecular investigation of protozoan diversity in streams

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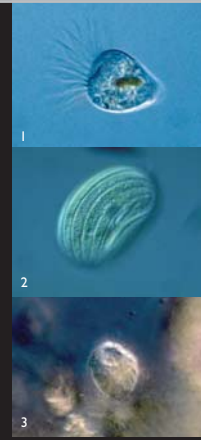


## What are protozoa, and why are they important?

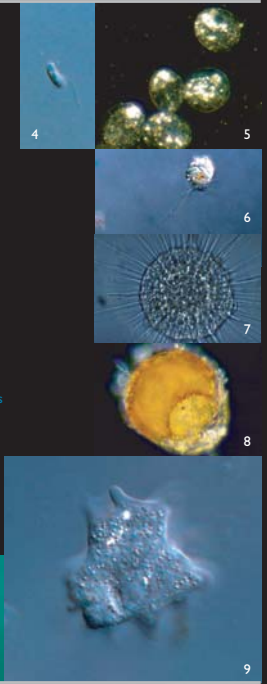
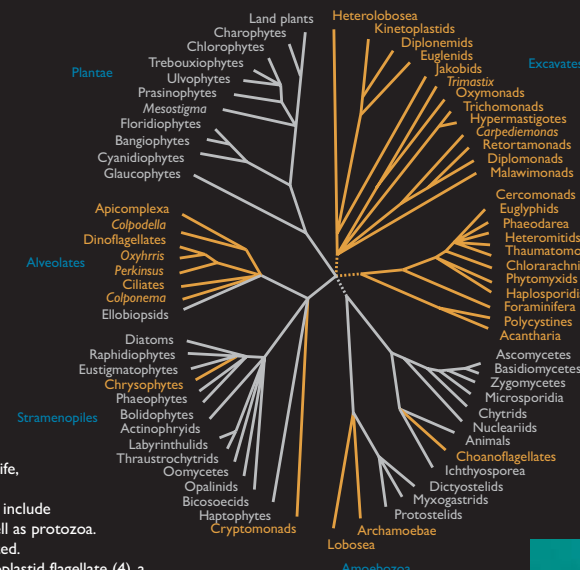
The term "protozoa" describes single-celled eukaryotic organisms with animal-like traits of motility and heterotrophy. The diversity of protozoa is enormous and fascinating; they are found virtually anywhere that liquid water is present<sup>(1,2)</sup>.

Protozoa are thought to be of major ecological importance<sup>(2)</sup> - together with photosynthetic protists, they are considered vital to "sustaining all life on planet Earth"<sup>(3)</sup>. Major predators of bacteria, they may control bacterial populations; they provide nutrition for other, larger protozoa and animals; they are integral components of nutrient and mineral transfer pathways, and are involved in decomposition of leaf litter - a vital resource in streams.

Understanding of protozoan ecology is very limited. This can be partly attributed to methodological difficulties - traditional methods for studying protozoa are based on microscopy, staining, fixing and culturing - methods which are difficult, time-consuming and often unreliable.



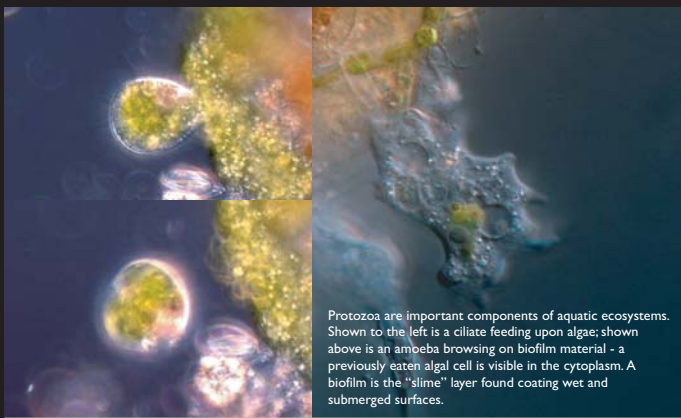
An evolutionary tree of eukaryotic life, based on DNA, biochemical and morphological evidence. Eukaryotes include animals, plants, fungi and algae, as well as protozoa. Main protozoan groups are highlighted. Illustrated are ciliates (1-3), a Kinetoplastid flagellate (4), a Euglenid flagellate (5), a Rhizarian testate amoeba (6) and heliozoan (7), testate (8) and naked Lobosean amoebae (9), and a Cryptomonad flagellate (10). Tree modified after Keeling et al. (2005).



## Research objectives

This research aims to test the following hypothesis: that molecular biological methods will allow description of protozoan diversity and ecology in streams.

