A sensitive genetic-based detection capability for *Didymosphenia geminata*

Interim Report

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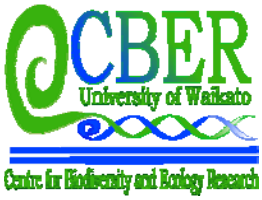

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Executive Summary

It is now well recognized that the increase in global transportation over the last two decades has brought with it an increased potential for the introduction of unwanted microorganisms (aquatic or terrestrial) that may have drastic effects on human and ecosystem health and agriculture. Until recently, surveillance for possible incursions and for post-incursion response has been extremely difficult due to microscopic size of the organisms and their ability to propagate asexually and spread through multiple vectors. Often, we know very little about the biology of the organism in question, requiring an extensive initial effort to gather the needed information to initiate an effective incursion response. Recently, highly sensitive molecular genetic tools, borrowed from the biomedical arena, have been adapted to meet the rising international biosecurity needs for cost-effective surveillance and monitoring. These techniques can be developed to detect and enumerate multiple target organisms with extreme sensitivity, specificity and high-throughput capacity.

Ongoing surveillance to monitor the distribution of *Didymosphenia geminata* (Lyngbe) Schmidt in New Zealand is a key component of Biosecurity New Zealand's incursion response. The present method for identifying didymo in waterbodies involves the collection of algal scrapings and drift net-enriched water samples, their transport to a laboratory and identification of didymo frustules within a subsample examined under a microscope. This is a time consuming, resource intensive and a subjective method that restricts our ability to sample a wide range of rivers and sites within each river. A highly sensitive and specific technique could dramatically increase our ability to analyse large numbers of samples in their entirety in a short period of time and to detect the alga at low levels, thus increasing the efficiency of our surveillance programme without greatly increasing costs. In addition, a low-level detection capability would enable better targeting of public awareness messages, enable geater confidence in the didymo status of high value sites requiring protection, and increase the probability of successful control, should a control tool be developed.

The Centre for Biodiversity and Ecology Research, University of Waikato, was commissioned by Biosecurity New Zealand to develop a sensitive molecular-based protocol capable of detecting *D. geminata* in environmental samples with extreme sensitivity. The specific requirements for this new methodology were that it has:

- Robust field capabilities from collection to quantification
- Species or strain level specificity that has been environmentally validated
- Extreme sensitivity for low-level detection
- A broad dynamic range of detection (> 5 orders of magnitude)
- The highest degree of reproducibility
- Efficient, cost-effective, rapid, high-throughput laboratory capability.

The research programme is divided into three discrete phases of six months each:

Phase 1 (reported here) -
a) design and test field collection and DNA extraction methods, and
b) identify a gene target to discriminate *D. geminata* and to develop a highly specific gene amplification detection protocol.

Phase 2 - take the amplification method to a higher resolution quantitative capability.

Phase 3 - adapt the protocols to a field-based high-throughput capacity and train end-users in their use and application.

This document reports on Phase 1 and preliminary Phase 2 results of the research programme.

Phase 1 - Field collection and DNA extraction methods

- The field collection and preservation procedures have been extremely effective at sampling *D. geminata* for genetic work using a variety to techniques including direct collection of *D. geminata* colonies for verification, rock swab samples where colonies were not visible and drift net samples.
- A new drift net was developed and tested specifically to target the collection of *D. geminata* cells. Initial tests showed that the net design was extremely effective at enriching for *D. geminata.* The net design has recently been adopted by the on-going *D. geminata* Delimiting Survey Programme.
- We successfully sampled *D. geminata* from at least three locations in all 11 known infected rivers (as of June 30/06) for type samples needed for the validation experiments.
- High quality DNA was extracted from all environmental samples and stabilised with two DNA preservatives. Subsequent tests of DNA stability of samples held in each of the DNA preservatives for extended periods of time indicated that the extraction buffer CTAB and ethanol preservatives were very effective over a number of weeks in the absence of freezing. The longevity of each sample is likely to be greater than five weeks, given that DNA can be successfully extracted for up to five weeks.

Phase 1 – Identification of gene target to discriminate *D. geminata*

- A unique genetic fingerprinting tool for *D. geminata* was developed and validated. The first gene to be examined as a signature for *D. geminata* was the gene encoding the small subunit ribosomal RNA (18S rRNA). The whole 18S rDNA gene was bi-directionally sequenced for *D. geminata* along with another New Zealand *Gomphoneis* sp., and compared to other closely related biraphid diatoms. All sequences were edited and aligned to produce a consensus sequence, which was used for subsequent phylogenetic analysis. It was determined that the 18S rRNA had the needed sequence variability to clearly distinguish *D. geminata* from all other known biraphid diatoms.
- Seven gene amplification (polymerase chain reaction – PCR) primers specific for *D. geminata* were designed and tested against pure *D. geminata* target and environmental samples known to contain *D. geminata.* The primer sets were triaged based on robustness and specificity to amplify their designated target. Two primer sets (long and short) were continued into intensive blind test validation. In all cases where an amplification occurred, the PCR product was then sequenced to confirm the specificity of the primers. A positive amplification was obtained from every sample known to contain *D. geminata*. No false positives were generated in any of the samples that did not contain *D. geminata*. Subsequent sequencing verified that environmental samples of *D. geminata* had amplified the correct sequence.
- Swab samples taken from rocks were very effective at detecting *D. geminata* in affected rivers on apparently bare substrate with minimum sample effort. Preliminary sensitivity tests revealed that the short primer set could detect as little as one pg of target DNA. This will be greater improved by at least 2-3 orders of magnitude when the procedure is taken to the quantitative level in Phase 2, which is currently underway.
- Primers were successfully tested on *D. geminata* samples collected from three foreign countries (USA, Canada, and UK) and found to be equally specific and sensitive.
- A global phylogeographic study of *D. geminata* to ascertain the origin(s) of the New Zealand strains is underway.

In summary, we have developed and validated a unique genetic fingerprinting tool for *D. geminata*. In concert, we developed field collection and preservation techniques specific for *D. geminata* along with genetic-based procedures that can now reliably detect *D. geminata* from a complex environmental community with a high degree of sensitivity. Recent work (Phase 2) has shown that the described methods will provide detection levels from <1 – 10,000 cells ml^{-1}. We contend that the genetic based detection approaches used in this study offer great promise to meet the increasing demands to monitor the global threat from invasive micro-organisms.

Introduction

The global increase in distribution and occurrence of harmful algal bloom (HAB) species (marine and freshwater) has become a matter of growing economic and ecological concern because of their possible negative impact on human and aquatic life. For the purposes of this report, we define HAB species as any alga that causes a decline in human or ecosystem health. Conservative estimates of $250 million globally each year are lost to commercial fisheries and tourism due to algal blooms while substantially less than this is spent on monitoring, which is often selective and inadequate (CENR, 2000). Too often, invasion has taken place and the invasive organisms have already established themselves by the time they are visually detectable, decreasing the likelihood and increasing the cost of successful mitigation strategies. Monitoring efforts have been hampered by a lack of tools for accurate and sensitive detection of harmful algae. Conventional microscopic methods for identifying and estimating abundance of HAB species are time-consuming, often require years of training, and are only effective when the target species is at relatively high concentrations and clearly distinguishable from the indigenous flora and fauna. Even then, many organisms cannot be positively identified to the species level and must be subjected to the examination of ultra-structure, demanding time-consuming preparation and costly high-powered microscopy. Immunological methods may be more specific, but are difficult to develop, can cross-react with native species, and are unreliable for background-level detection that is often critical to identification of invasive HAB species.

Developments in molecular genetic technology have revolutionised our approach to basic environmental microbial research. These new methods can provide high-resolution analysis of microbial species at specific taxonomic levels, while considerably decreasing time for analysis. Recent pioneering work in the Cary Lab at the University of Waikato and the University of Delaware, USA, has led the development of sensitive and quantitative molecular techniques for detection and enumeration of nine different marine and freshwater HAB species from environmental samples. The techniques involve using gene amplification technologies (polymerase chain reaction or PCR) that target specific diagnostic genes in the organism of interest. Recent developments now allow these amplification reactions to be quantified by quantitative real time PCR (QRT-PCR) allowing the concentrations of target gene (or cell number) within a sample to be calculated. These high-throughput methods provide greater sensitivity and specificity of detection and enumeration of HAB species, and are ideally suited for assisting routine monitoring in the field.

D. geminata – a pervasive opportunist

Algae attached to the substrate are a normal and crucial part of stream and river ecosystems because they provide a food source for grazing invertebrates, which in turn support higher trophic levels such as fish. However, under specific conditions of light, temperature, stream flow, and nutrient availability, they can proliferate to HABs where they cannot be controlled by grazers and degrade habitat for fish and other in-stream uses (Biggs 2000). *D. geminata* is a stalked diatom that may form persistent blooms of dense mucilaginous mats that can extend for several kilometres. Large blooms are associated with fluctuations in dissolved oxygen levels and pH and shifts in benthic invertebrate community composition which could significantly impact higher trophic levels, including fish (Larned, et al., 2006). *D. geminata* was noted in the 1800s in the boreal and mountain regions of Europe, and somewhat later in Great Britain, western Canada (Vancouver Island), China and Turkey and within the past 20 years, appears to be occurring more frequently in North American and Europe (Kilroy, 2004). In NZ, *D. geminata* was first reported in October 2004 on the South Island. It has since expanded its range from the lower Waiau and Mararoa Rivers to the upper

Waiau, Buller, Hawea, upper Clutha, Von, and Oreti Rivers in 2005, and subsequently to the Ahuriri, Aparima, and lower Waitaki rivers by June 2006 (*www.biosecurity.govt.nz/didymo*). Its potential for further spread within the South Island and spread to the North Island is serious.

Early detection of *D. geminata* in new locations in NZ could be confounded by the existence of morphologically similar gomphonemoid taxa (*Gomphoneis* and *Gomphonema* species; Biggs & Kilroy 2000), and potentially *Cymbella* species, that may coexist with *D. geminata*. *Gomphoneis* and *Gomphonema* are stalked diatoms that form a light-brown, soft, epilithic turf in gravel-bedded, clear-water streams and rivers that also support high-quality trout fisheries. These apparently indigenous taxa grow in the oligotrophic and mesotrophic conditions in which *D. geminata* also seems to thrive. Unambiguous morphological identification and discrimination of these species is technically demanding. Although *D. geminata* frustules are reportedly larger than those of the indigenous taxa (Kilroy 2004), diatoms are well known for reducing cell size with each successive division so that size might not always be a reliable diagnostic character. In addition, the sheer scale of sampling river-bed habitat that is suitable for invasion defeats the reliable early detection of new incursions of *D. geminata*.

Modern molecular genetic methods offer the potential for unambiguous identification, enumeration, and early detection of *D. geminata*. It also offers the potential to distinguish *D. geminata* from related species that are native to New Zealand. However, any identification system (genetic or morphological) must be well validated in the laboratory and field to avoid false positives caused by the indigenous genera. Prior to this study nothing was documented about the phylogenetics of the New Zealand stalked diatoms, nor about their culture in the laboratory. The molecular similarity of co-occurring native gomphonemoid diatoms had to be established in concert with *D. geminata* in order to develop a truly discriminating genetic assay.

Project objectives

Our objective was to develop a sensitive molecular-based detection method for *D. geminata* that can be used as the basis of an early detection protocol before new infestations are visible. Early detection offers the best chance of control or eradication of new incursions of the organism. We also sought to demonstrate and refine a model for developing highly sensitive, accurate diagnostics for any microorganisms of concern to Biosecurity New Zealand.

Our specific requirements for this new methodology were that it has:

- Robust field capabilities from cell collection to gene quantification
- Species or strain level specificity that has been environmentally validated
- Extreme sensitivity for low-level detection
- A broad dynamic range (> 5 orders of magnitude)
- The highest degree of reproducibility
- Efficient, cost-effective, rapid, high throughput laboratory capabilities

The overall method development programme is broken into three phases. Phase 1 is to develop methods for efficient sample collection, preservation and DNA extraction, and to design and implement a highly specific and sensitive PCR-based detection capability for *D. geminata*. Phase 2 is to design and validate a laboratory-based, high throughput QRT-PCR detection and enumeration assay for *D. geminata,* and to examine the phylogeography of D. geminata. Phase 3 is to design a compatible, long-term sampling and monitoring capability,

and adapt protocols for field-based instrumentation with continued testing, validation, and transfer of the technology to Biosecurity New Zealand.

This interim report will focus solely on the accomplishments made during Phase 1 of the project.

Phase 1

1. Develop protocols for collection and stabilisation of environmental samples of *D. geminata*
2. Develop protocols for the extracting DNA from *D. geminata*
3. Design and validate primers for the detection of *D. geminata.*

Future reports are planned to report results of Phase 2 and 3.

For those readers not fluent in molecular technology we have included a primer on gene amplification (PCR) methodologies and briefs on the primary constraints of applying these methods to environmental samples in Appendix 1.

Methods

1. Protocols for collection and stabilization of environmental samples of D. geminata

Check, Clean, Dry decontamination procedures developed for freshwater users by Biosecurity New Zealand's Didymo Incursion Response Programme (see *www.biosecurity.govt.nz/didymo*) were followed during field sampling to prevent the spread of *D. geminata*. Detailed field sampling protocols for genetic-based detection will be provided for the training program as part of the Phase 3 deliverable due in 2007. These protocols will include a stronger decontamination solution (at least 5% household bleach) used for a longer soaking time (at least fifteen minutes). This will ensure that in addition to killing all the *D. geminata* cells on reusable gear, all the DNA molecules will be fully denatured and thus unable to yield false positives through PCR amplification.

1.1. Water and benthic sampling

The purpose of this protocol was to test sampling procedures in combination with two common, low-cost preservatives that have proved successful in the past on environmental samples. These methods also needed to be complimentary to the molecular methods to be used later. We evaluated 70% ethanol and CTAB (cetyl trimethyl ammonium bromide extraction buffer) (Appendix 2) for their appropriateness as preservatives for the stabilisation of *D. geminata* DNA in environmental samples. On collection, algal samples were preserved in either 70% ethanol or in CTAB at 1X concentration. Immediately prior to use, 0.4% (w/v) 2-mercaptoethanol was added to the CTAB. In each case, a sample of no more than 1/10 the total volume was immersed in the preservative.

A. Benthic sampling. Individual colonies, or a clipping from the top of a dense mat, were plucked or cut from the surface of a rock with forceps or scissors and placed in sterile 14-mL Falcon tubes or 1.5 mL Eppendorf tubes. Swab samples were taken in some affected areas by wiping a cotton bud over wet surfaces of bare rock (Figure 1). To test the efficacy of swab sampling, it was essential that no colonies of *D. geminata* could be visually identified. After wiping the rock surface, the wetted swab was then placed in preservative in a sterile Eppendorf tube, the end of the swab was cut off, and the lid was sealed tightly. All tubes were sealed using Parafilm to ensure there was no spillage during transport. Once back in the laboratory, all tubes were kept at –20^{o}C until DNA extraction.

Figure 1. Collecting *D. geminata* by swabbing a bare rock from the lower Waitaki River.

B. Water column sampling. To sample the water column, we designed a "nested" plankton net specifically for recovery of suspended *D. geminata* (Figure 2). The nested design includes a pre-filter (250 µm) to collected larger unwanted debris. We used three nested 200-mm diameter plankton nets suspended in the water column of the Mararoa River at a site (SH 94 Bridge, MA3 Drift Net) that was badly affected with *D. geminata*. The three nested nets were made from 500, 250, and 47 µm Nitex mesh, with the coarsest mesh acting as a primary strainer, and the 250 µm mesh net acting as a secondary strainer and the main 47 µm net to capture the 100 x 30 µm *D. geminata* cells. We set the net in a water velocity of 0.30 m/s for 10 minutes (Figure 3). As the inside diameter of the net was 190 mm, the cross-sectional area of the water column being sampled was 0.009025 m^2.

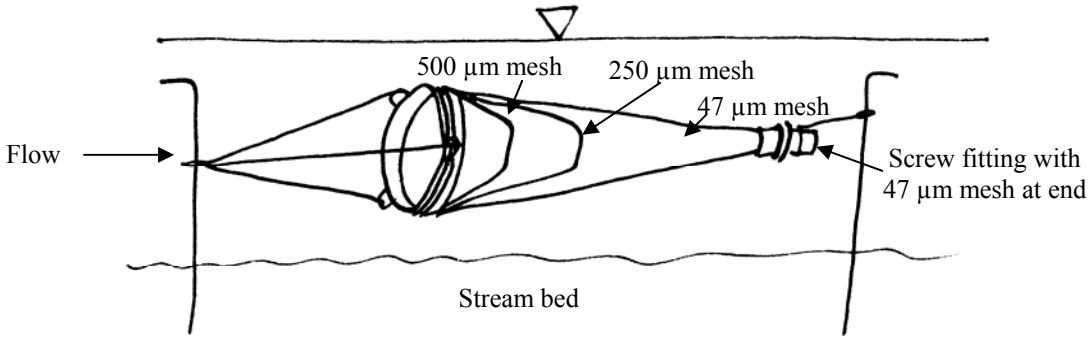

Figure 2. The design of a nested plankton 190-mm diameter drift net for sampling algal drift in the water column of streams and rivers.

Figure 3. The nested plankton net deployed in the Mararoa River.

1.2 Determine field collection thresholds and longevity

To address issues associated with sample stability, an experiment was carried out that investigated preservation of cells for extended periods of time. The same experiment addressed issues with DNA extraction efficiency over time. Methods for these experiments are addressed fully in Methods Section 2.2 below.

1.3. Extensive sampling of all known *D. geminata*-affected areas

From 9-12 May 2006, we sampled all 11 rivers (Buller, Ahuriri, Waitaki, Hawea, Clutha, Upper Waiau, Lower Waiau, Mararoa, Von, Oreti, and the Aparima) known to contain *D. geminata (www.biosecurity.govt.nz/didymo)*. Where possible, three sites were sampled to cover the affected sites of each river. At each site, a 2-5 g wet-weight sample of a colony was put into ethanol, and into CTAB. In addition, the surface of a submerged rock without visible colonies was swabbed with a dry, sterile cotton bud and the tip was cut into an Eppendorf tube filled with CTAB. The samples were stored at field temperatures (6-15^{o}C) for three days during transport back to the lab. DNA was extracted from all samples using the protocols described in Methods 2.1.

1.4. Sampling related species as controls

We sampled widely to find pure colonies of stalked algal species related to *D. geminata*. NIWA assisted in this sampling. Colonies of *Gomphoneis minuta* var *cassiae* were sampled from the Manganui-a-te-ao River, central North Island, and the Stony River, Taranaki. *Cymbella* spp. were collected from the Ahuriri and Aparima rivers. All samples were preserved in 70% ethanol, DNA was extracted by our standard protocol, and the DNA was subjected to a denaturing gradient gel electrophoresis (DGGE). The DGGE results were used to identify enriched samples of particular species for further cloning and sequencing. More species are currently being sought as representative specimens.

2. Protocols for extracting DNA from D. geminata

2.1. DNA extraction procedures

Central to our investigations is development of a reliable and robust DNA extraction technique for *D. geminata* and other diatoms. After some experimentation, we have refined the procedure to the following steps. For routine samples preserved initially in ethanol, we began at step 1. For samples preserved initially in CTAB, we began at step 2.

1) Tubes containing ethanol were centrifuged for 2 minutes at 13,200 rpm. The liquid was then decanted off, and the cells were re-suspended in 1ml of MilliQ water. The tubes were then spun again at 13,200 rpm for 2 minutes. The supernatant was then decanted, and the pellet in each tube was resuspended in 500 µl of CTAB.
2) Tubes were then briefly vortexed to ensure the CTAB was well mixed.
3) Tubes were placed in a 60ºC water bath for 20 minutes.
4) 500 µl of chloroform: isoamyl alcohol (24:1) was added to each tube, which was then inverted and vortexed. The tubes were then placed in a centrifuge for 15 minutes at 13,200 rpm.

5) 400 µl of the supernatant was then removed and placed in a new sterile Eppendorf tube. 400 µl of isopropyl alcohol and 200 µl of 5 M NaCl were added to the supernatant. The tubes were then inverted and left in the –80°C freezer for approximately 50 h to precipitate the DNA.
6) Once thawed, the tubes were then centrifuged for 30 minutes at 13,200 rpm. The supernatant was decanted, 500 µl of 70% ethanol was added, and the tube spun for 2 minutes at 13,200 rpm. The ethanol was then gently poured off leaving the DNA pellet in the tube.
7) The tubes were air-dried using the Savant Speed Vac at medium heat for at least 20 minutes until all the ethanol was removed.
8) The tubes were re-suspended in 20 µl of MilliQ water, and left to stand for at least 1 h before quantification. 1.5 µl of DNA was then quantified using the Nanodrop Spectrophotometer at 260 nm to determine relative recovery.

For samples that were either in a poor state of preservation or that had minimal recovery under normal extraction procedures (above), a phenol:chloroform extraction was performed as outlined below:

1) The volume of the tube was made up to 200 µl with MilliQ water.
2) Add equal volume of phenol: chloroform: isopropyl alcohol (25:24:1).
3) Mix well by inverting and vortexing, spin for 5 minutes at maximum speed (13,200 rpm).
4) Precipitate the supernatant by adding equal volume of isopropyl alcohol to the supernatant, and half the volume of 5 M NaCl.
5) Place the samples overnight in the –80ºC freezer.
6) Once thawed, the tubes were centrifuged for 30 minutes at 13,200 rpm. The liquid was decanted off, 500 µl of 70% ethanol was added to the DNA pellet, and each tube spun for 2 minutes at 13,200 rpm. The ethanol was then gently poured off.
7) The tubes were then air-dried using the Savant speed vac at medium heat for at least 20 minutes until all the ethanol was removed.
8) The tubes were then re-suspended in 20 µl of MilliQ water, and left to stand for at least 1 hour before quantification. 1.5 µl of DNA was then quantified using the Nanodrop Spectrophotometer.
9) Once quantified, the tubes were kept frozen in a –80ºC freezer for storage.

2.2. Validation of DNA extraction efficiency and recovery

A. Longevity. In order to transport samples obtained from distant field sites, we needed to establish the longevity and stability of DNA in whole *D. geminata* cells held under our two preservation methods (CTAB and 70% ethanol), as well as two reduced ethanol concentrations (50%, 25%), the latter equivalent to more commonly available grain alcohol. To evaluate stability under simulated travelling conditions, we tested DNA yield after storage at room temperature for 5 weeks. *D. geminata* was sampled from a rock with a dense mat of healthy looking *D. geminata* from the Mararoa River. The upper pigmented (cellular) layer of the *D. geminata* mat was clipped from the mat using tweezers and scissors. The sample was then placed in a sterile Falcon tube containing 50 ml of MilliQ water. The tube was then shaken vigorously for 1 minute. Immediately following shaking, the homogenate was filtered through a 500 µm net into a 50-ml sterile Falcon tube.

A 300-µl aliquot of the homogenate was then placed in each of 48 1.5-ml Eppendorf tubes. These tubes were grouped into six different sets to be sampled at weekly intervals (T_0, T_1, T_2, T_3, T_4, and T_5). Preservation treatments were either 1X CTAB or 70%, 50%, or 25%

ethanol. Each was added to the homogenate in a tube to make a final volume of 1 ml. Each treatment was performed in duplicate with a final concentration of 70% ethanol, 50% ethanol, 25% ethanol, or CTAB respectively. After adding the ethanol or CTAB, all eight T_0 tubes were placed immediately on ice and then transported by air to the University of Waikato.

DNA was extracted with our normal CTAB protocol immediately (T_0; within 5 h of collection) and at weekly intervals thereafter for 5 weeks (i.e., T_1, T_2, T_3, T_4, and T_5). After quantification, each tube was stored at -80ºC. Incubation temperature for samples before DNA extraction was recorded at 1-h intervals with an Onset temperature logger. Mean temperature over the 35 days of the experiment was 22.9°C (minimum 17.4, maximum 27.0, standard deviation 2.6°C).

B. Extraction sensitivity. To investigate the effect of cell density on extraction sensitivity and DNA yield from *D. geminata*, we extracted DNA by our usual protocol (Methods 2.1 above) from a range of cell densities. We created a dilution series of decreasing cell concentration from the drift net sample of *Synedra* and *D. geminata* cells from the Mararoa River and then extracted and quantified DNA yield. The original cell suspension was stirred continuously with a magnetic stirrer during the following procedure. From the suspension the following aliquot sizes were subsampled into a 14 ml Falcon tubes: 3.75 µL, 7.5 µL, 15 µL, 30 µL, 75 µL, 150 µL, 300 µL, 600 µL, 1.25 mL, 2.5 mL, and 5 mL and made up to a final volume of 5 mL with 70 % ethanol. They were shaken to ensure suspension and 1.5 mL was taken and put into and Eppendorf tube. DNA was extracted according to the normal CTAB method and measured on a Nanodrop spectrophotometer. Identification and cell counts were carried out using an inverted Olympus microscope (CK-2, Olympus) and Utermöhl settling chambers.

2.3. Extract DNA from *D. geminata* and related taxa

After successful development of DNA extraction protocols, 18S rDNA was extracted from *D. geminata* and related taxa as described in Methods Section 2.1 above.

3. Design and validate primers for low-level detection of D. geminata

3.1 Sequence *D. geminata* 18S rRNA gene

The aim of this portion of the study was to clone and sequence DNA from *D. geminata.* The 18S rRNA gene was obtained from a sample enriched in *D. geminata* collected from Redcliff Creek, Lower Waiua River, in December 2005. This initial sample was preserved in only Vodka and was processed 2 weeks after collection having been held a room temperature. DNA was extracted using the CTAB protocol (Methods 2.1 above). The entire 18S rRNA sequence was amplified using the polymerase chain reaction (PCR) with one set of universal eukaryotic-specific primers (EukA and EukB) (Table 6). PCR was carried out in 0.5-ml thin-walled tubes, containing a total volume of 50 µl comprising 33 µl of sterile distilled water, and a final concentration of 1X PCR buffer -$MgCl_2$ 50 mM KCl, 10 mM tris-HCl, pH 8.3 (Boehringer, Mannheim), 1.0 mM primer (Invitrogen Ltd), 2.5 mM $MgCl_2$, 2.0 mM of each dNTP (Boehringer, Mannheim), 0.5 units of *Taq* DNA polymerase (1 unit/µl) (Roche, Germany), and 2 µl of 10 ng/µl genomic DNA. One unit of Taq 1 is defined as the amount of enzyme that incorporates 10 nmol of total deoxyribonucleoside triphosphates into acid precipitate DNA within 30 minutes at 75°C under assay conditions. All PCR experiments

were evaluated with the inclusion in the experiment of a separate negative (which included distilled water instead of DNA) and a positive control (an easily amplifiable sample which works across successive PCR experiments). All PCR experiments were performed in an MJ Research Peltier thermal cycler under the following conditions: an initial denaturation period of one cycle at 94ºC for two minutes to denature the genomic DNA, followed by 35 cycles of 94ºC for 30 seconds to denature the DNA, 55°C for one minute to anneal the primer to the template DNA, and 72ºC for two minutes to let the DNA polymerase extend. A final extension period of 72ºC for 5 minutes followed to complete the PCR and the final products were then held at 4ºC until they were removed from the PCR machine.

PCR products were separated and visualized on a standard 1.0% 1X (SBA buffer) agarose (SeaKem LE), 5 mM EDTA (disodium salt) buffer gel, with ethidium bromide (0.1 mg/l) staining. Prior to loading, 5 µl of PCR product was pipetted and mixed directly with 3 µl loading dye (0.0083% bromophenol blue, 2.5% ficol (MW 400 000). The 5 µl mixture was then loaded in each well, in a 5 mM EDTA gel. Once loaded, PCR products were electrophoresed at 55 mA for at least 30 minutes. After electrophoresis, each gel was visualized in the presence of UV light in the Alpha Imager documentation system (Alpha Innotech Corp., San Leandro California). Five microlitres of (1 Kb Plus) base pair molecular weight ladder (prepared: 3 µl at a 1:10 dilution in TE buffer mixed with 2 µl of 1X loading buffer) (Invitrogen) was included in the outer lane of each gel and used as a size estimate comparison of individual amplification products. The successful PCR product was cloned using the TOPO TA cloning vector and propagated on LB media with Kanomycin to select for clones with inserts (Invitrogen).

Clones were picked and then screened for proper size insert (1.5 kb) using PCR amplifying from the flanking M13 regions following the manufacturers instructions (refer to TOPO TA Cloning kit for further details). The PCR product from 20 clones containing the correct size insert were then restricted with two restriction enzymes that recognise 4 base pairs cut sites (Mbo1 and Hae III) to identify those clones containing unique inserts. A representative of each restriction family was then propagated overnight in 5 ml of LB broth with Kanomycin and the plasmid extracted the following day using the Quick Clean Plasmid Miniprep kit following the manufacturer's instructions. Plasmid preparations were quantified using a Hoefer DyNAquant 200 fluorometer with Hoechst 33258 Dye at 1 mg/ml (Hoefer Scientific Instruments) prior to sequencing All direct sequencing was performed using the vector containing M13 sites for sequencing the insert termini and 608F/R and 1000 F/R for sequencing the internal regions of the gene. All reactions were performed in an MJ Peltier Thermal cycler, under the following conditions: 40 cycles of 95ºC for 20 seconds to denature the DNA, a set annealing temperature of 50ºC for 15 seconds to anneal the primer to the template DNA, and 60ºC for two minutes to let the DNA extend, the final products were then held at 4ºC for 10 minutes and removed from the PCR machine. All direct sequencing was achieved using the MegaBACE 500 DNA Analysis system, fitted with 40 cm capillary arrays (Amersham Biosciences, Buckinghamshire) loaded with a linear polyacrylamide Long Read Matrix (Amersham Biosciences, Buckinghamshire). DNA templates were prepared using DYEnamicET dye terminator chemistry (Amersham Biosciences, Buckinghamshire). See Appendix 3 for the full 18 S rRNA sequence. The equivalent sequence for *Gomphoneis minutae* var *cassiae* is shown in Appendix 4.

3.2. Sequence same regions in related taxa

In order to identify specific samples enriched in other diatoms that could potentially produce false positives, we used a diatom-specific DNA fingerprinting method (DGGE). This

method provides a rapid analysis of diatom community complexity and composition in a given sample.

Selected environmental samples were extracted using the CTAB protocol outlined above. PCR primers STRAM 9F and 517R-GC (Table 1) were used to amplify all environmental samples and the verified *D. geminata* 18S rRNA clone (Methods 3.1) as a control. PCR was carried out in 0.5-ml thin walled tubes, containing a total volume of 50 µl of 28.25 µl of sterile distilled water, and a final concentration of 1X PCR buffer -$MgCl_2$ 50 mM KCl, 10 mM tris-HCl, pH 8.3 (Boehringer, Mannheim), 1.0 mM primer (Invitrogen Ltd), 2.5 mM $MgCl_2$, 2.0 mM of each dNTP (Boehringer, Mannheim), 1.25 units of Taq DNA polymerase (1 unit/µl) (Roche, Germany), and 1 µl of 20 ng/µl genomic DNA. All PCRs were evaluated with the inclusion of a negative (which included distilled water instead of DNA) and a positive control (an easily amplifiable sample which works across successive PCRs). All PCR experiments were performed in an MJ Research Peltier thermal cycler under the following conditions: an initial denaturation period of one cycle at 94ºC for two minutes, followed by 20 cycles of 94ºC for 1 minute, 65°C for one minute to anneal the primer to the template DNA which was decreased by 0.5°C per cycle, and 72ºC for 2 minutes. This was then followed by 14 cycles of 94ºC for 1 minute, 55°C for 30 seconds, and 72ºC for one and half minutes. A final extension stage of 72ºC for 5 minutes followed to complete the experiment. The final products were then held at 4ºC until they were removed from the PCR machine. PCR products were then checked on a 1% (SBA buffer) gel in the procedure in Methods Section 3.1 above.

