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THE ORIGIN, GENETIC DIVERSITY AND TAXONOMY OF THE INVASIVE DIATOM *DIDYMOSPHENIA GEMINATA* (BACILLARIOPHYCEAE) IN NEW ZEALAND

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ABSTRACT

This thesis describes an investigation of the origin, genetic diversity and taxonomy of *Didymosphenia geminata*, in New Zealand. *D. geminata,* commonly known as “Didymo” or “Rock Snot”, is a freshwater diatom, a photosynthetic alga with a silica shell. It attaches to rocks and plants by its mucilaginous stalks, its large blooms often covering all available substrates and causing shifts in community structure. Although it was historically associated with high altitude, oligotrophic waters, it is now showing increased ecological tolerance. It has been increasing in abundance and range in Europe and North America and has been introduced into new areas including New Zealand, Iran and India. Since the first discovery of *D. geminata* in New Zealand in October 2004, it has spread rapidly across the South Island.

I have conducted a phylogeographic study of *D. geminata* samples from Europe, Asia, North America and New Zealand, using *D. geminata*-specific primers to amplify the internal transcribed spacer (ITS) region. I have also used these primers to amplify the small nuclear ribosomal DNA (nrDNA) subunit 18S to investigate the taxonomic placement of *D. geminata* within the pennate diatoms.

Results from this investigation indicate that *D. geminata* may belong to the Family Cymbellaceae. It appears that *D. geminata* may have been introduced though several different introduction events to North America from Europe and then to New Zealand from North America. These results can be used to inform strategies regarding the control and management of this invasive species, including lending support for continuation of the Biosecurity New Zealand program aimed at improving public, and especially freshwater users’, awareness and responsibility regarding *D. geminata* in New Zealand. This program is especially important as dispersal appears to be human-mediated. Limiting the number and source of introductions to an area can reduce the potential for increased genetic variation and thus adaptation to new environments.

PREFACE

This thesis begins with a Literature Review (Chapter 1), which describes the morphology, reproduction, ecology and classification of *D. geminata*. Chapter 1 also summarises records of the distribution of *D. geminata* worldwide and in New Zealand. A review on invasive species follows, which focuses on aquatic invaders and the genetic aspects of invasions, including phylogeography. Chapter 1 concludes with a brief summary of molecular markers used in phylogenetic analyses and provides details of the ITS region. Chapter 2 is presented as a research paper prepared for publication in a scientific journal. It presents the main findings of this research project, including the possible phylogeographic history of *D. geminata* and its taxonomic placement within the pennate diatoms. Chapter 3 provides a conclusion, including a summary of results, suggestions for further research and some final recommendations. References are found in Chapter 4.

The main findings presented in Chapter 2 were obtained through much trial and error due to the difficult nature of dealing with environmental samples. Results not presented in Chapter 2, including DNA extractions, PCR optimisation and post-PCR processing of DNA are found in the Appendices, together with some of the protocols used. The appendices also contain sequence alignments, pairwise distance matrices, phylogenetic trees and secondary structure analyses complementary to, but not included in, Chapter 2. It must be noted that 27 of the 46 sequences analysed in Chapter 2 were obtained through laboratory work conducted by myself in the course of the year. Technicians working on the development of *D. geminata*-specific primers (Cary et al. 2006; Cary et al. 2007) during 2006-2007 obtained the other 19 sequences. Table A.5 in Appendix 4 summarizes this information. The Appendices section concludes with a glossary containing some frequently used scientific terms in the context of this thesis.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS ii

ABSTRACT iii

PREFACE.. iv

TABLE OF CONTENTS v

LIST OF FIGURES viii

LIST OF TABLES x

CHAPTER 1

Introduction 1

1.1 Didymosphenia geminata Background 2

1.1.1 Morphology 3

1.1.2 Reproduction 6

1.1.3 Ecology 7

1.1.4 Ecological Impacts 8

1.2 History and Classification 10

1.2.1 Algal Taxonomy 10

1.2.2 Diatom Taxonomy 12

1.2.3 *Didymosphenia* Taxonomy 14

1.3 Recent Didymosphenia geminata Spread 18

1.3.1 Worldwide Spread 18

1.3.2 The New Zealand Invasion 23

1.4 Invasive Species 25

1.4.1 Invasion Success 26

1.4.2 Impacts of Invasions 28

1.4.3 Stages of Invasion 31
1.4.4 Genetic Factors in Invasions 33
1.4.5 Invasive Success: A Genetic Paradox 33
1.4.6 Role of Reproductive Systems 35
1.4.7 Phylogeography 37
1.4.8 Genetics in Control and Management of Invasive Species 37
1.5 Molecular Markers 39
1.5.1 Non-PCR-Based Methods 40
1.5.2 PCR-Based Methods 41
1.5.3 ITS Studies 45
1.6 Conclusion 47

CHAPTER 2

Research Paper 48
2.1 Abstract 48
2.2 Introduction 49
2.3 Materials and Methods 53
2.4 Results 65
2.5 Discussion 77

CHAPTER 3

Conclusion 88
3.1 Summary 88
3.2 Future Work 89
3.3 Concluding Recommendations 92

REFERENCES 94

APPENIDCES

APPENDIX 1: Didymosphenia geminata Worldwide Distribution. 113

APPENDIX 2: New Zealand Surveys and Sample Collection........ 116

APPENDIX 3: Protocols 122

DNA Extraction 122

PCR Conditions 125

PCR Cleanup Methods 128

DNA and PCR Quality and Quantity Indicators 129

Gel Extractions 130

Cloning.. 133

Single Cell Extraction.. 136

Media Recipes 138

Equipment... 139

Software.. 140

APPENDIX 4: Additional Results 141

PCR Optimization 141

Combination of Techniques Used to Obtain Sequences................ 145

Post-PCR DNA Processing.. 148

Testing Specificity of Primers... 152

Single Cell Extraction.. 153

QPCR Detection of *D. geminata* 154

18S and ITS Alignments 155

Pairwise Distances for Chapter 2 Alignments 180

Alignment and Tree Building Statistics.. 200

Additional Phylogenetic Trees 202

rRNA Secondary Folding Structures.. 208

APPENDIX 5: Glossary... 211

LIST OF FIGURES

CHAPTER 1

Figure 1.1: Light microscopy photographs of *D. geminata* cells................ 3

Figure 1.2: *Didymosphenia geminata* growing on riverbed substrate. 5

Figure 1.3: Tree of five eukaryote supergroups 11

Figure 1.4: Maximum Parsimony tree of heterokont algae....................... 12

Figure 1.5: Most parsimonious tree of diatoms 15

Figure 1.6: Classification of Bacillariaceae (pennate diatoms)................. 16

Figure 1.7: Worldwide distribution of *Didymosphenia geminata*............... 19

Figure 1.8: llustrations of invasive species mentioned in the text............. 30

Figure 1.9: Diagram of the nuclear ribosomal DNA gene region.............. 45

CHAPTER 2

Figure 2.1 (10): World map of sample locations used in this study.......... 54

Figure 2.2 (11): Map of New Zealand showing rivers infected with *D. geminata* (red) and sites of samples used in this project 55

Figure 2.3 (12): rDNA operon showing the 18S, 5.8S and 28S genes and the ITS1 and ITS2 regions. Arrows illustrate primers used...................... 59

Figure 2.4 (13): Microscope photographs of *D. geminata* sample cells ... 67

Figure 2.5 (14): Maximum Likelihood (ML) tree of the taxonomic placement of *D. geminata* based on 18S region of 58 raphid pennate diatoms (Bacillariophyceae). 73

Figure 2.6 (15): Maximum Likelihood (ML) tree for the 18S-ITS region for 29 *D. geminata* sequences 74

Figure 2.7 (16): Maximum Likelihood (ML) tree for the ITS region for 46 *D. geminata* sequences.. 75

Figure 2.8 (17): Maximum Likelihood (ML) tree for the population study of the ITS region of seven cloned sequences. 76

Figure 2.9 (18): Possible invasion succession of *D. geminata*................ 82

APPENDICES

Figure A.1(19): Photograph of drift net used for sampling *D. geminata* . 117

Figure A.2 (20): Gel comparing PCR products of small and large DNA regions using unspiked and spiked template. 142

Figure A.3 (21): Gel showing effects of varying primer concentrations .. 143

Figure A.4 (22): Gel of temperature gradient PCR products 144

Figure A.5 (23): Gels showing effects of DMSO levels on PCR............. 149

Figure A.6 (24): Gels showing post-gel extraction band migration and results of screening colonies... 150

Figure A.7 (25): Low Melting Point DNA extraction gel. 151

Figure A.8 (26): Gel showing tests of *D. geminata*-specific primers....... 153

Figure A.9 (27): Gels of PCR products from single cell DNA templates using different extraction methods. 154

Figure A.10 (28): Strict consensus Maximum Parsimony (MP) tree of the taxonomic placement of *D. geminata* based on partial 18S region of 58 raphid pennate diatoms (Bacillariophyceae). 202

Figure A.11 (29): Strict consensus Maximum Parsimony (MP) tree for the 18S-ITS region for 29 *D. geminata* sequences 203

Figure A.12 (30): Unrooted strict consensus Maximum Parsimony (MP) tree for the ITS region of seven cloned sequences 204

Figure A.13 (31): Unrooted strict consensus Maximum Parsimony (MP) tree for the ITS region. 205

Figure A.14 (32): Unrooted strict consensus Maximum Parsimony (MP) tree for the ITS region without P1 for 45 *D. geminata* sequences.......... 206

Figure A.15 (33): Maximum Likelihood (ML) tree for the ITS region withouth P1 for 45 *D. geminata* sequences... 207

Figure A.16 (34): Secondary structure of 5.8S....................................... 208

Figure A.17 (35): Secondary structure of ITS1....................................... 209

Figure A. 18 (36): Secondary structure of ITS2..................................... 210

LIST OF TABLES

CHAPTER 2

Table 2.1: Sample locations and primers used in this study 56

Table 2.2: Primer sequences used in amplification and sequencing........ 59

Table 2.3: GenBank accession numbers of species used 62

Table 2.4: PCR extension times and sizes of amplification products 64

Table 2.5: Statistics of the alignments generated to test phylogenetic relationships at different levels 69

APPENDICES

Table A.1 (6): International water bodies with *D. geminata*.................... 113

Table A.2 (7): Summary of New Zealand South Island waterways positive for *D. geminata* as at December 2007 119

Table A.3 (8): Survey details for New Zealand Surveys 2005-2007....... 121

Table A.4 (9): Table of methods and samples screened by S. Kelly...... 145

Table A.5 (10): Table of technician, extraction method, primers, multiple PCR products and sequencing template used for each sample............. 146

Table A.6(11): Output of 610 bp *D. geminata* ITS sequence alignment. 155

Table A.7 (12): Output of 1113 bp diatom alignment 161

Table A.8 (13): Pairwise distances of *D. geminata* ITS alignment 180

Table A.9 (14): Pairwise distances of *D. geminata* 18S-ITS alignment.. 186

Table A.10 (15): Pairwise distances of 18S diatom alignment 190

Table A.11 (16): Pairwise distances of population-level ITS alignment.. 199

Table A.12 (17): Model Test Statistics 200

Table A.13 (18): PhyML Maximum Likelihood Statistics 200

Table A.14 (19): Maximum Parsimony Statistics.................................... 201

CHAPTER 1: INTRODUCTION

Didymosphenia geminata (Lyngbye) M. Schmidt (Bacillariophyceae, Cymbellaceae) is a freshwater diatom, an alga commonly known as "Didymo" or "Rock Snot". In the last few decades in has been expanding in abundance, range and environmental tolerance and is causing increasing problems in Europe, North America and New Zealand (Kilroy 2004). In many instances, it has completely covered all available riverbed substrates, changing the natural composition of biodiversity in these areas. In New Zealand, *D. geminata* spread to over 50 rivers throughout the South Island in just four years (Larned *et al.* 2007).

Although *D. geminata* was first described by Lyngbye as early as 1819 (cited in Dawson 1973a, p. 65), little was known regarding its ecology, growth patterns, reproduction and distribution. Thus, management bodies and scientists were ill equipped to deal with the sudden invasion and proliferation of *D. geminata*. In response to the New Zealand invasion, Biosecurity New Zealand initiated a massive monitoring program and studies into the ecology of *D. geminata*, methods of control, means of dispersal and suitable environment prediction maps. As part of this response, the University of Waikato developed a highly sensitive detection method for *D. geminata* using quantitative real time polymerase chain reaction (QRT-PCR) (Cary *et al.* 2007), and launched a phylogeographic study to determine the 1) source and 2) number of introductions of *D. geminata* in New Zealand. The phylogeographic study is the focus of this thesis research project.

This literature review introduces *Didymosphenia geminata*, touching on its taxonomy, morphology, ecology, and recent spread around the world, including New Zealand. It summarises the impacts that invasive species can have, especially in isolated ecosystems such as those found in New Zealand and concludes with a description of phylogeographic genetic tools, focusing on the nuclear ribosomal (nr) DNA internal transcribed spacer (ITS) region.

1.1 *Didymosphenia geminata* Background

Didymosphenia geminata is a large freshwater diatom. Diatoms are algae; photosynthetic protists with silica cell walls (Graham & Wilcox 2000). They may occur as single cells or as chains. Diatoms are very successful evolutionarily, displaying high diversity and abundance in a wide variety of environments including freshwater, marine, air and moist terrestrial habitats. While there are currently 10 000 - 12 000 recognised diatom species from 285 genera (Round *et al.*1990), it is likely that many more species will be described in the future. Mann & Droop (1996) suggest there may be as many as 200 000 diatom species in existence. Diatom diversity may only be exceeded by green algal diversity and diatoms may be the most numerous eukaryotic aquatic organisms (Graham & Wilcox 2000).

Diatoms form an important part of the phytoplankton and periphyton community in rivers, lakes and oceans, providing a food source for macroinvertebrates and 20-25% of the global carbon fixation and atmospheric oxygen (Vanormelingen *et al.* 2008). Some species form large blooms and some produce toxins harmful to humans, marine mammals and fish, for example, *Pseudo-nitzschia pseudodelicatissima* and *Alexandrium catenella* (Bornet *et al.* 2005). Diatom species often show specificity for environmental conditions, enabling their use as environmental indicators of pH, salinity, conductivity, water quality and trophic status (Graham & Wilcox 2000). Diatom species assemblages may be unique to regions such as lakes, continents or hemispheres due to restricted geographic ranges (Kilroy *et al.* 2007; Vanormelingen *et al.* 2008). Because their shells are resistant to decay, they are useful in both lake history reconstruction and palaeoclimatic studies (Graham & Wilcox 2000).

1.1.1 Morphology

The word “diatom” means split in two, referring to the two siliceous shells, called frustules, which form the cell wall (Fig. 1.1). Frustules are composed of silicon dioxide (SiO_2) and possess unique shape and ornamentation, which influence behaviour and assist in diatom taxonomy. The top and bottom surface views of the frustule are referred to as the valve views while the side view is referred to as the girdle view (Fig. 1.1a). The top compartment (epitheca) of the frustule overlaps the bottom compartment (hypotheca) like a pillbox or a Petri dish (Fig. 1.1b) (Graham & Wilcox 2000; Stoermer & Julius 2003). The girdle consists of several linking bands, which vary in number, form and width depending on the species and which assist in species identification (Cox 1996). Both valves and girdle contain areolae, which are pores that enable exchange of molecules between the cell and its environment. Arrangement of pores can be linear, forming striae (Fig. 1.1d), which are visible as parallel lines under low magnification (Graham & Wilcox 2000).

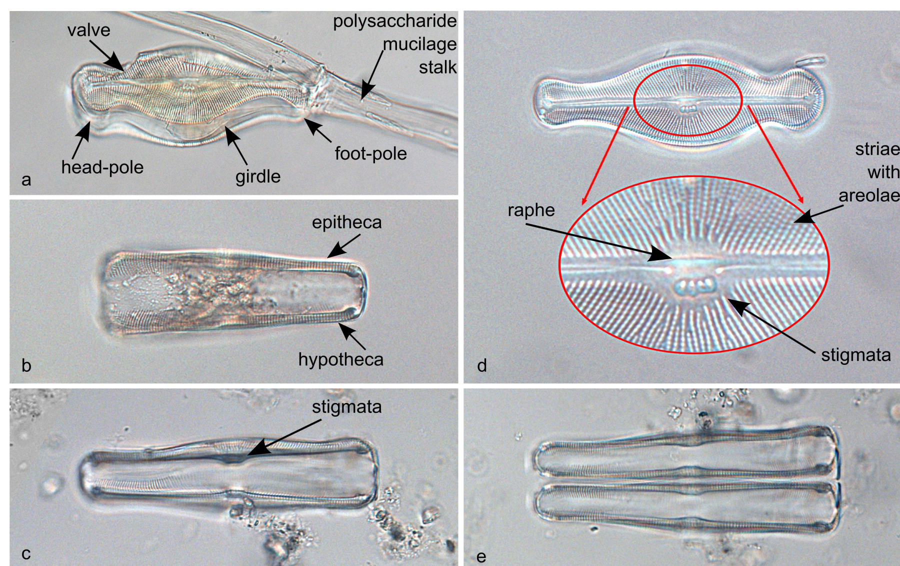


Figure 1.1: Light microscopy photographs of *D. geminata* cells from samples used in this project. a.) View of stalked cell showing valve and girdle. b.) Girdle view with chloroplast. c.) Girdle view without chloroplast. d.) Valve view with centre magnified to show raphe and stigmata. e.) Girdle view of vegetative cell division.

The shape of a diatom cell is either centric or pennate. Centric diatoms are radially symmetric in valve view and pennate diatoms are elongated and may be bilaterally symmetric or asymmetric in valve view. Pennate diatoms may possess a raphe system (Fig. 1.1d) or be araphid. The raphe is a longitudinal slit that occurs on one (monoraphid) or both (biraphid) valves. Polysaccharide based mucilage secreted from the raphe enables motility of the diatom over surfaces or fixture of cells to a substrate in the form of a stalk (Graham & Wilcox 2000). Isolated perforations called stigmata may be present in the centre of the valve (Fig. 1.1c, d). Stigmata have rounded external openings and slit-like internal openings. Diatoms are usually classified using frustule morphology. This method requires cleaning the frustules to remove any organic material, which also removes cell components that are useful for classification. Cox (1996) has developed a key to diatom identification using frustule and living components, including chloroplast shape and number, the presence and locations of pyrenoids and mucilaginous secretions.