Recent studies using polymerase chain reaction (PCR) to selectively amplify and analyse DNA have begun to illuminate micro-eukaryote biodiversity in different environments<sup>(3)</sup>. A single prior study has specifically measured diversity of only one of the many protozoan groups<sup>(4)</sup>. Understanding of protozoan diversity is necessary for effective management and restoration of stream environments.



Protozoa are important components of aquatic ecosystems. Shown to the left is a ciliate feeding upon algae; shown above is an amoeba browsing on biofilm material - a previously eaten algal cell is visible in the cytoplasm. A biofilm is the "slime" layer found coating wet and submerged surfaces.

## Methods

### The methodological challenge - design of protozoa-specific primers:

Because protozoa do not share a common evolutionary history, protozoa-specific PCR primers must separately target monophyletic groups within the protozoa. The ciliates form such a monophyletic group, are abundant, extremely diverse and perform important ecological functions, and were therefore targeted for analysis in this study.

### 1. Isolation and culturing

Biofilm samples collected from Auckland streams

Micromanipulation used to isolate single protozoan cells, to be grown into pure cultures

Cultures provide material for testing of DNA extraction and molecular methods

### 2. Molecular analysis

DNA extracted from stream biofilm samples

Eukaryote-specific PCR primers from literature used to amplify 18S ribosomal DNA sequences

PCR products cloned into bacterial vectors for analysis

Sequence identity determined by RFLP analysis and comparison with sequences in databases

### 3. Primer design and testing

Multiple alignment of 18S DNA sequences created

Sites conserved in ciliates but unconserved in non-target species identified and used as basis for primer design

Primers tested in PCRs on cultured protozoa and environmental DNA extracts

PCR products cloned and analysed by RFLP digestion and sequence analysis

### 4. Environmental diversity analysis

Best-performing primers used to amplify protozoan DNA from different streams

Effectiveness of restriction enzymes assessed by in-silico RFLP digestion

PCR products subject to RFLP digestion, profiles compared between streams

T-RFLP analysis to be used to compare ciliate diversity profiles between streams

## Results

### 1. Isolation and culturing

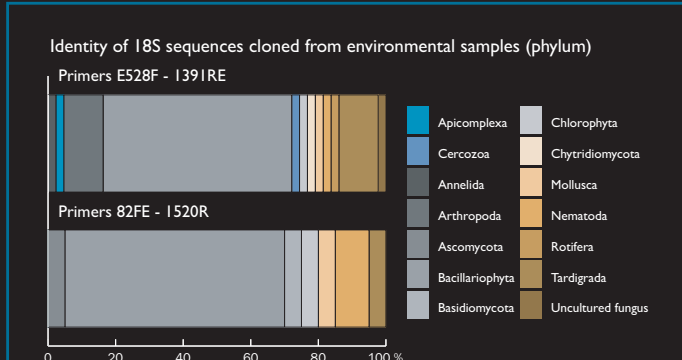
Pure cultures of representative protozoa have been established (illustrated below). DNA extraction and analysis methods have been tested on these isolates. Boiling of cultures was found to effectively release DNA to act as PCR template.



### 2. Molecular analysis

#### Can eukaryote-specific primers be used to detect protozoan diversity?

Eukaryote-specific primer combinations E528F-1391RE and 82FE-1520R failed to work well on cultured protozoa, and were ineffective for analysis of protozoan diversity in environmental samples:

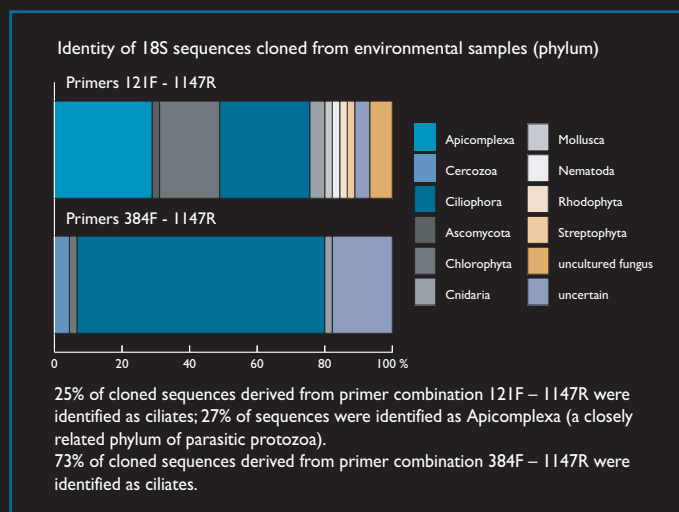


Only 4% of sequences detected by primer combination E528F - 1391RE were identified as protozoa (shown in blue). Primer combination 82FE - 1520R failed to detect any protozoan sequences.