Table 1. Primers targeting *D. geminata* and eukaryote-specific regions within the *D. geminata* 18S rDNA. *Primers used for sequencing the full 18S rDNA region.

Oligonucleotide	Sequence (5' to 3')	Specificity	Tm (^oC)
D602F	GTT GGA TTT GTG ATG GAA TTT GAA	*Didymosphenia*	52.4
D753R	AAT ACA TTC ATC GAC GTA AGT C	*Didymosphenia*	50.3
D1565F	CCT AGT AAA CGC AGA TCA TCA G	*Didymosphenia*	52.8
D1659F	GCT GGG GAT TGC AGC TA	*Didymosphenia*	55.0
D1670R	CAC CAG TAA AGG CAT TAG CTG	*Didymosphenia*	53.7
Euk 608F*	CGG TAA TTC CAG CTC CAA T	Euk universal	52.4
Euk608R*	TTG GAG CTG GAA TTA CCG	Euk universal	52.4
Euk1000F*	AAC GAA AGT TAG GGG ATC GA	Euk universal	53.3
Euk1000R*	TCG ATC CCC TAA CTT TCG TT	Euk universal	53.3
EukA*	AAC CTG G(TTGAT) CCT GCC AGT	Euk universal	49.0
EukB*	GAT CC(AT) TCT GCA GGT TCA CCT AC	Euk universal	52.0
STRAM9F	CTG CCA GTA GTC ATA CGC TC	Universal diatom	55.0
517R-GC	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GAC GGG GGA CCA GAC TTG CCC TC	Universal diatom DGGE	88.0

For the DGGE analysis, a gradient delivery system (Model 475, Bio-Rad Laboratories, NY, USA) was used to cast DGGE gels. Gels were composed of 6% acrylamide

with a denaturant concentration of 10-50% for expected amplicons of 850-1000 base pairs. Gels were loaded into an electrophoresis chamber (D GENE™ Denaturing Gel Electrophoresis system) containing 7 litres of 1X TAE buffer. Once gels were cast, they were allowed to set for at least 1 hour. Buffer inside the chamber was held at 60°C both prior and during loading. Between 15-20 µl of loading dye (0.05% BPB, 0.05% xylene cyanol and 70% glycerol diluted in 1X TAE) buffer was added to each PCR tube. 10-20 µl of PCR product was run in each well consisting of a maximum total volume in each well of 40 µl. Gels were electrophoresed for 5 hours at 140 V. All gels were stained in 500 ml of 1 mg/l ethidium bromide for 10 minutes, destained in 500 ml of distilled water for 10 minutes, and visualized on an AlphaImager 2000. Samples that contained a single strong non-*D. geminata* signal were then selected for continued sequence analysis. The 18S rDNAs were amplified, cloned, and sequenced in the manner outlined above. Unique diatom sequences were then aligned and edited manually using Sequencher ver 4.0 (Gene Codes Corporation). To obtain other diatom 18S rRNA sequences for phylogenetic analysis, the *D. geminata* 18S rDNA was blasted into the GenBank NCBI database (National Center for Biotechnology Information, *http://www.ncbi.nlm.nih.gov/*) to obtain the 18S rRNA sequences from other available closely related taxa not necessarily found in New Zealand. Other sequences included from GenBank were *Encyonema triangulatum*, *Gomphonema parvulum*, *Dickieia ulvacea*, *Anomoeoneis sphaerophora*, *Amphora montana*, *Eolimna subminuscula*, *Eolimna minima*. Each of these sequences was aligned with the other sequences.

All phylogenetic analyses were constructed using PAUP* Version 4.5b (Swofford, 1998) using Neighbor Joining (NJ), maximum parsimony (MP) and likelihood analysis (ML). NJ analysis was conducted on PAUP* using the Nei & Li (1979) distance coefficient which assumes random mating and equal rate divergence (Swofford, 1998). Distance was calculated by mean character differences, with break ties systematically taxon dependent. Maximum Parsimony and likelihood analysis were performed using the heuristic search of PAUP*, (Rogers-Swofford approximation method) (Swofford, 1998). One sequence obtained from GeneBank was used as a designated outgroup in all phylogenetic analyses to compare sequences obtained from this study to other relevant diatom genera. *Fragilaria striatula* was selected as an appropriate outgroup based on an initial blast analysis of a range of diatom genera, which suggested that they were most closely related to *D. geminata* from sequences held by GeneBank. Confidence measures using PAUP* (Swofford, 1998) such as bootstrapping were tested to assess for potential errors inherent in phylogeny. Bootstrap analysis was conducted using 1,000 pseudoreplicates to approximate the posterior probability of tree construction and to assess the support of individual clades and thus the relative confidence of the NJ, ML, and MP trees (Felsenstein, 1976).

3.3. Design of *D. geminata*-specific primers

The purpose of this protion of the study was to design PCR primers specific to *D. geminata*. Based on aligned sequences of *D. geminata* from New Zealand *Cymbella* spp., New Zealand *Gomphoneis* spp., and the other diatoms, five primers were designed to specifically amplify the 18S rDNA sequence of *D. geminata*. Several universal eukaryotic primers (Euk608F, Euk1000R, EukBR) and a diatom-specific primer (STRAM9F) were matched with our newly designed *D. geminata* primers (D602F, D753R, D1670R, D1565F, and D1659F) and tested in the PCR for their specificity and sensitivity to detect and amplify DNA from *D. geminata*. Our strategy here was to choose several alternative primer sets that would produce products of varying lengths. This is consistent with our initial objective of developing a low-level detection capability and later objective to move the protocols to a quantitative approach (QRT-PCR).

3.4. Validate primers against related species and environmental samples, establish sensitivity range of primers and optimise protocols for field samples

Our strategy was to develop the most sensitive detection capability by testing a variety of PCR primer sets made up of either *D. geminata*-specific primers or combinations with other universal or diatom specific primers. From these we would select, on the basis of specificity and sensitivity, primer sets to optimise and further validate.

An initial set of primer combinations was designed on the basis of PCR compatibility (Table 1 and Figure 12). Twelve *D. geminata*-specific combinations were initially tested. Primers were first optimised, using our *D. geminata* 18S rDNA plasmid as template DNA. PCR was conducted with the same procedure outlined in Methods 3.1 with only slight changes based on the Tm (melting point) of the individual primers (Table 1). From these initial tests, two primer sets were chosen, one producing a long amplicon (1073 base pairs) and one producing a short amplicon (170 base pairs), for further optimisation and validation.

The first step was to optimise the PCR reactions for each individual primer set. Experiments were run under varying annealing temperatures to establish the highest optimal temperature for each primer set, which was defined as the highest annealing temperature where the final product did not diminish significantly. We assumed that this temperature provided the highest specificity.

These experiments were then repeated for the short primer set only with a gradient of DNA template concentration. The aim was to test that at these highly specific annealing temperatures we had not diminished the sensitivity of the reaction to detect the *D. geminata* target by increasing the specificity with higher annealing temperatures. Here we titred the *D. geminata* 18S rDNA-containing plasmid at 10-fold increments to extinction. The same experiment was rerun with the addition of 20 ng of exogenous environmental DNA from which *D. geminata* was absent. This provided an assessment of the sensitivity of the two primer sets to detect their native target when in the presence of non-homologous DNA.

Once the reactions for each primer set had been optimised using the *D. geminata* 18S plasmid, the specificity of the primers was tested on cloned 18S rDNAs and environmental samples containing *D. geminata* and non-*D. geminata* taxa (Table 2). Each of the primer sets was tested against each of the environmental templates. Any positive amplification was sequenced directly to validate the specificity of the amplification. This was particularly important in assessing the ability of the primers to amplify *D. geminata* out of a complex community of other organisms. The short *D. geminata* specific primer pairs (170 base pairs; Table 2) were initially validated against both species-specific 18S rRNA-containing plasmids of related species and environmental samples (Table 3).

Table 2. Primer strategy of different primer combination sets, and estimated length of amplicons in base pairs (bp).

Primer Combination	Estimated length
D602F and D753R	170bp
Stram 9F and D753R	774bp
Euk 608 F and D753R	228bp
D602F and Euk 1000R	367bp
Stram 9F and D1670R	1079bp
Euk608F and D1670R	1129bp
D602F and 1670R	1073bp
D602F and Euk B R	1198bp
Euk 1000F and D1670R	741bp
D1565F and D1670R	110bp
D1565F and Euk B R	235bp
D1659F and Euk B R	141bp

Table 3. Initial environmental samples and species-specific plasmids tested during primer validation.

Sample	Taxa
AH-26	*Cymbella* sp. in environmental sample
AP-28	*Cymbella kappii* in environmental sample
AP-30	*Gomphonema* sp. in environmental sample
MAA001	*Gomphoneis* sp. 18S rRNA-containing plasmid
TAR009	*Gomphoneis* sp. 18S rRNA-containing plasmid
RAIFALLS 12	*Cymbella, Aulacoseira,* and *Spirogyra* in environmental sample
VR1-E1	*Didymosphenia* & other diatoms in environmental sample
VR2-E1	*Didymosphenia* & other diatoms in environmental sample
MA2-E1	*Didymosphenia* & other diatoms in environmental sample
VI-5	*Didymosphenia* sp. environmental sample (Vancouver Island)

3.5. Blind test comparisons using DGGE and sequencing

The final series of experiments in Phase 1 were blind specificity tests. The purpose of these tests was to validate the working primers on environmental samples with and without *D. geminata*. The DGGE diatom-specific protocol was utilised to screen all environmental samples from both *D. geminata* and non-*D. geminata*-affected areas. We used this method as

a first pass to provide evidence of *D. geminata* (or lack thereof). We then subjected 64 templates to PCR amplification with the two newly designed *D. geminata*-specific primers (D602F and D753R). The samples from which the templates were taken are shown in Appendix 5 by the shaded rows. In the event of a positive amplification, the amplicon was sequenced to verify that the target that had been amplified was *D. geminata* and only *D. geminata*. We used diatom-specific DGGE (Methods Section 3.2 above) as a blind test validation for each of the new PCR primer sets specific for *D. geminata* on 21 selected samples from New Zealand and 22 international samples. The DGGE clearly identified differentiated samples contained *D. geminata* from those containing other diatoms but not *D. geminata*. Each of these same samples was amplified using the newly designed *D. geminata*-specific primers. PCR products were then run out an agarose gel in the procedure outlined above to verify amplification. Positive amplifications were subsequently purified using a GenScript PCR cleanup kit (GeneScript, Inc.) and sequenced. Sequences were then aligned with the *D. geminata* plasmid sequenced previously to determine if the primers were specifically amplifying *D. geminata* 18S rDNA.

Results

1. Protocol for collection and stabilization of environmental samples

1.1. Field collection protocol for both water and surface samples

The field collection procedures outlined in the methods have been very effective in sampling benthic samples of *D. geminata* and other taxa. We were able to extract DNA from all sample types stabilised in either CTAB or 70% ethanol. These included picking likely looking *D. geminata* colonies or other periphytic algae from rocks, all samples of bare rock using cotton swabs, and the drift net samples of the water column. The nucleic acid yield of all these extractions are tabulated in Appendix 5. Successful DNA extraction was verified by gel electrophoresis by our standard protocol (see Methods 2.1 above) followed by Nanodrop spectrophotometry. Samples from the North Island were concentrated in the Taranaki area and the central North Island (Figure 4), whereas South Island samples were more widely distributed (Figure 5).

Of particular note was the success of our drift-net sampling rig. The nested drift net sampling excluded clumps of drifting stalk material and other debris whilst still allowing collection of algal cells. This is important because DNA from extraneous material can swamp the target DNA during the extraction process. In 10 minutes we sampled 5.65 m^3 of water and trapped about 1.39 cm^3 of algal cell material in the finest mesh (47 μm). The algal material was about 25% *D. geminata* cells; *Synedra* sp. comprised the majority of the sample, with fragments of *D. geminata* stalk material and short pieces of unidentified filamentous green algae.

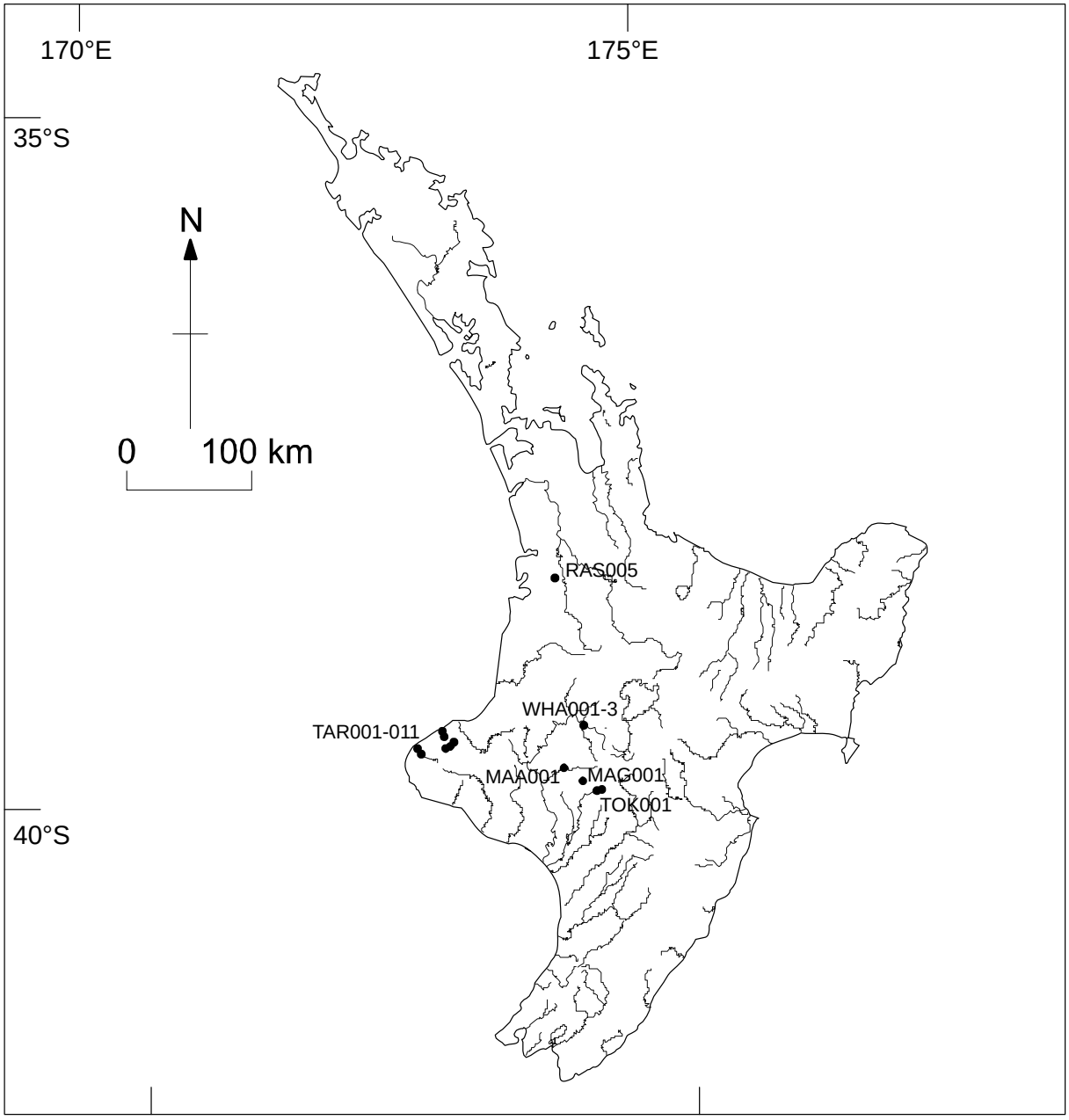

Figure 4. Location of sampling sites in the North Island, showing 6th order rivers and greater. See Appendix 5 for sample codes and river names.

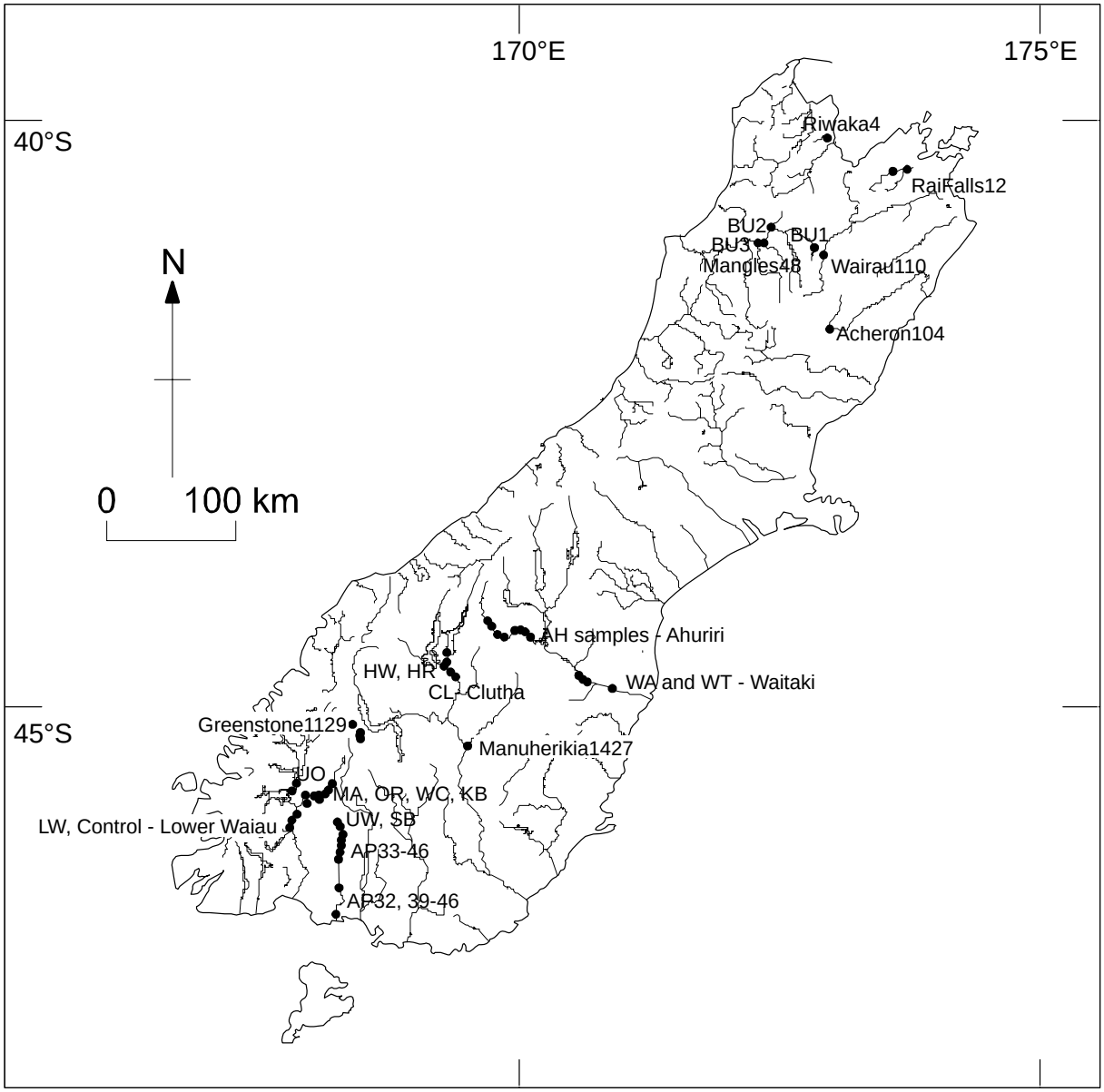

Figure 5. Location of sampling sites in the South Island, showing 6th order rivers and greater. See Appendix 5 for sample codes and river names.

1.2. Field collection thresholds and longevity

DNA was successfully extracted from all swab samples analysed so far. This demonstrates that the biofilm on apparently clean rocks in affected or clean rivers can easily be monitored for the presence of *D. geminata*. The further treatment and extraction of samples in the threshold and longevity experiments is described in Methods Section 2.2.

1.3. Extensively sample all known *D. geminata* affected areas

In total, we collected 103 samples from 11 rivers in the South Island (Table 4). *D. geminata* was visually determined to be present at all sites sampled but was sparse at the two lower sites in the Buller River as sampling followed a flood on 25 April that had scoured the river bed. DNA was extracted from samples at all *D. geminata*-affected sites (Appendix 5). At known affected sites, *D. geminata* DNA was found in all swabs of bare substrate with no apparent colonies of *D. geminata* or other algae (see Methods section 3.5). The Aparima River was inaccessible because of flooding on 11 May, so only one sample was obtained.

Table 4. Rivers sampled for *D. geminata* in the South Island, New Zealand, from 9 to 12 May 2006.

River	Number of sites	Number of samples
Ahuriri	3	12
Aparima	1	3
Buller	3	12
Clutha	3	12
Hawea	3	12
Upper Waiau	2	2
Lower Waiau	3	6
Mararoa	3	12
Oreti	3	12
Von	2	8
Waitaki	3	12

1.4. Sample all other related species as controls

New Zealand freshwater ecosystems contain many diatoms that may co-exist with *D. geminata* and that may be mistaken for *D. geminata* by the untrained eye. Detection of *D. geminata* on substrates is confounded by the existence of other morphologically similar co-existing taxa such as *Gomphoneis* and *Gomphonema* that also form light brown, epilithic turfs in clear-water, gravel-bedded streams and rivers. Other common components of epilithic algal communities include diatoms such as *Cymbella*, *Synedra*, and *Fragilaria*. Three native diatom genera were collected as controls: *Gomphoneis*, *Gomphonema*, and *Cymbella kappii*. DNA from all of these control species were extracted using the CTAB extraction method, and the 18S rRNA gene sequenced. The purpose of this sequencing was to acquire the 18S rRNA sequence to ensure that primers developed for *D. geminata* uniquely amplified the target.

The sequences of the control taxa were aligned with our *D. geminata* sequence. This alignment showed that the 18S rRNA contained several regions that are clearly unique and distinguishing to *D. geminata.* These regions provided suitable sites to design *D. geminata*-specific PCR primers for low-level detection. DGGE (see Methods 3.2 and Appendix 1) using diatom-specific primers successfully distinguished *D. geminata* from native *Gomphoneis* sp., and revealed a complex diatom community occurring with *D. geminata* in the Mararoa, Von, and Oreti rivers (Figure 6). These same DGGE profiles were used to identify specific samples enriched in one taxon, (e.g., Manganui-a-te-ao North Island sample shown in Figure 6).

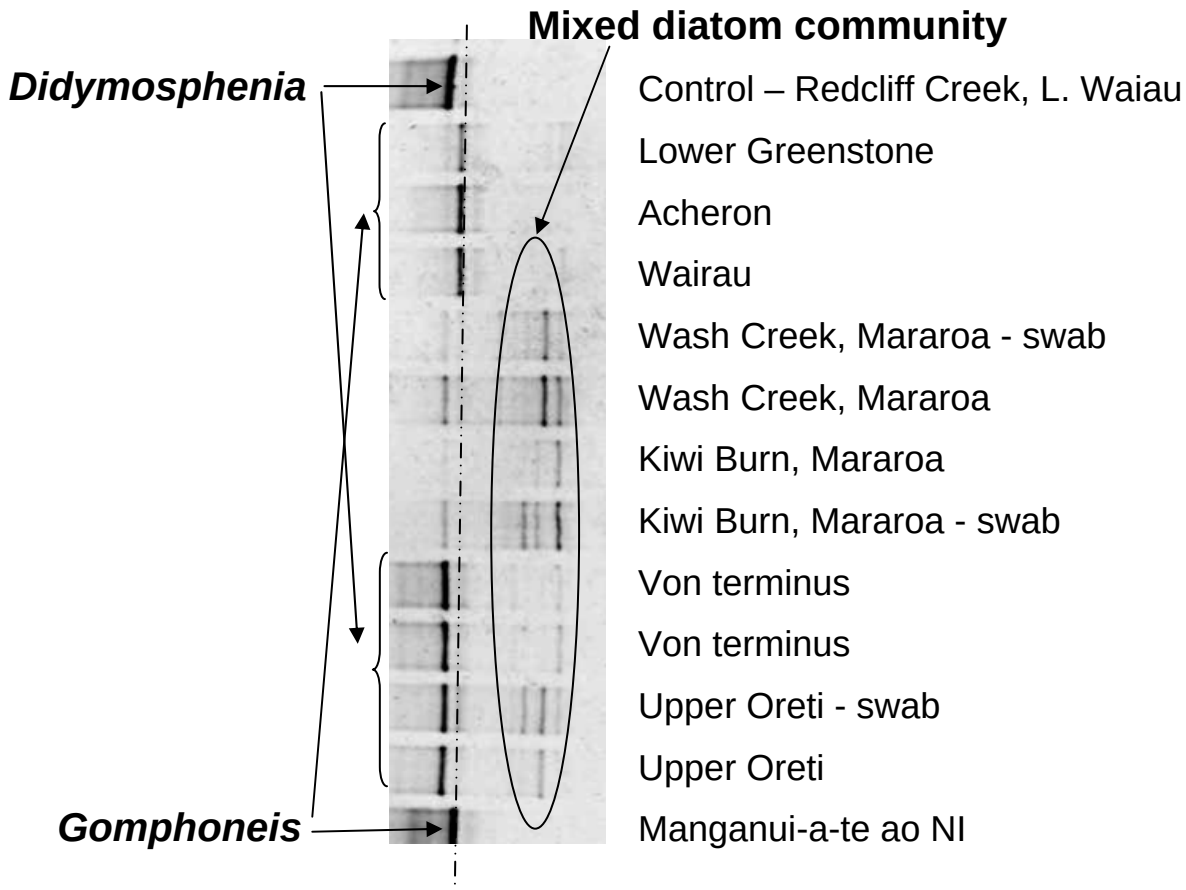

Figure 6. Discrimination of *D. geminata* and *Gomphoneis* sp. by denaturing gradient gel electrophoresis (DGGE). The dashed line identifies *Gomphoneis* sp.

2. Protocol for extraction of DNA from D. geminata

2.1. Extraction protocol experimentation

We collected a total of 307 environmental samples, and have so far extracted DNA from 182 of these (Appendix 5). Out of the 182, we obtained high-quality DNA that was PCR-amplifiable from all samples. These included samples preserved both in CTAB and ethanol regardless of sampling technique (swab, benthic sample, drift net collection). The extraction and preservation protocols developed in this study have performed extremely well. However, we have experienced some non-linearity with the extraction efficiency and yield at high cell and DNA concentrations.

2.2. Validation of DNA extraction efficiency and recovery

In the longevity experiment, DNA was successfully extracted from all 48 samples over a total period of 5 weeks in the DNA extraction validation experiment (see Methods 2.2). This suggests that the CTAB method of DNA extraction is very effective on *D. geminata*. However, overall DNA yields for longevity were inconsistent and not reproducible between replicates (Figure 7). No bias towards any particular preservative (ethanol or CTAB) was evident, with most samples spread over a wide range of yields between periods.

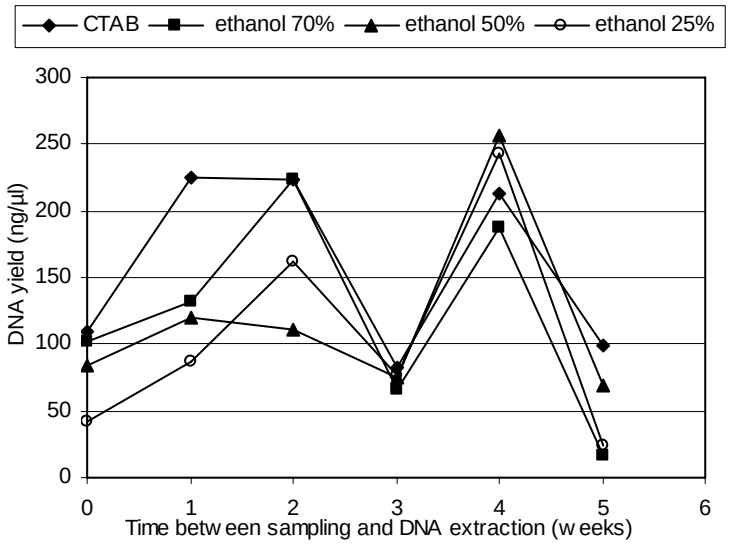

Figure 7. DNA yield (ng/µl) of different samples of *D. geminata* exposed to four different treatments (70% ethanol, 50% ethanol, 25% ethanol, and CTAB) over a period of 5 weeks.

In the extraction efficiency experiment, there was a linear relationship between the cell density and DNA yield (Figure 8). The results show that we can reliably extract DNA from as few as 6 cells/ml, and the lower detection limit might be even lower. Variable DNA quantitation occurs at this level because the lower limit of detection of the Nanodrop Spectrophotometer, on which the DNA yield was quantified, is about 5-10 ng/µL.

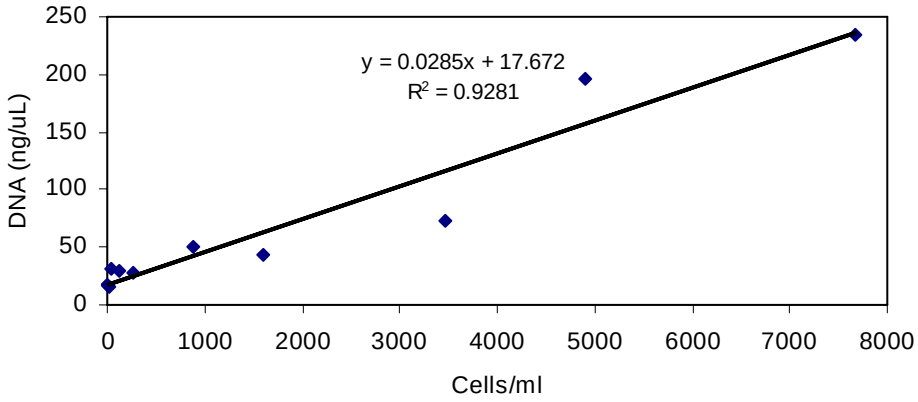

Figure 8. Relationship of DNA yield to cell density in a suspension of *Synedra* sp. and *D. geminata* cells collected by drift net sampling in the Mararoa River, Southland.

3. Design and validation of primers for low level detection of D. geminata

3.1. Sequence *D. geminata* 18S rRNA gene

The initial sample preserved in vodka produced high quality DNA using the CTAB extraction method. PCRs carried out on this enriched *D. geminata* sample using the eukaryotic 18S rRNA primers EukA and EukB were successful. From this enriched sample, we used PCRs to produce 20 clones with correct size inserts all with identical restriction patterns. We therefore only proceeded with three clones for further sequencing analysis. The whole 18S rDNA gene was sequenced for *D. geminata* using the vector-containing flanking M13 regions and internal priming sites 608 F/R and 1000 F/R. This produced complete bidirectional coverage of the whole 18S rDNA gene (Table 1). Sequencing reactions were attempted at least once in the forward and reverse direction on each clone to ensure accurate sequence information, and to assist in any ambiguous base calling. All sequences were edited and aligned to produce a consensus sequence, which was used for all subsequent analysis. The sequence of the inserts from the three clones with similar restriction patterns were 99.8% identical. The consensus sequence produced is the putative *D. geminata* 18S rDNA sequence for validation using PCR, DGGE, and further sequence analysis of other environmental samples. The full sequence of the *D. geminata* 18S rDNA is shown in Appendix 3.