Didymosphenia geminata Morphology

D. geminata is a bilaterally asymmetrical biraphid pennate diatom. In valve view, it has a unique bottle shape, while in girdle view it appears as a wedge (cuneate), being thinner at one end than the other (Fig. 1.1). *D. geminata* cells are very large (60-140 µm long and 25-43 µm wide). The centre of the valve is broad and lanceolate while the head and foot poles are capitate (Cox 1996). Cells are solitary and have mucilage stalks coming from the unlobed apical pore field at the narrower foot-pole (Fig. 1.1a). These stalks form particularly strong attachments to the substrate. *D. geminata* frustules do not possess septa on the girdle bands (Stoermer & Julius 2003). Striae are conspicuous and numerous, with 8-10 striae per 10 µm of frustule. Cells also possess short spines at the head pole on both valves (Cox 1996; Stoermer & Julius 2003). Stigmata, along with the nucleus, are ventral. Cells possesses one large, H-shaped chloroplast (Fig. 1.1b) positioned against one side of the girdle and lapping under both

valves (Cox 1996). The chloroplast contains a central lenticular, dorsal pyrenoid. Raphes are present on both valves and are dorsally deflected.

Macroscopically, although didymo looks slimy, it feels like wet cotton wool. It forms dense intertwined mats, often completely smothering the riverbed (Fig. 1.2). Its ends can turn white, looking like tissue paper. When rubbed it does not fall apart and it does not have a distinct odour (Global Invasive Species Database 2005). There are no known human health issues associated with *D. geminata*, although itchiness may occur after swimming in an affected river.

Figure 1.2: *Didymosphenia geminata* growing on riverbed substrate. (Pictures from Biosecurity New Zealand).

In New Zealand, there are several endemic species from the closely related genera of *Cymbella* in the Family Cymbellaceae and *Gomphoneis* and *Gomphonema* in the Family Gomphonemataceae. There are several distinguishable characteristics between these species. For example, stigmata are absent in *Cymbella* and single in *Gomphonema. Gomphoneis* girdle bands have septa. *Cymbella* appears bent in girdle view (Kociolek & Spaulding 2003). *D. geminata* cells are larger, thicker and form stronger attachments to the substrate than these related species (Kilroy 2004).

1.1.2 Reproduction

Sexual reproduction differs slightly among diatom species but has rarely been observed as it occurs over such a short time period. It is possible that *D. geminata* reproduces sexually in a similar manner to related diatoms such as *Cymbella* and *Gomphonema*, which undergo synchronous sexual reproduction in the spring due to increased temperature and irradiance (Edlund and Stoermer 1997). Pennate diatoms usually produce two amoeboid gametes of the same size (isogamous), which do not possess flagella. Pairing of the two parent cells within mucilage occurs before meiosis takes place. Fusion of two haploid nuclei forms an auxospore. When conditions are favourable, the auxospore germinates and divides by mitosis to form a cell with frustules of restored size (Graham & Wilcox 2000). Pennate freshwater diatoms may form resting cells, which enable survival in poor conditions. This may be important if the concentration of dissolved silicate drops. However, no resting spores have been observed for *D. geminata* (Graham & Wilcox 2000).

Diatoms reproduce vegetatively through mitotic cell division. The valves of the frustule separate, with each valve forming the epitheca of a new cell. The new hypotheca forms within the boundaries of the epitheca, causing the sizes of the cells to decrease with each cell division until they reach a minimum size. It is likely that sexual reproduction occurs at this point to restore cells to their maximum size (Graham & Wilcox 2000). In *D. geminata*, each vegetative division produces a branch in the mucilaginous stalk and intertwined stalks connect related clonal cells (Kilroy 2004). Diatom doubling times can range from 0.3 to 5.0 days (Cox 1996). Despite large amounts of populations being clonal, variability does exist within and between diatom cell lines and populations (Mann 1999) making it difficult to identify whether phenotypic variation has a genetic basis or is purely a morphological response to the environment (Stoermer & Julius 2003).

1.1.3 Ecology

Habitat – *D. geminata* is both epiphytic, attaching to plants, and epithilic, attaching to stones (Kilroy 2004). It grows in both lentic (running) and lotic (still) waters of rivers and lakes, preferring cool, oligotrophic waters (Kociolek & Spaulding 2003). It was historically located in high altitude waters with stable flows. Recently, blooms of *D. geminata* are occurring in lower altitude, warmer, deeper, nutrient-rich rivers, most significantly those with stable, controlled flows below dams or reservoirs. It can be extremely problematic in these areas, clogging up pipes and machinery (Kawecka & Sanecki 2003). In New Zealand, blooms are usually greatest in spring, followed by a decline, which may be caused by floods detaching stalks from the substrate. It increases again later in summer (Larned *et al.* 2007).

Flow Regime – Biomass may be influenced most by the length of time since the occurrence of a bed-mobilising flood (Larned *et al.* 2007). Stable flows probably assist in the attachment of *D. geminata* cells to the riverbed substrate, enabling it to become established. Subsequently, faster currents improve the delivery of nutrients to cells, facilitating growth (Kilroy 2004). *D. geminata* also exists on lake shorelines, where waves create turbulence (Kravtsova *et al.* 2004) and provide nutrients. *D. geminata* does not grow in slow moving or stationary water. Strong currents, as in periods of heavy rainfall and flooding, can dislodge and potentially reduce proliferations of *D. geminata*, but may also assist in its dispersal. Varying the flow rate in regulated waterways may help prevent massive blooms of *D. geminata*, which are expensive and labour intensive to remove manually (Larned et al. 2007). *D. geminata* may persist in rivers and lakes in low populations until the correct conditions enable expansion into blooms (Kilroy 2004).

Nutrients – While *D. geminata* was historically associated with oligotrophic waters, Larned *et al.* (2007) discovered that its growth was significantly limited by nitrogen and phosphorous. Furthermore, in New

Zealand low *D. geminata* biomass is associated with oligotrophic rivers and high biomass with enriched rivers, for example, the Aparima and Waiau Rivers respectively. This correlates to findings throughout Europe of large blooms in mesotrophic rivers or below dams, with low abundances in oligotrophic rivers (Kawecka & Sanecki 2003; Beltrami *et al*. 2008).

Light – As with other stalked diatoms in New Zealand, *D. geminata* requires high light levels for growth. It is most prevalent in east-west oriented reaches and at depths from 10 cm to 2.0 m. Greater biomass occurs in areas with more light (Kilroy 2004).

Geology and Climate – Geological factors may also play a part in determining the distribution of *D. geminata*. Sherbot & Bothwell (1993) suggest that catchments with lower buffer potential, such as those with volcanic geology, may be more suitable for *D. geminata* growth. They also found that all catchments containing *D. geminata* blooms had less than 180 frost-free days, suggesting that temperature may be an important factor. Observations that *D. geminata* is not found in spring-fed streams or near confluences of rivers with spring-fed streams suggest that some physical or chemical properties of groundwater may prevent *D. geminata* from establishing (Larned *et al.* 2007). *D. geminata* also appears to prefer waters high in calcium (Lindstrøm & Skulberg 2008).

1.1.4 Ecological Impacts

The lack of research on *D. geminata* makes it difficult to determine what the long-term effects of large blooms are on the waterways in which they persist. Nevertheless, some observations include: reduction in species diversity of benthic invertebrates through exclusion of other diatoms; interference in fish developmental stages; reduced dissolved oxygen; elimination of moss and macrophytes through complete coverage of all

substrates; persistence of stalk debris, which traps sediment; decline in fisheries and native fish populations due to reduction of their invertebrate food sources and suitable habitats. Loss of tourism and fisheries, bad taste and odour, and blockage of pipes, reservoirs and hydropower plants can have serious economic impacts (Kilroy 2004). While native algal blooms affect ecosystems in similar ways, albeit to a lesser extent, the greater dominance and persistence of *D. geminata* suggests that its blooms will cause much greater ecological and economic impacts.

D. geminata can cause changes in trophic interactions, habitat interactions and abundance and diversity of benthic macroinvertebrates. Larned *et al.* (2007) found that 25 galaxiids were at risk to impacts of *D. geminata*. These species forage, rest and spawn in benthic habitats, which *D. geminata* mats cover. Species that do not utilise *D. geminata* as a food source are negatively affected when it replaces alternative periphyton food sources. Native fish may be affected by this shift in food availability. In laboratory trials, Chironomidae (midge larvae) did not consume *D. geminata* cells or stalks, while all other herbivores (mayfly, caddisfly and gastropod species) consumed both. In a river monitoring program, *D. geminata* caused an increase in invertebrate diversity and abundance and a shift in organism assemblage away from predominantly pollution sensitive Ephemeroptera, Plecoptera and Trichopera (EPT) taxa towards crustaceans, worms and non-EPT insects.

High levels of photosynthesis can cause dissolved oxygen (DO) levels to fluctuate, which in turn causes wide diurnal swings in pH. In two rivers studied, abundance of *D. geminata* was correlated to wide daily pH ranges and high pH levels (over pH 9) (Larned et al. 2007). This may be problematic for native freshwater fish and shrimp, which are sensitive to changes in pH. Surprisingly, the concentration of DO increased from the surface of the mats to below them. This suggests there may be an assemblage of photoautotrophic organisms in the mats that utilise dissolved nutrients at the mat bases.

1.2 History and Classification

1.2.1 Algal Taxonomy

Classification of various algal groups was traditionally based on features such as colour and type of chlorophyll and presence and type of flagella. Rhodophytes (red algae with chlorophyll *a* and phycobilins) and chlorophytes (green algae with chlorophyll *a* and *b*) (Graham & Wilcox 2000; Ben Ali *et al.* 2001) diverged earliest, followed by the haptophytes and alveolates, which form monophyletic clades. The heterokont algae, also referred to as ochrophyta, are the most recently diverged (Cavalier-Smith 1998; Ben Ali *et al.* 2001). Ochrophyte plastids are derived from red algae (Frommolt *et al.* 2008) and were initially taken up by an ancestral cell, possibly a chrysophyte ancestor (Graham & Wilcox 2000), as an endsymbiont. Ochrophytes possess chlorophyll *a* and *c*, but the brown pigment fucoxanthin masks the green colour of chlorophyll to give an ochre appearance. Ochrophytes also contain β-carotine, (a yellow accessory pigment), stores of chrysolaminarin (a form of carbohydrate) and lipid reserves. Other algae with chlorophyll *a* and *c* include the cryptophytes, haptophytes and dinoflagellates (Ben Ali *et al.* 2001).

The heterokont algae belong to the stramenopiles (Ben Ali *et al.* 2001) (Fig. 3), which are protists with a distinct type of flagella that is modified or completely lost in some lineages (Graham & Wilcox 2000). There is now good molecular support for the monophyly of stramenopiles, as well as red algae, green algae, land plants, fungi and alveolates (Ben Ali *et al.* 2001). The alveolates (ciliates, dinoflagellates and apicomplexans) form a sister clade to the stramenopiles with weak support. Stramenopiles are currently grouped into a cluster of eukaryotes called the Chromalveolata, along with the alveolates, haptophytes and cryptophytes (Adl *et al.* 2005; Keeling *et al.* 2005) (Fig. 1.3). While each of these lineages is monophyletic, it is

unlikely that the grouping of the haptophytes and cryptophytes with the alveolates and stramenopiles is monophyletic (Frommolt *et al.* 2008).

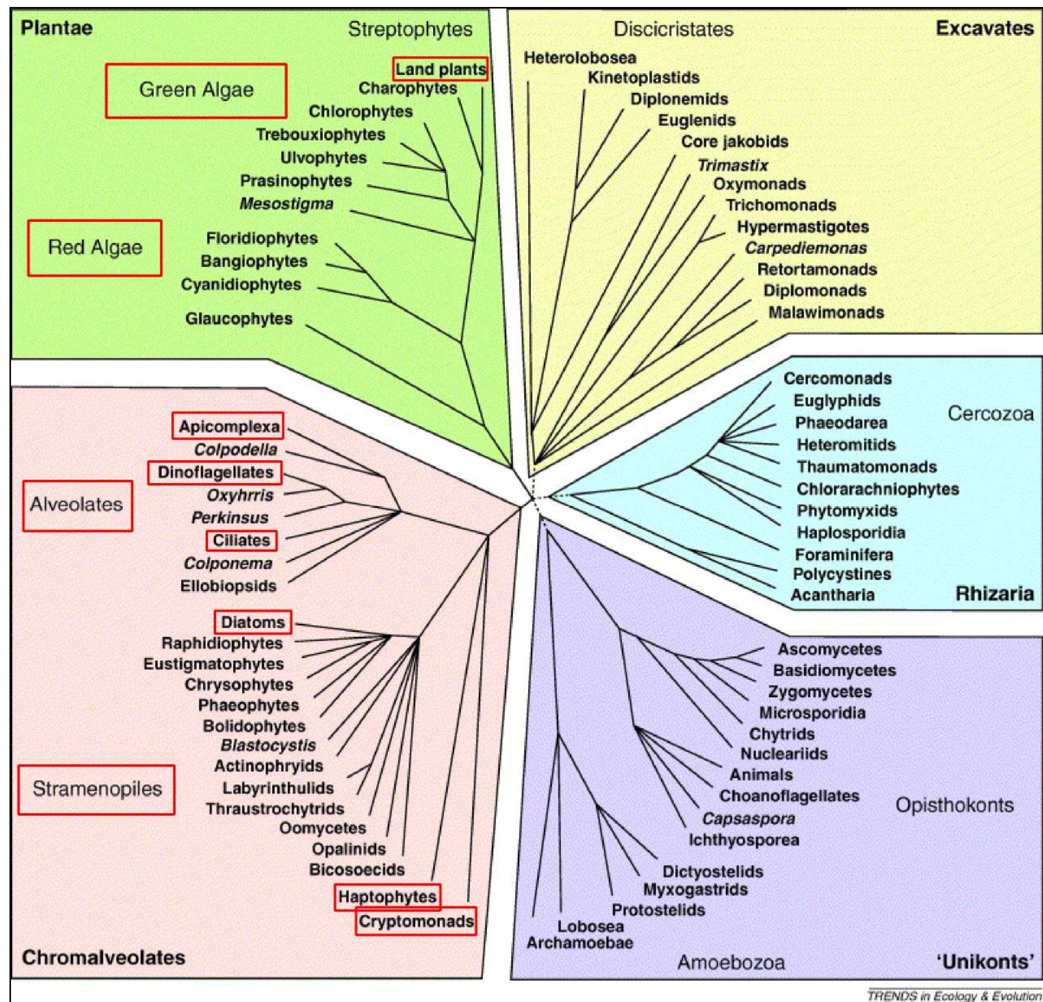


Figure 1.3: Tree of five eukaryote supergroups based on molecular, morphological and biochemical evidence. Adapted from Keeling *et al.* (2005). Red boxes indicate groups mentioned in the text.

Diatoms belong to the heterokont algal group, which is a monophyletic assemblage of diverse groups of algae for which the deeper branching relationships are unclear and further investigation is required. Using concatenated small subunit (SSU) ribosomal RNA (rRNA) and ribulose-bisphosphate carboxylase (*rbcL*) gene sequences, Anderson (2004) found weak support for heterokont algae being split into two monophyletic clades. One clade contained the chrysophytes, synurophytes and eustigmatophytes, while the sister clade contained diatoms (bacillariophytes) basal to the other heterokont algae. Other studies using SSU rRNA and large subunit (LSU) rDNA sequences place the diatoms

basal to the other heterokont algal groups (Ben Ali *et al.* 2002; Cavalier-Smith & Chao 2006) (Fig. 1.4).

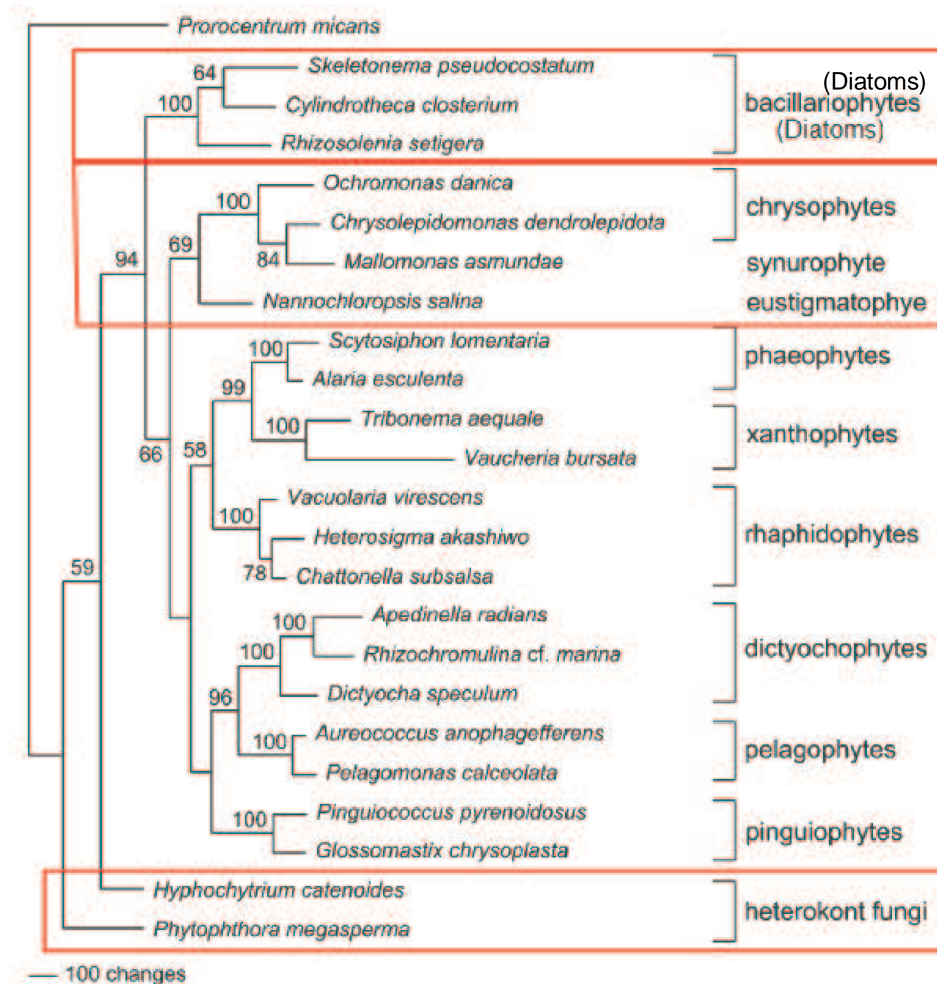


Figure 1.4: Maximum Parsimony tree of heterokont algae based on SSU and LSU rRNA sequences. Adapted from Ben Ali *et al.* (2002). Red boxes indicate groups mentioned in the text.