### 3. Primer design and testing

#### Can ciliate-specific primers be designed?

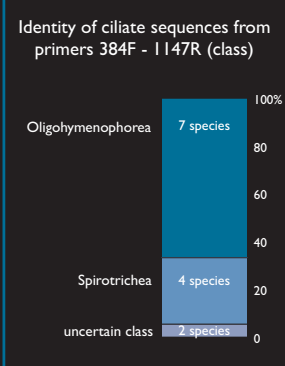
Six new PCR primers were designed to target the 18S rRNA gene of ciliates. All of these successfully amplified DNA from cultured ciliates, but not flagellates or amoebae. The two most reliable primer combinations were tested on environmental DNA extracts. Of these, primer combination 384F - 1147R was most ciliate-specific:



25% of cloned sequences derived from primer combination 121F - 1147R were identified as ciliates; 27% of sequences were identified as Apicomplexa (a closely related phylum of parasitic protozoa). 73% of cloned sequences derived from primer combination 384F - 1147R were identified as ciliates.

#### How much ciliate diversity can these primers detect?

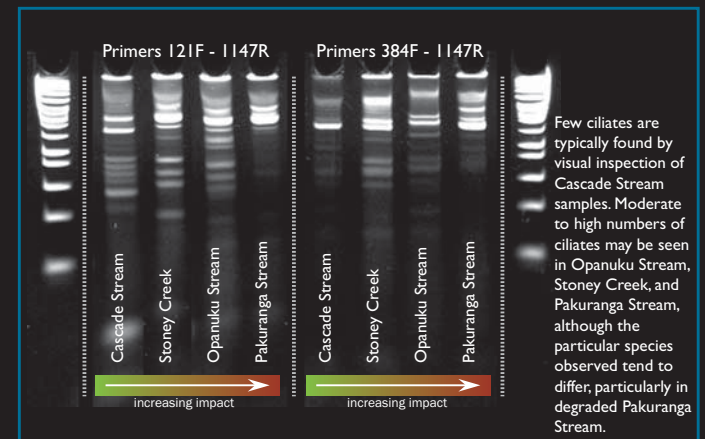
The phylum Ciliophora is divided into 11 classes. Sequences from classes Oligohymenophorea and Spirotrichea were detected in environmental samples. These primers have also been shown to amplify sequences from cultured Phyllopharyngean ciliates. Further work is needed to determine if these primers will detect sequences from the remaining classes of Ciliophora.



### 4. Environmental diversity analysis

#### Can differences in diversity be detected between different stream environments?

RFLP analysis of DNA amplified using primers 121F - 1147R and 384F - 1147R from streams in varying states of anthropogenic impact suggests differences in DNA sequence composition, and therefore protozoan diversity, between streams.



Few ciliates are typically found by visual inspection of Cascade Stream samples. Moderate to high numbers of ciliates may be seen in Opanuku Stream, Stoney Creek, and Pakuranga Stream, although the particular species observed tend to differ, particularly in degraded Pakuranga Stream.

## Discussion and conclusions

The molecular analysis used here shows PCR can be used to detect protozoa in streams, but existing eukaryote-specific PCR primers are inefficient for this purpose. New primers have therefore been designed to target the ciliates; while all of these have been used successfully on cultured ciliates, primer combination 384F - 1147R appears to be most effective, being most specific to ciliates.

RFLP analysis of PCR products from different stream environments suggests these primers can detect differences in diversity. The nature of between-stream differences will be further investigated using fluorescently labelled 384F and 1147R primers to carry out a T-RFLP analysis of ciliate diversity.

More complete molecular methods for protozoan ecology requires development of further PCR primers specific to other protozoan groups such as amoebae and flagellates; this can be achieved by following the same process described in this research.

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## References

1. Keeling et al., 2005; The tree of eukaryotes; TREE 20, 670-676. 2. Finlay & Esteban, 1998; Freshwater protozoa: biodiversity and ecological function; Biodiversity and Conservation 7, 1163-1186. 3. Corliss, 2004; Why the world needs protists; Journal of Eukaryotic Microbiology 51, 8-22. 4. Stoeck et al., 2006; A multiple PCR-primer approach to access the microeukaryotic diversity in environmental samples; Protist 157, 31-43. 5. Bass & Cavalier-Smith, 2004; Phylum-specific environmental DNA analysis reveals remarkably high global biodiversity of Cercozoa (Protozoa); International Journal of Systematics and Evolutionary Microbiology 54, 2393-2404.