3.2. Sequence same regions of related gomphonemoid taxa

DGGE was successfully used to identify environmental samples enriched in other diatom species (e.g., Figure 6 above). From these analyses several samples were selected for continued analysis. Following the same cloning protocol used for the *D. geminata* 18S rDNA, we extracted, screened with DGGE, cloned, and sequenced the full 18S rDNA sequence for a related *Gomphoneis* sp. from the North Island and *Cymbella* sp. from the Ahuriri River. These species were combined with seven other diatom species' 18S rRNA sequences available in the GenBank database and aligned with *D. geminata* in order to construct a phylogenetic tree. Using several inference programs we obtained and validated the topology of this tree (Figure 9). This same alignment was then used to identify variable areas around which we designed *D. geminata*-specific primers for PCR and probes for QRT-PCR (Phase 2b).

After close examination of the *D. geminata* 18S rRNA with other known closely related diatoms, it was clear that *D. geminata* contained the needed variability to develop appropriate species-specific primers. The full sequence of the 18S rDNA for New Zealand *Gomphoneis* from Stony River, Taranaki (TAR009; Appendix 5) is shown in Appendix 4. The resulting tree showed two distinct clades (Figure 9). These results extend the biraphid phylogeny suggested by Beszteri et al. (2001). *D. geminata* was positioned in the same clade as NZ *Gomphoneis* and *Gomphonema parvulum* obtained from GenBank, showing their close relatedness. The similarity between *D. geminata* and *Gomphoneis* and *Gomphonema parvulum* (95 and 96% respectively) and other biraphids can be seen in Table 6. New Zealand specimens of *Gomphonema* sp. and *Cymbella kappii* are currently being sequenced, and will be added to the tree.

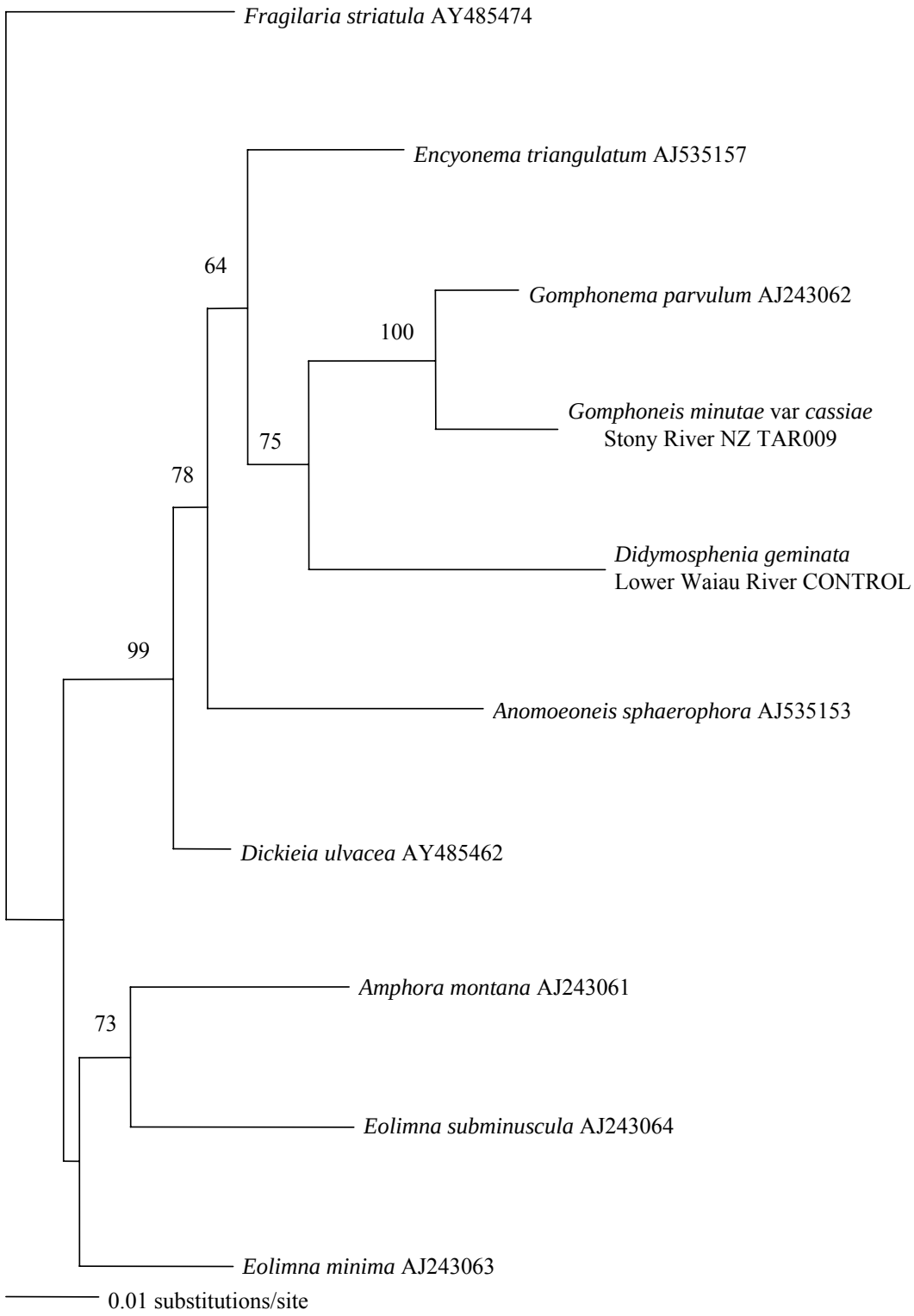

Figure 9. Phylogenetic tree, with bootstrapping support indicated, for New Zealand *D. geminata* and related biraphid diatoms developed from the maximum likelihood technique with PAUP* based on 18S rRNA sequences. Those with accession numbers were obtained from GenBank.

Table 5. Percent similarity of biraphid taxa to *D. geminata*.

	Didymosphenia geminata	*Gomphoneis* sp.	*Gomphonema parvulum*	*Cymbella* sp.	*Dickieia ulvacea*	*Eolimma minima*	*Amphora montana*	*Encyonema triangulatum*	*Anomoeoneis sphaerophora*	*Eolimma subminuscula*	*Fragilaria striatula*
Didymosphenia geminata	-										
Gomphoneis sp.	95	-									
Gomphonema parvulum	96	98	-								
Cymbella sp.	92	92	93	-							
Dickieia ulvacea	96	96	97	93	-						
Eolimma minima	94	95	96	92	97	-					
Amphora montana	94	95	96	91	97	97	-				
Encyonema triangulatum	94	96	97	93	97	96	95	-			
Anomoeoneis sphaerophora	93	95	95	91	96	95	95	95	-		
Eolimma subminuscula	92	92	93	89	94	95	95	92	92	-	
Fragilaria striatula	94	95	96	91	96	97	96	95	95	94	-

3.3. Design of *D. geminata*-specific primers

Based on the provisional alignments of the ten taxa, we were able to identify five areas of sequence variability that allowed us to design five *D. geminata*-specific primers (Table 6). The full 18S rDNA sequence, indicating primer positions across the whole sequence, is presented in Appendix 6. Our strategy was to use these five *D. geminata*-specific primers either together or in combination with other more universal primers (universal or diatom specific) to obtain the most specific and sensitive set for low level *D. geminata* detection. In total, 11 primers were used to cover the full 18S rDNA sequence (Figure 10 and Table 7). The requirements for low-level detection (Phase 1) and quantitation by QRT-PCR (Phase 2b and 3) are different but will be tested and optimised here collectively.

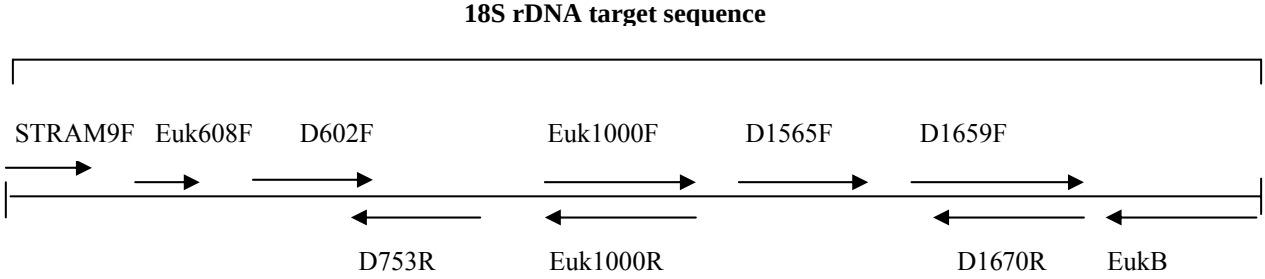

Figure 10. Primer positions indicated across the full 18S rDNA sequence.

Table 6. A) A representation of two sections of the full gene alignment where two of the *D. geminata*-specific short primer sets (D602F and D753R) were designed. B) IUB degenerate base code table used for the consensus sequences in Table 6A**.** The source of Table 6B is *http://www.cortec.ca/iubcodes.htm*.

A) Full gene alignment for D602F and D753R primers.

Taxon	D602F primer																														
Consensus sequence	T	C	G	T	A	G	T	T	G	R	A	Y	K	T	G	T	G	R	Y	D	N	N	D	Y	B	B	N	D	N	N	D	Y	Y
Amphora montana	T	C	G	T	A	G	T	T	G	G	A	T	T	T	G	T	G	G	T	G	-	G	T	C	C	C	T	G	A	G	G	T	C
Anomoeoneis sphaerophora	T	C	G	T	A	G	T	T	G	A	A	C	T	T	G	T	G	A	T	G	G	A	A	C	T	G	A	A	T	G	A	C	T
Cymbella	T	C	G	T	A	G	T	T	G	G	A	T	G	T	G	T	G	G	T	A	A	C	T	T	T	C	A	G	C	G	G	T	C
Dickieia ulvacea	T	C	G	T	A	G	T	T	G	G	A	T	T	T	G	T	G	G	T	G	-	G	T	T	T	C	T	G	A	T	G	T	C
Encyonema triangulatum	T	C	G	T	A	G	T	T	G	G	A	T	T	T	G	T	G	G	T	T	-	G	A	T	T	G	G	A	G	A	G	T	C
Eolimna minima	T	C	G	T	A	G	T	T	G	G	A	T	T	T	G	T	G	G	T	T	T	G	T	T	G	C	A	T	G	G	T	C	C
Eolimna subminuscula	T	C	G	T	A	G	T	T	G	G	A	T	T	T	G	T	G	G	T	G	C	G	A	C	T	G	C	G	G	C	G	T	C
Fragilaria striatula	T	C	G	T	A	G	T	T	G	G	A	T	T	T	G	T	G	G	T	T	G	G	G	T	C	C	G	T	C	G	G	T	C
Gomphonema parvulum	T	C	G	T	A	G	T	T	G	G	A	T	T	T	G	T	G	G	C	A	G	T	G	C	T	C	G	A	G	T	G	C	C
NZ *Gomphonesis* sp.	T	C	G	T	A	G	T	T	G	G	A	T	T	T	G	T	G	A	T	G	A	T	G	C	T	T	G	G	G	A	G	C	C
NZ *Didymosphenia geminata*	T	C	G	T	A	G	T	T	G	G	A	T	T	T	G	T	G	A	T	G	G	A	A	T	T	T	G	A	A	T	A	C	T

Taxon	D753R primer																														
Consensus sequence	A	A	G	C	A	R	R	C	T	T	A	Y	G	Y	Y	V	W	T	G	A	A	T	R	T	A	W	T	A	G	C	A	T
Amphora montana	A	A	G	C	A	G	G	C	T	T	A	C	G	C	C	G	T	T	G	A	A	T	A	T	A	T	T	A	G	C	A	T
Anomoeoneis sphaerophora	A	A	G	C	A	G	G	C	T	T	A	T	G	C	C	G	T	T	G	A	A	T	G	T	A	T	T	A	G	C	A	T
Cymbella	A	A	G	C	A	G	G	C	T	T	A	T	G	C	C	A	T	T	G	A	A	T	G	T	A	T	T	A	G	C	A	T
Dickieia ulvacea	A	A	G	C	A	G	G	C	T	T	A	T	G	C	C	G	T	T	G	A	A	T	G	T	A	T	T	A	G	C	A	T
Encyonema triangulatum	A	A	G	C	A	G	G	C	T	T	A	T	G	C	C	G	T	T	G	A	A	T	G	T	A	A	T	A	G	C	A	T
Eolimna minima	A	A	G	C	A	G	G	C	T	T	A	T	G	C	C	G	T	T	G	A	A	T	A	T	A	T	T	A	G	C	A	T
Eolimna subminuscula	A	A	G	C	A	G	G	C	T	T	A	T	G	C	C	G	T	T	G	A	A	T	A	T	A	T	T	A	G	C	A	T
Fragilaria striatula	A	A	G	C	A	G	G	C	T	T	A	T	G	C	C	G	T	T	G	A	A	T	A	T	A	T	T	A	G	C	A	T
Gomphonema parvulum	A	A	G	C	A	A	G	C	T	T	A	T	G	C	T	C	T	T	G	A	A	T	G	T	A	T	T	A	G	C	A	T
NZ *Gomphonesis* sp.	A	A	G	C	A	A	G	C	T	T	A	T	G	C	T	G	T	-	G	A	A	T	G	T	A	T	T	A	G	C	A	T
NZ *Didymosphenia geminata*	A	A	G	C	A	G	A	C	T	T	A	C	G	T	C	G	A	T	G	A	A	T	G	T	A	T	T	A	G	C	A	T

B) IUB code table for degenerate bases in the consensus sequences in Table 7A.

IUB (Degenerate Bases) Code Table											
IUB Code	N	V	B	H	D	K	S	W	M	Y	R
Bases	A,C,G,T	G,A,C	G,T,C	A,T,C	G,A,T	G,T	G,C	A,T	A,C	C,T	A,G

3.4. Validation of primers against related species and environmental samples, establish sensitivity range of primers, and optimise protocols for field samples

An initial series of PCR reactions allowed us to triage the possible primer combinations down to those that provided a successful robust amplification using the cloned *D. geminata* 18S rDNA as template (Figure 11). All but two initial PCR tests on the *D. geminata* plasmid with the different primer sets were successful based on amplicon length. Figure 11 shows that all primer sets produced visible bands. However, the bands for Euk608F and D753R, and D1659F and EukB were barely visible. Optimisation of the PCR reactions for each primer set will improve and ensure optimal specificity and sensitivity for their target. We found two groups of successful amplicons based on size (small 100-150 base pairs and large at 1068 base pairs) that contained the needed variability in both length and sequence. For the purposes of developing a low-level detection method to be resolved using gel electrophoresis (Phase 1), we would choose a primer set producing the largest amplicon possible. However, the requirements for QRT-PCR (Phase 2b and 3) are that the amplicon be much shorter (100-400 base pairs). From these initial experiments we chose one primer set producing a long amplicon (D602F and D1670R) and one producing a short amplicon (D602F and D753R) for further analysis and optimisation.

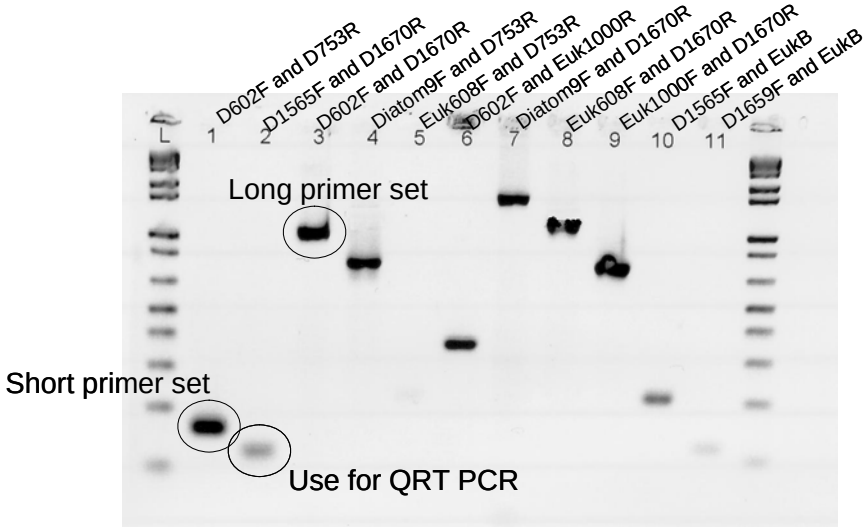

Figure 11. *D. geminata* primers tested in combination with each other and universals in a 1% agarose sodium boric acid (SBA) buffer gel screened on *D. geminata* plasmids.

PCR primer optimisation

As part of the PCR optimisation process, we tested specificity and sensitivity of primers. Increasing the annealing temperature of the PCR reaction can dramatically increase its specificity. This is, however, at the expense of the amount of final product produced. To test specificity, we increased the annealing temperature across a gradient produced on the PCR instrument for the long and short primer set. Our specific objective was to take the temperature high enough that the reaction does not take place at all (primer burn off). With

this full gradient we are able to pick a temperature where we have maximum specificity with maximum amplicon yield. Figure 12 shows the temperature gradients for each of two primer sets. The optimal annealing temp is usually about 5 to 8°C below burn off. For the short primer set (D602F and D753R), burn off occurred at 68.7°C, and thus we chose 60°C as the optimum annealing temperature. For the long primer set (D602F and D1670R), burn off occurred at 70.8°C, and thus we chose 65°C as the optimum annealing temperature.

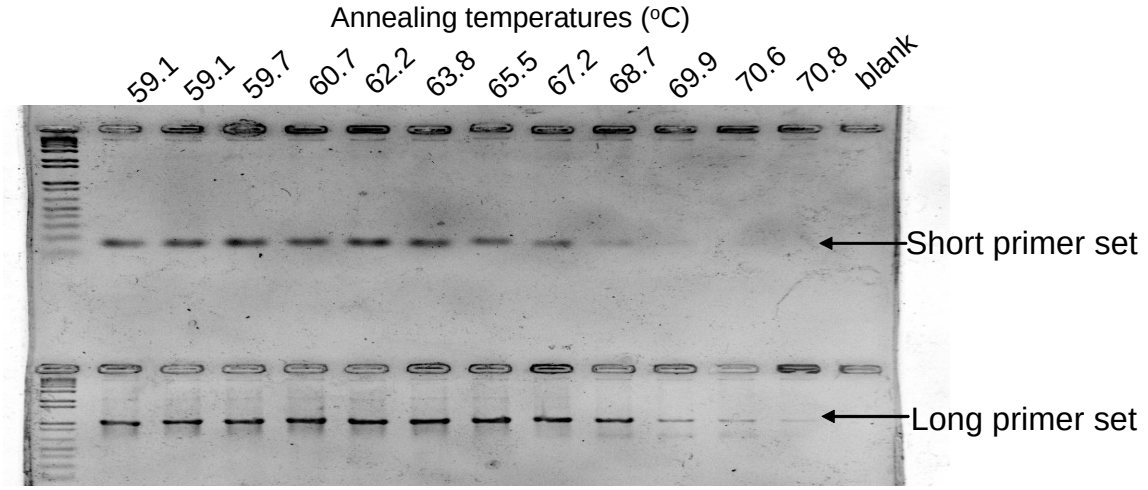

Figure 12. Annealing temperature gradient for long (D602F and D1670R) and short (D602F and D753R) primer sets for *D. geminata* in a 1% agarose sodium boric acid (SBA) buffer gel.

We proceeded to test sensitivity, given that we optimised the reaction conditions for each primer set (above). We then subjected these same primers to template at decreasing concentrations. Here again our object was to verify that given the new optimised reaction conditions we have not dramatically reduced the sensitivity of the primers to detect their target. Under optimal template conditions, the short primer set could detect 1 pg of DNA and the long primer set could detect 10 pg of DNA (Figure 13). From past experience, these primers are both extremely sensitive given the conditions of the reaction. Since the evaluation is being conducted visually on an agarose gel, our detection sensitivity is limited to PCR products ≥10 ng. When the method is moved to a QRT-PCR approach (Phase 2a and 2b), we can expect sensitivity to increase by 4 to 5 orders of magnitude.

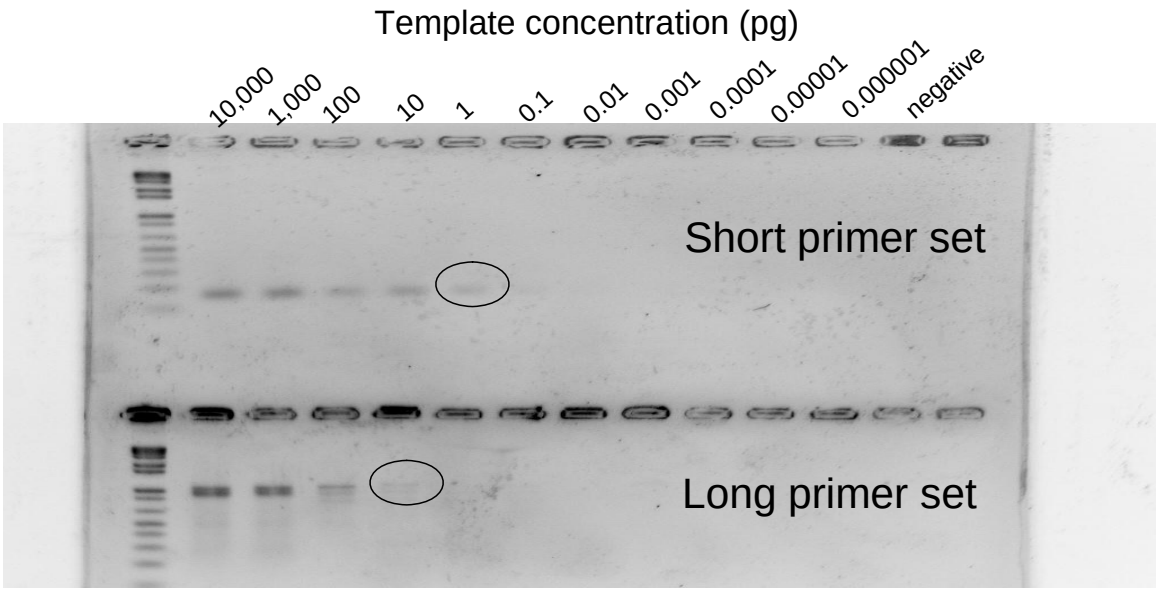

Figure 13. Visualized PCR reactions with titred *D. geminata* plasmid (10 ng to 1 ag) for long (D602F and D1670R) and short (D602F and D753R) primer sets for *D. geminata* in a 1% agarose sodium boric acid (SBA) buffer gel.

Preliminary primer validation experiments

Preliminary validation of the two primer sets began with amplification of *D. geminata* clones and *D. geminata*-containing environmental samples with both the long (1073 bp) and the short (170 bp) primer sets (Table 2). However, the long primer set failed to amplify some samples known to have *D. geminata*. The short primer set amplified all samples known to contain *D. geminata*. Thus, we proceeded to validate only the short primer set at this time (Figure 14). For the validation, we used four samples known to contain *D. geminata (*VR1-E1, VR2-E1, MA2-E1, VI-5, a *D. geminata* plasmid) and six thought to be *D. geminata*-free (AH-26, AP-28, AH-30, MAA001 Clone 2, TAROO9 Clone, RAIFALLS 12; see Appendix 5 for sample codes). In each case using the optimised reaction conditions, the short primer set only amplified samples with *D. geminata*. Where a positive amplification was obtained, the amplicon was sequenced directly and shown to be *D. geminata*.

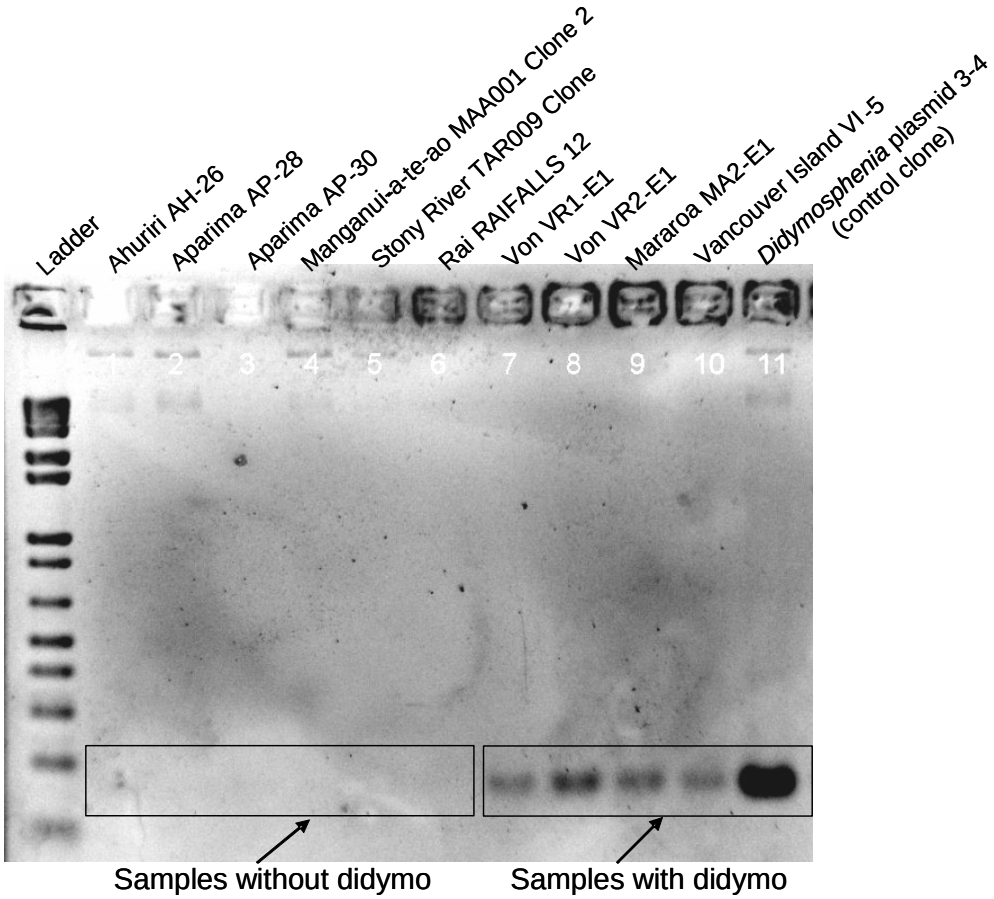

Figure 14. Preliminary primer validation of *D. geminata*-specific primers D602F and D753R used on environmental samples run in 1% agarose sodium boric acid (SBA) buffer gel with 18S rDNA obtained from PCR. *D. geminata* plasmid 3-4 was used as a control; sample codes are given in Appendix 5.

3.5. Blind test comparisons using DGGE and sequencing

All DGGE screening and sequencing validated the presence of the genetic marker in environmental samples with *D. geminata.* Subsequent sequencing verified environmental samples of *D. geminata* were amplifying the correct sequence they were designed for within the 18S rDNA region (Figure 15). Samples from sites known not to have *D. geminata* failed to amplify (as determined by gel electrophoresis) and therefore were not processed further.

Twenty-one environmental samples from New Zealand were chosen at random and screened using the *D. geminata*-specific primers DGGE, and sequencing (Figures 15A and B). A dark band under the label indicates a positive, e.g., VR1 (2) swab and WC1, and a blank in the same row indicates a negative, e.g., Pelorus 7 and Greenstone 1129. In all cases where *D. geminata* was shown to be present by DGGE analysis, amplification using the *D. geminata*-specific primers was successful. In cases where *D. geminata* was known not to occur and did not appear in the DGGE analysis, the amplifications using the *D. geminata*-specific primers were unsuccessful. Where the *D. geminata*-specific primers were successful, each amplification product was sequenced and shown to be *D. geminata*. This blind test survey showed that the *D. geminata* -specific short primers uniquely identified *D. geminata*.

In a second similar survey, 22 global environmental samples were surveyed using the same protocols (Figure 15B). All samples thought to contain *D. geminata* sp. were strongly positive with the short *D. geminata*-specific primer set. This indicates that the custom designed *D. geminata*-specific primers appear globally specific to *D. geminata*.

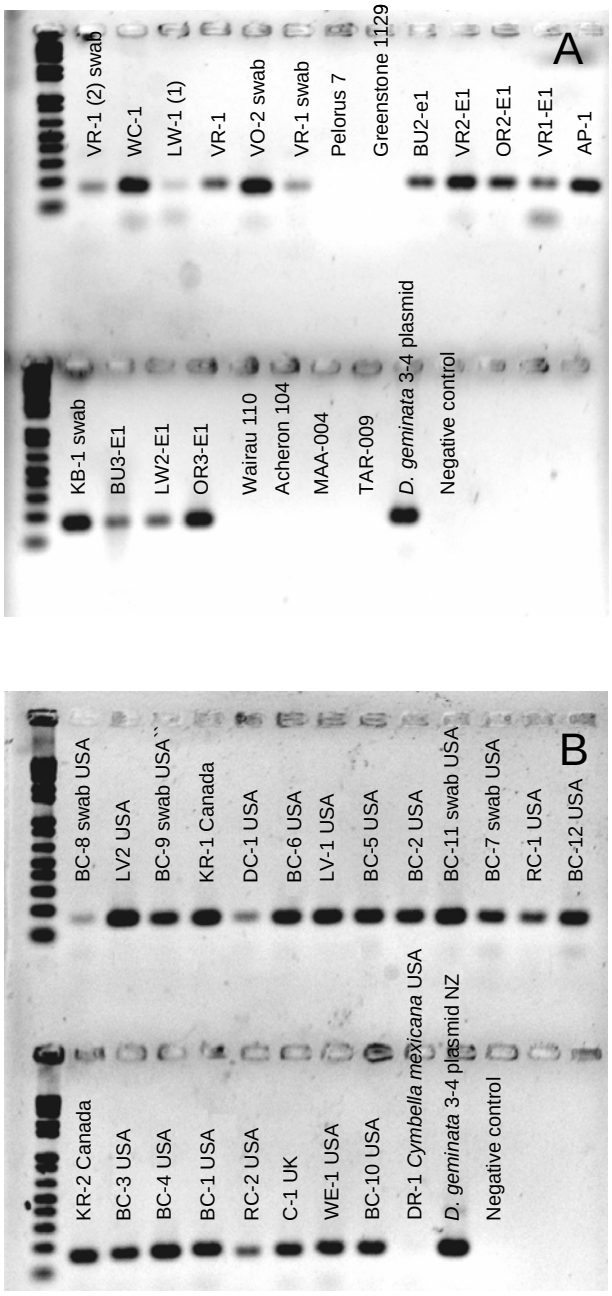

Figure 15. Blind test survey of miscellaneous environmental samples from (A) within New Zealand and (B) outside New Zealand whose *D. geminata* status was not known *a priori*. Samples used in these surveys are shaded in Appendix 5.

Conclusions

The primary objective of this study was to develop a highly sensitive detection and enumeration protocol to aid in the early detection of *D. geminata*. However, an additional intent was to use *D. geminata* as a model incursion to evaluate the feasibility of using molecular genetic approaches for early incursion surveillance of microorganisms in general. The results presented above represent the first phase of this ongoing study where we have designed robust and inexpensive sampling and DNA extraction protocols, sequenced the 18S rDNA of *D. geminata,* and designed, tested, and validated a highly sensitive PCR-based detection capability. These techniques are completely amenable to Real Time Quantitative PCR for direct enumeration (Phase 2a, b) and ultimately for the high throughput needs of a national surveillance programme. This entire process took six months to achieve and yet could have been considerably quicker had the needed DNA sequence data been available. If these new genetic tools were to become part of the arsenal of future incursion responses, it would be fitting to identify possible threat species and attain the needed data prior to an incursion.

1. Developed protocol for collection and stabilization of environmental samples

The validation process on all DNA extracts have shown that samples stabilised in CTAB and 70% ethanol yielded high quality DNA. Cells appear to be well stabilised, producing DNA of excellent quality and abundance as determined by DNA quantitation, DGGE, and sequencing. Direct immersion of field samples into the non-toxic CTAB buffer or 70% ethanol is therefore an effective tool for stabilisation of environmental periphyton samples. The DNA preserved in this fashion was stable for at least 5 weeks at room temperature making this method amenable to deep-field and worldwide collections. The overall yield inconsistencies seen in the longevity experiment will be further investigated. The methods are inexpensive and rapid, thus ideal for the high throughput requirements of a national surveillance programme.