1.2.2 Diatom Taxonomy

The phylogenetic relationships and classifications for diatoms are dynamic. To complicate diatom taxonomy further is the once popular ubiquity hypothesis, which proposed that large population sizes of microorganisms enabled ubiquitous dispersal, preventing geographic isolation and speciation (Finlay 2002; Vanormelingen *et al.* 2008). Morphological observations supported the appearance of low diversity and cosmopolitan species (Finlay 2002). However, with the advent of genetic comparisons

and finer morphological examination, there is mounting evidence that many species thought to be cosmopolitan may actually be cryptic or pseudo-cryptic species complexes, some of which may be narrowly endemic (Vanormelingen *et al.* 2008). Furthermore, some organisms with different morphotypes have been described as different species when their differences are only phenotypic (e.g. *Stephanodiscus yellowstonensis* and *S. niagarae*, Zechman *et al.* 1994). This increases confusion about species taxonomy, distributions and biodiversity. It is now suspected that subtle morphological variation may be important physiologically, ecologically or reproductively. Thus, total diatom diversity may be severely underestimated (Mann & Droop 1996; Vanormelingen *et al.* 2008).

Previously, classification of diatoms was based on the fossil record and frustule morphology. It divided diatoms into the radial, the araphid pennate and the raphid pennate diatoms (Medlin *et al.* 1993). However, differences in reproduction and plastid structure in the centrics and araphid pennates suggested that these groups were not monophyletic. Molecular phylogeny confirmed this suspicion (Medlin *et al.* 1993). Centric diatoms first appear in the fossil record in the Early Cretaceous (Medlin *et al.* 1993) 150 mya and probably originated prior to the Cretaceous. Araphid pennate diatoms appeared in the Late Cretaceous about 70 mya. Raphid pennate diatoms arose more recently in the middle Eocene about 40 mya (Medlin *et al.* 1993). Diatoms became prominent aquatic components from the Miocene 24 mya (Graham & Wilcox 2000).

Molecular clock calculations suggest that diatoms may have first appeared almost 250 mya (Sims *et al.* 2006), which is much earlier than they appear in the fossil record. One of the difficulties in reconstructing diatom phylogenetic histories is long branch lengths, caused by the long time since divergence of extant species from their common ancestors. Many species only exist in the fossil record and they may provide the missing links to understanding diatom history (Williams, 2007). However, as is becoming increasingly clear, minute differences in frustule structure can equate to large differences genetically and these details are not available from fossil remains. All published molecular diatom phylogenies show

similar relationships. The centric diatoms form the deepest branches but are polyphyletic, as are the araphids. Most radial centrics arose first, followed by bipolar centrics. Pennates followed and consist of the earlier formed araphids and the later formed raphids (Sorhannus 2004).

As a result of a detailed study using morphology, cytology and molecular data for 100 taxa, Medlin and Kaczmarska (2004) proposed dividing the diatoms into two subdivisions, Coscinodiscophytina (radial centrics) and Bacillariophytina, with Bacillariophytina being further divided into two Classes, Bacillariophyceae (pennates) and Mediophyceae (polar centrics). They also confirmed that the sister group to the diatoms is a group of unscaled, unsilicated biflagellated picoplankton, the Bolidophyceae. Based on 126 SSU rRNA sequences, Sorhannus (2004) split diatoms into six centric clades (including three radial and three bipolar centric clades), two araphid and three raphid clades (Fig. 1.5). The earliest raphid divergence was the *Eunotia* clade (Clade I, Fig. d), followed by the Bacillariales and Achnanthales clade (Clade J) and then a clade comprised of Cymbellales, Lyrellales, Naviculales, Thalassiophysales and Surirellales (Clade K). Williams and Kolciolek (2007) claim that all current diatom classification systems are based on paraphyletic groups and will need to be revised. Consequently, the diatom classification system is subject to debate and several systems are currently in use.

1.2.3 *Didymosphenia* Taxonomy

Didymosphenia M. Schmidt 1899 falls within the order Cymbellales D.G. Mann, *ord. nov.,* Class Bacillariophyceae. Cymbellales contains several families (Fig. 1.6) including Gomphonemataceae Kützing 1844 and Cymbellaceae Greville 1833. *Didymosphenia* was originally considered to belong to the Family Gomphonemataceae (Dawson 1973a, Round *et al.* 1990). Some authors (Kociolek & Stoermer 1988; Moffat 1994; Kociolek & Spaulding 2003) now consider *Didymosphenia* to belong to the Family Cymbellaceae based on the arrangement and structures of cytological

contents and because the apical pore field is not dissected by the distal raphe as in members of Gomphonemataceae. Thus, *Didymosphenia* species may resemble members of Gomphonemataceae through evolutionary parallelism (Kociolek & Stoermer 1988).

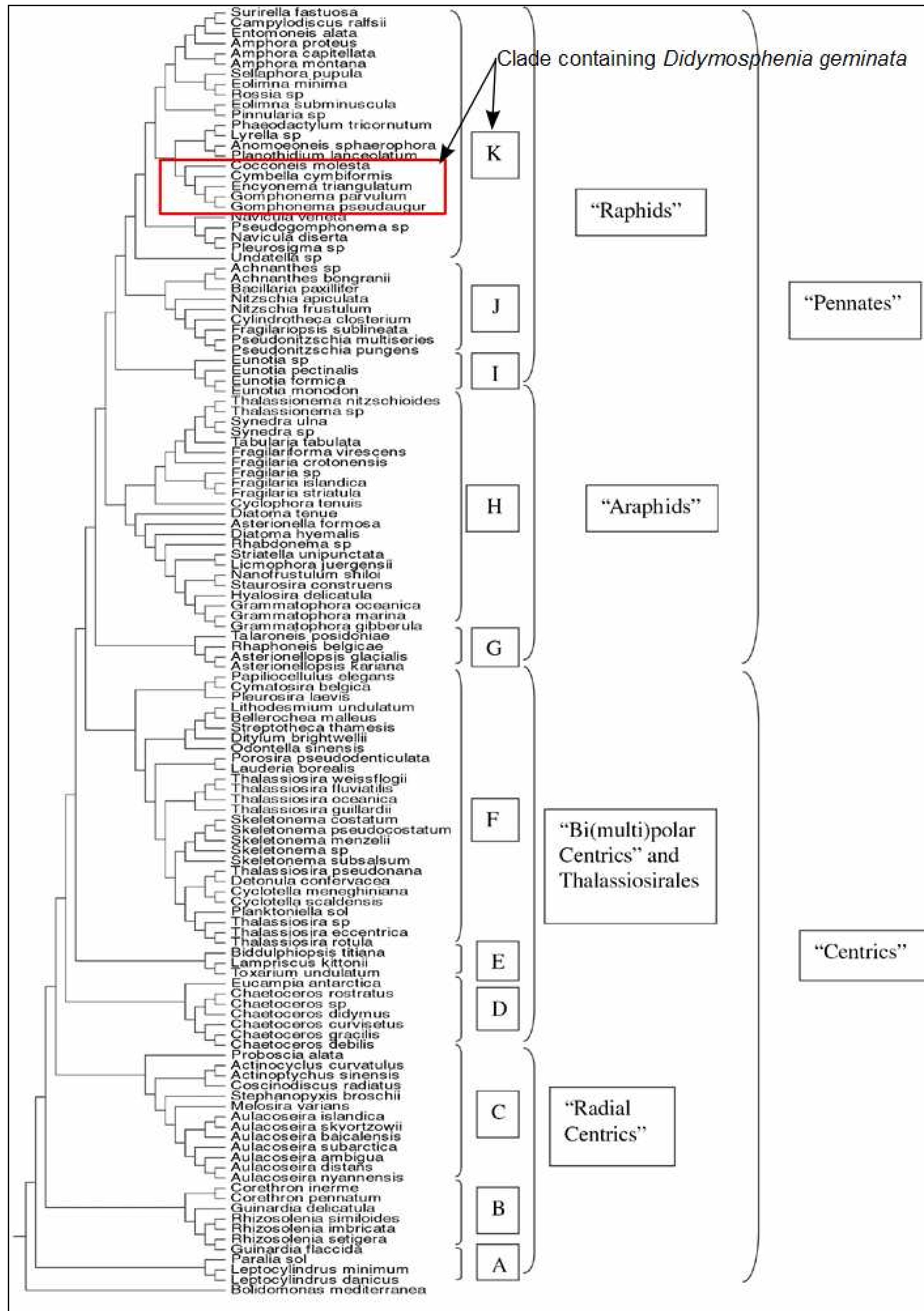


Figure 1.5: Most parsimonious tree of 126 SSU rRNA diatom sequences Adapted from Sorhannus (2004).

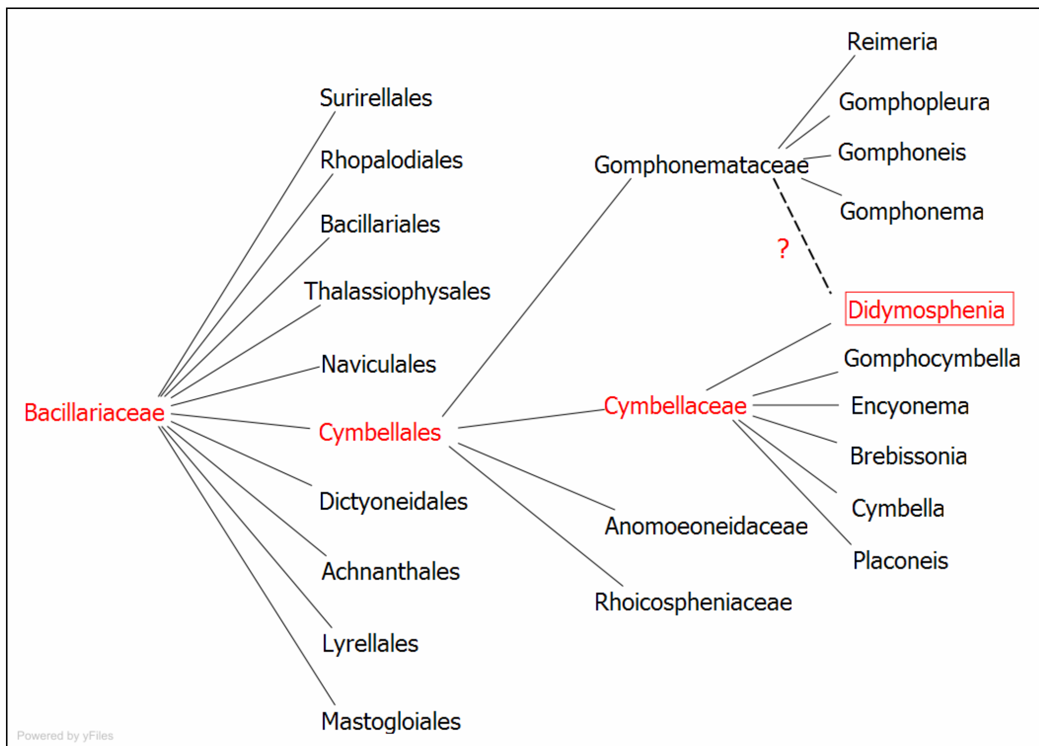


Figure 1.6: Classification of Bacillariaceae (pennate diatoms) based on the classification system of Round *et al.* (1990), while reflecting *Didymosphenia's* potential move to Cymbellaceae.

Didymosphenia geminata (Lyngbye) M. Schmidt may have first been described by Müller (cited in Dawson 1973a p. 65), as *Vorticella pyraria*. Lyngbye illustrated *D. geminata* in 1819, describing it as *Echinella geminata.* Agardh placed *E. geminata* in the genus *Gomphonema* as *G. geminatum* (Lyngb.) C. Ag in 1824 (Dawson 1973a). Didymosphenia was erected as a subgenus of *Gomphonema* C. Ag in 1899 by Martin Schmidt, who illustrated it in Adolf Schmidt's Atlas der Diatomaceenkunde (cited in Dawson 1973a). Didymosphenia was composed of three species and one variety, all of which had previously been regarded as varieties of one species, *Gomphonema geminatum* (Lyngb.) C. Ag. (Dawson 1973b). The taxa in this new subgenus were *D. geminata* (Lyngb.) M. Schm., *D. geminata* var. *stricta; D. curvirostrum* (Temp. et Brun) M. Schm. and *D. sibirica* (Grun).

Gomphonema C. Ag was since found to be a paraphyletic group of mostly epiphytic, wedge-shaped diatoms (Dawson 1973c). Hustedt (cited in

Dawson 1973a p. 66) raised *Didymosphenia* to the status of genus and Dawson (1973b, 1973c) validates this move from observations using scanning electron microscopy (SEM). Metzeltin & Lange-Bertalot (1995) further described *Didymosphenia* with five species distinguished by areolae structure. These are *D. clavaherculis* (Her.) nov. comb. (previously *D geminata* var. *stricta*); D. *siberica* (Grun.) M. Schmidt (sensu stricto); *D. curvata* (Skv. & Meyer) nov.stat.; *D. pumila* nov. spec; and *D. geminata* (Lynb.) M. Schmidt. *D. geminata* contains three morphotypes, *sensu stricto*, *capitata* and *subcapitata*. Other species described include *D. curvirostrum* (Tempère & Brun) M. Schmidt, *D. neocaledonica* Manguin, *D. lineata*, *D. dentata* Dorogostaisky and the fossil species *D. fossilis* Horikawa & Okuno (Mrozińska *et al.* 2006). *D. tatrensis* is the most recently described species of this genus (Mrozińska *et al.* 2006), occurring in the Western Carpathian Mountains of Poland and Slovakia. In total, 11 *Didymosphenia* species have now been described.

1.3 Recent *Didymosphenia geminata* Spread

1.3.1 Worldwide Spread

The first description of *Didymosphenia geminata* by Lyngbye in 1819 was from the Faroe Islands north of Scotland (Patrick & Reimer 1975). It was historically associated with boreal, montane or alpine locations (Kawecka & Sanecki) in Europe, Asia and North America (Beltrami *et al.* 2008). Due to the lack of references describing the ecology of *D. geminata* in the past, it is difficult to determine how much its range and ecological tolerances have changed in modern times. It is unclear to which regions this organism is native. However, findings of *D. geminata* in ice and sediment cores from several areas in Russia (Polyakova & Stein 2004; Miettinen *et al.* 2005), Finland (Sarmaja-Korjonen & Alhonen 1999) and North America (Stoermer 1993) suggest that it is native to these areas, in which it has been present for the last 8 000-120 000 years.

Cleve (cited in Kilroy 2004, p.6) lists records from Norway, Sweden, Finland, Ireland, Scotland, France, Switzerland, Spain, and Vancouver Island. Records do not indicate whether it is native to these regions, but *D. geminata* has existed in these regions for some time. However, in recent years it has been found in increasing abundance in these regions and in many new locations, often in mesotrophic waters and more temperate climates (Fig. 1.7). In many of these areas, it is one of the dominant components of the phytoplankton. The following section outlines some of the areas from which *D. geminata* has been recorded, including characteristics of some of the waterways in which it is found. More details of these waterways can be found in Table A.1 (Appendix 1).

Figure 1.7: Worldwide distribution of *Didymosphenia geminata* based on papers reviewed in this chapter and Table A.1, Appendix 1.

Russia – *D. geminata* may have been endemic to Lake Baikal, which is an aquatic diversity hotspot (Flower *et al.* 2004). Findings of *D. geminata* in ice cores from Peski, Russia (Miettinen *et al.* 2005) show that it was present in this area since at least the last interglacial period (the Eamian period) 120 000 years ago. Sediment cores taken from a bay in the Kara Sea, Arctic Ocean, contained *D. geminata* from approximately 15 000 years ago (Polyakova & Stein 2004). *D. geminata* also occurs in several rivers and lakes in the Far East Russian Primorsky region (Table A.1, Appendix 1), which has an extremely diverse algal flora and a large number of genera containing only a few species (Medvedeva 2001). Bacillariophyceae has the most diversity in this region.

Asia and the Middle East – *D. geminata* has been in headwaters of the Yangze River in the Qinghai-Xizang Plateau, Qinghai Province for many years (Li *et al.* 2002). There have been few studies in this region because of harsh conditions, which result in unique biological communities. Algae here survive high radiation, unpredictable light and low temperature.

The first record of *D. geminata* in India was in 2001 (Bhatt *et al.* 2005), where it was the most abundant species in phytoplankton and phytobenthic communities during monsoon and post-monsoon seasons. *D. geminata* occurred in other Himalayan rivers, but both trout and *D. geminata* were absent from Central Himalayan rivers. Thus, Bhatt et al. (2008) suspect that *D. geminata* was spread through trout distribution.

Most recently, *D. geminata* has been reported in Iran for the first time, high in the Alburz Mountain Range (Alimohammadian 2008). Interestingly, this is a limestone rich region (pers. obs; H. Alimohammadian pers.com.).

Continental Europe – The earliest records in Europe are from an early Holocene sediment core in Southern Finland, where *D. geminata* dominated the deepest diatom zone along with *Cymbella* species over 8000 years ago (Sarmaja-Korjonen & Alhonen 1999). It is common in Lapland tributaries (Korumlaynen 2008). In Norway, it has been present

for at least 150 years (Lindstrøm & Skulberg 2008). It formed large growths in the 1980s, but by 2003 it was found at lower levels (Miettinen 2006; Skulberg & Lillehammer, cited in Ellwood & Whitton 2007 p. 122). It has also become widespread in the Danube Basin in the last 20 years (Table A.1; Appendix 1).