2. Developed protocol for extraction of DNA from D. geminata

Our extraction procedures have worked consistently and reliably on all samples, with only a few that delivered very low DNA yields. Both ethanol and CTAB are suitable preservatives, and are not obviously affected by storage at ambient temperatures up to 26^{o}C for 5 weeks. Considering the short time that experimentation has been underway, we feel that we have made remarkable progress. Some challenges remain, especially in the area of DNA recovery at high cell densities. However, this was not the objective of this contract where we were commissioned to develop a low level detection capability. With both the collection and extraction protocols developed for *D. geminata,* the foundation was set for an extremely successful programme of research into reliable early detection.

3. Designed and validated primers for low level detection of D. geminata

We successfully sequenced the full 18S rRNA genes from *D. geminata* and two other biraphid diatoms from New Zealand and constructed a phylogenetic tree of relatedness with other known biraphid diatoms. These analyses provided the needed evidence that the 18S rRNA contained the required sequence heterogeneity from which *D. geminata* specific PCR

primers could be designed. Seven *D. geminata*- specific PCR primers were designed and tested against *D. geminata* template for specificity and robustness. Two pairs were selected for further evaluation. Repeated blind testing against environmental samples using DGGE and sequence analysis has validated both primer sets, and shown them to be highly specific for *D. geminata*. The shorter of the two primer sets was found to be the most sensitive at detecting *D. geminata* in preliminary tests. This same primer set was further shown to amplify *D. geminata* from all foreign samples currently in our possession, making the developed tool of international importance.

Having the ability to detect and monitor the very early phases of an incursion by a microorganism provides substantial benefits to any response effort. In the case of *D. geminata,* early detection in unaffected rivers will 1) enable better targeting of public awareness messages to urge freshwater users to Check Clean Dry to slow its spread, 2) increase the probability of successful control, should a control tool be developed, and 3) provide robust and simple sampling protocols using water samples compared to benthic scrapings that are easier for surveillance staff to perform. The genetic tools being developed for *D. geminata* are amenable to a high throughput capability that will dramatically increase our ability to analyze large numbers of samples in a short period of time, thus increasing the efficiency of any surveillance programme without greatly increasing costs. In addition, developing these basic genomic protocols will facilitate further studies on genetic diversity and phylogeography of *D. geminata* that could shed light on the number of introductions that have occurred in New Zealand and identify the country of origin.

Our research has shown that amplification of genetic material from a very small initial quantity offers the potential for the earliest possible detection of *D. geminata*. In a world-wide first, we have identified its unique genetic fingerprint, developed a field collection technique that can collect drifting algal cells, and developed laboratory procedures that can reliably identify and amplify genetic material of *D. geminata* uniquely, distinguishing it from related diatoms.

General recommendations

Our central goal in this project is to develop an efficient and cost-effective detection capability for *D. geminata* that can be easily incorporated into existing monitoring efforts in the laboratory and field. We believe that the approaches taken for *D. geminata* are directly applicable to any microorganism that is an incursion threat to New Zealand. Our work during Phase I of the project provided valuable insight into *D. geminata* and how this same genetic-based detection strategy might be employed on other potential micro-organism threats. From this initial research we see the need and opportunity to develop capabilities that would have broad reaching application for both the current *D. geminata* incursion and the possible incursion of other harmful microorganisms in the future. We suggest that there is a substantial need for:

1. **A high-resolution low-level enumeration capability.** In Phase 1, we have developed a highly specific genetic-based detection capability for *D. geminata* . We have validated this approach with both laboratory and field applications. We now have the ability to advance this approach to be able to both detect and to determine the actual number for cells present in a sample with an even greater degree of sensitivity. Through the use of quantitative real-time PCR (QRT-PCR) methodologies we have the ability to increase the sensitivity of the detection and enumerate to counts as low as 1 cell/ml. This can be achieved in two steps: 1) development of a relative enumeration capability to accurately compare samples, 2) development of a full quantification capability capable of delivering

actual cell numbers. Taking the method to the level of enumeration will require using an internal standard to control for extraction and amplification efficiency. Such a detection/enumeration tool will be essential for detecting and monitoring new incursions and assisting in monitoring the results of any mitigation effort.

2. **A national high throughput analytical capability.** We envisage that a comprehensive monitoring effort will include both field and laboratory-based components. The recent study on monitoring methods (Kilroy and Dale 2006) suggests that the use of drift nets would best sample rivers where *D. geminata* is in low concentration. As described in this report, this method of cell enrichment is perfectly suited for these genetic-based detection tools. The laboratory high-throughput capability would provide 24-hour turn around and would be used for routine monitoring and surveillance efforts. The field detection capability would be used to target high risk areas (e.g. those frequently fished or adjacent to affected streams), affected streams, spot checking of hot spots and for rapid incursion response. In addition, the genetic detection tools would also be well placed to monitor the short and long term effects of any mitigation effort. Such a capability would require a substantial initial investment in instrumentation and personnel training. Once in place the new facility would be able to rapidly take on pre-incursion monitoring on any new threat species and could easily be incorporated into current surveillance efforts.

3. **To determine the origin and frequency of *D. geminata* incursions.** Efforts should continue to be undertaken to investigate the genetic variability of *D. geminata* in New Zealand. The rationale for wanting to know more about *D. geminata* genetic variation within the NZ populations is three-fold: a) it may help determine the geographical origin of the population and whether there were multiple introductions from different locations, which in turn may help b) determine the pathway by which the organism was likely introduced so that we can analyse strengthening certain border controls to reduce further introductions and c) investigate potential sources of effective biocontrol agents.

4. **For pre-incursion awareness and response.** The design and use of these genetic detection techniques on new incursion species requires specific genetic information be known about the organism. Often, this information is not available and needs to be obtained from questionable samples, thus delaying development and implementation of these detection tools. Developing a pre-incursion database of possible threat species with the needed basic genetic information would provide a substantial head start for the design of genetic-based detection tools should the need arise.

5. **To develop multi-species detection capabilities**. The possible introduction of harmful microorganisms to New Zealand by land or sea is a continuous threat. Should support be available, one might envision multi-species micro-organism gene "chips" being developed from a pre-incursion database and used for routine monitoring and surveillance. These gene chips are now available in a cost-effective re-useable format that would provide the same detection sensitivity but for a range of target organisms. Such a chip could be customized to meet the current needs of a range of stakeholders requiring this capability. Once in place, the chips can be easily adapted as new target organisms present themselves. The technology in currently in place and affordable.

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Appendix 1. A primer on gene amplification (PCR) methodologies.

Approaches

Below is a brief primer on PCR and overviews of the methodological issues facing each of the milestones achieved in Phase 1. With our ultimate objective being to take the protocols to a high-resolution, high throughput, and quantitative level, each process, from collection to DNA amplification, must be extremely efficient, well controlled and highly validated. Hence, from the beginning of the program we have sought to develop each protocol with the highest quality assurance possible.

1. Polymerase Chain Reaction (PCR)

In this section, we will provide some fundamental information about the polymerase chain reaction (PCR) methodology and quantitative real time-PCR (QRT-PCR). While this knowledge may seem basic to some reviewers, we feel it is crucial to explain this technology for readers who may not have experience in the field of molecular biology. PCR is a method of amplifying specific fragments of DNA by primer extension. Analogous to "finding a needle in a haystack", the polymerase chain reaction "makes a haystack of needles". Each cycle of the reaction doubles the target sequence concentration, so that the fragment of interest can be amplified over a million fold after 30 cycles of the reaction. The particular stretch of DNA to be amplified is identified by a pair of DNA primers, or short oligonucleotides. Primers are complementary to unique stretches of DNA sequence and can be designed with different levels of specificity. Primers may be designed to amplify a target DNA sequence from a group of organisms, such as all eukaryotes or prokaryotes, or they may be specific to a class of organisms, such as the dinoflagellates or diatoms, or to a single species or strain. PCR is often used as a diagnostic assay. With extensive optimization amplification will occur only if the DNA from the organism is present in the reaction mix.

PCR reactions cycle between three steps, each at a defined temperature to achieve (i) dissociation of the DNA template, (ii) annealing of the primers to the template, and (iii) extension of the primers by the polymerase enzyme. The use of a thermostable polymerase enzyme allows the dissociation of the double stranded DNA and subsequent annealing or hybridisation of primers to the target sequence in a single tube with minimal loss of enzymatic activity (Mullis and Faloona, 1987). Conventional PCR typically requires about two hours to complete, and the amplification products are then analyzed by gel electrophoresis. The entire reaction is carried out in a thermal cycler, designed to cycle through and maintain very precise temperatures for each step in the reaction. Thermal cyclers are available with the capacity to run up to 384 reactions at a time, making this technology ideal for high-throughput diagnostic analysis of environmental samples.

2. Develop protocols for the collection and stabilization of environmental samples

D. geminata is a stalked diatom that forms dense, persistent mats and trailing strands attached to many different types of substrate, such as rocks and branches. Cells can also exist without stalks adpressed to substrates. A field collection survey was developed to sample visible colonies and to test surface substrates, using scraping and swabs of rocks. Techniques for sampling cells in the water column while excluding floating debris were also developed and tested.

The longevity and stability of DNA in transport and storage can often be problematic because DNA will often degrade if proper storage procedures are not followed. The specific requirements of DNA from *D. geminata* were unknown. Preferably, samples should be refrigerated or frozen as soon as possible in conjunction with stabilisation, and some

investigations have resorted to transport in liquid nitrogen to overcome loss of DNA stability. Unfortunately these processes are often impractical because of logistical constraints. Traditional preservative methods have included Lugol's fixative or 2% formaldehyde, but their inhibitory side effects for PCR make them impractical methods of stabilisation in this context. We therefore investigated alternative techniques of stabilisation for field samples of *D. geminata* samples over time for transport and short-term storage. There are many approaches that are available as possible alternatives for stabilisation, for example, direct sample immersion into reagents such as concentrated salt solution or a DNA extraction buffer. Both of these methods have been tested successfully on environmental samples in the field with exceptional results (see Coyne *et al*., 2001).

3. Develop protocols for extracting DNA from *D. geminata*

DNA extraction from environmental samples can provide a separate set of challenges. Co-precipitating substances such as polysaccharides or humic acids have been found to inhibit subsequent molecular applications. We have been very successful in extracting high quality DNA from environmental water and sediment samples using a CTAB protocol and used this method as our initial method for *D. geminata* DNA extraction and amplification. The method has been successfully used 24 for extracting DNA from other highly mucilaginous algal species (Raphidophyceae, see Coyne et al., 2005) and we were confident that it could be adapted to *D. geminata*. It also has the advantage in that it can be adapted to a standard 96-sample capacity to increase overall throughput.

4. Design and validate primers for low-level detection of *D. geminata*

Our aim was to design and validate PCR primers specific for *D. geminata*. We have extensive experience in designing PCR primers to assess the presence of bacterial and eukaryotic organisms in environmental samples at the strain, species, genus and class level. From this experience, we have developed very specific guidelines for the design of PCR primers for environmental targets. For the *D. geminata* PCR method, genus- or species-specific PCR primers were designed for the 18S ribosomal RNA (rRNA) gene sequence. This gene contains the necessary variability to distinguish between species, such as *D. geminata* and the other indigenous species. However, if strain-level detection were necessary, primers could easily be designed for the intergenic transcribed sequence (ITS) of the ribosomal operon. Both of these areas of the genome have conserved and variable regions, and are ideal for phylogenetic analysis or diagnostic PCR. In addition, algal species typically have tens to hundreds of copies of the ribosomal operon (Saito *et al*., 2002; Stryer, 1995). Because of this, we routinely are able to achieve amplification from DNA that has been diluted to less than 1 cell equivalent in the reaction mix, with a final sensitivity of detection on the order of 10 cells of target species per litre of environmental water (Coyne et al., 2001).

5. Denaturing Gradient Gel Electrophoresis (DGGE)

Since *D. geminata* has not been successfully cultured and the 18S rRNA gene had not been sequenced, a suite of culture-independent molecular approaches were to be used to obtain and verify the *D. geminata* rRNA gene sequence directly from environmental samples. This approach includes identification of clean samples of *D. geminata* and other related indigenous diatoms by denaturing gradient gel electrophoresis (DGGE), cloning and sequencing of the *D. geminata* 18S rRNA gene. This will be followed by design and testing of *D. geminata* species-specific primers for PCR amplification of the full-length gene from environmental samples. DGGE is used extensively in environmental community analysis to provide DNA "fingerprints" of eukaryotic and prokaryotic communities. DGGE separates

DNA fragments based on minor differences in sequence providing an overview of the dominant organisms in a given sample. In this process, a variable region within the 18S rDNA gene is amplified by PCR and fractionated by gel electrophoresis through a gradient of increasing chemical denaturant, producing a banding pattern where each band represents a unique species. Banding patterns produced by samples from multiple locations can be compared to identify the organism of interest. Bands may then be excised and sequenced. We routinely use DGGE as a tool to "fish" out short fragments of genes from organisms that have never been cultured. These small fragments can then be used to design PCR primers which, when paired with universal primers, allow us to amplify and retrieve the entire 18S rRNA gene. As the initial step, the DGGE method searches through many samples for those enriched in a given organism and free from other contaminating species. These enriched samples can then be cloned and the target organism more easily identified and sequenced. Prior to this project we developed a diatom-specific DGGE capability that will allow us to examine the diatom community within environmental samples. This provides a quick assessment of the presence of *D. geminata* and the structure and complexity of the diatom community in any environmental sample.

Once the target gene sequence is validated, *D. geminata* specific primers will be further evaluated for specificity and sensitivity of detection. Ground truthing and validation will be carried out with artificial and natural communities spiked with *D. geminata* DNA.

Appendix 2. Method of making CTAB (cetyl trimethyl ammonium bromide) extraction buffer.

CTAB (cetyl trimethyl ammonium bromide) buffer is also known as hexadecyl trimethyl ammonium bromide, and comprises 100 mM tris-HCL (pH 8.0), 1.4 M NaCl, 2% (w/v) cetyltrimethylammonium bromide (CTAB), 0.4%(v/v) ßmercaptoethanol, 1% (w/v) polyvinylpyrollidone, 20 mM EDTA (Dempster et al. 1999).

Method

Add 70 ml of 5M NaCl (pH 8.0), 10 ml 0.5M EDTA, and 12.5 ml 2M Tris (pH 8.0), in a glass Schott bottle. Add 5 g of CTAB gradually, if necessary heat solution to 50°C while stirring. Make up solution gradually to 250 ml with distilled water. Allow the solution to dissolve for at least 30 minutes, then autoclave.

Appendix 3. The full 18S rRNA nucleotide sequence of *D. geminata* from Redcliff Creek, Lower Waiau, Manapouri-Blackmount Road. Sample code: CONTROL (see Appendix 5 for details).

5'TTCTGCCAGTAGTCATACGCTCGTCTCAAAGATTAAGCCATGCATGTCTAAGTGTAAATATACAA
TTGTGAAACTGCGAATGGCTCATTATATCAGTTATAGTTTATTTGATAGTCCCTTACTACTTGGATA
ACCGTAGTAATTCTAGAGCTAATACTTGCATAAATATCCCTTCAAGGATAGTATTTATTAGATTTAA
ACCAACCCCTTCGGGGTGATGTGGTGATTCAAAATAAGTTTACGGATCGCATGGCTTTGCCGGCGA
CGGATCATTCAAGTTTCTGCCCTATCAGCTTTGGATGGTAGGGTATTGGCCTACCATGGCTTTAACG
GGTAACGGAGGATTAGGGTTTGATTCCGGAGAGGGAGCCTGAGAGACGGCTACCACATCCAAGGA
AGGCAGCAGGCGCGTAAATTACCCAATCCTGACACAGGGAGGTAGTGACAATAAATAACAATGCC
GAGCCCTTGTGGTTTGGCACTTGGAATGAGAACAACTCAAACCACTTAACGAGGATCAATTGGAGG
GCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTYGTTGCAGTT
AAAAAGCTCGTAGTTGGATTTGTGATGGAATTTGAATACTTTTAAAGTGTTTCAGAAACTGTCATCC
GTGGGTGGAATTTGTTTGGCATTAGGTTGTCAGRCAGAGGATGCCTATMCTTTACTGTGAAAAAAT
CAGTGCGTTCAAAGCAGACTTACGTCGATGAATGTATTAGCATGGAATAATAAGATAGGACCTTTG
TACTATTTTGTTGGTTTGTGTATAGAGGTAATGATTAAAAGGAACAGTTGGGGGTATTTGTATTCCA
TTGTCAGAGGTGAAATTCTTGGATTTTTGGAAGACAAACTACTGCGAARGCATTTACCAAGGATGT
TTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGATGATTAGATACCATCGTAGTCTTAACCAT
AAACTATGCCAACAAGGGATTGGTGGGGTTTCGTAATGTCCCCATCAGCACCTTAGGAGAAATCAA
AAGTTTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGGAAGGGC
ACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTTACCAGGTCCAGACA
TAGTGAGGATTGACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAG
TTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCGCTGCCTGCTAAATAGACTAG
TTAGTGATTTTCACTGATCRGGTCTTCTTAGAGGGACGTGCATTTTATTAGATGCAGGAAGATAGCG
GCAATAACAGGTCTGTGATGCCCTTAGATGATCTGGGCCGCACGCGCGCTACACTGATGTACTCAA
CGAGTTTTTCCTTGGCTGAGAAGCCTGGGTAATCTTTTAAACTTGCATCGTGATAGGGATAGATTAT
TGCAATTATTAATCTTGAACGAGGAATTCCTAGTAAACGCAGATCATCAGTCTGCATTGATTACGT
CCCTGCCCTTTGTACACACCGCCCGTCGCACCTACCGATTGAATGGTCCGGTGAAGGCTCGGGATT
GCAGCTAATGCCTTTACTGGTGTTGGTTTCAAGAACTTGTCTAAACCTTATCATTTAGAGGAAGGTG
AAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCAA 3'

Appendix 4. The full 18S rRNA nucleotide sequence for New Zealand *Gomphoneis minuta* var *cassiae* from Stony River, Taranaki (TAR009; Appendix 5).

5'TCAGGAACAGCTATGACCATGATTACGCCAAGCTCAGAATTAACCCTCACTAAAGGGACTAGTC
CTGCAGGTTTAAACGAATTCGCCCTTAACCTGGTTGATCCTGCCAGTAGTCATACGCTCGTCTCAAA
GATTAAGCCATGCACGTCTAAGTATAAATATATTACTGTGAAACTGCGAATGGCTCATTATATCAG
TTATAGTTTATTTGATAATCCCTTACTACTTGGATAATCGTAGTAATTCTAGAGCTAATACATGCAA
TAATACCCTTCTCGGGTAGTATTTATTAGATTTAAACCAACCCCTTTGGGGTGATGTGGTGATTCAT
AATAATTTTGCGAATCGCATGACCTAGTCGGCGATGGATCATTCAAGTTTCTGCCCTATCAGCTTTG
GATGGTAGGGTATTGGCCTACCATGGCTTTAACGGGTAACGGAGGATTAGGGTTTGATTCCGGAGA
GGGAGCCTGAGAGACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGTAAATTACCCAATCCTGA
TACAGGGAGGTAGTGACAATAAATAACAATGCCGGGCCTTTCAGGTCTGGCACTTGGAATGAGAA
CAACTCAAACCACTTATCGAGGATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCC
AGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTGTGATGATGCTT
GGGAGCCTCTTTTGGATTCCTTTACATTGTCATCTTTGGGAGGATTTTGTGTGGCATTAGCTTGTTGC
ATAGGGGATACTCAACGTTTACTGTGAAAAAATCAGCGCGTTCAAAGCAAGCTTATGCTGTGAATG
TATTAGCATGGAATAATAAGATAGGACCTTGGTACTATTTTGTTGGTTTGTGCACCGAGGTAATGA
TTAATAGGGATAGTTGGGGGTATTCGTATTCCATTGTCAGAGGTGAAATTCTTGGATTTTTGGAAG
ACGAACTACTGCGAAAGCATTTACCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCG
AAGATGATTAGATACCATCGTAGTCTTAACCATAAACTATGCCGACAAGGGATTGGTAGAGTTTCG
TAATGTCTCTATCAGCACCTTAGGAGAAATCATAAGTCTTTGGGTTCCGGGGGGAGTATGGTCGCA
AGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGA
CTCAACACGGGGAAACTTACCAGGTCCAGACATAGTGAGGATTGACAGATTGAGAGCTCTTTCTTG
ATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAA
CGAACGAGACCGCTGCCTGCTAAATAGCTCGGCTAGTGATTTTCACTGGCTTGAGCTTCTTAGAGG
GACGTGCATAATTTTAAATGCAGGAAGATAGCGGCAATAACAGGTCTGTGATGCCCTTAGATGTTC
TGGGCCGCACGCGCGCTACACTGATGTGTTCAAACGAGTTTTTCCTTGGCTGAGAAGCCTGGGCAA
TCTTTTGAACTCACATCGTGATAGGGATAGATTATTGCAATTATTGATCTTGAACGAGGAATTCCTA
GTAGACGCAAATCATCAATTTGCGTCGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCACCT
ACCGATTGAATGGTCCGGTGAAGACTCGGGATTGTGAGTGTTGCCTTTGCTGGTGATGTTTGCAAG
AACTTGTCTAAACCTTATCATTTAGAGGAAGGTGAAGTCGTAAACAAGGTTTCCGTAGGTGAACCT
GCAGAAT-3'

Appendix 5. Sample inventory for *D. geminata* and related species in the North and South Islands, New Zealand. A ? in the Genera column indicates that the presence of *D. geminata* in these samples has yet to be confirmed. A blank cell in the DNA column indicates that DNA extraction has not been attempted on these samples yet. Grey shaded rows indicate the 64 samples used in the blind test.

Appendix 5.

Country	River	Code	Date collected	Site	Taxon	Collector	DNA (ng/µL)	Northing/ Latitude	Easting/ Longitude	Sample type	order
NZ	Manganui-a-te-ao Rive	MAA001_1	28/12/2005	Orautaha, Meyers B	Gomphoneis	B Hicks	28.6	6207248	2703209	Benthic	1
NZ	Manganui-a-te-ao Rive	MAA003	28/12/2005	Orautaha, Meyers B	Gomphoneis	B Hicks	265.2	6207248	2703209	Benthic	2
NZ	Manganui-a-te-ao Rive	MAA004	28/12/2005	Orautaha, Meyers B	Gomphoneis	B Hicks	65.6	6207248	2703209	Benthic	3
NZ	Manganui-a-te-ao Rive	MAA005	28/12/2005	Orautaha, Meyers B	Gomphoneis	B Hicks	83.7	6207248	2703209	Benthic	4
NZ	Mangawhero Stream	MAG001	29/12/2005	Opposite Powderho	No didymo	B Hicks	82.2	6197550	2717765	Benthic	5
NZ	Waionganaiti Stream	TAR001	2/01/2006	Inglewood, Hursthou	No didymo	B Hicks	128.7	6228315	2614400	Benthic	6
NZ	Waionganaiti Stream	TAR002	2/01/2006	Inglewood, Hursthou	No didymo	B Hicks	98.0	6228315	2614400	Benthic	7
NZ	Waiongana Stream	TAR003	2/01/2006	Bedford Road	Gomphoneis	B Hicks	67.5	6224505	2611570	Benthic	8
NZ	Waiongana Stream	TAR004	2/01/2006	Bedford Road	No didymo	B Hicks	28.4	6224505	2611570	Benthic	9
NZ	Waiwhakaiho River	TAR005	2/01/2006	Peters Road (Leppe	Gomphoneis	B Hicks	0.0	6222815	2607860	Benthic	10
NZ	Waiwhakaiho River	TAR006	2/01/2006	Meeting of the Wate	Gomphoneis	B Hicks	14.1	6232929	2606594	Benthic	11
NZ	Kaihihi Stream	TAR007	2/01/2006	Okato	Gomphoneis	B Hicks	121.4	6223091	2585741	Benthic	12
NZ	Kaihihi Stream	TAR008	2/01/2006	Okato	Gomphoneis	B Hicks	19.2	6223091	2585741	Benthic	13
NZ	Stony River	TAR009	2/01/2006	Wiremu Road	Gomphoneis	B Hicks	22.8	6218320	2588897	Benthic	14
NZ	Te Henui Stream	TAR010	2/01/2006		Gomphoneis	B Hicks	197.7	6236716	2605002	Benthic	15
NZ	Te Henui Stream	TAR011	2/01/2006		No didymo	B Hicks		6236716	2605002	Benthic	16
NZ	Whakapapa River	WHA001	29/12/2005	Owhango	Gomphoneis	B Hicks	203.9	6242470	2717811	Benthic	17
NZ	Whakapapa River	WHA002	29/12/2005	Owhango	Gomphoneis	B Hicks	54.1	6242470	2717811	Benthic	18
NZ	Whakapapa River	WHA003	29/12/2005	Owhango	Gomphoneis	B Hicks	37.6	6242470	2717811	Benthic	19
NZ	Whangaehu River	WHE001	5-Feb-06	200 m upstream SH	No didymo	B Hicks		6190315	2731755	Benthic	20
NZ	Tokiahuru Stream	TOK001	5-Feb-06	75 m downstream S	No didymo	B Hicks	3.6	6191450	2728410	Benthic	21
NZ	Mangles River	MANGLES48	7-Feb-06		Gomphoneis	NIWA	62.7	5934400	2459200	Benthic	22
NZ	Lower Greenstone Riv	GREENSTONE1129	1-Feb-06	Carpark	Gomphoneis	NIWA	1056.4	5575586	2142726	Benthic	23
NZ	Acheron River	ACHERON104	1-Feb-06	Cableway	Gomphoneis	NIWA	191.2	5869899	2507092	Benthic	24
NZ	Rai River	RAIFALLS12	31-Jan-06	Rai Falls	No didymo	NIWA	85.6	5990942	2559017	Benthic	25
NZ	Riwaka River	RIWAKA4	31-Jan-06	Rugby Ground SH6	Gomphoneis	NIWA	89.7	6015645	2508739	Benthic	26
NZ	Manuherikia River	MANUHERIKIA1427	1-Feb-06	Alexandra Motor Ca	Gomphoneis	NIWA	56.3	5544775	2227679	Benthic	27
NZ	Wairau River	WAIRAU110	31-Jan-06	Dip Flat	Gomphoneis	NIWA	108.4	5925767	2503564	Benthic	28
NZ	Pelorus River	PELORUS7	1-Feb-06	Fishermans Flat	Gomphoneis	NIWA	54.64	5991520	2569697	Benthic	29
NZ	Rangitukia Stream	RAS005_2	24-Nov-05	Site B below road cu	Gomphoneis	B Hicks	2.1	6359915	2696850	Benthic	30
NZ	Lower Waiau	CONTROL	Dec-05	Redcliff Creek, Man	Didmyo	Bill Jarvie, Southland Fa		5487850	2093910	Benthic	31
NZ	Maraoa River	WC-1 swab	28-Feb-06	Wash Creek	Didmyo	Craig Cary	4505.7	5513795	2123057	Benthic swab	32
NZ	Maraoa River	WC-1	28-Feb-06	Wash Creek	Didmyo	Craig Cary	572.4	5513795	2123057	Benthic	33
NZ	Maraoa River	KB-1	28-Feb-06	Kiwi Burn	Didmyo	Craig Cary	2.6	5528507	2128179	Benthic	34
NZ	Maraoa River	KB-1 swab	28-Feb-06	Kiwi Burn	Didmyo	Craig Cary	89.1	5528507	2128179	Benthic swab	35
NZ	Von River	LW-1 (1)	28-Feb-06	Von Terminus,	Didmyo	Craig Cary	58.6	5559016	2150362	Benthic	36
NZ	Von River	LW-1 (2)	28-Feb-06	Von Terminus,	Didmyo	Craig Cary	47.3	5559016	2150362	Benthic	37
NZ	Upper Oreti River	UO-1 swab	28-Feb-06	Windley access	Didmyo	Craig Cary	93.1	5508237	2130481	Benthic swab	38
NZ	Upper Oreti River	UO-1	28-Feb-06	Windley access	Didmyo	Craig Cary	54.7	5508237	2130481	Benthic	39
NZ	Upper Oreti River	UO-2 swab	28-Feb-06	Windley access	Didmyo	Craig Cary	92.7	5508237	2130481	Benthic swab	40
NZ	Upper Oreti River	UO-3	28-Feb-06	Mt. Nicholas bridge	Didmyo	Craig Cary	261.9	5531281	2134470	Benthic	41
NZ	Upper Oreti River	UO-3 swab	28-Feb-06	Mt. Nicholas bridge	Didmyo	Craig Cary	4.5	5531281	2134470	Benthic swab	42
NZ	Upper Oreti River	UO-4	28-Feb-06	Mt. Nicholas bridge	Didmyo	Craig Cary	309.8	5531281	2134470	Benthic	43
NZ	Maraoa River	SB-1 swab (1)	28-Feb-06	Station bridge	Didmyo	Craig Cary	13.6	5510798	2117636	Benthic swab	44
NZ	Maraoa River	SB-1 swab (2)	28-Feb-06	Station bridge	Didmyo	Craig Cary	50.4	5510798	2117636	Benthic swab	45
NZ	Maraoa River	SB-1	28-Feb-06	Station bridge	Didmyo	Craig Cary	73.8	5510798	2117636	Benthic	46
NZ	Von River	VR-1	28-Feb-06	Von River	Didmyo	Craig Cary	238.2	5553567	2145501	Benthic	47
NZ	Von River	VR-1 (1) swab	28-Feb-06	Von River	Didmyo	Craig Cary	80.5	5553567	2145501	Benthic swab	48
NZ	Von River	VR-1 (2) swab	28-Feb-06	Von River	Didmyo	Craig Cary	385.9	5553567	2145501	Benthic swab	49
NZ	Von River	VR-2 swab	28-Feb-06	Von River	Didmyo	Craig Cary	19.4	5553567	2145501	Benthic swab	50
NZ	Hawea River	HR-1 (1)	11-Mar-06	Hawea River	Didmyo	Stu	144.1	5608213	2209119	Benthic	51
NZ	Hawea River	HR-1 (2)	11-Mar-06	Hawea River	Didmyo	Stu	422.7	5608213	2209119	Benthic	52
NZ	Hawea River	HR-1 Swab	11-Mar-06	Hawea River	Didmyo	Stu	18.1	5608213	2209119	Benthic swab	53
NZ	Waitaki River	WA-1	7-Apr-06	Waitaki	Didmyo	Stu Sutherlar	86.09	5589100	2341291	Benthic	54
NZ	Waitaki River	WA-2	25-Apr-06	Waitaki	Didmyo	Stu Sutherlar	406.54	5589100	2341291	Benthic	55
NZ	Waitaki River	WA-2 swab	25-Apr-06	Waitaki	Didmyo	Stu Sutherlar	130.44	5589100	2341291	Benthic swab	56
NZ	Aparima River	AP-1	27-Mar-06	Aparima River	Didmyo	Stu Sutherlar	40.57	5485471	2130293	Benthic	57
NZ	Aparima River	AP-2	27-Mar-06	Aparima River	?	Stu Sutherlar	149.65	5485471	2130293	Benthic	58
NZ	Aparima River	AP-2 swab	27-Mar-06	Aparima River	?	Stu Sutherlar	129.69	5485471	2130293	Benthic swab	59
NZ	Ahuriri River	AH-1	6-Apr-06	SH8 road bridge	?	Cathy Kilroy		5633415	2269962	Site 3, Benthic	60

Appendix 5.