The first find of *D. geminata* in Southern Poland was in the 1960s in oligotrophic alpine waters in low abundance. At some of these locations, *D. geminata* appears to have disappeared in conjunction with eutrophication (Kawecka & Sanecki 2003). It then appeared in Vistula River tributaries in the 1990s. In the San River, it was rare in mountain sites but abundant below the Myczkowce and Solina reservoirs, where it formed such large masses that it plugged water filters in supply systems (Kawecka & Sanecki 2003). *D. geminata* has since spread to other, mostly oligotrophic-mesotrophic, Polish rivers (Noga 2003). Interestingly, it was found in the Czarna Orawa River and its right-bank tributaries, which have high velocities, cold temperatures and clay or loam geology (Noga 2003). It did not occur in the left-bank tributaries, which are deeper, browner, slower, warmer and originate in peat land.

There is no information on how long *D. geminata* has been present in Turkish waters, where it has become prevalent in even lowland, eutrophic waters (Table 1. Appendix A.1). Interestingly, *D. geminata* was rare in the oligotrophic Lake Cildir (Akbulut & Yildiz 2002), but dominant in the fast-flowing eutrophic Sana River (Kolayli *et al.* 1998).

Great Britain – *D. geminata* blooms have occurred in the northern and western United Kingdom for at least 150 years and have not increased in biomass or geographic range (Antoine & Benson-Evans 1984; Spaulding & Elwell 2007). A study found the morphology of cells from Wales and England most similar, and those from Scotland considerably longer. Cells from Great Britain showed greater variability and wider range in length and breadth than those from previous descriptions of European and American populations (Antoine & Benson-Evans 1984).

North America – A study of diatoms in karst ice caves of the Northern Yukon Territory, Canada, in the 1980s and 1990s found that *D. geminata* was abundant (over 80%) in the sea ice and the cryogenic calcite powders in Grande Caverne Glacée (Lauriol *et al.* 2006). Paleolimology shows *D. geminata* once occurred throughout the oligotrophic upper lakes. Stoermer (1993) recorded it only in Lake Superior, due to decline in lake quality with European settlement, and describes it as sensitive to ecological change. Since this time, *D. geminata* has become prevalent in many new areas throughout the United States and Canada.

Although *D. geminata* was recorded on Vancouver Island, Canada, in the 1800s (Cleve, cited in Spaulding & Elwell 2007, p. 7), nuisance blooms were first noted on Vancouver Island in the 1980s (Sherbot & Bothwell 1993). From the Heber River in 1989, *D. geminata* spread to more than half of the islands rivers in five years (Sherbot & Bothwell 1993; Kirkwood *et al.* 2007). Nuisance blooms since declined (Bothwell *et al.* 2008) but *D. geminata* continued to spread to other waterways. In a study of the Canadian Kootenai River's degraded river ecosystem, *D. geminata* comprised the bulk of the biovolume (Holderman *et al.* 2004).

In the United States, *D. geminata* has expanded its range to include Alaska, Idaho; Colorado, Washington; Oregon; California; Montana; South Dakota; Tennessee; Arkansas; New York State; Vermont; Virginia; Delaware and Utah (LaPerriere 1989; Edwardson *et al.* 2003; Opsahl *et al.* 2003; Holderman *et al.* 2004; Oberholster *et al.* 2005; New York State Department of Environmental Conservation 2007a; Spaulding & Elwell 2007; Spaulding et al. 2008; Kumar *et al.* 2009). *D. geminata* is now present in the Delaware tailwaters, which contain important East Coast trout fisheries (New York State Department of Environmental Conservation 2007b). It is also in the Batten Kill, NY, which is a popular stream for trout fishing, canoeing, kayaking and tubing (New York State Department of Environmental Conservation 2007a). *D. geminata* was observed along the Cache la Poudre River, Colorado, where it dominated sites contaminated by coal tar residue (Oberholster *et al.* 2005).

Iceland –*D. geminata* formed large blooms in west and southwest regions in the 1990s. It has since declined or become stable, although it is now present in most lowland regions (Jonsson *et al.*, cited in Spaulding & Elwell 2007, p. 9; Jonsson *et al.* 2008).

1.3.2 The New Zealand Invasion

The first and only known occurrence of *D. geminata* in the Southern Hemisphere is in New Zealand. It was discovered during a routine periphyton survey of the South Island's Waiau and Mararoa Rivers in October 2004 (Kilroy 2004). The National Institute of Water & Atmospheric Research (NIWA) and Environment Southland have monitored this river since 1997, suggesting that *D. geminata* was introduced shortly before this survey, or was present at very low levels prior to October 2004. During the summer of 2004-2005, *D. geminata* mats covered a 150 km stretch of river and persisted during autumn and winter 2005. Biosecurity New Zealand (BNZ) declared *D. geminata* an "unwanted organism" in November 2004.

After the first *D. geminata* discovery, BNZ established a system of ongoing passive surveillance (OPS) for reporting suspected *D. geminata* finds by public and local organizations (Duncan *et al.* 2005). No more *D. geminata* was found until September 2005, when it was found in the Buller, Hawea, Oreti, upper Waiau and Clutha Rivers (Duncan *et al.* 2005). Further positive samples were identified from Von River, Lake Dunstan and Lake Manapouri. Thus, in less than one year, *D. geminata* was observed to spread from one river system to three independent catchments (Duncan *et al.* 2005). *D. geminata* has not yet been found in the North Island.

After *D. geminata* findings above, BNZ planned to conduct quarterly surveys. Appendix 2 provides information regarding these surveys, including a summary of *D. geminata* finds (Tables A.2 & A.3). Most sites with *D. geminata* had clear water (Duncan 2006b). In 2006, *D. geminata* was found in several new rivers in popular fishing spots and rivers suited for *D. geminata* growth and establishment (Duncan & Wilkins 2006). In

2007 came the first report of *D. geminata* from Westland and the second from Nelson. Both locations are important for trout fishing (Duncan 2007). *D. geminata* was also found in the Kakanui River. This caused concern for DOC because one of its tributaries, the Kauru Stream, is home to Kokopu (*Galaxias prognathus*), a rare long-jawed galaxiid. *D. geminata* may interfere with the Kokopu's habitat and food sources (Duncan 2007).

The specially designed sampling drift net (Appendix 2) and DNA testing developed by Cary *et al.* (2006) was highly efficient, enabling detection of cells in the water column when they were not detected in benthic samples. However, as *D. geminata* continued to be detected through the OPS system, the surveys may not have been providing enough coverage. Alternatively, the surveys may not have been frequent enough, with *D. geminata* establishing between surveys (Duncan 2007). In 2007 dead cells were found in samples from the North Island during a routine monitoring program. Thorough re-sampling and an audit found cross-contamination in a laboratory supplying sample containers (Didymo National Delimiting Survey 2007), emphasizing the importance of decontamination procedures and the sensitivity of the DNA detection method.

***D. geminata* Long-Term Management Programme**

Estimates of the potential economic impacts *D. geminata* will have in New Zealand if there is no Government intervention are $58-285 million from 2004-2011 (Didymo Long-Term Management Programme 2007), affecting biodiversity, recreation, cultural values and the economy. Objectives of the long-term management program are to slow aquatic pest spread, mitigate impacts, keep the North Island 'didymo-free' as long as possible and protect valued species and sites at risk. It continues to target freshwater users' behavior with the slogan "Check, Clean, Dry". Monitoring for abundance and spread of *D. geminata* will be important, as it can provide information on preferred environments and natural factors that promote blooms or reduce biomass. The desired outcome is to reduce the impacts of *D. geminata* (Didymo Long-Term Management Programme 2007).

1.4 Invasive Species

Species invasions are fundamental to the history of life on earth. Invasions occur when introduced species become established outside their native ranges and cause ecological and economic damage (Alpert *et al.* 2000). Anthropogenic activities cause a greater increase in long-distance dispersal more frequently than found in nature, causing widespread ecosystem changes (Coleman 1996). Invasive species are second only to landscape fragmentation and habitat destruction as a threat to biodiversity (Provan *et al.* 2005). Invasive species have major economic impacts worldwide and cause losses over $125 billion annually in the US alone (Allendorf & Lundquist 2003). While habitat disturbance often enables terrestrial invasive species that are adapted to such conditions to outcompete natives (Byers 2002), aquatic environments are more stable, encountering less stress and may therefore provide additional opportunities for establishment of exotic species (Coleman 1996). However, aquatic invaders receive less publicity than terrestrial invaders.

Well known aquatic invasive species include brown trout (*Salmo trutta*), the zebra mussel (*Dreissena polymorpha*), water flea (*Daphnia lumholtzi*), green crab (*Carcinus maenas*), alligator weed (*Alternanthera philoxeroides*), water hyacinth (*Eichhornia crassipes*) and several seaweeds (*Codium fragile, Undaria pinnatifida*). Phytoplankton invasions are rarely documented, largely due to poor prior knowledge of the diversity and abundance of the range of phytoplankton in a region (Féral 2002). In the case of diatoms, poor knowledge of diversity may stem from their small size, their high diversity, the difficulty of distinguishing species morphologically, the difficulty of conducting mating experiments with diatoms (Mann & Droop 1996) and the ideas behind the ubiquity hypothesis (Finlay 2002). The ubiquity hypothesis proposes that local extinction through processes such as predation and competition could not occur in the microbial world, due to large population sizes and ubiquitous dispersal. However, increasing evidence suggests endemism is more

widespread among diatoms than was previously thought, making invasions and local extinctions feasible (Kilroy *et al.* 2007). Another problem facing research of diatom invasions is the difficulty in pinpointing the time of arrival of diatoms in a region, as they often persist in low, undetectable numbers until reaching nuisance proportions (Edwards *et al.* 2001). Several diatoms are known to have invaded the Great Lakes through transportation in ships' ballast water (Mills *et al.* 1994), for example, *Thalassiosira baltica* (Edlund *et al.* 2000). Another example of an invasive diatom is *Coscinodiscus wailesii,* which was first reported in the English Channel in 1977 and has since spread to and persisted in the North Sea (Edwards *et al.* 2001). *Asterionella formosa* is a diatom that appears in sediment records only after European arrival in New Zealand but is now widespread throughout New Zealand lakes (Vanormelingen *et al.* 2008). The invasion of *D. geminata* has recently drawn considerable attention due to its sudden rapid spread and formation of nuisance blooms worldwide (Kilroy 2004).

1.4.1 Invasion Success

Many studies attempt to predict the likelihood of invasive success of species or the invasibility of a habitat, with the goal of improving control and preventing successful invasions. Successful invaders have traits such as rapid, efficient dispersal mechanisms, broad native ranges, short generation times, and phenotypic plasticity enabling response to environmental variables (Alpert *et al.* 2000). Invaders may be successful if they initially evolved in more competitive environments, or if the new location does not have any natural enemies to keep their populations in check (Sax & Brown 2000). It has been proposed that species invasions may be more successful in communities with low species diversity because there is likely to be less competition, predation or pathogens among native species. However, the opposite has often been observed (Allen *et al.* 2006). Alpert *et al.* (2000) therefore propose that the most

likely predictors of invasibility are disturbance, low levels of competition and minimal environmental stress.

Invasive species are often most successful in disturbed habitats (Lee & Gelembiuk 2008), where invaders that have adapted to survival in highly disturbed habitats thrive. Streams and rivers occur in the upper ranges of disturbance and productivity indices, suggesting they have a high potential for dominance by invasive species (Kilroy *et al.* 2007). It is in these waterbodies, especially in human disturbed reaches, that *D. geminata* has been most problematic. Kilroy *et al.* (2007) suggest that, in the absence of disturbance, endemics can coexist with invasive species if invaders have different niche requirements.

Invasion success may also be correlated to phylogenetic relatedness. Although introduced species closely related to native species are more likely to establish in the new environments (Duncan & Williams 2002), introduced species less related to native species are more likely to be highly invasive. While introduced species closely related to natives may share traits that enable pre-adaptation to the new environment (Duncan & Williams 2002), non-relatedness may enable invasive species to escape local enemies and utilise different resources, reducing competition. There are also less coevolved defences against non-related invasives by community members, such as prey (Duncan & Williams 2002; Strauss *et al.* 2006b). This explains why impacts from species invasions are so large in isolated islands, lakes and river basin ecosystems, including high rates of extinction. Isolated species may not be able to adapt to invasive species rapidly enough to prevent extinction due to low genetic variation, small populations and limited habitat availability (Strauss *et al.* 2006a).

1.4.2 Impacts of Invasions

Invasions disrupt established communities and alter habitats. However, not all impacts are negative (Strayer *et al.* 2006). Invasive species can facilitate native and other exotic species, although often at the cost of other species. Simberloff & Von Holle (1999) suggested the possibility of an 'invasional meltdown' occurring, where invasive species facilitate the survival of other invasive species, resulting in a rapidly increasing number of invasions and their impacts. For example, Chase & Knight (2006) suggest that the success of the invasive aquatic plant Eurasian watermilfoil (*Myriophyllum spicatum*) may be facilitated by the introduction of molluscivorous fish, which prey on snails controlling the plant's biomass.

Invasive species can facilitate other species by reducing competition or predation, providing novel habitats and nutrients and functionally replacing natives. For example, the introduction of the European green crab (*Carcinus maenas*) preferentially preys on native clams, reducing their competitive advantage over an introduced clam (Grosholz 2002). In New Zealand, the introduced brown trout (*Salmo trutta*) reduces predation on algae by preying on native herbivores and enabling increase in algal biomass (Townsend 1996). The zebra mussel (*Dreissena polymorpha*) provides limiting resources and increases habitat complexity on muddy lagoon floors, providing shelter and food for native and non-native invertebrates (Zaiko *et al.* 2007).

Invasive facilitation of native species can cause conflicting management options, especially where endemic or endangered species are concerned (Rodriguez 2006). For example, in the USA, the saltcedars (*Tamarix* sp.) have replaced native riparian vegetation that was lost through agriculture, grazing and urbanization and now provide nesting habitat for the endangered native willow flycatcher (*Empidonax trailii extimus*), thus functionally replacing native species (Zavaleta *et al.* 2001). Recovery of native communities following removal of invasive species may be difficult if

invasive species have functionally replaced native species that are no longer present in the community (Gleeson *et al.* 2006).

Community effects of invaders may include a shift towards species that utilise the invader as a food source (Strayer *et al.* 2006), as seen with the shift towards crustaceans and worms in *D. geminata* invaded rivers (Larned et al. 2007). Communities may also shift towards species resistant to the invader. Invaders may be drivers of biodiversity loss in native communities. For example, *Ceratophyllum demersum*, an invasive hornwart in New Zealand, can establish in naturally occurring gaps of native aquatic plants, from which it grows more vigorously than the native plants (Hofstra *et al.* 1995). This can cause complete replacement of native plants. Invaders may enable their diseases, parasites or predators to arrive and establish. This can limit the invader, but may also have devastating effects on the native community (Strayer *et al.* 2006). Communities are also affected by the influence invaders have on abiotic factors, such as nutrient cycling, chemical concentrations, soil characteristics, sedimentation rates, erosion, fire regimes and hydrology. Impacts of invaders may change over time and after the immediate decline of native species post-invasion, diversity may return to original levels.

Two opposing theories exist regarding the interaction between disturbances and invasive species resulting in biodiversity loss. The first suggests that human-induced disturbances drive ecological change, providing opportunities for invasive species to out-compete native ones, resulting in biodiversity loss (Suarez & Tsutsui 2008). The second suggests that invasions result indirectly from habitat modification and then drive native species loss themselves (Didham *et al.* 2005). The most likely scenario may be that of interacting drivers, whereby effects of disturbance and invasive species are additive or synergistic (Didham *et al.* 2005).

Endemic species are especially at risk of extinction during invasions. Endemism is highly developed in ancient lakes, such as Lake Baikal, and on islands, such as New Zealand, and is mostly associated with isolated ancient habitats, although speciation can occur relatively rapidly (Mann

1999). A survey of freshwater habitats in New Zealand (Kilroy *et al.* 2007) found that endemic diatoms were most abundant in low disturbance sites and low-productivity environments. Diatoms in these environments may face low rates of competitive displacement, as there are limited resources for reproduction and dispersal. The findings of unique diatom assemblages in isolated areas highlight the importance of conservation in these areas and the need to protect isolated areas from invasions (Vanormelingen *et al.* 2008).

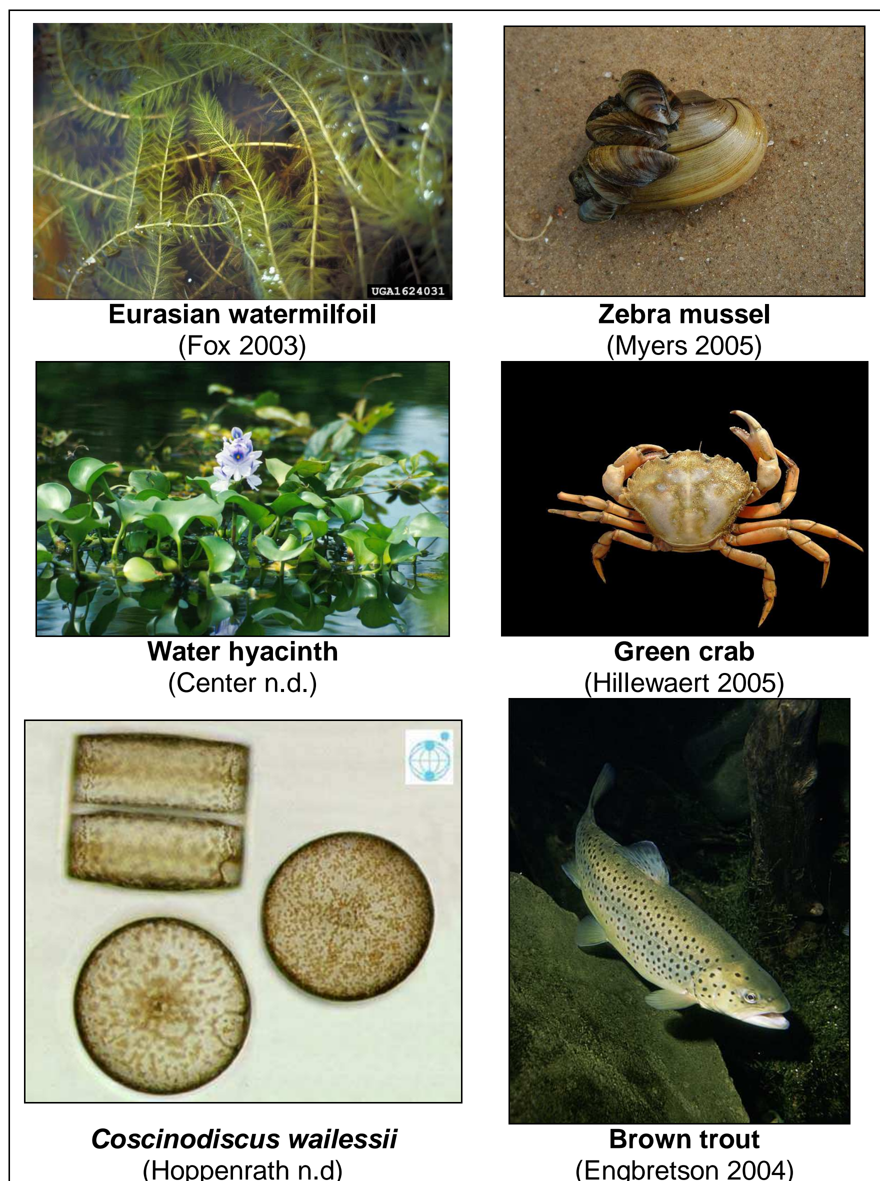


Figure1.8: Ilustrations of some invasive species mentioned in the text.

1.4.3 Stages of Invasion

The First Stage: Arrive, Survive, Establish

A non-indigenous species must survive and establish on arrival in a new area. Invasions often involve long-distance dispersal into areas in which they have not occurred before (Alpert *et al.* 2000). Diatom dispersal occurs through water currents and potentially by animal, bird, wind or human assistance (Vanormelingen *et al.* 2008). There is little evidence for animal and wind dispersal, and the major impact of dispersal by humans in recent years suggests that non-human dispersal was limited in the past. Some fossil records confirm this, where species thought to be cosmopolitan only appear in sediments after human arrival in the area. Human dispersal of diatoms and other aquatic invaders occurs via ship ballast water, aquarium releases, agriculture (e.g. shellfish transplantations) and recreation (Roman & Darling 2007; Vanormelingen *et al.* 2008). The introduction of *D. geminata* in India was likely to have been through the introduction of trout to the Himalayas (Bhatt *et al.* 2008).

Increased propagule pressure (the number of introductions and the number of individuals introduced) increases the likelihood of successful dispersal and establishment (Roman & Darling 2007). Propagule pressure is important in predicting establishment, perhaps because genetic diversity reduces population bottlenecks (Roman & Darling 2007). Multiple introductions (separate dispersal events over time) enable production of new genetic combinations, bringing together variation from different, often completely separate, source populations (Dlugosch & Parker 2008). For example, introduced water hyacinth *Eichhornia creassipes* in China is thought to have greater variation than other clonal invasive aquatic plants in China due to multiple introductions (Ren *et al.* 2003).

The Second Stage: Spread and Replacement

Once established, successful invaders spread and replace native species. A lag time often occurs between arrival and spread. Species can exist in low, undetectable numbers until they suddenly explode in number (Allendorf & Lundquist 2003). This may be due to small populations growing exponentially until they are suddenly detected. Alternatively, expansion may only be possible after an adaptive breakthrough is reached. This breakthrough may occur through hybridization, new introductions or mutations (Suarez & Tsutsui 2008). There may also be a lag time before the effects of invaders can be seen (Strayer *et al.* 2006).

If a species is introduced to a new area, from which it spreads, it is expected that the new populations will be genetically uniform or decreasing in genetic variation. For example, the extremely low genetic variation found in a RAPD analysis of the highly invasive aquatic alligator weed (*Alternanthera philoxeroides*) in China suggests that it had one introduction and that it reproduces vegetatively (Xu *et al.* 2003). If a species is introduced to multiple new areas from different populations in its native range, it is likely that these specific genotypes will be initially localised (Suarez & Tsutsui 2008). Multiple introductions may be haphazard and isolated, and traits of each population may not be optimal for its location. However, over time, admixture will optimise the traits to their appropriate locations. In the long-term, multiple introductions may result in hybridization between populations, extreme phenotypes or adaptation to new habitats and microenvironmental conditions, increasing the species' ability to spread. Therefore, it is important to confine movement and importation of invasive species already present in an area (Dlugosch & Parker 2008; Suarez & Tsutui 2008).

1.4.4 Genetic Factors in Invasions

Recent invasions offer unique opportunities to study evolutionary changes over observable time scales, including adaptation of invaders and native community responses to invasions (Lee & Mitchell-Olds 2006; Gilchrist & Lee 2007). Changes brought about by invasions occur much more rapidly than changes caused by natural dispersal, necessitating rapid adaptation of natives in the face of extinction (Allendorf & Lundquist 2003). Altered selection pressures can cause rapid evolution in both native and introduced species. Each stage in an invasion exerts unique selection pressures, which filter out individuals that are not able to survive and adapt. Changes correlated with geographical variation can result in speciation (Strayer *et al.* 2006). Understanding the genetics of invasive and native species may assist in understanding why and how species become invasive, the susceptibility of communities to invasion and the effects of invasion on genetic composition of the community (Allendorf & Lundquist 2003). Standard ecological observations cannot definitively determine genetic diversity, number of introductions or source populations of invasive species. Studies of genetic variation can determine the history of invasive species using high-resolution markers and provide risk assessments by predicting if a nonindigenous species is likely to become invasive based on population size, population genetic diversity, and the potential for gene flow, adaptation and hybridization (Allendorf & Lundquist 2003; Provan *et al.* 2005).

1.4.5 Invasive Success: A Genetic Paradox

There is conflict between current genetic theories and the success of invasive species. Invaders were thought to have characteristically low levels of genetic variation compared to source populations (Provan *et al.* 2005) due to establishment from a small number of individuals. This founder effect should reduce the ability of new populations to adapt to new

environments. In contrast, invasive species outperform genetically diverse, locally well-adapted native populations (Allendorf & Lundquist 2003).

Some theories for this unexpected success have been proposed. One theory is that some alleles that are deleterious in source populations may be favourable in new environments (Barrett *et al.* 2008) Also, loss of genetic diversity may cause loss of deleterious alleles or genetic changes (Roman & Darling 2007). Furthermore, many fitness-related traits are quantitative, with additive variation, and loss of diversity at one locus will not cause loss of the trait altogether (Dlugosch & Parker 2008).

Another theory is that lack of natural enemies in the new environment improves survival rate (Sax & Brown 2000) and requires less expenditure on defences, leaving more energy available for growth and reproduction. Furthermore, under normal growth conditions, invasive species may out-compete native species because they are not spending resources on long-term adaptations for local conditions (Allendorf & Lundquist 2003). Native species, on the other hand, are better able to survive particular events in the long-term, such as fire, drought or storms. Many invasive plants can reproduce by asexual reproduction, avoiding inbreeding depression (Gleeson *et al.* 2006), but short-term success in low diversity invaders may be outweighed later if their lack of genetic variation prevents adaptation to new diseases or environments (Suarez & Tsutsui 2008). This may explain why some invasive species are continually colonizing new areas in order to survive (Gray 1987).

Another theory for the success of invasive species with low diversity is that they may be generalists,able to survive in a wide range of habitats. A species may also appear to adapt to the new environment through phenotypic plasticity (Lee 2002). It is difficult to differentiate between phenotypic plasticity and genetic adaptation. Non-genetic changes include resource allocation, morphology, physiology and gene expression (Strayer *et al.* 2006). Genetic adaptation can evolve rapidly, including reduction of chemical defences due to lack of natural enemies, diversion of resources to growth and reproduction, matching life-history traits to environmental

conditions, utilization of new food sources, and acquisition of defence against local enemies (Dlugosch & Parker 2008).

Finally, introduced populations may not undergo as large a loss in genetic diversity as was previously expected (Roman & Darling 2007). Many studies have discovered invasive populations with genetic diversity equal or greater than that in their native populations. Diversity loss through founder effects and genetic drift is minimized if a large number of propagules are introduced. Admixture of genotypes from several source populations following multiple introductions can result in intraspecific hybridization, increasing the genetic diversity of the invasive population (Roman & Darling 2007). Furthermore, low genetic diversity in an invasive population may reflect low genetic diversity within the source population rather than a bottleneck effect. For example, the highly invasive freshwater plant *Egeria densa* had similar low levels of genetic variability in South America and the invaded USA (Carter & Sytsma 2001) based on RAPD analysis. This highlights the importance of investigating genetic diversity of both invaded and source populations where possible.

1.4.6 Role of Reproductive Systems

Reproductive systems shape the genetic variation within populations and influence genetic recombination, gene flow, distribution of genetic diversity and effective population size (Barrett *et al.* 2008). Loss of mating types in founder populations affects obligatory outcrossing species, limiting the chance of success, and increasing the effects of genetic drift. However, outcrossing enables genetic recombination and evolution, and thereby, adaptation. Uniparental reproduction involving asexual reproduction, as common with *D. geminata*, provides reproductive assurance, enabling rapid population growth even when founder populations are small. Clonal reproduction prevents inbreeding depression associated with selfing, preserving multilocus associations. However, it also prevents admixture

and recombination, minimizing the effects of multiple introductions. Successful clonal invaders (e.g. *Alternanthera philoxeroides, eichhornia crassipes*) demonstrate a range of phenotypic plasticity, enabling them to colonize wide ranges (Barrett *et al.* 2008). Clonal reproduction does not necessitate low genetic diversity, as illustrated by the invasive clonal plant *Solidago canadensis* in China. This plant possesses extremely high genetic diversity, with no identical genotypes found in an ISSR analysis (Dong *et al.* 2006).

Interspecies hybridization has been instrumental in the success of several severe plant invasions. Hybridization often occurs between the invader and a native species and can increase heterozygosity levels and genetic diversity, enabling rapid adaptation to new conditions and increase in invasiveness (Gleeson *et al.* 2006). For example, the cordgrass hybrid *Spartina anglica,* produced by hybridization between the invasive northern American *Spartina alterniflora* and the native *Spartina maritima*, now dominates many estuarine marshes in the British Isles, while its parent species maintain limited distributions (Thompson 1991). *Potamogeton* species, including the invasive pondweed *Potamogeton crispus,* frequently form hybrids and this, together with a wide range of phenotypic plasticity, makes their taxonomic classification difficult (Kaplan 2002). *P. crispus* has the potential to hybridize with the native New Zealand *P. ochreatus* producing a hybrid more vigorous than either parent species (Champion & Clayton 2000).

Intraspecific hybridization in populations containing combined, genetically diverse source populations can also increase invasiveness (Lee 2002). Intraspecific hybridization may be especially serious in aquatic algal species, with the potential to form new, toxic, vigorously blooming strains (Kooistra *et al.* 2001). For example, the harmful flagellate alga *Fibrocapsa japonica* from geographically separate native populations contains low levels of genetic differentiation. The high level of intra-individual heterozygosity throughout its invasive range suggests that there may have been an intraspecies hybridization event between introduced and native

parents, which caused the sudden appearance of blooms (Kooistra *et al.* 2001).

1.4.7 Phylogeography

Phylogeography, a relatively new area of study, has been useful in determining pathways of spread of invasive species. John C. Avise coined 'phylogeography' in 1987 to describe the study of the geographic distributions of genealogical lineages (Avise 1998). Phylogeography elucidates population processes over space and time (Hare 2001). Information on factors that produced the current geographical distribution is sought, such as bottlenecks, range and population expansions, migration, gene flow, hybridization, geographical barriers, habitat fragmentation, colonisation, effects of climatic changes, dates of divergence and size of ancestral populations (Bermingham & Moritz 1998). Combined with other disciplines, such as geography and ecology (Avise 1998), phylogeography can provide a fuller picture of the forces acting on populations than would be obtainable by other means. Accumulating many phylogeographic studies for a number of co-distributed species in a given geographical area ('comparative phylogeography') reveals underlying processes that produce observable patterns (Avise 1998; Arbogast & Kenagy 2001). This knowledge will be especially important in the area of conservation of global biodiversity (Avise 1994).

1.4.8 Genetics in Control and Management of Invasive Species

Population genetic tools can be used to distinguish invasive from non-invasive strains if they have different genetic profiles. These tools can also be used to assess the likelihood of invaders developing resistance to control agents based on the genetic variation present within the invasive

population. While biological control may be the only chance for reducing some invasive species, this may have mixed success, with closely related native species, community structures and processes often being affected. More research and a great deal of care are required when considering such an approach for control and management of invasive species (Allendorf & Lundquist 2003).

Eradication is most successful and cost-effective in the lag phase, during which the species is in low abundance, found in limited locations and has not yet developed adaptations to local conditions (Allendorf & Lundquist 2003). Thus, early detection of invaders in small numbers is important. For example, early detection for improved eradication is the principle behind routine QPCR screening of water samples for *D. geminata* in New Zealand's North Island (Cary *et al.* 2006). Nevertheless, prevention of introductions is more efficient than trying to eradicate them once established. The boundaries in this area become grey when non-indigenous species are economically important, for example, trout fishing. Risk analyses and management possibilities must be thoroughly explored in these instances (Allendorf & Lundquist 2003).

1.5 Molecular Markers

Many molecular markers are now available for phylogenetic studies and they target either specific or anonymous DNA regions (Liu & Cordes 2004). The ideal molecular marker is that from noncoding DNA under neutral selection (Parker *et al.* 1998). Mutations in DNA regions not under selection can accumulate at a faster rate. Mutations in genes under selection accumulate more slowly because mutations affect function, potentially reducing the fitness of the organism. Slowly evolving markers are useful for investigating ancient events, while rapidly evolving, highly variable markers are useful for investigating recent events (Hare 2001). A good molecular marker is easy to amplify and has sufficient variation for the desired comparison but can still be aligned unambiguously without the phylogenetic signal being lost (Zechman *et al.* 1994; Baldwin *et al.* 1995). Markers do not necessarily provide the same level of analysis across taxa. For example, a marker that shows generic-level comparison in angiosperms may be so variable in diatoms that it is only useful for population level analyses (Zechman *et al.* 1994).

This section focuses on molecular markers commonly used in phylogeography, especially that of diatoms, which is central to this project. Phylogeographic markers generally target intraspecific, population-level variation (Avise 1998). Most phylogeographic studies of animals use mitochondrial (mt) DNA and plant studies use chloroplast (cp) and nuclear DNA (Beheregaray 2008). Phylogeographic studies of diatoms frequently use ITS regions (Créach *et al.* 2006; Godhe *et al.* 2006; Casteleyn et al. 2009).

As the evolution of an organism is the result of many gene genealogies, using only one genetic marker may provide misleading results (Hare 2001). Studies utilising several markers provide more reliable datasets and a clearer picture of a species' history (Avise 1998). In an ideal situation,

markers across multiple unlinked loci are used. Problems with using only one marker include not being able to identify interspecific hybridization, pseudogenes and natural selection (Bermingham & Moritz 1998). Another means of increasing confidence in datasets includes using large sample sizes from as many populations as practical, although the number of markers used affects results more than the number of individuals used (Medina *et al.* 2006). Greater numbers of markers and individuals are needed to assign organisms into reliable groups (cluster analyses) when genetic variation is great. Lower numbers of markers are required for determining relationships among isolated as opposed to panmictic populations (Medina *et al.* 2006).

1.5.1 Non-PCR-Based Methods

Allozymes – Before the advent of new sequencing methods, phylogenetic studies predominantly used non-PCR (polymerase chain reaction)-based techniques. Allozymes are co-dominant molecular markers that have been used since the 1960s. Allozyme studies investigate allelic differences in nuclear enzymes (Parker *et al.* 1998). These are identified by gel electrophoresis (isozymes), which separates proteins by size, shape and charge. Enzyme-specific staining enables visualisation of the allozyme positions as coloured bands. Homozygotes usually have one band, while multiple bands indicate heterozygosity (Avise 1994). While this method is fairly inexpensive and easy to use, allozymes often show insufficient variation for low-level genetic analyses, especially in species that have experienced genetic bottlenecks. Additionally, they may not be selectively neutral.

Restriction Fragment Length Polymorphisms (RFLPs) – RFLPs use restriction enzymes to cut DNA at recognition sites. Mutations in recognition sites cause creation of different length DNA fragments, which

can be visualised on a gel (Parker *et al.* 1998). These were the first markers used for population-level studies (Avise 1998). RFLPs were initially obtained from extracted genomic DNA and were hybridized with probes for visualization of desired bands (Avise 1994). Now they can be obtained from a PCR product, which is small enough to enable visualization of fragments without probes (Liu & Cordes 2004). While RFLPs do not always have great resolutionary power, they are useful co-dominant markers (Liu & Cordes 2004). An RFLP analysis of total cpDNA was used to investigate intraspecific and intrapopulational variation in the red algal genus *Ceramium* (Wattier *et al.* 2001).

1.5.2 PCR-Based Methods

PCR-based methods can target specific or anonymous DNA regions. Advantages are that only a small amount of DNA is required and degraded DNA can be used as a template if amplification targets a small region. DNA sequence variation provides the finest scale of resolution, supplying detailed information, such as small sized insertions and deletions that would not be large enough to detect using other techniques. Advantages of sequencing methods include ease of adding sequences to analyses at any time and obtaining large amounts of precise data from a short DNA region (Olmstead & Palmer 1994). Sequencing avoids variations being undetected due to having similar migration in gels or redundant genetic code (Parker *et al.* 1998). The biggest disadvantage is that single gene sequences may not represent evolutionary history accurately (Parker *et al.* 1998). PCR-based methods also often require prior knowledge and optimization conditions to achieve adequate results (Olmstead & Palmer 1994). Developing new primers for amplification and sequencing can become costly and time-consuming.