Country	River	Code	Date collected	Site	Taxon	Collector	DNA (ng/µL)	Northing/ Latitude	Easting/ Longitude	Sample type	order
NZ	Ahuriri River	AH-2	5-Apr-06	Glenburn Station, d/	?	Cathy Kilroy		5630294	2276144	Site 1 Benthic	61
NZ	Ahuriri River	AH-3	6-Apr-06	SH8 road bridge	?	Cathy Kilroy		5633415	2269962	Site 3, 10 minu	62
NZ	Ahuriri River	AH-4	6-Apr-06	Gorge WL recordinç	?	Cathy Kilroy		5631842	2249975	Site 5, Drift 10	63
NZ	Ahuriri River	AH-5	6-Apr-06	u/s Gorge bridge	?	Cathy Kilroy		5634611	2247706	Site 6, 1 min w	64
NZ	Ahuriri River	AH-6	5-Apr-06	Glenburn Station, d/	?	Cathy Kilroy		5630294	2276144	Site 1, 10 min v	65
NZ	Ahuriri River	AH-7	6-Apr-06	Glenburn Station, u/	?	Cathy Kilroy		5632703	2274669	Site 2, 10 minu	66
NZ	Ahuriri River	AH-8	6-Apr-06	SH8 road bridge	?	Cathy Kilroy		5633415	2269962	Site 3, 1 min w	67
NZ	Ahuriri River	AH-9	6-Apr-06	Gorge WL recordinç	?	Cathy Kilroy		5631842	2249975	Site 5, 10 L filte	68
NZ	Ahuriri River	AH-10	6-Apr-06	u/s Gorge bridge	?	Cathy Kilroy		5634611	2247706	Site 6, 10 sec v	69
NZ	Ahuriri River	AH-11	5-Apr-06	Glenburn Station, d/	?	Cathy Kilroy		5630294	2276144	Site 1, 1 min w	70
NZ	Ahuriri River	AH-12	6-Apr-06	Glenburn Station, u/	?	Cathy Kilroy		5632703	2274669	Site 2, 1 min w	71
NZ	Ahuriri River	AH-13	6-Apr-06	SH8 road bridge	?	Cathy Kilroy		5633415	2269962	Site 3, Water 1	72
NZ	Ahuriri River	AH-14	6-Apr-06	Opposite Killermont	?	Cathy Kilroy		5627749	2255902	Site 4, 10 sec v	73
NZ	Ahuriri River	AH-15	6-Apr-06	u/s Gorge bridge	?	Cathy Kilroy		5634611	2247706	Site 6, 10L wat	74
NZ	Ahuriri River	AH-16	5-Apr-06	Glenburn Station, d/	?	Cathy Kilroy		5630294	2276144	Site 1, 10 dec v	75
NZ	Ahuriri River	AH-17	6-Apr-06	Glenburn Station, u/	?	Cathy Kilroy		5632703	2274669	Site 2, Water 1	76
NZ	Ahuriri River	AH-18	6-Apr-06	SH8 road bridge	?	Cathy Kilroy		5633415	2269962	Site 3, 10L Wa	77
NZ	Ahuriri River	AH-19	6-Apr-06	Opposite Killermont	?	Cathy Kilroy		5627749	2255902	Site 4, 10L drift	78
NZ	Ahuriri River	AH-20	6-Apr-06	u/s Gorge bridge	?	Cathy Kilroy		5634611	2247706	Site 6, 10L filte	79
NZ	Ahuriri River	AH-21	6-Apr-06	Glenburn Station, u/	?	Cathy Kilroy		5632703	2274669	Site 2, 10L Wa	80
NZ	Ahuriri River	AH-22	5-Apr-06	Glenburn Station, d/	?	Cathy Kilroy		5630294	2276144	Site 1, 10L Wa	81
NZ	Ahuriri River	AH-23	6-Apr-06	SH8 road bridge	?	Cathy Kilroy		5633415	2269962	Site 3, 10L filte	82
NZ	Ahuriri River	AH-24	6-Apr-06	Opposite Killermont	?	Cathy Kilroy		5627749	2255902	Site 4, 10L filte	83
NZ	Ahuriri River	AH-25	6-Apr-06	Gorge WL recordinç	?	Cathy Kilroy		5631842	2249975	Site 5, 10 sec d	84
NZ	Ahuriri River	AH-26	5-Apr-06	Glenburn Station, d/	Cymbella sp.	Cathy Kilroy	111.06	5630294	2276144	Site 1, T3 St.5	85
NZ	Ahuriri River	AH-27	5-Apr-06	Glenburn Station, d/	?	Cathy Kilroy		5630294	2276144	Site 1, T5 St. 2	86
NZ	Aparima River	AP-3	7-Apr-06	Access 600 m N of	?	Cathy Kilroy		5458942	2131626	Site 1, 10 sec d	87
NZ	Aparima River	AP-4	7-Apr-06	Access opposite Sir	?	Cathy Kilroy		5462541	2131551	Site 2, 10 Litre	88
NZ	Aparima River	AP-5	7-Apr-06	2 km u/s Etal Strear	?	Cathy Kilroy		5473428	2131791	Site 3, Benthic	89
NZ	Aparima River	AP-6	8-Apr-06	access opposite Go	?	Cathy Kilroy		5467421	2131703	Site 4, 10 min d	90
NZ	Aparima River	AP-7	8-Apr-06	d/s Wreys Bush - M	?	Cathy Kilroy		5480678	2135012	Site 5, 1 min dı	91
NZ	Aparima River	AP-8	7-Apr-06	Access 600 m N of	?	Cathy Kilroy		5458942	2131626	Site 1, 10 sec d	92
NZ	Aparima River	AP-9	7-Apr-06	Access opposite Sir	?	Cathy Kilroy		5462541	2131551	Site 2, 10 Litre	93
NZ	Aparima River	AP-10	7-Apr-06	1 km u/s Etal Strear	?	Cathy Kilroy		5473428	2131791	Site 3, 10 sec d	94
NZ	Aparima River	AP-11	8-Apr-06	access opposite Go	?	Cathy Kilroy		5467421	2131703	Site 4, 1 min dı	95
NZ	Aparima River	AP-12	8-Apr-06	d/s Wreys Bush - M	?	Cathy Kilroy		5480678	2135012	Site 5, 10 sec d	96
NZ	Aparima River	AP-13	7-Apr-06	Access 600 m N of	?	Cathy Kilroy		5458942	2131626	Site 1, 10L drift	97
NZ	Aparima River	AP-14	7-Apr-06	Access 600 m N of	?	Cathy Kilroy		5458942	2131626	Site 1, 10 min d	98
NZ	Aparima River	AP-15	7-Apr-06	Access opposite Sir	?	Cathy Kilroy		5462541	2131551	Site 2, Benthic	99
NZ	Aparima River	AP-16	8-Apr-06	access opposite Go	?	Cathy Kilroy		5467421	2131703	Site 4, 10 sec d	100
NZ	Aparima River	AP-17	8-Apr-06	d/s Wreys Bush - M	?	Cathy Kilroy		5480678	2135012	Site 5 10L wate	101
NZ	Aparima River	AP-18	7-Apr-06	Access 600 m N of	?	Cathy Kilroy		5458942	2131626	Site 1, 10L Drif	102
NZ	Aparima River	AP-19	7-Apr-06	Access 600 m N of	?	Cathy Kilroy		5458942	2131626	Site 1, 1 min dı	103
NZ	Aparima River	AP-20	7-Apr-06	Access opposite Sir	?	Cathy Kilroy		5462541	2131551	Site 2, 1 min dı	104
NZ	Aparima River	AP-21	8-Apr-06	access opposite Go	?	Cathy Kilroy		5467421	2131703	Site 4, 10L drift	105
NZ	Aparima River	AP-22	8-Apr-06	d/s Wreys Bush - M	?	Cathy Kilroy		5480678	2135012	Site 5, 10L filte	106
NZ	Aparima River	AP-23	7-Apr-06	Access 600 m N of	?	Cathy Kilroy		5458942	2131626	Site 1, 10L filte	107
NZ	Aparima River	AP-24	7-Apr-06	Access 600 m N of	?	Cathy Kilroy		5458942	2131626	Site 1, 1 min dı	108
NZ	Aparima River	AP-25	7-Apr-06	Access opposite Sir	?	Cathy Kilroy		5462541	2131551	Site 2, 10 sec d	109
NZ	Aparima River	AP-26	8-Apr-06	access opposite Go	?	Cathy Kilroy		5467421	2131703	Site 4, 10 litre f	110
NZ	Aparima River	AP-27	8-Apr-06	access opposite Go	?	Cathy Kilroy		5467421	2131703	Site 4, benthic	111
NZ	Aparima River	AP-28	8-Apr-06	d/s Wreys Bush - M	Cymbella kappii	Cathy Kilroy	92.69	5480678	2135012	Site 5, benthic	112
NZ	Aparima River	AP-30	8-Apr-06	d/s Dunrobin Bridge	Gomphonema sp.	Cathy Kilroy		5485364	2130206	Site 6, 10L drift	113
NZ	Aparima River	AP-31	8-Apr-06	d/s Dunrobin Bridge	?	Cathy Kilroy		5485364	2130206	Site 6, 10L filte	114
NZ	Aparima River	AP-32	25-Apr-06	u/s Thornbury road	?	Cathy Kilroy / Neil Blair		5424480	2131080	Site A, benthic	115
NZ	Aparima River	AP-33	25-Apr-06	u/s Otautau road bri	?	Cathy Kilroy / Neil Blair		5441020	2123795	Site B, benthic	116
NZ	Aparima River	AP-34	25-Apr-06	u/s Wreys Bush roa	?	Cathy Kilroy / Neil Blair		5452702	2131777	Site C, benthic	117
NZ	Aparima River	AP-35	25-Apr-06	Access 600 m N of	?	Cathy Kilroy / Neil Blair		5458942	2131626	Site 1, benthic	118
NZ	Aparima River	AP-36	25-Apr-06	Access opposite Sir	?	Cathy Kilroy / Neil Blair		5462541	2131551	Site 2, benthic	119
NZ	Aparima River	AP-37	25-Apr-06	access opposite Go	?	Cathy Kilroy / Neil Blair		5467421	2131703	Site 4, benthic	120

Appendix 5.

Country	River	Code	Date collected	Site	Taxon	Collector	DNA (ng/µL)	Northing/ Latitude	Easting/ Longitude	Sample type	order
NZ	Aparima River	AP-38	25-Apr-06	2 km u/s Etal Strean	?	Cathy Kilroy / Neil Blair		5473428	2131791	Site 3, benthic	121
NZ	Aparima River	AP-39	25-Apr-06	u/s Thornbury road	?	Cathy Kilroy / Neil Blair		5424480	2131080	Site A, 10 L dri	122
NZ	Aparima River	AP-40	25-Apr-06	u/s Thornbury road	?	Cathy Kilroy / Neil Blair		5424480	2131080	Site A, 10 sec	123
NZ	Aparima River	AP-41	25-Apr-06	u/s Thornbury road	?	Cathy Kilroy / Neil Blair		5424480	2131080	Site A, 1 min d	124
NZ	Aparima River	AP-42	25-Apr-06	u/s Thornbury road	?	Cathy Kilroy / Neil Blair		5424480	2131080	Site A, 10 min	125
NZ	Aparima River	AP-43	25-Apr-06	u/s Otautau road bri	?	Cathy Kilroy / Neil Blair		5441020	2123795	Site B, 10 L dri	126
NZ	Aparima River	AP-44	25-Apr-06	u/s Otautau road bri	?	Cathy Kilroy / Neil Blair		5441020	2123795	Site B, 10 sec	127
NZ	Aparima River	AP-45	25-Apr-06	u/s Otautau road bri	?	Cathy Kilroy / Neil Blair		5441020	2123795	Site B, 1 min d	128
NZ	Aparima River	AP-46	25-Apr-06	u/s Otautau road bri	?	Cathy Kilroy / Neil Blair		5441020	2123795	Site B, 10 min	129
NZ	Aparima River	AP-47	25-Apr-06	u/s Wreys Bush roa	?	Cathy Kilroy / Neil Blair		5452702	2131777	Site C, 10 L dri	130
NZ	Aparima River	AP-48	25-Apr-06	u/s Wreys Bush roa	?	Cathy Kilroy / Neil Blair		5452702	2131777	Site C, 10 sec	131
NZ	Aparima River	AP-49	25-Apr-06	u/s Wreys Bush roa	?	Cathy Kilroy / Neil Blair		5452702	2131777	Site C, 1 min d	132
NZ	Aparima River	AP-50	25-Apr-06	u/s Wreys Bush roa	?	Cathy Kilroy / Neil Blair		5452702	2131777	Site C, 10 min	133
NZ	Aparima River	AP-51	25-Apr-06	Access 600 m N of	?	Cathy Kilroy / Neil Blair		5458942	2131626	Site 1, 10 L dri	134
NZ	Aparima River	AP-52	25-Apr-06	Access 600 m N of	?	Cathy Kilroy / Neil Blair		5458942	2131626	Site 1, 10 sec	135
NZ	Aparima River	AP-53	25-Apr-06	Access 600 m N of	?	Cathy Kilroy / Neil Blair		5458942	2131626	Site 1, 1 min dı	136
NZ	Aparima River	AP-54	25-Apr-06	Access 600 m N of	?	Cathy Kilroy / Neil Blair		5458942	2131626	Site 1, 10 min	137
NZ	Aparima River	AP-55	25-Apr-06	Access opposite Sir	?	Cathy Kilroy / Neil Blair		5462541	2131551	Site 2, 10 L dri	138
NZ	Aparima River	AP-56	25-Apr-06	Access opposite Sir	?	Cathy Kilroy / Neil Blair		5462541	2131551	Site 2, 10 sec	139
NZ	Aparima River	AP-57	25-Apr-06	Access opposite Sir	?	Cathy Kilroy / Neil Blair		5462541	2131551	Site 2, 1 min dı	140
NZ	Aparima River	AP-58	25-Apr-06	Access opposite Sir	?	Cathy Kilroy / Neil Blair		5462541	2131551	Site 2, 10 min	141
NZ	Aparima River	AP-59	25-Apr-06	2 km u/s Etal Strean	?	Cathy Kilroy / Neil Blair		5473428	2131791	Site 3, 10 L dri	142
NZ	Aparima River	AP-60	25-Apr-06	2 km u/s Etal Strean	?	Cathy Kilroy / Neil Blair		5473428	2131791	Site 3, 10 sec	143
NZ	Aparima River	AP-61	25-Apr-06	2 km u/s Etal Strean	?	Cathy Kilroy / Neil Blair		5473428	2131791	Site 3, 10 min	144
NZ	Aparima River	AP-62	25-Apr-06	access opposite Go	?	Cathy Kilroy / Neil Blair		5467421	2131703	Site 4, 10 L dri	145
NZ	Aparima River	AP-63	25-Apr-06	access opposite Go	?	Cathy Kilroy / Neil Blair		5467421	2131703	Site 4, 10 sec	146
NZ	Aparima River	AP-64	25-Apr-06	access opposite Go	?	Cathy Kilroy / Neil Blair		5467421	2131703	Site 4, 1 min dı	147
NZ	Aparima River	AP-65	25-Apr-06	access opposite Go	?	Cathy Kilroy / Neil Blair		5467421	2131703	Site 4, 10 min	148
NZ	Aparima River	AP-66	25-Apr-06	Access 600 m N of	?	Cathy Kilroy / Neil Blair		5458942	2131626	Site 1, textured	149
NZ	Aparima River	AP-67	25-Apr-06	Access 600 m N of	?	Cathy Kilroy / Neil Blair		5458942	2131626	Site 1, textured	150
NZ	Aparima River	AP-68	25-Apr-06	Access opposite Sir	?	Cathy Kilroy / Neil Blair		5462541	2131551	Site 2, textured	151
NZ	Aparima River	AP-69	25-Apr-06	Access opposite Sir	?	Cathy Kilroy / Neil Blair		5462541	2131551	Site 2, textured	152
NZ	Aparima River	AP-70	25-Apr-06	2 km u/s Etal Strean	?	Cathy Kilroy / Neil Blair		5473428	2131791	Site 3, textured	153
NZ	Aparima River	AP-71	25-Apr-06	access opposite Go	?	Cathy Kilroy / Neil Blair		5467421	2131703	Site 4, textured	154
NZ	Aparima River	AP-72	25-Apr-06	Access 600 m N of	?	Cathy Kilroy / Neil Blair		5458942	2131626	Site 1, glass sli	155
NZ	Aparima River	AP-73	25-Apr-06	Access opposite Sir	?	Cathy Kilroy / Neil Blair		5462541	2131551	Site 2, glass sli	156
NZ	Aparima River	AP-74	25-Apr-06	Access opposite Sir	?	Cathy Kilroy / Neil Blair		5462541	2131551	Site 2, glass sli	157
NZ	Aparima River	AP-75	25-Apr-06	2 km u/s Etal Strean	?	Cathy Kilroy / Neil Blair		5473428	2131791	Site 3, glass sli	158
NZ	Aparima River	AP-76	25-Apr-06	access opposite Go	?	Cathy Kilroy / Neil Blair		5467421	2131703	Site 4, glass sli	159
NZ	Aparima River	AP-77	25-Apr-06	d/s Wreys Bush - M	?	Cathy Kilroy / Neil Blair		5480678	2135012	Site 5, glass sli	160
NZ	Aparima River	AP-78	25-Apr-06	d/s Dunrobin Bridge	?	Cathy Kilroy / Neil Blair		5485364	2130206	Site 6, glass sli	161
NZ	Aparima River	AP-79	25-Apr-06	2 km u/s Etal Strean	?	Cathy Kilroy / Neil Blair		5473428	2131791	Site 3, ceramic	162
NZ	Aparima River	AP-80	25-Apr-06	access opposite Go	?	Cathy Kilroy / Neil Blair		5467421	2131703	Site 4, ceramic	163
NZ	Aparima River	AP-81	25-Apr-06	Access 600 m N of	?	Cathy Kilroy / Neil Blair		5458942	2131626	Site 1, plastic s	164
NZ	Aparima River	AP-82	25-Apr-06	Access opposite Sir	?	Cathy Kilroy / Neil Blair		5462541	2131551	Site 2, plastic s	165
NZ	Aparima River	AP-83	25-Apr-06	Access opposite Sir	?	Cathy Kilroy / Neil Blair		5462541	2131551	Site 2, plastic s	166
NZ	Aparima River	AP-84	25-Apr-06	2 km u/s Etal Strean	?	Cathy Kilroy / Neil Blair		5473428	2131791	Site 3, plastic s	167
NZ	Aparima River	AP-85	25-Apr-06	access opposite Go	?	Cathy Kilroy / Neil Blair		5467421	2131703	Site 4, plastic s	168
NZ	Aparima River	AP-86	25-Apr-06	d/s Wreys Bush - M	?	Cathy Kilroy / Neil Blair		5480678	2135012	Site 5, plastic s	169
NZ	Aparima River	AP-87	25-Apr-06	d/s Dunrobin Bridge	?	Cathy Kilroy / Neil Blair		5485364	2130206	Site 6, plastic s	170
NZ	Ahuriri River	AH-29	6-Apr-06	u/s Gorge bridge	?	Cathy Kilroy et al		5634611	2247706	Site 6, 10 min	171
NZ	Ahuriri River	AH-30	6-Apr-06	u/s Gorge bridge	?	Cathy Kilroy et al		5634611	2247706	Site 6, 10L filte	172
NZ	Ahuriri River	AH-31	17-Apr-06	Glenburn Station, d/	?	Matt Dale		5630294	2276144	Site 1, 10 L wa	173
NZ	Ahuriri River	AH-32	17-Apr-06	Glenburn Station, d/	?	Matt Dale		5630294	2276144	Site 1, 10 sec v	174
NZ	Ahuriri River	AH-33	18-Apr-06	Glenburn Station, u/	?	Matt Dale		5632703	2274669	Site 2, 10L Wa	175
NZ	Ahuriri River	AH-34	18-Apr-06	Glenburn Station, d/	?	Matt Dale		5630294	2276144	Site 1, Texture	176
NZ	Ahuriri River	AH-35	18-Apr-06	Glenburn Station, d/	?	Matt Dale		5630294	2276144	Site 1, Texture	177
NZ	Ahuriri River	AH-36	18-Apr-06	Glenburn Station, u/	?	Matt Dale		5632703	2274669	Site 2, Texture	178
NZ	Ahuriri River	AH-37	18-Apr-06	Glenburn Station, u/	?	Matt Dale		5632703	2274669	Site 2, Texture	179
NZ	Ahuriri River	AH-38	18-Apr-06	Glenburn Station, u/	?	Matt Dale		5632703	2274669	Site 2, Texture	180

Appendix 5.

Country	River	Code	Date collected	Site	Taxon	Collector	DNA (ng/µL)	Northing/ Latitude	Easting/ Longitude	Sample type	order
NZ	Ahuriri River	AH-39	18-Apr-06	SH8 road bridge	?	Matt Dale		5633415	2269962	Site 3, Texture	181
NZ	Ahuriri River	AH-40	18-Apr-06	SH8 road bridge	?	Matt Dale		5633415	2269962	Site 3, Texture	182
NZ	Buller River	BU1-E1	9-May-06	Upper Buller bridge,	Didmyo	Brendan Hicl	197.65	5935560	2494386	Benthic	183
NZ	Buller River	BU1-E2	9-May-06	Upper Buller bridge,	Didmyo	Brendan Hicl	125.58	5935560	2494386	Benthic	184
NZ	Buller River	BU1-C	9-May-06	Upper Buller bridge,	Didmyo	Brendan Hicl	54.61	5935560	2494386	Benthic	185
NZ	Buller River	BU1-C Swab	9-May-06	Upper Buller bridge,	?	Brendan Hicl	204.56	5935560	2494386	Benthic swab	186
NZ	Buller River	BU2-E1	9-May-06	Owens River reserv	Didmyo	Brendan Hicl	206.81	5946425	2464210	Benthic	187
NZ	Buller River	BU2-E2	9-May-06	Owens River reserv	Didmyo	Brendan Hicl	215.15	5946425	2464210	Benthic	188
NZ	Buller River	BU2-C	9-May-06	Owens River reserv	Didmyo	Brendan Hicl	180.44	5946425	2464210	Benthic	189
NZ	Buller River	BU2-C Swab	9-May-06	Owens River reserv	?	Brendan Hicl	465.94	5946425	2464210	Benthic swab	190
NZ	Buller River	BU3-E1	9-May-06	Upstream of Murchi	Didmyo	Brendan Hicl	140.54	5934822	2455586	Benthic	191
NZ	Buller River	BU3-E2	9-May-06	Upstream of Murchi	Didmyo	Brendan Hicl	281.95	5934822	2455586	Benthic	192
NZ	Buller River	BU3-C	9-May-06	Upstream of Murchi	Didmyo	Brendan Hicl	106.42	5934822	2455586	Benthic	193
NZ	Buller River	BU3-C Swab	9-May-06	Upstream of Murchi	Didmyo	Brendan Hicl	369.14	5934822	2455586	Benthic swab	194
NZ	Lower Waiau	LW2-E1	12-May-06	Rakatu Wetland	Didmyo	Brendan Hicl	114.78	5491878	2093689	Benthic	195
NZ	Lower Waiau	LW2-E2	13-May-06	Rakatu Wetland	Didmyo	Brendan Hicl	127.71	5491878	2093689	Benthic	196
NZ	Lower Waiau	LW2-C	14-May-06	Rakatu Wetland	Didmyo	Brendan Hicl	196.61	5491878	2093689	Benthic	197
NZ	Lower Waiau	LW2-C Swab	15-May-06	Rakatu Wetland	?	Brendan Hicl	167.37	5491878	2093689	Benthic swab	198
NZ	Oreti River	OR2-E1	11-May-06	Upstream of Site 3	Didmyo	Brendan Hicl	83.02	5518543	2129157	Benthic	199
NZ	Oreti River	OR2-E2	11-May-06	Upstream of Site 3	Didmyo	Brendan Hicl	82.01	5518543	2129157	Benthic	200
NZ	Oreti River	OR2-C	11-May-06	Upstream of Site 3	Didmyo	Brendan Hicl	71.48	5518543	2129157	Benthic	201
NZ	Oreti River	OR2-C Swab	11-May-06	Upstream of Site 3	?	Brendan Hicl	186.17	5518543	2129157	Benthic swab	202
NZ	Oreti River	OR3-E1	11-May-06	Upstream of	Didmyo	Brendan Hicl	92.91	5508309	2130406	Benthic	203
NZ	Oreti River	OR3-E2	11-May-06	Upstream of	Didmyo	Brendan Hicl	356.1	5508309	2130406	Benthic	204
NZ	Oreti River	OR3-C	11-May-06	Upstream of	Didmyo	Brendan Hicl	160.33	5508309	2130406	Benthic	205
NZ	Oreti River	OR3-C Swab	11-May-06	Upstream of	?	Brendan Hicl	45.04	5508309	2130406	Benthic swab	206
NZ	Maraoa River	MA1-E1	11-May-06	Wood Burn,	Didmyo	Brendan Hicl	39.66	5520772	2124105	Benthic	207
NZ	Maraoa River	MA1-E2	11-May-06	Wood Burn,	Didmyo	Brendan Hicl	61.22	5520772	2124105	Benthic	208
NZ	Maraoa River	MA1-C	11-May-06	Wood Burn,	Didmyo	Brendan Hicl	42.76	5520772	2124105	Benthic	209
NZ	Maraoa River	MA1-C Swab	11-May-06	Wood Burn,	?	Brendan Hicl	50	5520772	2124105	Benthic swab	210
NZ	Maraoa River	MA2-E1	11-May-06	Dale Creek, Te	Didmyo	Brendan Hicl	52.16	5510782	2117628	Benthic	211
NZ	Maraoa River	MA2-E2	11-May-06	Dale Creek, Te	Didmyo	Brendan Hicl	267.98	5510782	2117628	Benthic	212
NZ	Maraoa River	MA2-C	11-May-06	Dale Creek, Te	Didmyo	Brendan Hicl	138.61	5510782	2117628	Benthic	213
NZ	Maraoa River	MA2-C Swab	11-May-06	Dale Creek, Te	?	Brendan Hicl	135.41	5510782	2117628	Benthic swab	214
NZ	Maraoa River	MA3-E1	12-May-06	SH 94 bridge, The	Didmyo	Brendan Hicl	52.87	5506003	2110802	Benthic	215
NZ	Maraoa River	MA3-E2	12-May-06	SH 94 bridge, The	Didmyo	Brendan Hicl	167.37	5506003	2110802	Benthic	216
NZ	Maraoa River	MA3-C	12-May-06	SH 94 bridge, The	Didmyo	Brendan Hicl	115.28	5506003	2110802	Benthic	217
NZ	Maraoa River	MA3-C Swab	12-May-06	SH 94 bridge, The	?	Brendan Hicl	139.8	5506003	2110802	Benthic swab	218
NZ	Maraoa River	MA3 Drift Net	13-May-06	SH 94 bridge, The	Didmyo	Brendan Hicl	173.05	5506003	2110802	Drift net (500,	219
NZ	Von River	VR1-E1	11-May-06	Most Upstream,	Didmyo	Brendan Hicl	95.38	5554758	2146258	Benthic	220
NZ	Von River	VR1-E2	11-May-06	Most Upstream,	Didmyo	Brendan Hicl	38.96	5554758	2146258	Benthic	221
NZ	Von River	VR1-C	11-May-06	Most Upstream,	Didmyo	Brendan Hicl	45.56	5554758	2146258	Benthic	222
NZ	Von River	VR1-C Swab	11-May-06	Most Upstream,	?	Brendan Hicl	169.21	5554758	2146258	Benthic swab	223
NZ	Von River	VR2-E1	11-May-06	Downstream, Von	Didmyo	Brendan Hicl	161.46	5556772	2146666	Benthic	224
NZ	Von River	VR2-E2	11-May-06	Downstream, Von	Didmyo	Brendan Hicl	129.01	5556772	2146666	Benthic	225
NZ	Von River	VR2-C	11-May-06	Downstream, Von	Didmyo	Brendan Hicl	141.77	5556772	2146666	Benthic	226
NZ	Von River	VR2-C Swab	11-May-06	Downstream, Von	?	Brendan Hicl	258.15	5556772	2146666	Benthic swab	227
NZ	Hawea River	HW1-E1	11-May-06	200m below the	Didmyo	Brendan Hicl	42.4	5614904	2212442	Benthic	228
NZ	Hawea River	HW1-E2	11-May-06	200m below the	Didmyo	Brendan Hicl	57.55	5614904	2212442	Benthic	229
NZ	Hawea River	HW1-C	11-May-06	200m below the	Didmyo	Brendan Hicl	123.4	5614904	2212442	Benthic	230
NZ	Hawea River	HW1-C Swab	11-May-06	200m below the	?	Brendan Hicl	99.23	5614904	2212442	Benthic swab	231
NZ	Hawea River	HW2-E1	11-May-06	Downstream of	Didmyo	Brendan Hicl	450.82	5610704	2212344	Benthic	232
NZ	Hawea River	HW2-E2	11-May-06	Downstream of	Didmyo	Brendan Hicl	304.14	5610704	2212344	Benthic	233
NZ	Hawea River	HW2-C	11-May-06	Downstream of	Didmyo	Brendan Hicl	202.92	5610704	2212344	Benthic	234
NZ	Hawea River	HW2-C Swab	11-May-06	Downstream of	?	Brendan Hicl	157.72	5610704	2212344	Benthic swab	235
NZ	Hawea River	HW3-E1	11-May-06	Just above the	Didmyo	Brendan Hicl	286.99	5607646	2208387	Benthic	236
NZ	Hawea River	HW3-E2	11-May-06	Just above the	Didmyo	Brendan Hicl	154.49	5607646	2208387	Benthic	237
NZ	Hawea River	HW3-C	11-May-06	Just above the	Didmyo	Brendan Hicl	96.59	5607646	2208387	Benthic	238
NZ	Hawea River	HW3-C Swab	11-May-06	Just above the	?	Brendan Hicl	117.4	5607646	2208387	Benthic swab	239
NZ	Clutha River	CL1-E1	11-May-06	Downstream	Didmyo	Brendan Hicl	262.76	5607044	2208621	Benthic	240

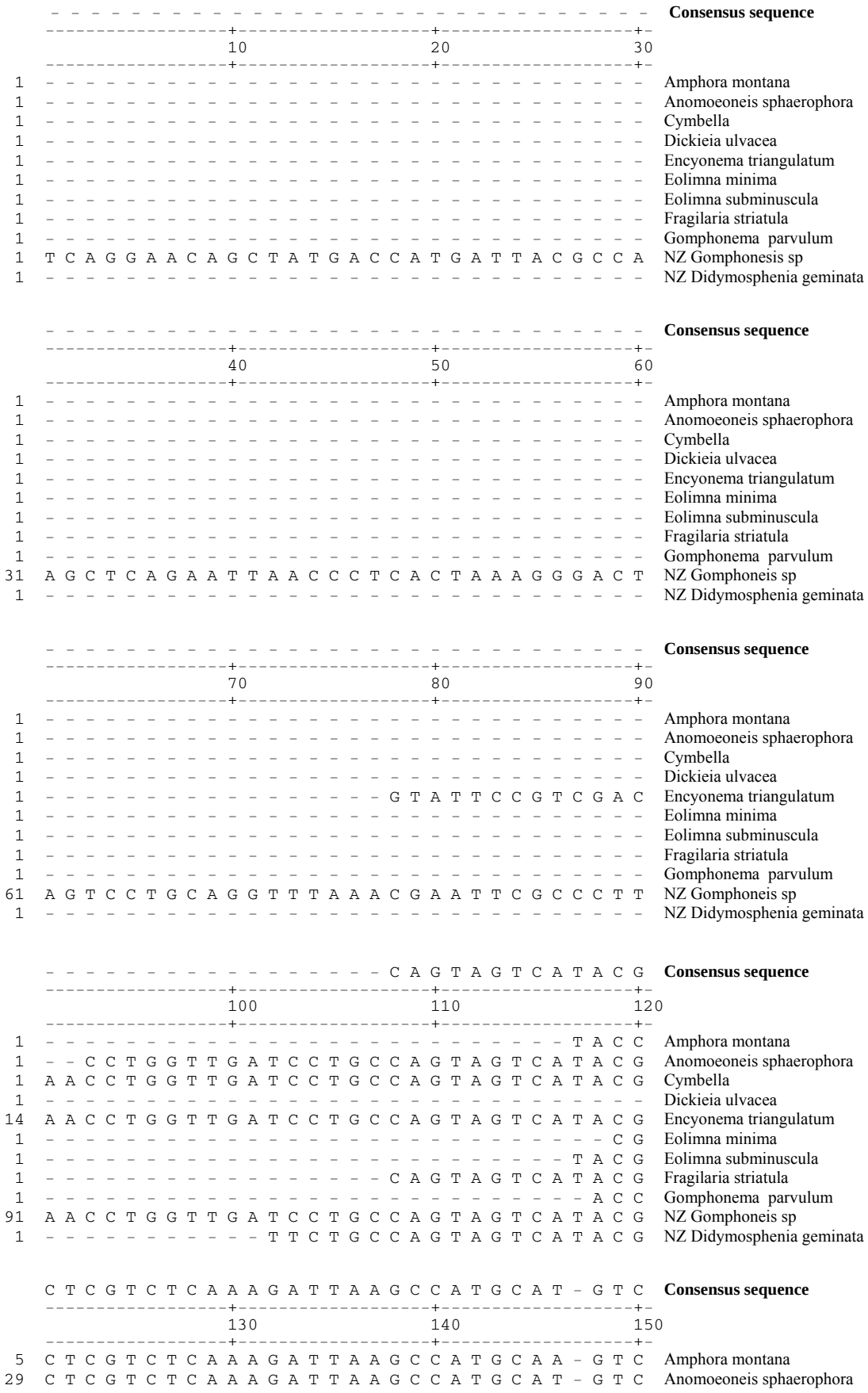
Appendix 5.