Markers of Anonymous DNA Regions

Random Amplified Polymorphic DNAs (RAPDs) – Some algal population studies (Lewis *et al.* 1997; Gabrielsen *et al.* 2002) have used dominant RAPD markers, which have demonstrated a high level of diversity within blooms and between populations. RAPD procedures use short primers (10 bp) of random sequences to amplify DNA (Williams *et al.* 1990; Parker *et al.* 1998). Polymorphism is seen as presence or absence of annealing sites for the primers or as indels between the sites. Advantages of RAPDs are that no prior knowledge of a target gene is required and only a small amount of DNA is needed for analysis (Godhe *et al.* 2006). However, these markers are not usually co-dominant and may produce artificial amplification products due to low annealing temperatures, reducing reproducibility (Liu & Cordes 2004).

Amplified fragment length polymorphisms (AFLPs) – AFLP techniques involve digestion of genomic DNA with restriction enzymes, followed by ligation of adaptors of known sequences to the ends of the DNA fragments. Fragments are then amplified by PCR. Polymorphisms occur when there are base substitutions at the PCR primer binding sites (as in RAPDs), at restriction sites or as indels between restriction sites (as in RFLPs) (Liu & Cordes 2004). AFLPs are dominant markers. Their advantages include high levels of polymorphism, no requirement of prior molecular knowledge, good reproducibility due to high annealing temperatures and they are relatively economic to run. Disadvantages include requiring special equipment or labelling techniques for electrophoretic analysis (Liu & Cordes 2004).

Microsatellite Markers – Variable number tandem repeats (VNTRs) of non-coding DNA units occur throughout the nuclear genome and include microsatellite sequences (2-6 bp per unit), also known as simple sequence repeats (SSRs) (Parker *et al.* 1998). After amplification, repeats of different sizes can be separated by gel electrophoresis. Co-dominant microsatellite markers provide the most sensitive population-level analytical tool (Godhe *et al.* 2006). Their level of variation makes them useful for paternity analyses and determining population genetic structure (Zhang & Hewitt 2003). Other advantages are their small size, genomic distribution, and high levels of polymorphism (Liu & Cordes 2004). However, microsatellite markers are often only useful for the species or very closely related species for which they were developed. They can be time-consuming and expensive to develop for new studies and universal primers can only be used if the flanking region containing primer sites is highly conserved (Parker *et al.* 1998). Microsatellite markers developed for *Ditylum brightwellii*, a centric diatom, revealed genetic diversity among individuals from a highly stratified water body (Rynearson & Armbrust 2004). Based on microsatellite data, the invasive seaweed *Codium fragile* ssp. *tomentosoides*, appears to have spread to the Mediterranean and the North Atlantic from two separate introductions (Provan *et al.* 2004).

Intersimple Sequence Repeats (ISSRs) – Primers made from SSRs are used to generate PCR products of anonymous SSR loci called ISSRs. Advantages of ISSRs are their high polymorphism and relatively simple, quick, economical use (Rakoczy-Trojanowska & Bolibok 2004). However, ISSRs are dominant markers. Bornet *et al.* (2005) developed sequence characterized amplified region (SCAR) markers using intersimple sequence repeats (ISSR) to determine unique polymorphic regions for toxic species of the diatoms *Pseudo-nitzschia pseudodelicatissima* and *Alexandrium catenella*. These were used to design primers for detecting these toxic species during monitoring.

Markers of Specific DNA Regions

Organeller Markers – After PCR amplification, these markers can involve analysis using RFLPs or sequencing. Organellar DNA occurs in the chloroplasts and mitochondria. This DNA is inherited cytoplasmically, usually by maternal transmission and can provide information on hybridization, introgression, and founder effects (Parker *et al.* 1998). The first phylogeographic studies used mtDNA (Avise 1998). This remains the preferred material for animal phylogeographic studies (Beheregaray 2008) because it is haploid, mtDNA in animals evolves rapidly, mtDNA genes are single copy and intraindividual mtDNA sequences are homogenous (Avise 1998; Hare 2001). mtDNA markers commonly used are cytochrome *b* (*cob*) and cytochrome oxidase subunit 1 (*cox1*).

Plant mitochondrial DNA evolves slowly, making it less suitable for population-level studies. Chloroplast DNA is highly conserved and often evolves too slowly to be used for intraspecific comparisons (Avise 1994). However, some regions have been used successfully, such as the tRNA intergenic region (*trn*T-*trn*L) (Taberlet *et al.* 1991) and the ribosomal protein gene cluster (*rps11-rps2*) in green algae (Provan *et al.* 2004). Mitochondrial and chloroplast markers are relatively unexplored in diatoms (Alverson 2008). However, they may prove useful in the future as it was found that *Cox1* shows intraspecific variation in some species of the diatom *Sellaphora* (Evans *et al.* 2007).

Nuclear Markers – Nuclear DNA markers provide a more complete picture of population histories than can be achieved using only uniparentally inherited organellar DNA (Hare 2001). Portions of the nuclear ribosomal RNA (nrRNA) gene cluster (Fig. 1.8) are the most widely sequenced DNA marker in diatoms (Alverson 2008). rRNA gene numbers vary in unicellular algae from 1-1000 copies per cell (Créach *et al.* 2006). The 5.8S and small subunit (SSU) rRNA 18S genes are most conserved

and are useful for interspecific comparisons (Beszteri *et al.* 2001). The large subunit (LSU) 28S rDNA followed by the internal transcribed spacer (ITS) regions are least conserved and are often useful for intraspecific comparisons (Alverson 2008). This level of variation is species-dependent, as some species have identical ITS regions over a wide geographic range while others may show variation within a population or over small spatial scales (Godhe *et al.* 2006). In some species, such as the diatom *Sellaphora pupula*, the ITS region may be so divergent that only very closely related demes can be aligned (Mann 1999).

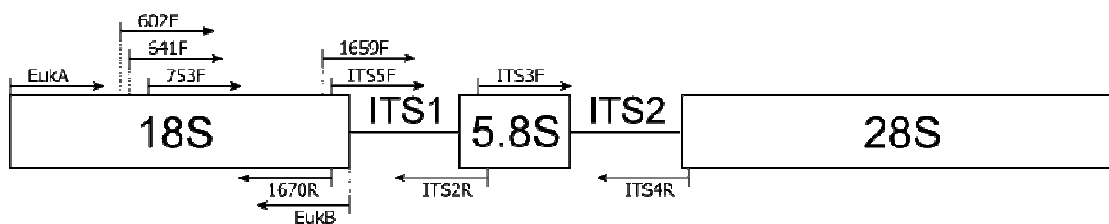


Figure 1.9: Diagram of the nuclear ribosomal DNA gene region, including the 18S, 5.8S and 28S genes, the ITS1 and ITS2 regions and primers used in this study.

1.5.3 ITS Studies

As this project focuses on the ITS region of *D. geminata,* this section describes the ITS region in more detail and illustrates some previous ITS investigations in diatoms. ITS regions occur hundreds to thousands of times throughout the genome and are under concerted evolution (Behnke *et al.* 2004). The high copy number of the rRNA operon is probably to meet transcriptional requirements (Alverson & Kolnick 2005). Within the rRNA operon, ITS1 occurs between the 18S gene and the 5.8S gene and ITS2 between the 5.8S gene and the 28S gene (Fig. 1.8). Behnke *et al.* (2004) found that major variations among species occurred in the stem regions of helices in secondary structures, while deletions occurred in loop regions. ITS transcripts are thought to be important in rRNA maturation, but are not incorporated into mature ribosomes. It is therefore likely that

they are under some evolutionary constraint, although less than the ribosomal subunit regions. Some characters may be involved in non-independent evolution, as base pairing in the secondary structure may result in compensatory mutations. Sites directly opposite mutations in the stem structure may need to mutate to maintain structural integrity and functionality (Baldwin *et al.* 1995).

Advantages of the ITS marker include: 1) its small size, facilitating PCR and sequencing, 2) its high variability, which is often enough for population-level studies, 3) its conserved flanking rRNA genes, providing sites for primers, and 4) point mutations rather than large length polymorphisms (Zechman *et al.* 1994). The main disadvantage is that intraindividual polymorphism can occur, affecting phylogenies. Multiple copies of the rRNA operon in the genome mean that non-functional ITS paralogues can exist. Concerted evolution should homogenize intraindividual variations in interbreeding populations. Non-homogenization can occur if there has been a recent hybridization event or if a hybrid is asexual. Chimeric repeat-types can also occur (Baldwin *et al.* 1995). There were 0.57-1.81% intragenomic polymorphic sites in the 18S rDNA gene of *Skeletonema* diatom strains, suggesting that the mutation or speciation rate is exceeding that of concerted evolution or that concerted evolution has not yet proceeded to completion (Alverson & Kolnick 2005). This intragenomic variation is comparable to that obtained from whole genome sequencing projects (e.g. the diatom *Thalassiosira pseudonana* displays 0.75% polymorphism and no chimeric DNA sequences).

ITS variation is not uniform within the diatoms. Zechman *et al.* (1994) conducted the first ITS analysis of diatoms by investigating the level at which this marker could be useful in resolving phylogenetic relationships. They found that the ITS region could be used at the species-level in the centric diatom genus *Stephanodiscus*, but only at the family level for Thalassiosiraceae. In the *Sellaphora pupula* species complex, Behnke et al. (2004) found demes that were not interfertile had such divergent ITS sequences that alignment was difficult, while those that were interfertile had only slight sequence diversity. While there was some intraindividual

variation, this was less than the interindividual variation and did not affect phylogenies.

QPCR probes targeting ITS1 were developed to determine the distribution of a cosmopolitan benthic diatom, *Navicula phyllepta* (Créach *et al.* 2006) and to detect extremely low levels of the invasive *D. geminata* in New Zealand rivers (Cary *et al.* 2007). Orsini *et al.* (2004) found that different ITS types of the diatom *Pseudo-nitzschia delicatissima* in the Gulf of Naples can occur in the water column at the same time and these may represent reproductively isolated species.

1.6 Conclusion

With invasive aquatic plants causing losses of $US 100 million annually, 20-30% of exotics becoming invasive and most eradication attempts failing (Pimentel *et al.* 2001), better measures of understanding and preventing invasions are needed. Limited data on invasive aquatic species (Provan *et al.* 2005) hinders their control and management. Population genetics, along with other population-level studies, are vital for biosecurity and conservation (Corin *et al.* 2007). Phylogeography can uncover pathways of spread and levels of variation in introduced populations. Invasions will become increasingly important in New Zealand waterways as humans continue to increase dispersal of exotic species, such as *D. geminata*. Understanding genetic variation in *D. geminata,* including its likely source and number of introductions, should assist in informed decision making regarding its control and management.

CHAPTER 2: RESEARCH PAPER

The origin, genetic diversity and taxonomy of the invasive diatom *Didymosphenia geminata* (Bacillariophyceae) in New Zealand

2.1 Abstract

Didymosphenia geminata is an invasive freshwater diatom that has become highly invasive over the last few decades. It forms massive blooms that completely cover the riverbed substrate, causing shifts in community structure. Although historically associated with high altitude, oligotrophic waters, it is now showing increased ecological tolerance, being found in warmer, mesotrophic waters. It has been increasing in abundance and range in Europe and North America and has been introduced to New Zealand, Iran and India. New Zealand is the only Southern Hemisphere location where it is found. However, the origin and genetic diversity of New Zealand's *D. geminata* was unknown. This study investigates the phylogeographic history of *D. geminata* in New Zealand using the small nuclear ribosomal DNA subunit 18S and internal transcribed spacer (ITS) region. Additionally, as there have been no molecular taxonomic investigations of the genus *Didymosphenia*, the 18S region was used to determine the placement of *D. geminata* within the pennate diatoms (Bacillariophyceae). The taxonomic analysis suggests that *D. geminata* belongs to the Family Cymbellaceae, which may be paraphyletic. Phylogeographic results suggest that there have been several introductions of *D. geminata* from Europe to North America, and then from North America to New Zealand. This has implications for control and management of this invasive species.

2.2 Introduction

Invasive species are one of the greatest threats to biodiversity (Provan *et al.* 2005) because they induce widespread ecosystem changes, disrupt established communities and alter habitats (Strayer *et al.* 2006). In particular, anthropogenic activities are responsible for an increased rate and distance of dispersal of invasive species in recent times (Coleman 1996). The rate of invasion of aquatic species is rapidly rising (Roman & Darling 2007). Although there are several well-known aquatic invaders, such as the zebra mussel (*Dreissena polymorpha*), water flea (*Daphnia lumholtzi*), water hyacinth (*Eichhornia crassipes*) and seaweed (*Codium fragile*), much less is known about diatom invaders, which are rarely documented.

Diatom dispersal occurs through water currents and potentially by animal, bird, wind or human assistance (Vanormelingen *et al.* 2008). Human dispersal occurs via ship ballast water, aquarium releases, aquaculture (e.g. shellfish transplantations) and recreation such as fishing (Roman & Darling 2007; Vanormelingen *et al.* 2008). Several diatoms are known to have invaded the Great Lakes through transportation in ships' ballast water (Mills *et al.* 1994). Furthermore, while some diatoms appear to be native to a particular area, they may have only invaded the area following European arrival. For example, *Asterionella formosa* is a diatom that appears in sediment records only after European arrival in New Zealand and is now widespread throughout New Zealand lakes (Vanormelingen *et al.* 2008). Another example of an invasive diatom is *Coscinodiscus wailesii,* a marine diatom that was first reported in the English Channel in 1977 and has since spread to and persisted in the North Sea (Edwards *et al.* 2001). The findings of unique diatom assemblages in isolated areas highlight the importance of conservation in these areas and the need to protect them from exotic species invasions (Vanormelingen *et al.* 2008).

The freshwater alga, *Didymosphenia geminata* (Lyngbye) M. Schmidt (Bacillariophyceae, Cymbellaceae) is an invasive freshwater diatom that has received much publicity in recent years due to its widespread, often devastating effects on river ecosystems (Larned *et al.* 2007). It is a large, asymmetrical, biraphid diatom. It attaches strongly to benthic rocks and plants in rivers, streams and lakeshores by its long mucilaginous stalks (Kilroy 2004). Historically, *D. geminata* was associated with cool, oligotrophic waters (Kociolek & Spaulding 2003) in boreal, montane or alpine locations (Kawecka & Sanecki 2003) in Europe, Asia and North America (Patrick & Reimer 1975; Li *et al.* 2003; Flower *et al.* 2004; Beltrami *et al.* 2008). Ice and sediment cores from Siberia, Finland and northern North America (Stoermer 1993; Sarmaja-Korjonen & Alhonen 1999; Polyakova & Stein 2004; Miettinen *et al.* 2005) suggest that *D. geminata* is native to these areas, where it has been present for the past 8000-120 000 years.

Currently, there is disagreement over the familial placement of *D. geminata.* Dawson (1973a) and Round *et al.* (1990) placed *D. geminata* in the Family Gomphonemataceae, based on traditional frustule morphology. Other genera in Gomphonemataceae include *Gomphoneis, Gomphonema*, *Gomphopleura* and *Reimeria* (Round *et al.* 1990). Kociolek & Stoermer (1988) and Moffat (1994) place *D. geminata* in the Family Cymbellaceae, based on the arrangement and structures of cytological contents and because the distal raphe does not dissect the apical pore field as in members of Gomphonemataceae. Other genera in Cymbellaceae include *Brebissonia, Cymbella, Encyonema*, *Gomphocymbella* and *Placoneis*. Currently, ten extant and one fossil *Didymosphenia* species are recognised (Mrozińska *et al.* 2006). There have been no genetic taxonomic investigations within the genus *Didymosphenia*.

Blooms of *D. geminata* are now occurring at lower altitudes in warmer, nutrient-rich rivers, most significantly those with stable, controlled flows below dams or reservoirs (Gágyorová & Marvan 2002; Kawecka & Sanecki 2003; Kolmakov *et al.* 2008). It frequently forms mats that completely cover all available substrates. Blooms clog water filters in

supply systems (Kawecka & Sanecki 2003) and cause major shifts in species compositions (Larned *et al.* 2007). The reason for the sudden spread of *D. geminata* is currently unknown. The paucity of studies investigating its ecology in the past makes it difficult to determine how much its range and ecological tolerance has changed in recent times. However, blooms are increasing in severity and abundance across Europe and North America (Kawecka & Sanecki 2003; Şahin 2003; Kirkwood *et al.* 2007). It has now been sighted in many locations in the USA (Idaho, Utah, Virginia, Tennessee, Montana, California, South Dakota, Arkansas, Alaska and Colorado) and Canada (Edwardson *et al.* 2003; Opsahl *et al.* 2003; Oberholster *et al.* 2005; Spaulding & Elwell 2007; Kirkwood *et al.* 2007). In Europe, *D. geminata* is now found in many countries including Norway, UK, Poland, Turkey, and Switzerland and is widespread throughout the Danube Basin (Antoine & Benson-Evans 1984; Kawecka & Sanecki 2003; Şahin 2003; Robinson & Kawecka 2005; Szabo *et al.* 2005; Beltrami *et al.* 2008; Lindstrøm & Skulberg 2008).

D. geminata has also recently been reported for the first time in several locations, such as India (Bhatt *et al.* 2005; Bhatt *et al.* 2008), Iran (Alimohammadian 2008) and New Zealand (Kilroy 2004). At present, the South Island of New Zealand is the only location in the Southern Hemisphere where *D. geminata* has been found. It was initially discovered in the lower Waiau and Mararoa Rivers in Southland in 2004 and then in 2005, it was found in the Buller River in Nelson and in several rivers in the Otago region. It has now spread to over 50 rivers throughout all regions of the South Island (Larned *et al.* 2007). The source of these introductions is unknown. This invasion offers opportunities to study the spread and genetic changes of this species over a relatively short time scale.

Phylogeography has become an important means of determining the geographic pattern of spread of an invasive species, enabling determination of source populations, vectors, pathways of spread and levels of variation in introduced populations (Avise 1998; Suarez & Tsutsui 2008). This information can assist in management and control of invasive species (Corin *et al.* 2007). Phylogeographic studies have been conducted

on several invasive aquatic species, such as the highly invasive marine green alga *Codium fragile* subspecies *tomentosoides* (Verbruggen *et al.* 2007). Within the diatoms, phylogeographic studies include *Ditylum brightwellii*, *Navicula phyllepta* and *Skeletonema marinoi* Sarno et Zingone (Rynearson & Armbrust 2000; Créach *et al.* 2006; Godhe *et al.* 2006). These diatom studies used microsatellites, the internal transcribed spacer region 1 (ITS1) and the nuclear ribosomal DNA (nrDNA) large subunit (LSU) and ITS regions respectively. The ITS regions in the nrDNA gene cluster are frequently used for phylogeographic population studies (Zechman *et al.* 1994; Hung *et al.* 2004; Créach *et al.* 2006). They have also been used in assessing the intraspecific genetic variation in blooms of the toxic diatom species *Pseudo-nitzschia delicatissima* (Orsini *et al.* 2004). The level of variation of the ITS region is species-dependent (Godhe *et al.* 2006), enabling species-level comparisons for some species but only family-level comparisons for others.

The purpose of this study was to investigate the geographical origin of *D. geminata* in New Zealand using the intraspecific variation in the ITS region. A phylogeographic analysis was conducted using the ITS region of *D. geminata* samples from New Zealand, Europe (Iceland, Norway, Poland, UK), the Middle East (Iran) and North America (Canada, USA). The analysis of ITS sequences suggests that the invasive history of *D. geminata* has included multiple introductions worldwide. Additionally, a phylogenetic analysis using the partial 18S region was conducted to investigate the taxonomic placement of *D. geminata* among the pennate diatoms. Results suggest *D. geminata* belongs to the pennate diatom Family Cymbellaceae.

2.3 Materials and Methods

Collection and identification of environmental samples

37 *D. geminata* samples were collected from 27 rivers worldwide. (Fig. 2.1; Table 2.1). Samples were stored in 70% ethanol and sent to the University of Waikato, New Zealand, in response to a request by Biosecurity New Zealand in May 2006. More intensive sampling was carried out in New Zealand (Fig. 2.2; Table 2.1), where benthic or water column samples were collected from four South Island rivers. Benthic samples were collected by swabbing a rock surface as described by Cary *et al.* (2006). Water column samples were collected using a drift net specifically designed by Cary *et al.* (2006) for sampling the water column for *D. geminata* (Fig. A.1; Appendix 2). In brief, the drift net was deployed in the upper half of the water column for 10 minutes. The sampling unit was removed from the net and inverted into a 110 mL Elkay sampling container. 2 mL of the concentrated suspended material was placed into a 14 mL graduated falcon tube and ethanol was added to a final concentration of 70%. All samples were frozen at -20 ℃ on arrival i n the laboratory.

Positive identification of *D. geminata* in all samples was performed first by microscopy using an Olympus System Microscope BX51 and later verified using a PCR-based genetic detection method developed by Cary *et al.* (2007). Photographs of cells (Fig. 2.4) were taken using a Nikon Digital Sight DS-U1 camera managed by Image-Pro Plus software.

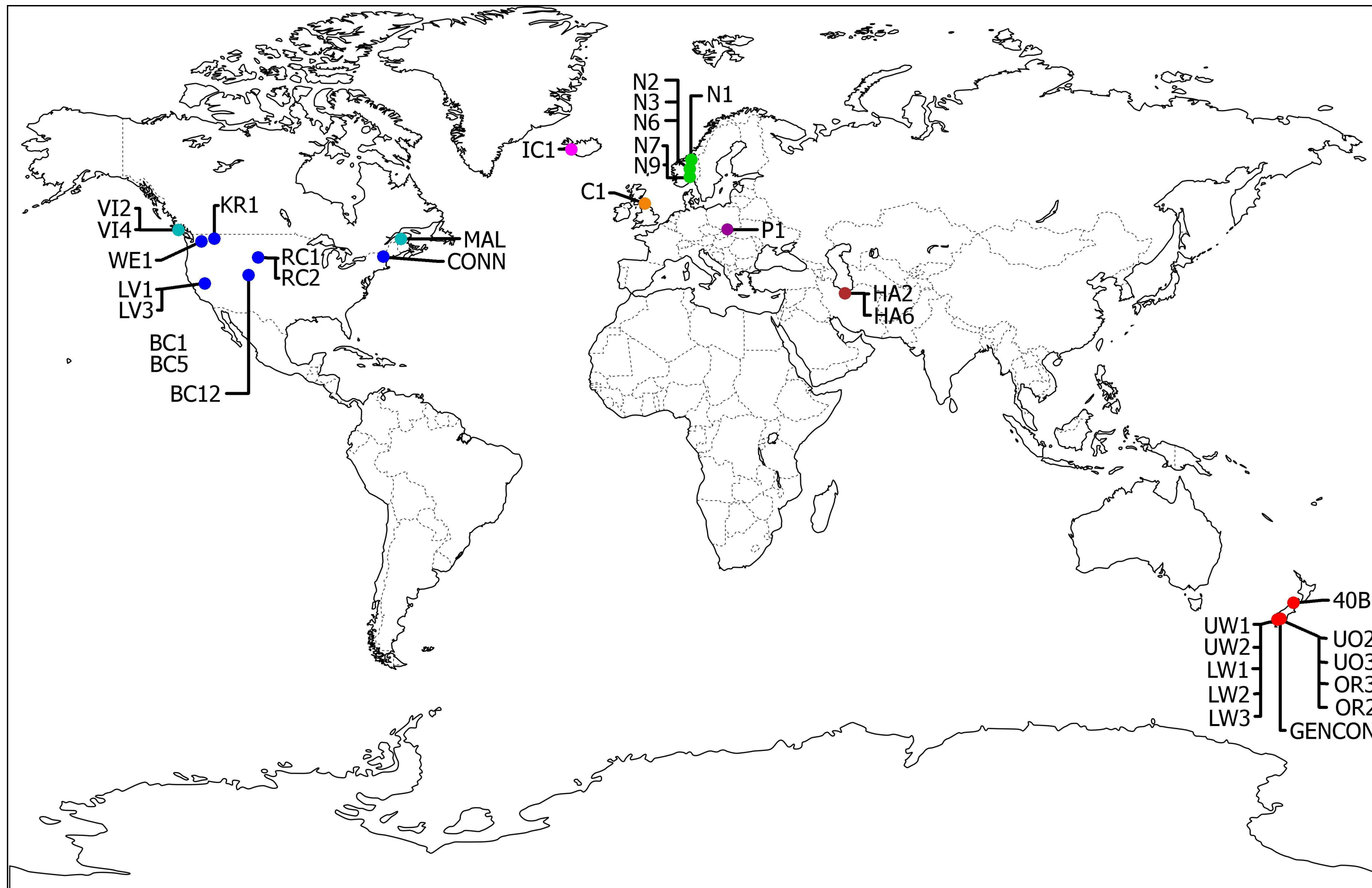


Figure 2.1 (10): World map of sample locations used in this study. 37 *D. geminata* samples were collected from 8 countries worldwide.

Figure 2.2 (11): Map of New Zealand showing rivers infected with *D. geminata* (red) and sites of samples used in this project. Information on affected rivers was obtained from the Didymo Samples Database (MAF Biosecurity New Zealand 2009). Map sourced from Topographic Map 265-2 South Island. Crown Copyright Reserved.

Table 2.1: Sample locations and amplification primers used in this study.

Country	Region	Water Body	Sample Code	Northing	Easting	Forward Primers
Canada	British Columbia	Vancouver Island	VI2	49°40 ' 48.5300" N	125°03' 57.2800" W	641F
	British Columbia	Vancouver Island	VI4	49°18' 18.5 800" N	124°33' 12.9300" W	641F
	Quebec	Matapedia River	MAL	47°58' 23.4100" N	66° 56' 54.2300" W	EukA/602F
Iceland	Leirarsveit	Laxa River	IC1	64°25' 54.1800" N	21°37' 52.9800" W	1659F
Iran	Tehran	Havir River	HA6	35°41' 05.6000" N	52° 26' 59.0000" E	602F
	Tehran	Tar River	HA2	35°43' 33.4000" N	52°10' 03 .5000" E	602F
New Zealand	Southland	Lower Waiau River	LW1	39°12' 38.1884" S	195°25' 03.7147" E	602F
	Southland	Lower Waiau River	LW2	39°14' 12.3971" S	195°24' 22.6520" E	602F
	Southland	Lower Waiau River	LW3	39°16' 54.9321" S	195°23' 27.9031" E	602F
	Southland	Mararoa River	GENCON	38°59' 46.8872" S	195°40' 30.8929" E	602F
	Southland	Oreti River	OR2	38°56' 41.4501" S	195° 43' 59.0750" E	602F
	Southland	Oreti River	OR3	39°01' 47.5899" S	195° 46' 14.2710" E	602F
	Southland	Upper Oreti River	UO2	39°01' 49.3080" S	195°46' 17.8308" E	602F
	Southland	Upper Oreti River	UO3	38°49' 35.4074" S	195°45' 41.1825" E	602F
	Southland	Upper Waiau River	UW1	39°02' 33.7812" S	195°22' 08.4767" E	602F
	Southland	Upper Waiau River	UW2	39°05' 32.4001" S	195°21' 17.4957" E	602F
	West Coast	Buller River	40B	34°41' 01.7659" S	198 °27' 32.9863" E	602F
Norway	Dokka sentrum	Dokka	N7	60°50' 17.8700" N	10 °03' 38.5800" E	EukA/1659F
	Isielva, Bjørum sag	Sandviksvassdraget	N9	59°56' 26.3800" N	10°25' 33.6600" E	1659F
	Setninga	Atna	N2	61°47' 26.2700" N	10°29' 24.930 0" E	602F

Country	Region	Water Body	Sample Code	Northing	Easting	Forward Primers
	Tisleia	Tisleia	N6	60°52' 09.3200" N	9°12' 59.83 00" E	EukA/602F
	Trondheim	Nidelva	N1	63°23' 45.8900" N	10°23' 08 .0300" E	EukA/753F
	Vinstra sentrum	Vinstra	N3	61°35' 07.9700" N	9°4 2' 46.9900" E	1659F
Poland	Southern Poland	Dunajec River	P1	49°27' 0 0.0000" N	20°20' 00.0000" E	EukA/1659F
UK	Northumberland	River Coquet	C1	55°20' 15.1100" N	1°53' 54.0100" W	602F
USA	-	Prosser Creek	PRS	N/A	N/A	EukA/1659F
	-	Virginia Lake Creek	VIR	N/A	N/A	1659F
	California	Lee Vining Creek	LV1	37°57' 45.0000" N	119°16' 31.0000" W	602F
	California	Lee Vining Creek	LV3	37°57' 34.7200" N	119°16' 30.5700" W	641F
	Colorado	South Boulder Creek	BC12	39°55' 27.0000" N	105°16' 35.0000" W	641F
	Montana	Boulder Creek	BC1	N/A	N/A	602F
	Montana	Boulder Creek	BC5	N/A	N/A	602F
	Montana	Kootenai River	KR1	48°21' 57.4400" N	115° 19' 22.3700" W	EukA/1659F
	Vermont/New Hampshire	Conneticut River	CONN	44°36' 45.4000" N	71°33' 16.5400" W	641F
	South Dakota	Rapid Creek	RC1	44°04' 30.0000" N	10 3°29' 30.0000" W	602F
	South Dakota	Rapid Creek	RC2	44°03' 18.5700" N	10 3°24' 15.7000" W	EukA/1659F
	Washington	Wenatchee River	WE1	47°40' 35.0400" N	120°43' 58.0800" W	1659F

DNA extraction

1 mL of suspended sample was centrifuged and the supernatant removed. The pellet was washed once in 1 mL H_2O and re-centrifuged to remove residual ethanol. Total genomic DNA was extracted using the PureLink Genomic DNA Kit as per the manufacturer's instructions (Invitrogen). Samples with problematic extractions were extracted using a more robust protocol. Briefly, material was prepared for extraction as above, followed by lysis in the presence of 350 µL lysis buffer (final concentrations: 700 mM Tris-HCl, pH 9; 1% sodium dodecyl sulphate; 50 mM EDTA, pH 8; 100 mM NaCl) and 10 µL of 60 µg μL^{-1} proteinase K at 55 ℃ for 2 hrs and then at 37 ℃ overnight. Purification of DNA from the ly sate was performed by adding 5 M LiCl:chloroform (1:2) for 45 min. DNA was precipitated with an equal volume of isopropanol at room temperature and centrifuged at 16.1 × *g* for 10 min. The pellets were air dried and re-suspended in 10 µL H_2O. Samples extracted by this method were BC12, HA2, HA6, KR1, N1, N7, N9, PRS, RC2 (Table A.5; Appendix 4). The quality and quantity of all DNA extracts were assessed by gel electrophoresis on 1-1.5% TAE agarose gels and using a NanoDrop, Spectrophotometer ND1000, respectively. Gels were stained with ethidium bromide and then visualised using an Alpha Imager (Alpha Innotech Corporation).

PCR, cloning and sequencing

A portion of the 18S rRNA gene and the full ITS1-5.8S-ITS2 (ITS) region (Fig. 2.3) were amplified using *D. geminata*-specific forward primers (602F, 641F, 753F, 1659F) and the universal ITS4 reverse primer (Table 2.2). Some samples (Table 2.2) were amplified by nested PCR, using EukA-ITS4 primers followed by *D. geminata*-specific-ITS4 primers. PCR was carried out on a Peltier Thermal Cycler PTC-200 (BioRad) or an Eppendorf Mastercycler ep. 50 µL reactions contained final concentrations of 1 x PCR buffer-$MgCl_2$, 2.5 mM $MgCl_2$, 0.1 mM dNTP, 0.04 µg μL^{-1} bovine serum albumin (BSA), 0.2 µM of each primer, 0.028 U Platinum *Taq* DNA Polymerase (Invitrogen, New Zealand) and 0.1 to 10 ng of template DNA.

Cycling conditions were as follows: 94 ℃ for 2 min , 40 cycles of 94 ℃ for 30 s, 60 ℃ for 30 s, 72 ℃ for 55 s-120 s (Table 2 .4) and a final step at 72 ℃ for 5-30 min.

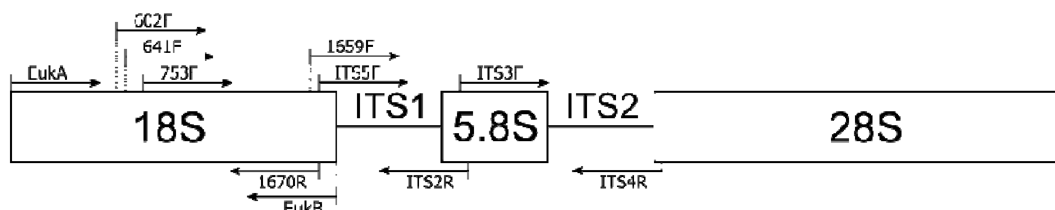


Figure 2.3 (12): rDNA operon showing the 18S, 5.8S and 28S genes and the ITS1 and ITS2 regions. Arrows illustrate the positions and directions of primers used in this study.

Table 2.2: Primer sequences used in PCR amplification and sequencing. *D. geminata* specific primers include 602F, 641F, 753F, 1659F and 1670R. Seq=Sequencing.

Primer Name	Primer Use	Sequence (5'-3')	Reference
EukA	PCR	AACCTGGTTGATCCTGCCAGT	Medlin *et al.* (1988)
EukB	PCR/Seq	GATCCATCTGCAGGTTCACCTAC	Medlin *et al.* (1988)
602F	PCR/Seq	GTTGGATTTGTGATGGAATTTGAA	Cary *et al.* (2007)
641F	PCR/Seq	TCAGAAACTGTCATCCGTGGGTG	Cary *et al.* (2007)
753F	PCR/Seq	GACTTACGTCGATGAATGTATT	Cary *et al.* (2007)
1659F	PCR/Seq	GCTGGGGATTGCAGCTA	Cary *et al.* (2007)
1670R	PCR/Seq	CACCAGTAAAGGCATTAGCTG	Cary *et al.* (2007)
ITS2	Seq	GCTGCGTTCTTCATCGATGC	White *et al.* (1990)
ITS3	Seq	GCATCGATGAAGAACGCAGC	White *et al.* (1990)
ITS4	PCR/Seq	TCCTCCGCTTATTGATATGC	White *et al.* (1990)
ITS5	Seq	GGAAGTAAAAGTCGTAACAAGG	White *et al.* (1990)
M13F	Seq	GTAAAACGACGGCCAG	Invitrogen (2004)
M13R	Seq	CAGGAAACAGCTATGAC	Invitrogen (2004)
T7	Seq	TAATACGACTCACTATAGGG	Invitrogen (2004)
T3	Seq	ATTAACCCTCACTAAAGGGA	Invitrogen (2004)

The PCR products were either sequenced directly or cloned and then sequenced. All PCR products were either cleaned using ExoSAP-IT (USB

Corporation, USA) prior to sequencing directly or were cloned immediately following PCR. Samples that produced multiple PCR products (40B, BC1, BC12, CONN, GENCON, KR1, LV3, MAL, N1, N6, PRS, RC2, VI4) were gel purified using the freeze and squeeze method (Thuring *et al.* 1975) before cloning (Table A.4; Appendix4). Briefly, the desired PCR products (Table 2.4) were excised from a 0.7% agarose gel under UV light following electrophoresis, wrapped in parafilm and frozen at -20 °C. DNA extraction from the gel was as follows. Exudate was collected from the gel into 1.5 mL microcentrifuge tubes by applying firm, constant pressure to the wrapped, frozen bands. DNA was then extracted from the exudate with phenol:chloroform (1:1), followed by two extractions with chloroform. DNA was precipitated with 0.1 volumes of 3 M sodium acetate and 0.6 volumes of isopropanol at -20 °C overnight. DNA was collected at 4 °C for 30 minutes at 16.1 × *g* with a bench top centrifuge (Eppendorf 5415R, Germany). DNA pellets were washed once with 70% ethanol chilled at -20 °C, air-dried and re-suspended in 10 µL H_2O.