Country	River	Code	Date collected	Site	Taxon	Collector	DNA (ng/µL)	Northing/ Latitude	Easting/ Longitude	Sample type	order
NZ	Clutha River	CL1-E2	11-May-06	Downstream	Didmyo	Brendan Hicl	177.33	5607044	2208621	Benthic	241
NZ	Clutha River	CL1-C	11-May-06	Downstream	Didmyo	Brendan Hicl	88.64	5607044	2208621	Benthic	242
NZ	Clutha River	CL1-C Swab	11-May-06	Downstream	?	Brendan Hicl	98.71	5607044	2208621	Benthic swab	243
NZ	Clutha River	CL2-E1	11-May-06	Below bridge to	Didmyo	Brendan Hicl	140.12	5601916	2215495	Benthic	244
NZ	Clutha River	CL2-E2	11-May-06	Below bridge to	Didmyo	Brendan Hicl	85.16	5601916	2215495	Benthic	245
NZ	Clutha River	CL2-C	11-May-06	Below bridge to	Didmyo	Brendan Hicl	90.26	5601916	2215495	Benthic	246
NZ	Clutha River	CL2-C Swab	11-May-06	Below bridge to	?	Brendan Hicl	131.83	5601916	2215495	Benthic swab	247
NZ	Clutha River	CL3-E1	11-May-06	Sheep Farm, that	Didmyo	Brendan Hicl	158.02	5597921	2219981	Benthic	248
NZ	Clutha River	CL3-E2	11-May-06	Sheep Farm, that	Didmyo	Brendan Hicl	182.12	5597921	2219981	Benthic	249
NZ	Clutha River	CL3-C	11-May-06	Sheep Farm, that	Didmyo	Brendan Hicl	133.85	5597921	2219981	Benthic	250
NZ	Clutha River	CL3-C Swab	11-May-06	Sheep Farm, that	?	Brendan Hicl	134.44	5597921	2219981	Benthic swab	251
NZ	Clutha River	Clutha Swab	11-May-06	Albert town bridge,	?	Brendan Hicl	152.53	5607200	2208120	Benthic swab	252
NZ	Waitaki River	WT1-E1	10-May-06	Just below Otiake C	Didmyo	Brendan Hicl	77.77	5599036	2315979	Benthic	253
NZ	Waitaki River	WT1-E2	10-May-06	Just below Otiake C	Didmyo	Brendan Hicl	175.46	5599036	2315979	Benthic	254
NZ	Waitaki River	WT1-C	10-May-06	Just below Otiake C	Didmyo	Brendan Hicl	169.55	5599036	2315979	Benthic	255
NZ	Waitaki River	WT1-C Swab	10-May-06	Just below Otiake C	?	Brendan Hicl	134.96	5599036	2315979	Benthic swab	256
NZ	Waitaki River	WT2-E1	10-May-06	Side channel, Oteka	Didmyo	Brendan Hicl	85.76	5596387	2318669	Benthic	257
NZ	Waitaki River	WT2-E2	10-May-06	Side channel, Oteka	Didmyo	Brendan Hicl	160.88	5596387	2318669	Benthic	258
NZ	Waitaki River	WT2-C	10-May-06	Side channel, Oteka	Didmyo	Brendan Hicl	65.19	5596387	2318669	Benthic	259
NZ	Waitaki River	WT2-C Swab	10-May-06	Side channel, Oteka	?	Brendan Hicl	213.21	5596387	2318669	Benthic swab	260
NZ	Waitaki River	WT3-E1	10-May-06	Priests Rd extensio	Didmyo	Brendan Hicl	125.9	5594678	2322895	Benthic	261
NZ	Waitaki River	WT3-E2	10-May-06	Priests Rd extensio	Didmyo	Brendan Hicl	99.66	5594678	2322895	Benthic	262
NZ	Waitaki River	WT3-C	10-May-06	Priests Rd extensio	Didmyo	Brendan Hicl	201.52	5594678	2322895	Benthic	263
NZ	Waitaki River	WT3-C Swab	10-May-06	Priests Rd extensio	?	Brendan Hicl	181.83	5594678	2322895	Benthic swab	264
NZ	Ahuriri River	AH1-E1	10-May-06	Below Longslip Stat	Didmyo	Brendan Hicl	121.27	5632360	2249323	Benthic	265
NZ	Ahuriri River	AH1-E2	10-May-06	Below Longslip Stat	Didmyo	Brendan Hicl	175.71	5632360	2249323	Benthic	266
NZ	Ahuriri River	AH1-C	10-May-06	Below Longslip Stat	Didmyo	Brendan Hicl	72.02	5632360	2249323	Benthic	267
NZ	Ahuriri River	AH1-C Swab	10-May-06	Below Longslip Stat	?	Brendan Hicl	306.55	5632360	2249323	Benthic swab	268
NZ	Ahuriri River	AH2-E1	10-May-06	Killermont Station	Didmyo	Brendan Hicl	90.14	5627802	2256049	Benthic	269
NZ	Ahuriri River	AH2-E2	10-May-06	Killermont Station	Didmyo	Brendan Hicl	84.75	5627802	2256049	Benthic	270
NZ	Ahuriri River	AH2-C	10-May-06	Killermont Station	Didmyo	Brendan Hicl	108.01	5627802	2256049	Benthic	271
NZ	Ahuriri River	AH2-C Swab	10-May-06	Killermont Station	?	Brendan Hicl	306.25	5627802	2256049	Benthic swab	272
NZ	Ahuriri River	AH3-E1	10-May-06	Just upstream of La	Didmyo	Brendan Hicl	169.8	5633336	2270230	Benthic	273
NZ	Ahuriri River	AH3-E2	10-May-06	Just upstream of La	Didmyo	Brendan Hicl	117.62	5633336	2270230	Benthic	274
NZ	Ahuriri River	AH3-C	10-May-06	Just upstream of La	Didmyo	Brendan Hicl	23.31	5633336	2270230	Benthic	275
NZ	Ahuriri River	AH3-C Swab	10-May-06	Just upstream of La	?	Brendan Hicl	99.49	5633336	2270230	Benthic swab	276
NZ	Upper Waiau	UW1	29-May-06	Yerex Reach	Didmyo	Bill Jarvie, Sc	52.72	5514300	2095000	Benthic	277
NZ	Upper Waiau	UW2	29-May-06	Balloon Loop	Didmyo	Bill Jarvie, Sc	63.99	5509000	2092600	Benthic	278
NZ	Lower Waiau	LW1	29-May-06	Whare Creek acces	Didmyo	Bill Jarvie, Sc	79.45	5494600	2095300	Benthic	279
NZ	Lower Waiau	LW3	29-May-06	Jericho access	Didmyo	Bill Jarvie, Sc	51.18	5487100	2091300	Benthic	280
CAN	Vancouver Island	VI-1	7-Apr-06	Puntledge River, HV	Didmyo	John Clayton	62.76	49°40'48.53"N	125°03'57.28"W	Benthic	281
CAN	Vancouver Island	VI-2	7-Apr-06	Puntledge River, HV	Didmyo	John Clayton	143.25	49°40'48.53"N	125°03'57.28"W	Benthic	282
CAN	Vancouver Island	VI-3	7-Apr-06	Little Qualicum Rive	Didmyo	John Clayton	104.28	49°18'18.58"N	124°33'12.93"W	Benthic	283
CAN	Vancouver Island	VI-4	7-Apr-06	Little Qualicum Rive	Didmyo	John Clayton	154.2	49°18'18.58"N	124°33'12.93"W	Benthic	284
CAN	Vancouver Island	VI-5	7-Apr-06	Little Qualicum Rive	Didmyo	John Clayton	159.62	49°18'18.58"N	124°33'12.93"W	Benthic	285
USA	Wenatchee River, WA	WE-1	1-May-06	River Mile 35.6	Didmyo	Chris Coffin	33.91	47.6764 N	120.7328 W	Benthic	286
USA	Boulder Creek, MO	BC-1	16-May-06		Didmyo	Craig Cary	182.05			Benthic	287
USA	Boulder Creek, MO	BC-2	15-May-06		Didmyo	Craig Cary	110.66			Benthic	288
USA	Boulder Creek, MO	BC-3	15-May-06		Didmyo	Craig Cary	327.86			Benthic	289
USA	Boulder Creek, MO	BC-4	15-May-06		Didmyo	Craig Cary	26.56			Benthic	290
USA	Boulder Creek, MO	BC-5	15-May-06		Didmyo	Craig Cary	113.28			Benthic	291
USA	Boulder Creek, MO	BC-6	15-May-06		Didmyo	Craig Cary	40.13			Benthic	292
USA	Boulder Creek, MO	BC-7 Swab	15-May-06		?	Craig Cary	13.86			Benthic swab	293
USA	Boulder Creek, MO	BC-8 Swab	15-May-06		?	Craig Cary	27.04			Benthic swab	294
USA	Boulder Creek, MO	BC-9 Swab	15-May-06		?	Craig Cary	387			Benthic swab	295
USA	Boulder Creek, MO	BC-10	15-May-06		Didmyo	Craig Cary	50.03			Benthic	296
USA	Boulder Creek, MO	BC-11 Swab	15-May-06		?	Craig Cary	348.03			Benthic swab	297
USA	Boulder Creek, CO	BC-12	24-Apr-06	South Boulder Cree	Didmyo	Sarah Spaul	188.76	39.9325 N	105.27639 W	Benthic	298
USA	Lee Vining Creek, CA	LV-1	26-Jun-06	Near Mono Lake	Didmyo	Andy Rost	56.97	37 57 45 N	119 16 31 W	Benthic	299
USA	Lee Vining Creek, CA	LV-2	26-Jun-06	Near Mono Lake	Didmyo	Andy Rost	70.59	37 57 45 N	119 16 31 W	Benthic	300

Appendix 5.

Country	River	Code	Date collected	Site	Taxon	Collector	DNA (ng/µL)	Northing/ Latitude	Easting/ Longitude	Sample type	order
USA	Rapid Creek, SD	RC-1	10-May-06	32km west of Rapid	Didmyo	Jeff Shearer	171.76	44 04 30 N	103 29 30 W	Benthic	301
USA	Rapid Creek SD	RC-2	12-May-06	Near Rapid city	Didmyo	Aaron Larsor	61.49	44.055157234 N	103.404361134 W	Benthic	302
UK	River Coquet	C-1	4-May-06	Upstream Rowhope	Didmyo	M. Kelly	100.51			Benthic	303
USA	Drum Canal	DC-1	19-May-06	Upstream of Drum F	Didmyo	Peter Pryfog	264.27			Benthic	304
CAN	Kootenai River	KR-1	27-Oct-04	Site 13-4	Didmyo	A. Genung a	33.63			Benthic	305
CAN	Kootenai River	KR-2	27-Oct-04	Site 13-4	Didmyo	A. Genung a	45.52			Benthic	306
USA	Deschutes River	DR-1	7-May-06	Terrebonne, Old dia	Cymbella mexicana var j	Sarah Spaul	44.5	44.35306 N	121.17667 W	Benthic	307

Appendix 6. Sequence alignment of 18S rRNA region in 11 biraphid diatom species from within and outside New Zealand.



```
 31  C T T G T C T C A A A G A T T A A G C C A T G C A G - G T C  Cymbella
  1  - - - - - - - - - - - - - - - - - - - - - T G C A C - G T C  Dickieia ulvacea
 44  C T C G T C T C A A A G A T T A A G C C A T G C A C - G T C  Encyonema triangulatum
  3  C T C G T C T C A A A G A T T A A G C C - T G C A T - G T C  Eolimna minima
  5  C T C G T C T C A A A G A T T A A G C C A T G C A T - G T C  Eolimna subminuscula
 14  C T C G T C T C A A A G A T T A A G C C A T G C A T - G T C  Fragilaria striatula
  4  C T C G T C T C A A A G A T T A A G C C A T G C A C T G T C  Gomphonema  parvulum
121  C T C G T C T C A A A G A T T A A G C C A T G C A C - G T C  NZ Gomphoneis sp
 20  C T C G T C T C A A A G A T T A A G C C A T G C A T - G T C  NZ Didymosphenia geminata

     T A A G T A T A A A T A T T T T A C T G T G A A A C T G C G  Consensus sequence
     -----------------+-----------------+-----------------+-
                      160               170               180
     -----------------+-----------------+-----------------+-
 34  T A A G T A T A A A T A T T T T A C T T T G A A A C T G C G  Amphora montana
 58  T A A G T A T A A A T A C T T T A C T G T G A A A C T G C G  Anomoeoneis sphaerophora
 60  T A A G T A C A A A T A T T T C A C T G T G A A A C T G C G  Cymbella
  9  T A A G T A T A A A T A T A T C A C T T T G A A A C T G C G  Dickieia ulvacea
 73  T A A G T A T A A A T A T A T C A C T G T G A A A C T G C G  Encyonema triangulatum
 31  T A A G T A T A A A T C T T T T A C T T T G A A A C T G C G  Eolimna minima
 34  T A A G T A T A A A T C T T T T A C T T T G A A A C T G C G  Eolimna subminuscula
 43  T A A G T A T A A A T C T T T T A C T T T G A A A C T G C G  Fragilaria striatula
 34  T A A G T A T A A A T A T T A T A T T G T G A A A C T G C G  Gomphonema  parvulum
150  T A A G T A T A A A T A T A T T A C T G T G A A A C T G C G  NZ Gomphoneis sp
 49  T A A G T G T A A A T A T A C A A T T G T G A A A C T G C G  NZ Didymosphenia geminata

     A A T G G C T C A T T A T A T C A G T T A T A G T T T A T T  Consensus sequence
     -----------------+-----------------+-----------------+-
                      190               200               210
     -----------------+-----------------+-----------------+-
 64  A A T G G C T C A T T A T A T C A G T T A T A G T T T A T T  Amphora montana
 88  A A T G G C T C A T T A T A T C A G T T A T A G T T T C T T  Anomoeoneis sphaerophora
 90  A A T G G C T C A T T A T A T C A G T A A T A G T T T A T T  Cymbella
 39  A A T G G C T C A T T A T A T C A G T T A T A G T T T A T T  Dickieia ulvacea
103  A A T G G C T C A T T A T A T C A G T T A T A G T T T A T T  Encyonema triangulatum
 61  A A T G G C T C A T T A T A T C A G T T A T A G T T T A T T  Eolimna minima
 64  A A C G G C T C A T T A T A T C A G T T A T A G T T T A T T  Eolimna subminuscula
 73  A A C G G C T C A T T A T A T C A G T T A T A G T T T A T T  Fragilaria striatula
 64  A A T G G C T C A T T A T A T C A G T T A T A G T T T A T T  Gomphonema  parvulum
180  A A T G G C T C A T T A T A T C A G T T A T A G T T T A T T  NZ Gomphoneis sp
 79  A A T G G C T C A T T A T A T C A G T T A T A G T T T A T T  NZ Didymosphenia geminata

     T G A T A G T C C C T T A C T A C T T G G A T A A C C G T A  Consensus sequence
     -----------------+-----------------+-----------------+-
                      220               230               240
     -----------------+-----------------+-----------------+-
 94  T G A T A G T C C C T T A C T A C T T G G A T A A C C G T A  Amphora montana
118  T G A T A G T C C C T T A C T A C T T G G A T A A C C G T A  Anomoeoneis sphaerophora
120  T G A T A A T C C C T T T A C T A C T T G G A T A A C T G T A  Cymbella
 69  T G A T A G T C C C T T A C T A T T T G G A T A A C C G T A  Dickieia ulvacea
133  T G A T A G T C C C T T A C T A C T T G G A T A A C C G T A  Encyonema triangulatum
 91  T G A T A G T C C C T T A C T A C T T G G A T A A C C G T A  Eolimna minima
 94  T G A T A G T C C C T T A C T A C T C G G A T A A C C G T A  Eolimna subminuscula
103  T G A T A G T C C C T T A C T A C T T G G A T A A C C G T A  Fragilaria striatula
 94  T G A T A G T C C C T T A C T A C T T G G A T A A C C G T A  Gomphonema  parvulum
210  T G A T A A T C C C T T A C T A C T T G G A T A A T C G T A  NZ Gomphoneis sp
109  T G A T A G T C C C T T A C T A C T T G G A T A A C C G T A  NZ Didymosphenia geminata
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     G T A A T T C T A G A G C T A A T A C A T G C G T C A A T A  Consensus sequence
     -----------------+-----------------+-----------------+-
                      250               260               270
     -----------------+-----------------+-----------------+-
124  G T A A T T C T A G A G C T A A T A C A T G C G T C A A T A  Amphora montana
148  G T A A T T C T A G A G C T A A T A C A T G C G T T A A T A  Anomoeoneis sphaerophora
150  G T A A T T C T A G A G C T A A T A C A T G T G T A A A T G  Cymbella
 99  G T A A T T C T A G A G C T A A T A C A T G C G T C A A T A  Dickieia ulvacea
163  G T A A T T C T A G A G C T A A T A C A T G C G T C A A T A  Encyonema triangulatum
121  G T A A T T C T A G A G C T A A T A C A T G C G T C A A T A  Eolimna minima
124  G T A A T T C T A G A G C T A A T A C G T G C G T C A A T A  Eolimna subminuscula
133  G T A A T T C T A G A G C T A A T A C A T G C G T C A A C A  Fragilaria striatula
124  G T A A T T C T A G A G C T A A T A C A T G C A T C A A T A  Gomphonema parvulum
240  G T A A T T C T A G A G C T A A T A C A T G C A A T A A T A  NZ Gomphoneis sp
139  G T A A T T C T A G A G C T A A T A C T T G C A T A A A T A  NZ Didymosphenia geminata

     - C C C T T C T G G G G T A G T A T T T A T T A G A T T G A  Consensus sequence
     -----------------+-----------------+-----------------+-
                      280               290               300
     -----------------+-----------------+-----------------+-
154  - C C C C T C T G G G G T A G C A T T T A T T A G A T G G A  Amphora montana
178  - C C C T T C T T G G G G T A G C A A T T A T T A G A T T G A  Anomoeoneis sphaerophora
180  - C C C T T C T G G G G T A G T A T T T A T T A G A T T G A  Cymbella
129  - C C C T T C T G G G G T A G T A T T T A T T A G A T T G A  Dickieia ulvacea
193  - C C C T T C T G G G G T A G T A T T T A T T A G A T T G A  Encyonema triangulatum
151  - C C C T T C T G G G G T A G T A T T T A T T A G A T G G A  Eolimna minima
154  - C T C T T C - G G A G T A G T A T T T A T T A G A T G G A  Eolimna subminuscula
163  - C C T T T - - - G G G T G G T A T T T A T T A G A A T T A  Fragilaria striatula
154  - C C C T T C T C G G G G T A G T A T T T A T T A G A T T G A  Gomphonema parvulum
270  - C C C T T C T C G G G T A G T A T T T A T T A G A T T T A  NZ Gomphoneis sp
169  T C C C T T C A A G G A T A G T A T T T A T T A G A T T T A  NZ Didymosphenia geminata

     A A C C A A C C C C T T C - - - - G G G G T G A T G T G G T  Consensus sequence
     -----------------+-----------------+-----------------+-
                      310               320               330
     -----------------+-----------------+-----------------+-
183  A A C C A A C C C C T T C - - - - G G G G T G A T G T G G T  Amphora montana
207  A A C C A A C C C C T T C - - - - G G G G T G A T G T G G T  Anomoeoneis sphaerophora
209  A A C T T A A C C C T T C - - - - - - - G G T G A T G T G G T  Cymbella
158  A A C C A A C C C C T T C - - - - G G G G T G A T G T G G T  Dickieia ulvacea
222  A A C C A A C C C C T C A - - - - C G G G T G A T G T G G T  Encyonema triangulatum
180  A A C C A A C C C C T T C - - - - G G G G T G A T G T G G T  Eolimna minima
182  A A C C A A C T G C T T C - - - - G G C A T G A T G T G G T  Eolimna subminuscula
189  A A C C A A C C T C T G C T T G C A G G G G T G A T G T G G T  Fragilaria striatula
183  A A C C A A C C C C T T C - - - - G G G G T G A T G T G G T  Gomphonema parvulum
299  A A C C A A C C C C T T T - - - - G G G G T G A T G T G G T  NZ Gomphonesis sp
199  A A C C A A C C C C T T C - - - - G G G G T G A T G T G G T  NZ Didymosphenia geminata

     G A T T C A T A A T A A G T T T G C G G A T C G C A T G G C  Consensus sequence
     -----------------+-----------------+-----------------+-
                      340               350               360
     -----------------+-----------------+-----------------+-
209  G A A T C A T A A T A A G T T T G C G G A T C G C - - - - -  Amphora montana
233  G A T T C A T A A T A A G C T T G C G A A T C G C A C A G C  Anomoeoneis sphaerophora
233  G A C T C A T A A T A A G T T T G C G G A T T G C A T G G A  Cymbella
184  G A T T C A T A A T A A G C T T G C G G A T C G C A T G G C  Dickieia ulvacea
248  G A T T C A T A A T A A G - T T G C G A A T C G C A C G G C  Encyonema triangulatum
206  G A T T C A T A A T A A G C T T G C G G A T C G C A C G G G  Eolimna minima
208  G A T T C A T A A T A A G C T T G C G G A T C G C A T G G -  Eolimna subminuscula
219  G A A T C A T A A T A A G T T T G C G A A T C G C A C G G C  Fragilaria striatula
209  G A T T C A T A A T A A G T T T G C G A A T C G C A T G A C  Gomphonema parvulum
325  G A T T C A T A A T A A T T T T G C G A A T C G C A T G A C  NZ Gomphoneis sp
225  G A T T C A A A A T A A G T T T A C G G A T C G C A T G G C  NZ Didymosphenia geminata
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     T T - - T G C C G G C G A T G G A T C A T T C A A G T T T C  Consensus sequence
     -----------------+------------------+------------------+-
                      370                380                390
     -----------------+------------------+------------------+-
234  - - - - C G G T G G C G A T G G A T C A T T C A A G T T T C  Amphora montana
263  C T - - A G C T G G C G A T G G A T C A T G C A A G T T T C  Anomoeoneis sphaerophora
263  T T - - T T C C G G T G A T G G A T C A T T C A A G T T T C  Cymbella
214  T T - - T G C C G G C G A T G G A T C A T T C A A G T T T C  Dickieia ulvacea
277  C T - - T G C T G G C G A T G G A T C A T T C A A G T T T C  Encyonema triangulatum
236  C T T - T G C C G G C G A T G G A T C A T T C A A G T T T C  Eolimna minima
237  C C T - A G C C G G C G A C G G A T C A T T C A A G T T T C  Eolimna subminuscula
249  T T T A T G C T G G C G A T G G A T C A T T C A A G T T T C  Fragilaria striatula
239  T T - - T G T C G G C G A T G G A T C A T T C A A G T T T C  Gomphonema parvulum
355  C T - - A G T C G G C G A T G G A T C A T T C A A G T T T C  NZ Gomphoneis sp
255  T T - - T G C C G G C G A C G G A T C A T T C A A G T T T C  NZ Didymosphenia geminata

     T G C C C T A T C A G C T T T G G A T G G T A G G G T A T T  Consensus sequence
     -----------------+------------------+------------------+-
                      400                410                420
     -----------------+------------------+------------------+-
260  T G C C C T A T C A G C T T T G G A C G G T A A G G T A T T  Amphora montana
291  T G C C C T A T C A G C T T T G G A T G G T A G G G T A T T  Anomoeoneis sphaerophora
291  T G C C C T A T C A G C T T T G G A T G G T A G G G T A T T  Cymbella
242  T G C C C T A T C A G C T T T G G A T G G T A G G G T A T T  Dickieia ulvacea
305  T G C C C T A T C A G C T T T G G A T G G T A G G G T A T T  Encyonema triangulatum
265  T G C C C T A T C A G C T T T G G A A G G T A G G G T C T T  Eolimna minima
266  T G C C C T A T C A G C T T T G G A C G G T A C T G T A T T  Eolimna subminuscula
279  T G C C C T A T C A G C T T T G G A T G G T A G G G T A T T  Fragilaria striatula
267  T G C C C T A T C A G C T T T G G A T G G T A G G G T A T T  Gomphonema parvulum
383  T G C C C T A T C A G C T T T G G A T G G T A G G G T A T T  NZ Gomphoneis sp
283  T G C C C T A T C A G C T T T G G A T G G T A G G G T A T T  NZ Didymosphenia geminata

     G G C C T A C C A T G G C T T T A A C G G G T A A C G G A G  Consensus sequence
     -----------------+------------------+------------------+-
                      430                440                450
     -----------------+------------------+------------------+-
290  G G C T T A C C G T G G C T T T A A C G G G T A A C G G G G  Amphora montana
321  G G C C T A C C A T G G C T T T C A C G G G T A A C G G A G  Anomoeoneis sphaerophora
321  G C C C T A C C A T T G C T T T A A C G G G T A A C G G A G  Cymbella
272  G G C C T A C C A T G G C T T T A A C G G G T A A C G G A G  Dickieia ulvacea
335  G G C C T A C C A T G G C T T T A A C G G G T A A C G G A G  Encyonema triangulatum
295  G G C C T A C C T T G G C T T T A A C G G G T A A C G G A T  Eolimna minima
296  G G A G T A C C G T G G C G G T A A C G G G T A A C G G A A  Eolimna subminuscula
309  G G C C T A C C A T G G C T T T A A C G G G T A A C G G G A  Fragilaria striatula
297  G G C C T A C C A T G G C T T T A A C G G G T A A C G G A G  Gomphonema parvulum
413  G G C C T A C C A T G G C T T T A A C G G G T A A C G G A G  NZ Gomphoneis sp
313  G G C C T A C C A T G G C T T T A A C G G G T A A C G G A G  NZ Didymosphenia geminata

     G A T T A G G G T T T G A T T C C G G A G A G G G A G C C T  Consensus sequence
     -----------------+------------------+------------------+-
                      460                470                480
     -----------------+------------------+------------------+-
320  A A T T A G G G T T C G A T T C C G G A G A G G G A G C C T  Amphora montana
351  G A T T A G G G T T T G A T T C C G G A G A G G G A G C C T  Anomoeoneis sphaerophora
351  G A T T A G G G T T T R A T T C C G G A G A G G G A G C C T  Cymbella
302  G A T T A G G G T T T G A T T C C G G A G A G G G A G C C T  Dickieia ulvacea
365  G A T T A G G G T T T G A T T C C G G A G A G G G A G C C T  Encyonema triangulatum
325  A A T T A G G G T T T G A T T C C G G A G A G G G A G C C T  Eolimna minima
326  A A T T A G G G T T T G A C A C C G G A G A G G G A G C C T  Eolimna subminuscula
339  A A T T A G G G T T T G A T T C C G G A G A G G G A G C C T  Fragilaria striatula
327  G A T T A G G G T T T G A T T C C G G A G A G G G A G C C T  Gomphonema parvulum
443  G A T T A G G G T T T G A T T C C G G A G A G G G A G C C T  NZ Gomphoneis sp
343  G A T T A G G G T T T G A T T C C G G A G A G G G A G C C T  NZ Didymosphenia geminata
```

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     G A G A G A C G G C T A C C A C A T C C A A G G A A G G C A  Consensus sequence
     -----------------+-----------------+-----------------+-
                      490               500               510
     -----------------+-----------------+-----------------+-
350  G A G A G A C G G C T A C C A C A T C C A A G G A A G G C A  Amphora montana
381  G A G A G A C G G C T A C C A C A T C C A A G G A A G G C A  Anomoeoneis sphaerophora
381  G A G A G A C G G C T A C C A C A T C C A A G G A A G G C A  Cymbella
332  G A G A G A T G G C T A C C A C A T C C A A G G A A G G C A  Dickieia ulvacea
395  G A G A G A C G G C T A C C A C A T C C A A G G A A G G C A  Encyonema triangulatum
355  G A G A G A C G G C T A C C A C A T C C A A G G A A G G C A  Eolimna minima
356  G A G A G A C G G C T A C C A C A T C C A A G G A A G G C A  Eolimna subminuscula
369  G A G A G A C G G C T A C C A C A T C C A A G G A A G G C A  Fragilaria striatula
357  G A G A G A C G G C T A C C A C A T C C A A G G A A G G C A  Gomphonema parvulum
473  G A G A G A C G G C T A C C A C A T C C A A G G A A G G C A  NZ Gomphoneis sp
373  G A G A G A C G G C T A C C A C A T C C A A G G A A G G C A  NZ Didymosphenia geminata

     G C A G G C G C G T A A A T T A C C C A A T C C T G A C A C  Consensus sequence
     -----------------+-----------------+-----------------+-
                      520               530               540
     -----------------+-----------------+-----------------+-
380  G C A G G C G C G T A A A T T A C C C A A T C C T T G A C A C  Amphora montana
411  G C A G G C G C G T A A A T T A C C C A A T C C T G A C A C  Anomoeoneis sphaerophora
411  G C A G G C G C G T A A A T T A C C C A A T C C T G A C A C  Cymbella
362  G C A G G C G C G T A A A T T A C C C A A T C C T G A C A C  Dickieia ulvacea
425  G C A G G C G C G T A A A T T A C C C A A T C C T G A C A C  Encyonema triangulatum
385  G C A G G C G C G T A A A T T A C C C A A T C C T G A C A C  Eolimna minima
386  G C A G G C G C G T A A A T T A C C C A A T C C T T G A C A C  Eolimna subminuscula
399  G C A G G C G C G T A A A T T A C C C A A T C C T G A C A C  Fragilaria striatula
387  G C A G G C G C G T A A A T T A C C C A A T C C T G A C A C  Gomphonema parvulum
503  G C A G G C G C G T A A A T T A C C C A A T C C T G A T A C  NZ Gomphoneis sp
403  G C A G G C G C G T A A A T T A C C C A A T C C T G A C A C  NZ Didymosphenia geminata

     A G G G A G G T A G T G A C A A T A A A T A A C A A T G C C  Consensus sequence
     -----------------+-----------------+-----------------+-
                      550               560               570
     -----------------+-----------------+-----------------+-
410  A A G G A G G T A G T G A C A A T A A A T A A C A A T G C C  Amphora montana
441  A G G G A G G T A G T G A C A A T A A A T A A C A A T G C T  Anomoeoneis sphaerophora
441  A G G G A G G T A G T G A C A A T A A A T A A C A A T G C T  Cymbella
392  A G G G A G G T A G T G A C A A T A A A T A A C A A T G C C  Dickieia ulvacea
455  A G G G A G G T A G T G A C A A T A A A T A A C A A T G G C  Encyonema triangulatum
415  A G G G A G G T A G T G A C A A T A A A T A A C A A T G C C  Eolimna minima
416  A A G G A G G T A G T G A C A A T A A A T A A C A A T G C C  Eolimna subminuscula
429  A G G G A G G T A G T G A C A A T A A A T A A C A A T G C C  Fragilaria striatula
417  A G G G A G G T A G T G A C A A T A A A T A A C A A T G C C  Gomphonema parvulum
533  A G G G A G G T A G T G A C A A T A A A T A A C A A T G C C  NZ Gomphoneis sp
433  A G G G A G G T A G T G A C A A T A A A T A A C A A T G C C  NZ Didymosphenia geminata

     G G G C C T T T - T A G G T C T G G C A C T T G G A A T G A  Consensus sequence
     -----------------+-----------------+-----------------+-
                      580               590               600
     -----------------+-----------------+-----------------+-
440  G G G C C T T C - T A G G T C T G G C A A T T G G A A T G A  Amphora montana
471  G G G C C T A T C T A G G T C T G G C A C T T G G A A T G A  Anomoeoneis sphaerophora
471  G G G C C T T A - C A G G T T T G T C A C T T G G A A T G A  Cymbella
422  G G G C C T T T C T A G G T C T G G C A C T T G G A A T G A  Dickieia ulvacea
485  G G G C C T T A - C A G G T C T G G C A C T T G G A A T G A  Encyonema triangulatum
445  G G G C C T T T G T A G G T C T G G C A A T T G G A A T G A  Eolimna minima
446  G G G C C A T T - A C G G T C T G G C A A T T G G A A T G A  Eolimna subminuscula
459  G G G C C T T T A T A G G T C T G G C A A T T G G A A T G A  Fragilaria striatula
447  G G G C C T T T - C A G G T C T G G C A C T T G G A A T G A  Gomphonema parvulum
563  G G G C C T T T - C A G G T C T G G C A C T T G G A A T G A  NZ Gomphoneis sp
463  G A G C C C T T G T - G G T T T G G C A C T T G G A A T G A  NZ Didymosphenia geminata
```

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     G A A C A A T T T A A A C C C C T T A T C G A G G A T C A A  Consensus sequence
     ----------------+-----------------+-----------------+-
                     610               620               630
     ----------------+-----------------+-----------------+-
469  G A A C A A T T T A A A C C C C T T A T C G A G G A T C A A  Amphora montana
501  G A A C A A T T T A A A T C T C T T T T C G A G G A T C A A  Anomoeoneis sphaerophora
500  G T A C A A T T C A A A C T A C T T A A C A A G G A T C A A  Cymbella
452  G A A C A A T T T A A A C C C C T T A T C G A G G A T C A A  Dickieia ulvacea
514  G A A C A A T T C A A A C C A C T T A T C G A G G A T C G A  Encyonema triangulatum
475  G A A C A A T T T A A A C C C C T T A T C G A G G A T C C A  Eolimna minima
475  G A A C A A T T T A A A C C C C T T A T C G A G G A T C A A  Eolimna subminuscula
489  G A A C A A T T T A A A C C C C T T A T C G A G G A C C A A  Fragilaria striatula
476  G A A C A A C T C A A A C C A C T T A T C G A G G A T C A A  Gomphonema  parvulum
592  G A A C A A C T C A A A C C A C T T A T C G A G G A T C A A  NZ Gomphoneis sp
492  G A A C A A C T C A A A C C A C T T A A C G A G G A T C A A  NZ Didymosphenia geminata

     T T G G A G G G C A A G T C T G G T G C C A G C A G C C G C  Consensus sequence
     ----------------+-----------------+-----------------+-
                     640               650               660
     ----------------+-----------------+-----------------+-
499  T T G G A G G G C A A G T C T G G T G C C A G C A G C C G C  Amphora montana
531  T T G G A G G G C A A G T C T G G T G C C A G C A G C C G C  Anomoeoneis sphaerophora
530  T T G G A G G G C A A G T C T G G T G C C A G C A G C C G C  Cymbella
482  T T G G A G G G C A A G T C T G G T G C C A G C A G C C G C  Dickieia ulvacea
544  T T G A A G T G C A A G T C T G G T G C C A G C A G C C G G  Encyonema triangulatum
505  A T G G A G G G C A A G T C T G G T G C C A G C A G C C G C  Eolimna minima
505  T T G G A G G G C A A G T C T G G T G C C A G C A G C C G C  Eolimna subminuscula
519  T T G G A G G G C A A G T C T G G T G C C A G C A G C C G C  Fragilaria striatula
506  T T G G A G G G C A A G T C T G G T G C C A G C A G C C G C  Gomphonema  parvulum
622  T T G G A G G G C A A G T C T G G T G C C A G C A G C C G C  NZ Gomphoneis sp
522  T T G G A G G G C A A G T C T G G T G C C A G C A G C C G C  NZ Didymosphenia geminata
                                                          |
     G G T A A T T C C A G C T C C A A T A G C G T A T A T T A A  Consensus sequence
     ----------------+-----------------+-----------------+-
                     670               680               690
     ----------------+-----------------+-----------------+-
529  G G T A A T T C C A G C T C C A A T A G C G T A T A T T A A  Amphora montana
561  G G T A A T T C C A G C T C C A A T A G C G T A T A T T A A  Anomoeoneis sphaerophora
560  G G T A A T T C C A A C T C C A A T A G C G T A T A T T A A  Cymbella
512  G G T A A T T C C A G C T C C A A T A G C G T A T A T T A A  Dickieia ulvacea
574  G G T A A T T C C A G C T C A A A T A G C G T A T A T T A A  Encyonema triangulatum
535  G G T A A T T C C A G C T C C A T T A G C G T A T A T T A A  Eolimna minima
535  G G T A A T T C C A G C T C C A A T A G C G T A T A T T A A  Eolimna subminuscula
549  G G T A A T T C C A G C T C C A A T A G C G T A T A T T A A  Fragilaria striatula
536  G G T A A T T C C A G C T C C A A T A G C G T A T A T T A A  Gomphonema  parvulum
652  G G T A A T T C C A G C T C C A A T A G C G T A T A T T A A  NZ Gomphoneis sp
552  G G T A A T T C C A G C T C C A A T A G C G T A T A T T A A  NZ Didymosphenia geminata
     ---------------Euk608F------------|

     A G T T G T T G C A G T T A A A A A G C T C G T A G T T G G  Consensus sequence
     ----------------+-----------------+-----------------+-
                     700               710               720
     ----------------+-----------------+-----------------+-
559  A G T T G T T G C A G T T A A A A A G C T C G T A G T T G G  Amphora montana
591  A G T T G T T G C A G T T A A A A A G C T C G T A G T T G A  Anomoeoneis sphaerophora
590  A G T T G T T G C A G T T A A A A A G C T C G T A G T T G G  Cymbella
542  A G T T G T T G C A G T T A A A A A G C T C G T A G T T G G  Dickieia ulvacea
604  A G T T G T T G C A G T T A A A A A G C T C G T A G T T G G  Encyonema triangulatum
565  C G T T G T T G C A G T T A A A A A G C T C G T A G T T G G  Eolimna minima
565  A G T T G T T G C A G T T A A A A A G C T C G T A G T T G G  Eolimna subminuscula
579  A G T T G T T G C A G T T A A A A A G C T C G T A G T T G G  Fragilaria striatula
566  A G T T G T T G C A G T T A A A A A G C T C G T A G T T G G  Gomphonema  parvulum
682  A G T T G T T G C A G T T A A A A A G C T C G T A G T T G G  NZ Gomphoneis sp
582  A G T Y G T T G C A G T T A A A A A G C T C G T A G T T G G  NZ Didymosphenia geminata
                                                    |-------
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     A T T T G T G G T G G G T T T C G G G G G T C C T T T - - -  Consensus sequence
     -----------------+-----------------+-----------------+-
                      730               740               750
     -----------------+-----------------+-----------------+-
589  A T T T G T G G T G - G T C C C T G A G G T C C G A T - - -  Amphora montana
621  A C T T G T G A T G G A A C T G A A T G A C T C A T C A - -  Anomoeoneis sphaerophora
620  A T G T G T G G T A A C T T T C A G C G G T C A T T T - - -  Cymbella
572  A T T T G T G G T G - G T T T C T G A T G T C C A A T - - -  Dickieia ulvacea
634  A T T T G T G G T T - G A T T G G A G A G T C C T C T - - -  Encyonema triangulatum
595  A T T T G T G G T T T G T T G C A T G G T C C C A A T A T T  Eolimna minima
595  A T T T G T G G T G C G A C T G C G G C G T C C A T T - - -  Eolimna subminuscula
609  A T T T G T G G T T G G G T C C G T C G G T C C G G C A C A  Fragilaria striatula
596  A T T T G T G G C A G T G C T C G A G T G C C T T T T - T -  Gomphonema  parvulum
712  A T T T G T G A T G A T G C T T G G G A G C C T C T T - T -  NZ Gomphoneis sp
612  A T T T G T G A T G G A A T T T G A A T A C T T T T A A - -  NZ Didymosphenia geminata
     --------------D602F--------------|

     - - - T - T T G G T T C T T T T A G G - T - C T G C C A T C  Consensus sequence
     -----------------+-----------------+-----------------+-
                      760               770               780
     -----------------+-----------------+-----------------+-
615  - - - T - C C G G T A C T T T T G G G G A - C T G C C A T C  Amphora montana
649  - - - - - - T T G A T G T C A G A A A G C T - T T G T C A A T  Anomoeoneis sphaerophora
647  - - - - - - T T G A C T T C T C T A G G - - - T A A C C A T C  Cymbella
598  - - - T - T T G G T A C A A T C G G A G A - C C G C C A T C  Dickieia ulvacea
660  - - - T - T T G G T A C T T T C A T G A T - C A G C C A T C  Encyonema triangulatum
625  A T G T - T G G G T G C T G T T G C G A G - G A G C C A T C  Eolimna minima
622  - T A T - T T G G T T C T G C C G T G G T T G T G C C A T C  Eolimna subminuscula
639  T T G T G C G G G T A C T T G A T G G A C T C G T C C A T C  Fragilaria striatula
624  - - - - - - T G G A C G C T C T G A G - - C A C A G C C A T C  Gomphonema  parvulum
740  - - - - - - T G G A T T C C T T T A - - - C A T T G T C A T C  NZ Gomphoneis sp
640  - - - - - - A G T G T T T C A G A A A - - - - C T G T C A T C  NZ Didymosphenia geminata

     C T T G G G T G G A A - T C T G T G T G G C A T T A G G T T  Consensus sequence
     -----------------+-----------------+-----------------+-
                      790               800               810
     -----------------+-----------------+-----------------+-
640  C T T G G G T G G A T - C C T G T G T G G C A T T A G G T T  Amphora montana
673  T T T G A G T G G A A - C T T G C A T G G C A T T A G G T T  Anomoeoneis sphaerophora
669  T T T G G G T G A A A - C C A A T G T G G C A T T A G T T T  Cymbella
623  C T T G G G T G G A A - T C T G T G T G G C A T T A G G T T  Dickieia ulvacea
685  C T T G G G T G G A A A C C T G T G T G G C A T T A G G T T  Encyonema triangulatum
653  C T T G G G T G G A A - T C T G T G T G G C A T T A G G T T  Eolimna minima
650  C T T G G G T G G A T - T C T G T G T G G C A T T T A G T T  Eolimna subminuscula
669  C T T G G G T G G A A - T C T G T G T G G C A T T A A G T T  Fragilaria striatula
647  C T T G G G A G G A A - T T C G T G T G G C A T T A G G T T  Gomphonema  parvulum
762  T T T G G G A G G A T - T T T G T G T G G C A T T A G C T T  NZ Gomphoneis sp
661  C G T G G G T G G A A - T T T G T T T G G C A T T A G G T T  NZ Didymosphenia geminata

     G T C G T G C A G G G G A T G C C C A T C G T T T A C T G T  Consensus sequence
     -----------------+-----------------+-----------------+-
                      820               830               840
     -----------------+-----------------+-----------------+-
669  G T C G C G C A G G G G A T G C C C A T C G T T T A C T G T  Amphora montana
702  G T T G T G T A G G G G A T G C T C A T C G T T T A C T G T  Anomoeoneis sphaerophora
698  G T C C C G T T G G T G A T T C C C A T C A T T T A C T G T  Cymbella
652  G T C G T G C A G G G G A T G C C C A T C G T T T A C T G T  Dickieia ulvacea
715  G T C G T G C T G G G G A T G C T C A T C A T T T A C T G T  Encyonema triangulatum
682  G T C G C G C A G G G G A T G C C C A T C G T T T A C T G T  Eolimna minima
679  G T C G T G C A G G G G A C - C C C A T C G T T T A C T G T  Eolimna subminuscula
698  G T C G T G C A G G G G A T G C C C A T C G T T T A C T G T  Fragilaria striatula
676  G T T G C A T G G G G G A T A C T C A A C A T T T A C T G T  Gomphonema  parvulum
791  G T T G C A T A G G G G A T A C T C A A C G T T T A C T G T  NZ Gomphoneis sp
690  G T C A G R C A G A G G A T G C C T A T M C T T T A C T G T  NZ Didymosphenia geminata
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```
     G A A A A A A T C A G C G C G T T C A A A G C A G G C T T A  Consensus sequence
     -----------------+-----------------+-----------------+-
                      850               860               870
     -----------------+-----------------+-----------------+-
699  G A A A A A A T T A G A G T G T T C A A A G C A G G C T T A  Amphora montana
732  G A A A A A A T C A G C G C G T T C A A A G C A G G C T T A  Anomoeoneis sphaerophora
728  G A A A A A A T C A G T G C G T T C A A A G C A G G C T T A  Cymbella
682  G A A A A A A T C A G C G C G T T C A A A G C A G G C T T A  Dickieia ulvacea
745  G A A A A A A T C A G C G C G T T C A A A G C A G G C T T A  Encyonema triangulatum
712  G A A A A A A T T A G A G T G T T C A A A G C A G G C T T A  Eolimna minima
708  G A A A A A A T T A G A G T G T T C A A A G C A G G C T T A  Eolimna subminuscula
728  G A A A A A A T T A G A G T G T T C A A A G C A G G C T T A  Fragilaria striatula
706  G A A A A A A T C A G C G C G T T C A A A G C A A G C T T A  Gomphonema  parvulum
821  G A A A A A A T C A G C G C G T T C A A A G C A A G C T T A  NZ Gomphoneis sp
720  G A A A A A A T C A G T G C G T T C A A A G C A G A C T T A  NZ Didymosphenia geminata
                                                  |----------

     T G C C G T T G A A T G T A T T A G C A T G G A A T A A T A  Consensus sequence
     -----------------+-----------------+-----------------+-
                      880               890               900
     -----------------+-----------------+-----------------+-
729  C G C C G T T G A A T A T A T T A G C A T G G A A T A A T G  Amphora montana
762  T G C C G T T G A A T G T A T T A G C A T G G A A T A A T A  Anomoeoneis sphaerophora
758  T G C C A T T G A A T G T A T T A G C A T G G A A T A A T A  Cymbella
712  T G C C G T T G A A T G T A T T A G C A T G G A A T A A T A  Dickieia ulvacea
775  T G C C G T T G A A T G T A A T A G C A T G G A A T A A T A  Encyonema triangulatum
742  T G C C G T T G A A T A T A T T A G C A T G G A A T A A T G  Eolimna minima
738  T G C C G T T G A A T A T A T T A G C A T G G A A T A A T A  Eolimna subminuscula
758  T G C C G T T G A A T A T A T T A G C A T G G A A T A A T G  Fragilaria striatula
736  T G C T C T T G A A T G T A T T A G C A T G G A A T A A T A  Gomphonema  parvulum
851  T G C T G T - G A A T G T A T T A G C A T G G A A T A A T A  NZ Gomphoneis sp
750  C G T C G A T G A A T G T A T T A G C A T G G A A T A A T A  NZ Didymosphenia geminata
     ---------D753R-----------------|

     A G A T A G G A C C T T G G T A C T A T T T T G T T G G T T  Consensus sequence
     -----------------+-----------------+-----------------+-
                      910               920               930
     -----------------+-----------------+-----------------+-
759  A G A T A G G A C C T C G G T A C T A T T T T G T T G G T T  Amphora montana
792  A G A T A G G A C T T G T G T A C T A T T T T G T T G G T T  Anomoeoneis sphaerophora
788  T G A T A G G A C C T C G G T A C T A T T T T G T T G G T T  Cymbella
742  A G A T A G G A C C T T G G T A C T A T T T T G T T G G T T  Dickieia ulvacea
805  A G A T A G G A C C T T G G T A C T A T T T T G T T G G T T  Encyonema triangulatum
772  A G A T A G G A C C T T G G T A C T A T T T T G T T G G T T  Eolimna minima
768  A G A T A G G T C T T T G G T C C T A T T T T G T T G G T T  Eolimna subminuscula
788  A G A T A G G A C C T T G G T A C T A T T T T G T T G G T T  Fragilaria striatula
766  A G A T A G G A C T T T A G T A C T A T T T T G T T G G T T  Gomphonema  parvulum
880  A G A T A G G A C C T T G G T A C T A T T T T G T T G G T T  NZ Gomphoneis sp
780  A G A T A G G A C C T T T G T A C T A T T T T G T T G G T T  NZ Didymosphenia geminata

     T G C G C A C C G A G G T A A T G A T T A A T A G G G A C A  Consensus sequence
     -----------------+-----------------+-----------------+-
                      940               950               960
     -----------------+-----------------+-----------------+-
789  T G C G C A C T G G G G T A A T G A T T A A T A G G G A C A  Amphora montana
822  T G T G T A C G C A G G T A A T G G T T A A T A G G G A C A  Anomoeoneis sphaerophora
818  T G T G C A C C A A G G T A A T G A T T A A T A G G G A T A  Cymbella
772  T G C G C A C C G A G G T A A T G A T T A A T A G G G A C A  Dickieia ulvacea
835  T G T G C A C C G A G G T A A T G A T T A A T A G G G A C A  Encyonema triangulatum
802  T G C G C A C C G A G G T A A T G A T T A A T A G G G A C A  Eolimna minima
798  T G C G G A C C A G A G A A A T G A T T A A C A G G G A C A  Eolimna subminuscula
818  T G C G C A C C G A G G T A A T G A T T A A T A G G G A C A  Fragilaria striatula
796  T G C G T A T T A A G G T A A T G A T T A A T A G G G A T A  Gomphonema  parvulum
910  T G T G C A C C G A G G T A A T G A T T A A T A G G G A T A  NZ Gomphoneis sp
810  T G T G T A T A G A G G T A A T G A T T A A A A G G A A C A  NZ Didymosphenia geminata
```

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      G T T G G G G G T A T T C G T A T T C C A T T G T C A G A G  Consensus sequence
      -----------------+-----------------+-----------------+-
                       970               980               990
      -----------------+-----------------+-----------------+-
  819 G T T G T G G G T A T T C G T A T T C C A T T G T C A G A G  Amphora montana
  852 G T T G G G G G T A T T C G T A T T C C A T T G T C A G A G  Anomoeoneis sphaerophora
  848 G T T G G G G G T A T T T G T A T T C C A T T G T C A G A G  Cymbella
  802 G T T G G G G G T A T T C G T A T T C C A T T G T C A G A G  Dickieia ulvacea
  865 G T T G G G G G T A T T C G T A T T C C A T T G T C A G A G  Encyonema triangulatum
  832 G T T G G G G G T A T T C G T A T T C C A T T G T C A G A G  Eolimna minima
  828 G T T G G G G G T A T T C G T A T T C C A T T G T C A G A G  Eolimna subminuscula
  848 G T T G G G G G T A T T C G T A T T C C A T T G T C A G A G  Fragilaria striatula
  826 G T T G G G G G T A T T C G T A T T C C A T T G T C A G A G  Gomphonema  parvulum
  940 G T T G G G G G T A T T C G T A T T C C A T T G T C A G A G  NZ Gomphoneis sp
  840 G T T G G G G G T A T T T G T A T T C C A T T G T C A G A G  NZ Didymosphenia geminata

      G T G A A A T T C T T G G A T T T T T G G A A G A C G A A C  Consensus sequence
      -----------------+-----------------+-----------------+-
                       1000              1010              1020
      -----------------+-----------------+-----------------+-
  849 G T G A A A T T C T T G G A T T T C T G G A A G A C G A A C  Amphora montana
  882 G T G A A A T T C T T G G A T T T C T G G A A G A C G A A C  Anomoeoneis sphaerophora
  878 G T G A A A T T C T T G G A T T T T T G G A A G A C A A A C  Cymbella
  832 G T G A A A T T C T T G G A T T T C T G G A A G A C G A A C  Dickieia ulvacea
  895 G T G A A A T T C T T G G A T T T T T G G A A G A C G A A C  Encyonema triangulatum
  862 G T G A A A T T C T T G G A T T T C T G G A A G A C G A A C  Eolimna minima
  858 G T G A A A T T C T T G G A T T T C T G G A A G A C G A A C  Eolimna subminuscula
  878 G T G A A A T T C T T G G A T T T T T G G A A G A C G A A C  Fragilaria striatula
  856 G T G A A A T T C T T G G A T T T T T G G A A G A C G A A C  Gomphonema  parvulum
  970 G T G A A A T T C T T G G A T T T T T G G A A G A C G A A C  NZ Gomphoneis sp
  870 G T G A A A T T C T T G G A T T T T T G G A A G A C A A A C  NZ Didymosphenia geminata

      T A C T G C G A A A G C A T T T A C C A A G G A T - G T T T  Consensus sequence
      -----------------+-----------------+-----------------+-
                       1030              1040              1050
      -----------------+-----------------+-----------------+-
  879 T A A T G C G A A A G C A T T T T C C A A G G A T - G T T T  Amphora montana
  912 T A C T G C G A A A G C A T T T A C C A A G G A T - G T T T  Anomoeoneis sphaerophora
  908 T A C T G C G A A A G C A T T T A C C A A G G A T T G T T T  Cymbella
  862 T A C T G C G A A A G C A T T T A C C A A G G A T - G T T T  Dickieia ulvacea
  925 T A C T G C G A A A G C A T T T A C C A A G G A T T G T T T  Encyonema triangulatum
  892 T A C T G C G A A A G C A T T T A C C A A G G A T - G T T T  Eolimna minima
  888 T A C T G C G A A A G C A T T T A C C A A G G A T - G T T T  Eolimna subminuscula
  908 T A C T G C G A A A G C A T T T A C C A A G G A T - G T T T  Fragilaria striatula
  886 T A C T G C G A A A G C A T T T A C C A A G G A T - G T T T  Gomphonema  parvulum
 1000 T A C T G C G A A A G C A T T T A C C A A G G A T - G T T T  NZ Gomphoneis sp
  900 T A C T G C G A A R G C A T T T A C C A A G G A T - G T T T  NZ Didymosphenia geminata

      T C A T T A A T C A A G A A C G A A A G T T A G G G G A T C  Consensus sequence
      -----------------+-----------------+-----------------+-
                       1060              1070              1080
      -----------------+-----------------+-----------------+-
  908 T C G T T A A T C A A G A A C G A A A G T T A G G G G A T C  Amphora montana
  941 T C A T T A A T C A A G A A C G A A A G T T A G G G G A T C  Anomoeoneis sphaerophora
  938 T C A T T A A T C A A G A A C G A A A G T T A G G G G A T C  Cymbella
  891 T C A T T A A T C A A G A A C G A A A G T T A G G G G A T C  Dickieia ulvacea
  955 T C A T T A A T C A A G A A C G A A A G T T A G G G G A T C  Encyonema triangulatum
  921 T C A T T A A T C A A G A A C G A A A G T T A G G G G A T C  Eolimna minima
  917 T C A T T A A T C A A G A A C G A A A G T T A G G G G A T C  Eolimna subminuscula
  937 T C A T T A A T C A A G A A C G A A A G T T A G G G G A T C  Fragilaria striatula
  915 T C A T T A A T C A A G A A C G A A A G T T A G G G G A T C  Gomphonema  parvulum
 1029 T C A T T A A T C A A G A A C G A A A G T T A G G G G A T C  NZ Gomphoneis sp
  929 T C A T T A A T C A A G A A C G A A A G T T A G G G G A T C  NZ Didymosphenia geminata
                           |--------------Euk1000F/R----------
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      G A A G A T G A T T A G A T A C C A T C G T A G T C T T A A  Consensus sequence
      -----------------+-----------------+-----------------+-
                       1090              1100              1110
      -----------------+-----------------+-----------------+-
  938 G A A G A T G A T T A G A T A C C A T C G T A G T C T T A A  Amphora montana
  971 G A A G A T G A T T A G A T A C C A T C G T A G T C T T A A  Anomoeoneis sphaerophora
  968 G A A G A T G A T T A G A T A C C A T C G T A G T C T T A A  Cymbella
  921 G A A G A T G A T T A G A T A C C A T C G T A G T C T T A A  Dickieia ulvacea
  985 G A A G A T G A T T A G A T A C C A T C G T A G T C T T A A  Encyonema triangulatum
  951 G A A G A T G A T T A G A T A C C A T C G T A G T C T T A A  Eolimna minima
  947 G A A G A T G A T T A G A T A C C A T C G T A G T C T T A A  Eolimna subminuscula
  967 G A A G A T G A T T A G A T A C C A T C G T A G T C T T A A  Fragilaria striatula
  945 G A A G A T G A T T A G A T A C C A T C G T A G T C T T A A  Gomphonema  parvulum
 1059 G A A G A T G A T T A G A T A C C A T C G T A G T C T T A A  NZ Gomphoneis sp
  959 G A A G A T G A T T A G A T A C C A T C G T A G T C T T A A  NZ Didymosphenia geminata
      ---|

      C C A T A A A C T A T G C C G A C A A G G G A T T G G T G G  Consensus sequence
      -----------------+-----------------+-----------------+-
                       1120              1130              1140
      -----------------+-----------------+-----------------+-
  968 C C A T A A A C T A T G C C G A C A A G A G A T T G G G A A  Amphora montana
 1001 C C A T A A A C T A T G C C A A C A A A G G A T T G G T G G  Anomoeoneis sphaerophora
  998 C C A T A A A C T A T G C C A A C A A G G G A T T G G T G G  Cymbella
  951 C C A T A A A C T A T G C C G A C A A G G G A T T G G T G G  Dickieia ulvacea
 1015 C C A T A A A C T A T G C C G A C A A G G G A T T G G T G G  Encyonema triangulatum
  981 C C A T A A A C T A T G C C G G T T A G G G A T T G G C A G  Eolimna minima
  977 C C A T A A A C T A T G C C G A C A A G G G A T T G G C G G  Eolimna subminuscula
  997 C C A T A A A C T A T G C C G A C A A G G G A T T G G C - G  Fragilaria striatula
  975 C C A T A A A C T A T G C C G A C A A G G G A T T G G T G G  Gomphonema  parvulum
 1089 C C A T A A A C T A T G C C G A C A A G G G A T T G G T A G  NZ Gomphoneis sp
  989 C C A T A A A C T A T G C C A A C A A G G G A T T G G T G G  NZ Didymosphenia geminata

      G G T T T C G T A A T G T C T C C A T C A G C A C C T T A T  Consensus sequence
      -----------------+-----------------+-----------------+-
                       1150              1160              1170
      -----------------+-----------------+-----------------+-
  998 A G T T T C G T T A C G T C T C C C T C A G C A C C T T A G  Amphora montana
 1031 G G T C T C G T T A T G T C C C C A T C A G C A C T T T A T  Anomoeoneis sphaerophora
 1028 G G C C T C G T A A T G A G C C C A T C A G C A C C T T A A  Cymbella
  981 G G T C T C G T T A C G T C T C C A T C A G C A C C T T A T  Dickieia ulvacea
 1045 A G T T T C G T A A T G T C T C C A T C A G C A C C T T A T  Encyonema triangulatum
 1011 G G C T T C G T T A T G T C T C T G T C A G C A C C T T A T  Eolimna minima
 1007 G G T C T C G T T A C G T C T C C G T C A G C A C C T T A T  Eolimna subminuscula
 1026 G T C G T T G T A T T G A C T C C G T C A G C A C C T T A T  Fragilaria striatula
 1005 G G T T T C G T A A T G T C T C C A T C A G C A C C T T A G  Gomphonema  parvulum
 1119 A G T T T C G T A A T G T C T C T A T C A G C A C C T T A G  NZ Gomphoneis sp
 1019 G G T T T C G T A A T G T C C C C A T C A G C A C C T T A G  NZ Didymosphenia geminata

      G A G A A A T C A T - A A G T T T T T G G G T T C C G G G G  Consensus sequence
      -----------------+-----------------+-----------------+-
                       1180              1190              1200
      -----------------+-----------------+-----------------+-
 1028 G A G A A A T C A T - A A G T T T T T G G G T T C C G G G G  Amphora montana
 1061 G A G A A A T C A C - A A G T T T T T G G G T T C C G G G G  Anomoeoneis sphaerophora
 1058 G A G A A A T C A T - A A G T C T T T G G G T T C C G G G G  Cymbella
 1011 G A G A A A T C A C - A A G T T T T T G G G T T C C G G G G  Dickieia ulvacea
 1075 G A G A A A T C A C - A A G T T T T T G G G T T C C G G G G  Encyonema triangulatum
 1041 G A G A A A T C A T T A A A C T T T T G G G T T C C G G G G  Eolimna minima
 1037 G A G A A A T C A C - A A G T T T T T G G G T T C C G G G G  Eolimna subminuscula
 1056 G A G A A A T C A C - A A G T C T T T G G G T T C C G G G G  Fragilaria striatula
 1035 G A G A A A T C A T - A A G T C T T T G G G T T C C G G G G  Gomphonema  parvulum
 1149 G A G A A A T C A T - A A G T C T T T G G G T T C C G G G G  NZ Gomphoneis sp
 1049 G A G A A A T C A A - A A G T T T T T G G G T T C C G G G G  NZ Didymosphenia geminata
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      G G A G T A T G G T C G C A A G G C T G A A A C T T A A A G  Consensus sequence
      -----------------+-------------------+-------------------+-
                       1210                1220                1230
      -----------------+-------------------+-------------------+-
1057  G G A G T A T G G T C G C A A G G C T G A A A C T T A A A G  Amphora montana
1090  G G A G T A T G G T C G C A A G G C T G A A A C T T A A A G  Anomoeoneis sphaerophora
1087  G G A G T A T G G T C A C A A G G C T G A A A C T T A A A G  Cymbella
1040  G G A G T A T G G T C G C A A G G C T G A A A C T T A A A G  Dickieia ulvacea
1104  G G A G T A T G G T C G C A A G G C T G A A A C T T A A A G  Encyonema triangulatum
1071  G G A G T A T G G T C G C A A G G C T G A A A C T T A A A G  Eolimna minima
1066  G G A G T A T G G T C G C A A G G C T G A A A C T T A A A G  Eolimna subminuscula
1085  G G A G T A T G G T C G C A A G G C T G A A A C T T A A A G  Fragilaria striatula
1064  G G A G T A T G G T C G C A A G G C T G A A A C T T A A A G  Gomphonema  parvulum
1178  G G A G T A T G G T C G C A A G G C T G A A A C T T A A A G  NZ Gomphoneis sp
1078  G G A G T A T G G T C G C A A G G C T G A A A C T T A A A G  NZ Didymosphenia geminata

      A A A T T G A C G G A A G G G C A C C A C C A G G A G T G G  Consensus sequence
      -----------------+-------------------+-------------------+-
                       1240                1250                1260
      -----------------+-------------------+-------------------+-
1087  A A A T T G A C G G A A G G G C A C C A C C A G G A G T G G  Amphora montana
1120  A A A T T G A C G G A A G G G C A C C A C C A G G A G T G G  Anomoeoneis sphaerophora
1117  A A - - T T G C G G A A G G G C A C C A C C A G G A G T G G  Cymbella
1070  A A A T T G A C G G A A G G G C A C C A C C A G G A G T G G  Dickieia ulvacea
1134  A A A T T G A C G G A A G G G C A C C A C C A G G A G T G G  Encyonema triangulatum
1101  A A A T T G A C G G A A G G G C A C C A C C A G G A G T G G  Eolimna minima
1096  A A A T T G A C G G A A G G G C A C C A C C A G G A G T G G  Eolimna subminuscula
1115  A A A T T G A C G G A A G G G C A C C A C C A G G A G T G G  Fragilaria striatula
1094  A A A T T G A C G G A A G G G C A C C A C C A G G A G T G G  Gomphonema  parvulum
1208  A A A T T G A C G G A A G G G C A C C A C C A G G A G T G G  NZ Gomphoneis sp
1108  A A A T T G A C G G A A G G G C A C C A C C A G G A G T G G  NZ Didymosphenia geminata

      A G C C T G C G G C T T A A T T T G A C T C A A C A C G G G  Consensus sequence
      -----------------+-------------------+-------------------+-
                       1270                1280                1290
      -----------------+-------------------+-------------------+-
1117  A G C C T G C G G C T T A A T T T G A C T C A A C A C G G G  Amphora montana
1150  A G C C T G C G G C T T A A T T T G A C T C A A C A C G G G  Anomoeoneis sphaerophora
1145  A G C - T A C G G C T T A A T T T G A C T C A A C A C G G G  Cymbella
1100  A G C C T G C G G C T T A A T T T G A C T C A A C A C G G G  Dickieia ulvacea
1164  A G C C T G C G G C T T A A T T T G A C T C A A C A C G G G  Encyonema triangulatum
1131  A G C C T G C G G C T T A A T T T G A C T C A A C A C G G G  Eolimna minima
1126  A G C C T G C G G C T T A A T T T G A C T C A A C A C G G G  Eolimna subminuscula
1145  A G C C T G C G G C T T A A T T T G A C T C A A C A C G G G  Fragilaria striatula
1124  A G C C T G C G G C T T A A T T T G A C T C A A C A C G G G  Gomphonema  parvulum
1238  A G C C T G C G G C T T A A T T T G A C T C A A C A C G G G  NZ Gomphoneis sp
1138  A G C C T G C G G C T T A A T T T G A C T C A A C A C G G G  NZ Didymosphenia geminata

      A A A A C T T A C C A G G T C C A G A C A T A G T G A G G A  Consensus sequence
      -----------------+-------------------+-------------------+-
                       1300                1310                1320
      -----------------+-------------------+-------------------+-
1147  A A A A C T T A C C A G G T C C A G A C A A A G T G A G G A  Amphora montana
1180  G A A A C T T A C C A G G T C C A G A C A T A G T G A G G A  Anomoeoneis sphaerophora
1174  A A A A C T T A C C A G G T C C A G A C A T A G T T A A G G  Cymbella
1130  A A A A C T T A C C A G G T C C A G A C A T A G T G A G G A  Dickieia ulvacea
1194  A A A A C T T A C C A G G T C C A G A C A T A G T G A G G A  Encyonema triangulatum
1161  A A A A C T T A C C A G G T C C A G A C A T A G T G A G G A  Eolimna minima
1156  A A A A C T T A C C A G G T C C A G A C A T A G T G A G G A  Eolimna subminuscula
1175  A A A A C T T A C C A G G T C C A G A C A T A G T G A G G A  Fragilaria striatula
1154  G A A A C T T A C C A G G T C C A G A C A T A G T G A G G A  Gomphonema  parvulum
1268  G A A A C T T A C C A G G T C C A G A C A T A G T G A G G A  NZ Gomphoneis sp
1168  G A A A C T T A C C A G G T C C A G A C A T A G T G A G G A  NZ Didymosphenia geminata
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      T T G A C A G A T T G A G A G C - - - T C T T T C T T G A T  Consensus sequence
      -----------------+-------------------+-------------------+-
                       1330                1340                1350
      -----------------+-------------------+-------------------+-
1177  T T G A C A G A T T G A G A G C - - - T C T T T C T T G A T  Amphora montana
1210  T T G A C A G A T T G A G A G C - - - T C T T T C T T G A T  Anomoeoneis sphaerophora
1204  T T T A C A T A G T T A G A A G A C G T C T T T C T T T G T  Cymbella
1160  T T G A C A G A T T G A G A G C - - - T C T T T C T T G A T  Dickieia ulvacea
1224  T T G A C A G A T T G A G A G C - - - T C T T T C T T G A T  Encyonema triangulatum
1191  T T G A C A G A T T G A G A G C - - - T C T T T C T T G A T  Eolimna minima
1186  T T G A C A G A T T G A G A G C - - - T C T T T C T T G A T  Eolimna subminuscula
1205  T T G A C A G A T T G A G A G C - - - T C T T T C T T G A T  Fragilaria striatula
1184  T T G A C A G A T T G A G A G C - - - T C T T T C T T G A T  Gomphonema  parvulum
1298  T T G A C A G A T T G A G A G C - - - T C T T T C T T G A T  NZ Gomphoneis sp
1198  T T G A C A G A T T G A G A G C - - - T C T T T C T T G A T  NZ Didymosphenia geminata

      T C T A T - G G G T G G T G G T G C A T G G C C G T T C T T  Consensus sequence
      -----------------+-------------------+-------------------+-
                       1360                1370                1380
      -----------------+-------------------+-------------------+-
1204  T C T T T - G G G T G G T G G T G C A T G G C C G T T C T T  Amphora montana
1237  T C T A T - G G G T G G T G G T G C A T G G C C G T T C T T  Anomoeoneis sphaerophora
1234  T T T A T T G G G T G G T G G T - C A T G G C C G T T C T T  Cymbella
1187  T C T A T - G G G T G G T G G T G C A T G G C C G T T C T T  Dickieia ulvacea
1251  T C T A T - G G G T G G T G G T G C A T G G C C G T T C T T  Encyonema triangulatum
1218  T C T A T - G G G T G G T G G T G C A T G G C C G T T C T T  Eolimna minima
1213  T C T A T - G G G T G G T G G T G C A T G G C C G T T C T T  Eolimna subminuscula
1232  T C T A T - G G G T G G T G G T G C A T G G C C G T T C T T  Fragilaria striatula
1211  T C T A T - G G G T G G T G G T G C A T G G C C G T T C T T  Gomphonema  parvulum
1325  T C T A T - G G G T G G T G G T G C A T G G C C G T T C T T  NZ Gomphoneis sp
1225  T C T A T - G G G T G G T G G T G C A T G G C C G T T C T T  NZ Didymosphenia geminata

      A - G T T G G T G G A G T G A T T T G T C T G G T T A A T T  Consensus sequence
      -----------------+-------------------+-------------------+-
                       1390                1400                1410
      -----------------+-------------------+-------------------+-
1233  A - G T T G G T G G A G T G A T T T G T C T G G T T A A T T  Amphora montana
1266  A - G T T G G T G G A G T G A T T T G T C T G G T T A A T T  Anomoeoneis sphaerophora
1263  A T G T T T G T G G T G T G - - T T G T C T G G - - A A T C  Cymbella
1216  A - G T T G G T G G A G T G A T T T G T C T G G T T A A T T  Dickieia ulvacea
1280  A - G T T G G T G G A G T G A T T T G T C T G G T T A A T T  Encyonema triangulatum
1247  A - G T T G G T G G A G T G A T T T G T C T G G T T A A T T  Eolimna minima
1242  A - G T T G G T G G A G T G A T T T G T C T G G T T A A T T  Eolimna subminuscula
1261  A - G T T G G T G G A G T G A T T T G T C T G G T T A A T T  Fragilaria striatula
1240  A - G T T G G T G G A G T G A T T T G T C T G G T T A A T T  Gomphonema  parvulum
1354  A - G T T G G T G G A G T G A T T T G T C T G G T T A A T T  NZ Gomphoneis sp
1254  A - G T T G G T G G A G T G A T T T G T C T G G T T A A T T  NZ Didymosphenia geminata

      C C G T T A A C G A A C G A G A C C G C T G C C T G C T A A  Consensus sequence
      -----------------+-------------------+-------------------+-
                       1420                1430                1440
      -----------------+-------------------+-------------------+-
1262  C C G T T A A C G A A C G A G A C C C C T G C C T G C T A A  Amphora montana
1295  C C G T T A A C G A A C G A G A C C G C T G C C T G C T A A  Anomoeoneis sphaerophora
1289  C C T G T A A C G A - - C A G A C C G C T G C C T G C T A A  Cymbella
1245  C C G T T A A C G A A C G A G A C C G C T G C C T G C T A A  Dickieia ulvacea
1309  C C G T T A A C G A A C G A G A C C G C T G C C T G C T A A  Encyonema triangulatum
1276  C C G T T A A C G A A C G A G A C C C C T G C C T G C T A A  Eolimna minima
1271  C C G T T A A C G A A C G A G A C C C C T G C C T G C T A A  Eolimna subminuscula
1290  C C G T T A A C G A A C G A G A C C C G T G C C T G C T A A  Fragilaria striatula
1269  C C G T T A A C G A A C G A G A C C G C T G C C T G C T A A  Gomphonema  parvulum
1383  C C G T T A A C G A A C G A G A C C G C T G C C T G C T A A  NZ Gomphoneis sp
1283  C C G T T A A C G A A C G A G A C C G C T G C C T G C T A A  NZ Didymosphenia geminata
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      A T A G - T T C A G T T A G T G A - T T T T C A C - - T G A  Consensus sequence
      -----------------+------------------+------------------+-
                       1450               1460               1470
      -----------------+------------------+------------------+-
1292  A T A G - T C C T T T G A G T G A - T T T T C A C - - T G A  Amphora montana
1325  A T A G C A C T A G T - A G T G A - T T T T C A C - - T G C  Anomoeoneis sphaerophora
1317  A T A G - T T C T T T A A G T T A T T T G C A A C C T T A C  Cymbella
1275  A T A G - T T C C G T T A A G T G A - A T T T C A C - - T G G  Dickieia ulvacea
1339  A T A G - T G C A G T T A G T G A - T T T T C A C - - T G A  Encyonema triangulatum
1306  A T A G - T C C A G T T A G T G A - T T T T C A C - - T G G  Eolimna minima
1301  A T A G - T T C C G T G A G T G A - A T T T C A C - - T G A  Eolimna subminuscula
1320  A T A G - T C G G G T T A A T G A A T T T T C A T - - T G G  Fragilaria striatula
1299  A T A G - C T C G G T G A G T G A - T T T T C A C - - T G A  Gomphonema  parvulum
1413  A T A G - C T C G G C T A G T G A - T T T T C A C - - T G G  NZ Gomphoneis sp
1313  A T A G - A C T A G T T A G T G A - T T T T C A C - - T G A  NZ Didymosphenia geminata

      C T T G A G C T T C T T A G A G G G A C G T G C A T T T T A  Consensus sequence
      -----------------+------------------+------------------+-
                       1480               1490               1500
      -----------------+------------------+------------------+-
1318  T T T G G A C T T C T T A G A G G G A C G T G C A T T C T A  Amphora montana
1351  A T G G G G C T T C T T A G A G G G A C G T G C A T T T T A  Anomoeoneis sphaerophora
1346  T T T G A T C T T C T T A G A G G G A C G T G C A T T T T -  Cymbella
1301  C G A G G A C T T C T T A G A G G G A C G T G C A T T T T A  Dickieia ulvacea
1365  C T C G A G C T T C T T A G A G G G A C G T G C A T T T T T  Encyonema triangulatum
1332  C C C G G G C T T C T T A G A G G G A C G T G C A T T C T A  Eolimna minima
1327  C G A G A A C T T C T T A G A G G G A C G T G C A T T C T A  Eolimna subminuscula
1347  C T A C A A C T T C T T A G A G G G A C G T G C G T T T T A  Fragilaria striatula
1325  C T T G A G C T T C T T A G A G G G A C G T G C A T T T T T  Gomphonema  parvulum
1439  C T T G A G C T T C T T A G A G G G A C G T G C A T A A T T  NZ Gomphoneis sp
1339  T C R G G T C T T C T T A G A G G G A C G T G C A T T T T A  NZ Didymosphenia geminata

      T T A G A T G C A G G A A G A T A G C G G C A A T A A C A G  Consensus sequence
      -----------------+------------------+------------------+-
                       1510               1520               1530
      -----------------+------------------+------------------+-
1348  T T A G A T G C A G G A A G A T A G G G G C A A T A A C A G  Amphora montana
1381  T T A G A T G C A G G A A G A T A G C G G C A A T A A C A G  Anomoeoneis sphaerophora
1375  T T A G A T G C A G T A A G G T A G C G G C A A T A A C A G  Cymbella
1331  T T A G A T G C A G G A A G A T A G C G G C A A T A A C A G  Dickieia ulvacea
1395  T T A G A T G C A G G A A G A T A G C G G C A A T A A C A G  Encyonema triangulatum
1362  T T A G A T G C A G G A A G A T A G G G G C A A T A A C A G  Eolimna minima
1357  T T A G A T G C A G G A A G A T A G G G G C A A T A A C A G  Eolimna subminuscula
1377  T A A A A C G C A G G A A G A T A C G G G C A A A A A C A G  Fragilaria striatula
1355  T T A G A T G C A G G A A G A T A G C G G C A A T A A C A G  Gomphonema  parvulum
1469  T T A A A T G C A G G A A G A T A G C G G C A A T A A C A G  NZ Gomphoneis sp
1369  T T A G A T G C A G G A A G A T A G C G G C A A T A A C A G  NZ Didymosphenia geminata

      G T C T G T G A T G C C C T T A G A T G T T C T G G G C C G  Consensus sequence
      -----------------+------------------+------------------+-
                       1540               1550               1560
      -----------------+------------------+------------------+-
1378  G T C T G T G A T G C C C T T A G A T G T T C T G G G C C G  Amphora montana
1411  G T C T G T G A T G C C C T T A G A T G T T C T G G G C C G  Anomoeoneis sphaerophora
1405  G T C T G T G A T G C C C T T A G A T G T T C T G G G C C G  Cymbella
1361  G T C T G T G A T G C C C T T A G A T G T T C T G G G C C G  Dickieia ulvacea
1425  G T C T G T G A T G C C C T T A G A T G T T C T G G G C C G  Encyonema triangulatum
1392  G T C T G T G A T G C C C T T A G A T G T T C T G G G C C G  Eolimna minima
1387  G T C T G T G A T G C C C T T A G A T G T T C T G G G C C G  Eolimna subminuscula
1407  G T C T G T G A T G C C C T T A G A T G T C C T G G G C C G  Fragilaria striatula
1385  G T C T G T G A T G C C C T T A G A T G T T C T G G G C C G  Gomphonema  parvulum
1499  G T C T G T G A T G C C C T T A G A T G T T C T G G G C C G  NZ Gomphoneis sp
1399  G T C T G T G A T G C C C T T A G A T G A T C T G G G C C G  NZ Didymosphenia geminata
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      C A C G C G C G C T A C A C T G A T G C A T T C A A - C G A  Consensus sequence
      -----------------+-----------------+-----------------+-
                       1570              1580              1590
      -----------------+-----------------+-----------------+-
1408  C A C G C G C G C T A C A C T G A T G C A A T C A A - C G A  Amphora montana
1441  C A C G C G C G C T A C A C T G A T G T A T T C A A - C G A  Anomoeoneis sphaerophora
1435  C A C G C G C G C T A C A C T G A T G T G T T C A A - C A A  Cymbella
1391  C A C G C G C G C T A C A C T G A T G C A T T C A A - C G A  Dickieia ulvacea
1455  C A C G C G C G C T A C A C T G A T G C A T T C A A - C G A  Encyonema triangulatum
1422  C A C G C G C G C T A C A C T G A T G C A T T C A A - C G A  Eolimna minima
1417  C A C G C G C G C T A C A C T G A T G C A T T C A A - C G A  Eolimna subminuscula
1437  C A C G C G C G C T A C A C T G A T G C A T G C A A - C G A  Fragilaria striatula
1415  C A C G C G C G C T A C A C T G A T G T G T T C A A - C G A  Gomphonema  parvulum
1529  C A C G C G C G C T A C A C T G A T G T G T T C A A A C G A  NZ Gomphoneis sp
1429  C A C G C G C G C T A C A C T G A T G T A C T C A A - C G A  NZ Didymosphenia geminata

      G T T T T T C C T T G G C T G A G A A G C C T G G G C A A T  Consensus sequence
      -----------------+-----------------+-----------------+-
                       1600              1610              1620
      -----------------+-----------------+-----------------+-
1437  G T T A - - C C T T G G C C G A G A G G C C T G G G C A A T  Amphora montana
1470  G T T T T T C C T T G G C T G A G A A G T C T G G G C A A T  Anomoeoneis sphaerophora
1464  G T T T - - C C T T G A C T G A G A A G T C T G G G C A A T  Cymbella
1420  G T G T T T C C T T G G C T G A G A A G C C T G G G C A A T  Dickieia ulvacea
1484  G T C C T A C C T T G G C T G A G A A G C C T G G G C A A T  Encyonema triangulatum
1451  G T T T T T C C T T G G C C G A G A G G C C T G G G C A A T  Eolimna minima
1446  G T T T T A C C T T G G C C G A G A G G C C T G G G C A A T  Eolimna subminuscula
1466  G T T T - A C C T T G G C C G A G A G G C C T G G G A A A T  Fragilaria striatula
1444  G T T T T T C C T T G G C T G A G A A G C C T G G G C A A T  Gomphonema  parvulum
1559  G T T T T T C C T T G G C T G A G A A G C C T G G G C A A T  NZ Gomphoneis sp
1458  G T T T T T C C T T G G C T G A G A A G C C T G G G T A A T  NZ Didymosphenia geminata

      C T T T T G A A C G T G C A T C G T G A T A G G G A T A G A  Consensus sequence
      -----------------+-----------------+-----------------+-
                       1630              1640              1650
      -----------------+-----------------+-----------------+-
1465  C T T T T G A A C C T G C A T C G T G A T A G G G A T A G A  Amphora montana
1500  C T T G T A A A C G T G C A T C G T G A T A G G G A T A G A  Anomoeoneis sphaerophora
1492  C T T C T G A A A C C C C T G C G T G T T A G G G A T A G A  Cymbella
1450  C T T T T G A A C G T G C A T C G T G A T A G G G A T A G A  Dickieia ulvacea
1514  C T T T T G A A T G T G C A T C G T G A T A G G G A T A G A  Encyonema triangulatum
1481  C T T T T G A A C G T G C A T C G T G A T A G G G A T A G A  Eolimna minima
1476  C T T T T G A A C G T G C A T C G T G A T A G G G A T A G A  Eolimna subminuscula
1495  C T G T - G A A C G T G C A T C G T G A T A G G G A T A G A  Fragilaria striatula
1474  C T T G T G A A C T C G C A T C G T G A T A G G G A T A G A  Gomphonema  parvulum
1589  C T T T T G A A C T C A C A T C G T G A T A G G G A T A G A  NZ Gomphoneis sp
1488  C T T T T A A A C T T G C A T C G T G A T A G G G A T A G A  NZ Didymosphenia geminata

      T T A T T G C A A T T A T T A A T C T T G A A C G A G G A A  Consensus sequence
      -----------------+-----------------+-----------------+-
                       1660              1670              1680
      -----------------+-----------------+-----------------+-
1495  T T A T T G C A A T T A T T A A T C T T G A A C G A G G A A  Amphora montana
1530  T T A T T G C A A T T A T T A A T C T T G A A C G A G G A A  Anomoeoneis sphaerophora
1522  T T A T T G C A A T T C T T A A T T T T G A A C G G G G G A  Cymbella
1480  T T A T T G C A A T T A T T A A T C T T G A A C G A G G A A  Dickieia ulvacea
1544  T T A T T G C A A T T A T T A A T C T T G A A C G A G G A A  Encyonema triangulatum
1511  T T A T T G C A A T T A T T A A T C T T G A A C G A G G A A  Eolimna minima
1506  T T A T T G C A A T T A T T A A T C T T G A A C G A G G A A  Eolimna subminuscula
1524  T T A T T G C A A T T A T T A A T C T T G A A C G A G G A A  Fragilaria striatula
1504  T T A T T G C A A T T A T T G A T C T T G A A C G A G G A A  Gomphonema  parvulum
1619  T T A T T G C A A T T A T T G A T C T T G A A C G A G G A A  NZ Gomphoneis sp
1518  T T A T T G C A A T T A T T A A T C T T G A A C G A G G A A  NZ Didymosphenia geminata
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      T T C C T A G T A A A C G C A G - A T C A T C A A T C T G C  Consensus sequence
      -----------------+-----------------+-----------------+-
                       1690              1700              1710
      -----------------+-----------------+-----------------+-
1525  T T C C T A G T A A A C G C A G - A T C A T C A A T C T G C  Amphora montana
1560  T T C C T A G T A A A C G C A G - A T C A T C A G T C T G C  Anomoeoneis sphaerophora
1552  T T C C T C G T C C C C G C G C C A T C A T A C G T T T G C  Cymbella
1510  T T C C T A G T A A A C G C A G - A T C A T C A A T C T G C  Dickieia ulvacea
1574  T T C C T A G T A A A C G C A G - A T C A T C A A T C T G C  Encyonema triangulatum
1541  T T C C T A G T A A A C G C A G - T T C A T C A A A C T G C  Eolimna minima
1536  T T C C T A G T A A A C G C A G - A T C A T C A A T C T G C  Eolimna subminuscula
1554  T T C C T A G T A A A C G C A G - A T C A T C A G T C T G C  Fragilaria striatula
1534  T T C C T A G T A G A C G C A G - A T C A T C A A T C T G C  Gomphonema  parvulum
1649  T T C C T A G T A G A C G C A A - A T C A T C A A T T T G C  NZ Gomphoneis sp
1548  T T C C T A G T A A A C G C A G - A T C A T C A G T C T G C  NZ Didymosphenia geminata
        |-----------------D1565F-----------------|

      A T T G A T T A C G T C C C T G C C C T T T G T A C A C A C  Consensus sequence
      -----------------+-----------------+-----------------+-
                       1720              1730              1740
      -----------------+-----------------+-----------------+-
1554  A T T G A T T A C G T C C C T G C C C T T T G T A C A C A C  Amphora montana
1589  A T T G A T T A C G T C C C T G C C C T T T G T A C A C A C  Anomoeoneis sphaerophora
1582  A T T G A T T A C G T C C C T G C C C T T T G T A C A C A C  Cymbella
1539  A T T G A T T A C G T C C C T G C C C T T T G T A C A C A C  Dickieia ulvacea
1603  A T T G A T T A C G T C C C T G C C C T T T G T A C A C A C  Encyonema triangulatum
1570  A T T G A T T A C G T C C C T G C C C T T T G T A C A C A C  Eolimna minima
1565  A T T G A T T A C G T C C C T G C C C T T T G T A C A C A C  Eolimna subminuscula
1583  A T T G A T T A C G T C C C T G C C C T T T G T A C A C A C  Fragilaria striatula
1563  G T C G A T T A C G T C C C T G C C C T T T G T A C A C A C  Gomphonema  parvulum
1678  G T C G A T T A C G T C C C T G C C C T T T G T A C A C A C  NZ Gomphoneis sp
1577  A T T G A T T A C G T C C C T G C C C T T T G T A C A C A C  NZ Didymosphenia geminata

      C G C C C G T C G C A C C T A C C G A T T G A A T G G T C C  Consensus sequence
      -----------------+-----------------+-----------------+-
                       1750              1760              1770
      -----------------+-----------------+-----------------+-
1584  C G C C C G T C G C A C C T A C C G A T T G G A T G G T C C  Amphora montana
1619  C G C C C G T C G C A C C T A C C G A T T G A A T G G T C C  Anomoeoneis sphaerophora
1612  C G - C C G T C T G A C C T A A A G A T T G A A T G G T C A  Cymbella
1569  C G C C C G T C G C A C C T A C C G A T T G A A T G G T C C  Dickieia ulvacea
1633  C G C C C G T C G C A C C T A C C G A T T G A A T G G T C C  Encyonema triangulatum
1600  C G C C C G T C G C A C C T A C C G A T T G A A T G G T C C  Eolimna minima
1595  C G C C C G T C G C A C C T A C C G A T T G A A T G G T C C  Eolimna subminuscula
1613  C G C C C G T C G C A C C T A C C G A T T G A A T G G T C C  Fragilaria striatula
1593  C G T C C G T C G C A C C T A C C G A T T G A A T G G T C C  Gomphonema  parvulum
1708  C G C C C G T C G C A C C T A C C G A T T G A A T G G T C C  NZ Gomphoneis sp
1607  C G C C C G T C G C A C C T A C C G A T T G A A T G G T C C  NZ Didymosphenia geminata

      G G T G A A G C C T C G G G A T T G T G G C C A G T G - C C  Consensus sequence
      -----------------+-----------------+-----------------+-
                       1780              1790              1800
      -----------------+-----------------+-----------------+-
1614  G G T G A A G C C T C G G G A T T G T G G C C A G T G - C C  Amphora montana
1649  G G T G A A G C C T C G G G A T T G C G G C C T C T G C C C  Anomoeoneis sphaerophora
1641  G G T G A A G G G T T G G G A T T T T A A C A A G T G - T G  Cymbella
1599  G G T G A A G C C T C G G G A T T G T G G C C A A T G - C C  Dickieia ulvacea
1663  G G T G A A G C C T C G G G A T T G T G A T C A G T G - C C  Encyonema triangulatum
1630  G G T G A A G C C T C G G G A T T G T G G C C A G T G - C C  Eolimna minima
1625  G G T G A A G C C T C G G G A T T G T G G C C G G T G - C C  Eolimna subminuscula
1643  G G T G A A G C C T C G G G A T T G T G A T G A G T T T C C  Fragilaria striatula
1623  G G T G A A G A C T C G G G A T T G T G A G T G T T T G - C C  Gomphonema  parvulum
1738  G G T G A A G A C T C G G G A T T G T G A G T G T T T G - C C  NZ Gomphoneis sp
1637  G G T G A A G G C T C G G G A T T G C A G C T A A T G C C -  NZ Didymosphenia geminata
                |-----------D1659F--------------|
                                    |-D1670R---------------
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      T T T A C T G G T G - T T G G T T G C G A G A A C T T G T C  Consensus sequence
      -----------------+-----------------+-----------------+-
                       1810              1820              1830
      -----------------+-----------------+-----------------+-
1643  T T T A C T G G T G - T T G G T T G C G A G A A C T T G T C  Amphora montana
1679  T T C A C T G G T T - G G G G T T G T G A G A A C T T G T C  Anomoeoneis sphaerophora
1670  T T T A T T G G T G - A T G G T T G G G A G A A C T T G T T  Cymbella
1628  T T T A T T G G T G - T T G G T T G C A A G A A C T T G T C  Dickieia ulvacea
1692  T T T A C T G G T G - C T G G C T G C A A G A A C T T G T C  Encyonema triangulatum
1659  T T T A C T G G T G - T T G G C T G C G A G A A C T T G T C  Eolimna minima
1654  T T T A C T G G T G - C C G G T T G C G A G A A C T T G T C  Eolimna subminuscula
1673  T T T A T T G G G G A T T T G T C                          Fragilaria striatula
1652  T T T A C T G G T G - A T G T T T G C A A G A A C T T G T C  Gomphonema  parvulum
1767  T T T G C T G G T G - A T G T T T G C A A G A A C T T G T C  NZ Gomphoneis sp
1666  T T T A C T G G T G - T T G G T T T C A A G A A C T T G T C  NZ Didymosphenia geminata
      ------------------|

      T A A A C C T T A T C A T T T A G A G G A A G G T G A A G T  Consensus sequence
      -----------------+-----------------+-----------------+-
                       1840              1850              1860
      -----------------+-----------------+-----------------+-
1672  T G A A C C T T A T C A T C T A G A G G A A G G T G A A G A  Amphora montana
1708  T G A A C C T T A T C A T T T A G A G G A A G G T G A A G T  Anomoeoneis sphaerophora
1699  T A A A C C T T C T C A T T T A G A G G A C G G T G A A G T  Cymbella
1657  T A A A C C T T A T C A T T T A G                          Dickieia ulvacea
1721  T A A A C C T T A T C A T T T A G A G G A A G G T G A A G T  Encyonema triangulatum
1688  T A A A C C T T A T C A T T T A G A G G A A G G T G A A G T  Eolimna minima
1683  T A A A C C T T A T C A T T T A G A G G A A G G T G A A G A  Eolimna subminuscula
1689                                                            Fragilaria striatula
1681  T A A A C C T T A T C A T T T A G A G G A A G G T G A A G T  Gomphonema  parvulum
1796  T A A A C C T T A T C A T T T A G A G G A A G G T G A A G T  NZ Gomphoneis sp
1695  T A A A C C T T A T C A T T T A G A G G A A G G T G A A G T  NZ Didymosphenia geminata

      C G T A A - C A A G G T T T C C G T A G G T G A A C C T G C  Consensus sequence
      -----------------+-----------------+-----------------+-
                       1870              1880              1890
      -----------------+-----------------+-----------------+-
1702  C G T A A - C A A G G T T T C C G T A G G T G A A C C T G    Amphora montana
1738  C G T A A - C A A G G T T T C C G T A G G T G A A C C T G C  Anomoeoneis sphaerophora
1729  T G T A A - C A A G G T T T C C G T A G G T G G A C C T G A  Cymbella
1673                                                            Dickieia ulvacea
1751  C G T A A - C A A G G T T T C C G T A G G T G A A C C T G C  Encyonema triangulatum
1718  C G T A A - C A A G G T T T C C G T A G G T G A A C C T G C  Eolimna minima
1713  C G T A A - C A A G G T T T C C G T A G G T G A A C C T G C  Eolimna subminuscula
1689                                                            Fragilaria striatula
1711  C G T A A - C A A G G T T T C C G T A G G T G A A C C T G C  Gomphonema  parvulum
1826  C G T A A A C A A G G T T T C C G T A G G T G A A C C T G C  NZ Gomphoneis sp
1725  C G T A A - C A A G G T T T C C G T A G G T G A A C C T G C  NZ Didymosphenia geminata

      A G A A G X X X X X X X X                                 Consensus sequence
      -----------------+-------
                       1900
      -----------------+-------
1729                                                            Amphora montana
1767  A G A T G G T T C A T G T                                 Anomoeoneis sphaerophora
1758  T G A A G G A T C A A                                     Cymbella
1673                                                            Dickieia ulvacea
1780  A G A A G G A T C A                                       Encyonema triangulatum
1747  A G A A G                                                 Eolimna minima
1742  A G A A G G                                               Eolimna subminuscula
1689                                                            Fragilaria striatula
1740  A G A A G                                                 Gomphonema  parvulum
1856  A G A A T                                                 NZ Gomphoneis sp
1754  A G A A G G A T C A A                                     NZ Didymosphenia geminata
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