PCR products were cloned using a TOPO TA cloning kit (Invitrogen, New Zealand) according to the manufacturer's instructions at half reaction volumes. Plasmids were extracted from successful transformants using the Quickclean 5M Miniprep Kit (GenScript, USA). Sequencing was performed by the Waikato DNA Sequencing Facility using BigDye Terminator v3.1 Cycle Sequencing (Applied Biosystems) and a 3130xl Genetic Analyzer (Applied Biosystems). Plasmid based primers M13F and M13R or T3 and T7 (TOPO TA cloning kit, Invitrogen, New Zealand) were used for sequencing cloned samples and the original set of PCR primers were used for sequencing non-cloned samples. Internal primers ITS2, ITS3, ITS5, EukB, 1659F and 1670R (Table 2.2, Fig. 2.3) were used to gain the internal sequence for long regions. All ITS1-5.8S-ITS2 regions were bidirectionally sequenced for each sample. Sequence cycling was as follows: 96 °C for 60 sec; 35 cycles of 96 °C for 10 sec, 50 °C for 10 sec, 60 °C for 180 sec; and a final step of 72 °C for 10 min.

Phylogenetic Analysis

Sequences were edited and aligned with Clustal W (Chenna *et al.* 2003) using the Geneious Pro v.4.5 software package (Biomatters Ltd., New Zealand). A blastn search (Altschul *et al.* 1997) using *D. geminata* ITS and 18S rDNA sequences was performed to obtain similar sequences of taxa from GenBank (Benson *et al.* 2008). Four alignments were constructed for analyses at varying hierarchical levels.

Taxonomic Investigation: A partial 18S alignment (Table A.7) was generated to test the phylogenetic placement of *D. geminata* at family level. This alignment was made of partial 18S rDNA sequences from seven *D. geminata* samples, 50 Bacillariaceae (raphid pennate diatom) species from GenBank (Accession Nos: Table 2.3) and one other Bacillariaceae species, *Gomphoneis minuta* var. *cassiae* (Cary *et al.* 2006). Three Fragilariaceae (araphid pennate diatom) species *Asterionella formosa, Fragilaria delicatissima,* and *Synedra fragilaroides* (Accession Nos: Table 2.3) from GenBank were included to form the outgroup.

Phylogeographic Investigation: An alignment at the species level contained all 29 *D. geminata* sequences for which the entire partial 18S-ITS2 region was obtained and included two outgroup taxa: *Cymbella proxima* (Accession No. AM502017) and *Gomphoneis minuta* var. *cassiae* (Cary *et al.* 2006). At the intraspecific level, an alignment was constructed consisting of the ITS region for 46 *D. geminata* sequences. A final alignment at population level consisted of the ITS region from 7 colonies of the clone library from the New Zealand Buller River (40B) PCR product.

Phylogenetic trees were constructed for each of the four alignments above. Maximum Parsimony (MP) trees were constructed in PAUP* (version 4.0b10) (Swofford, 2003; Sinauer Associates) using a fast heuristic search and a starting tree obtained by stepwise addition with random sequence addition. Bootstrap resampling was performed using 1000 replicates. Model Test 3.7 (Posada & Crandall 1998) was used to select the most appropriate substitution model prior to building Maximum Likelihood (ML) trees, which were then built using PhyML (Guindon & Gascuel 2003)

within Geneious Pro v 4.5. (Biomatters Ltd., New Zealand) Bootstrap resampling was performed using 100 replicates.

Table 2.3: GenBank accession numbers of species used in the 18S diatom alignment.

Order	Family	Species	Accession No.
Achnanthales	Achnanthaceae	*Achnanthes brevipes*	AY485476
		Achnanthes minutissima	AJ866992
		Achnanthes sp. CCAP1001/1	AY485496
	Achnanthidiaceae	*Achnanthidium cf. longipes*	AY485500
		Achnanthidium minutissimum	AM502032
	Cocconeidaceae	*Cocconeis pediculus*	AM502010
		Cocconeis placentula	AM502013
Bacillariales	Bacillariaceae	*Cylindrotheca closterium*	DQ178394
		Nitzschia closterium f. minutissima	EF553459
		Pseudo-nitzschia multiseries	NPU18241
		Pseudo-nitzschia pungens	NPU18240
Cymbellales	Anomoeoneidaceae	*Anomoeoneis sphaerophora*	AJ535153
	Cymbellaceae	*Cymbopleura naviculiformis*	AM502004
		Cymbella affinis	AM502018
		Cymbella aspera	AM502016
		Cymbella cymbiformis	AJ535156
		Cymbella lanceolata	AM502026
Cymbellales	Cymbellaceae	*Cymbella proxima*	AM502017
		Cymbella proxima	AM502017
		Encyonema caespitosum	AM502035
		Encyonema minutum	AM501961
		Encyonema triangulatum	AJ535157

Order	Family	Species	Accession No.
		Placoneis elginensis	AM501953
		Placoneis hambergii	AM502030
		Placoneis sp. AT-220.09	AM502014
	Gomphonemataceae	*Gomphoneis minuta* var. *cassiae*	Cary *et al.* 2006
		Gomphonema acuminatum	AM502019
		Gomphonema affine	AM502033
		Gomphonema cf. angustatum	AM502005
		Gomphonema cf. parvulum	AM501995
		Gomphonema micropus	AM501964
		Gomphonema parvulum	AJ243062
		Gomphonema productum	AM501993
		Gomphonema pseudaugur	AB085833
		Gomphonema truncatum	AM501956
Eunotiales	Eunotiaceae	*Eunotia formica*	AM502040
Fragilariales	Fragilariaceae	*Asterionella formosa*	AM712617
		Fragilaria delicatissima	AM497721
		Synedra fragilaroides	EF193001
Lyrellales	Lyrellaceae	*Lyrella atlantica*	AJ544659
Naviculales	Amphipleuraecae	*Amphiprora alata*	AY485497
		Frustulia vulgaris	AM502038
Naviculales	Diadesmidaceae	*Diadesmis gallica*	AJ867023
	Naviculaceae	*Eolimna subminuscula*	AJ243064
		Navicula lanceolata	AY485484
		Pseudogomphonema sp. p382	AJ535152
	Neidiaceae	*Neidium affine*	AM501955
	Phaeodactylaceae	*Phaeodactylum tricornutum*	EF553458

Order	Family	Species	Accession No.
	Pinnulariaceae	*Pinnularia subcapitata*	AM501979
	Sellaphoraceae	*Sellaphora laevissima*	AJ544656
		Sellaphora pupula	AJ544651
	Stauroneidaceae	*Craticula molestiformis*	AM501977
		Stauroneis anceps	AM502008
Surirellales	Entomoneidaceae	*Entomoneis cf. alata*	AJ535160
		Entomoneis sp. CCMP1693	EF585586
	Surirellaceae	*Cymatopleura elliptica*	AJ867030
		Surirella brebissoni	AJ867029
		Surirella fastuosa var	AJ535161
Thalassiophysales	Catenulaceae	*Amphora montana*	AJ243061
		Amphora pediculus	AM501960

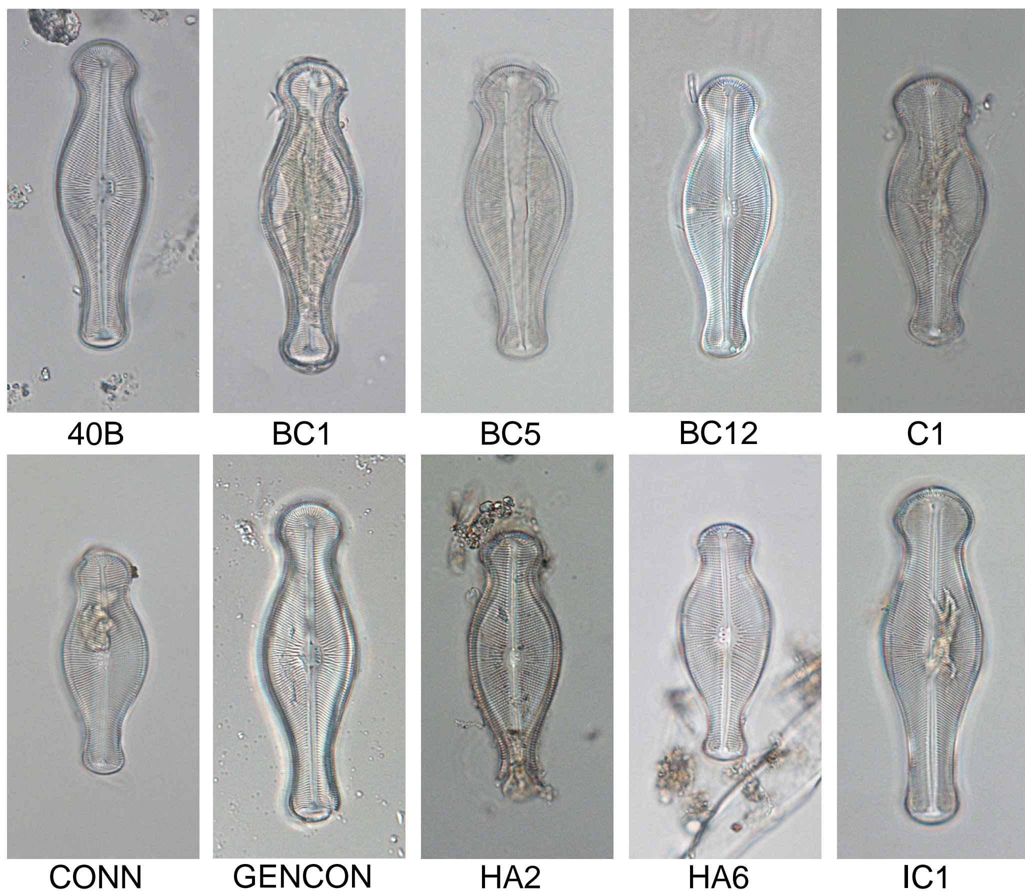
Table 2.4: PCR extension times and sizes of amplification products for primer sets.

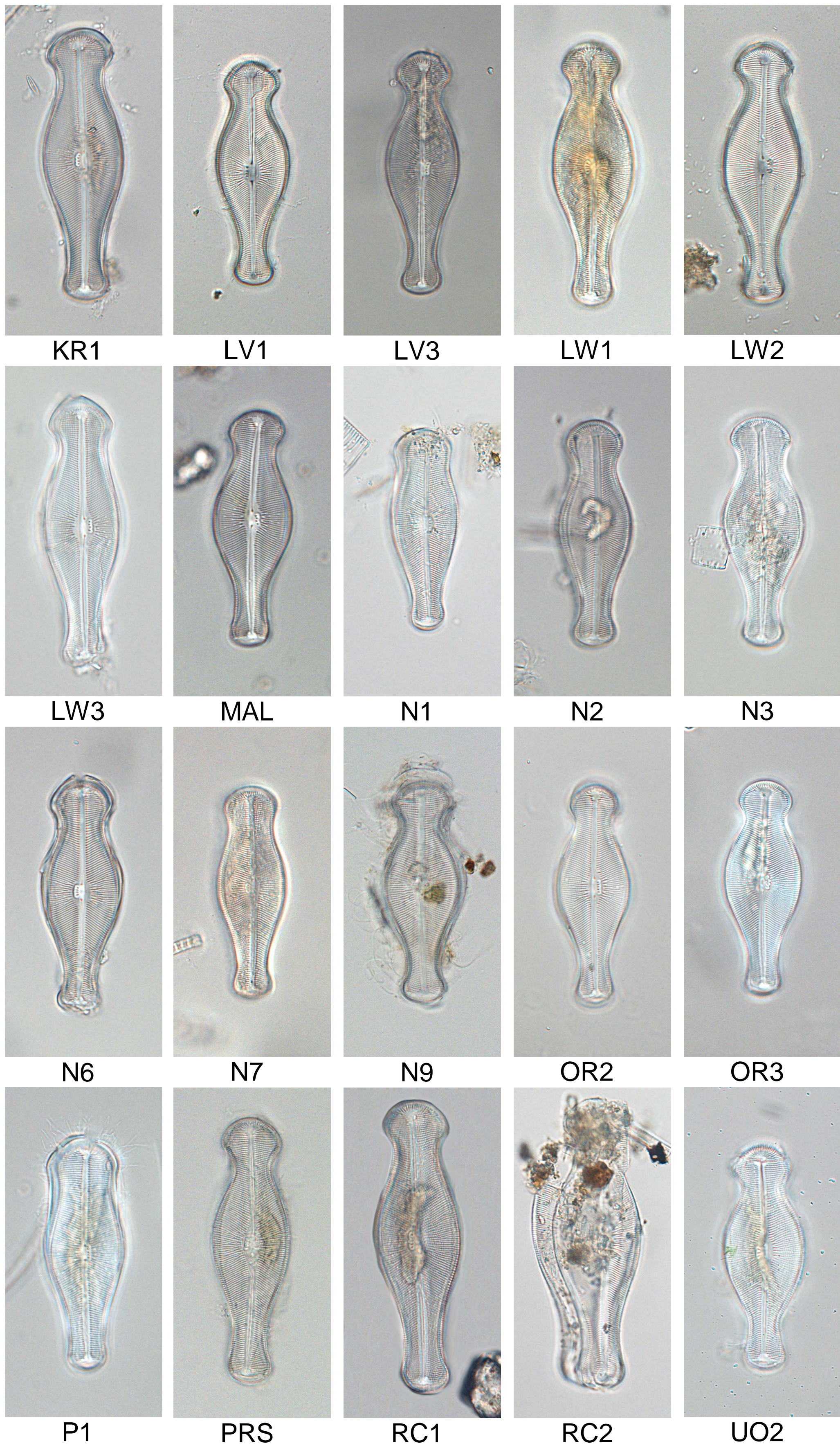
Amplification Product	PCR Extension Time (s)	Product size (bp)
602F-ITS4R	105	1773
641F-ITS4R	105	1735
753F-ITS4R	105	1637
1659F-ITS4R	55	737-740
EukA-ITS4R	120	2375

2.4 Results

Identification

All samples were positively identified as containing *D. geminata* using the PCR detection method developed by Cary *et al.* (2006). *D. geminata* cells were positively identified by microscopy in 35 of 37 samples. Photographs (Fig. 2.4) show variation in length (99-156 µm), width (35-48 µm) and cell shape. The number of stigmata (isolated perforations in the valve centre), which can be used for classification purposes, ranged from two to five.




KR1
LV1
LV3
LW1
LW2
LW3
MAL
N1
N2
N3
N6
N7
N9
OR2
OR3
P1
PRS
RC1
RC2
UO2

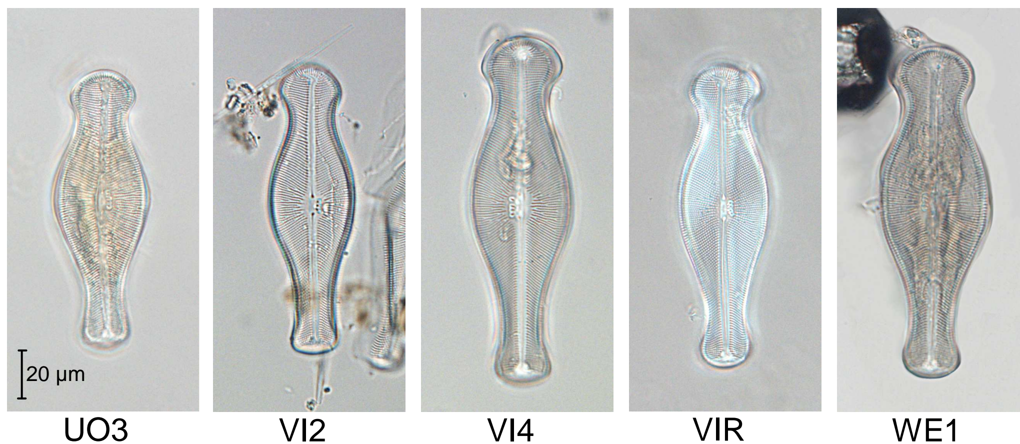


Figure 2.4 (13): Microscope photographs of *D. geminata* cells from 35 samples in this study.

DNA extraction, PCR, cloning and sequencing

As DNA was extracted from environmental samples that each contained a diversity of species, *D. geminata* DNA was amplified using at least one *D. geminata*-specific primer. *D. geminata*-specific PCR products ranged in length from 737 bp (1659F-ITS4R) to 2375 bp (EukA-ITS4R) depending on the primer set used (Table 2.4). As all samples were environmental samples that represent the community of organisms present at the time of sampling (Valentini *et al.* 2009), failure of amplification of some of the kit-extracted DNA may have been caused by various inhibitors present in samples from different locations.

PCR products represented DNA from a population of *D. geminata* cells, rather than DNA from one individual. Samples directly sequenced without cloning had variable quality, which may have been due to multiple ITS variants being present within the population or due to interference from secondary folding of PCR products. As a result, PCR products from most (22 of 37) samples were cloned (Appendix 4, Table A.5). Cloning efficiency of the large PCR products (1773-1637 bp) was low. Cloning efficiency of the 737-740 bp PCR products was as expected according to the manufacturer's guidelines.

Partial 18S-ITS sequences were obtained for each of 27 samples, with an additional sequence obtained for each of two samples (BC5 and LW1). Due to difficulties in cloning and amplifying this entire region, sequences of only the ITS region from a further 10 samples were obtained. At least one bidirectional ITS sequence was obtained for each of the 37 *D. geminata* samples collected. In addition, a second ITS colony from the clone library was sequenced for four samples (BC5, GENCON, P1 and LW1). As the two P1 sequences were identical, only one was used in phylogenetic analyses. Seven cloned 40B colonies were sequenced for a population-level study.

Alignments

Taxonomic Investigation: The 18S alignment was 1111 bp long and contained 61 sequences with pairwise identity of 90.0% and 55.9% identical sites (Table 2.5). Examination of the alignment (Appendix 4, Table A.7) at *D. geminata*-specific binding sites confirmed the choice of these regions for primers as no other taxa had similar sequences in these regions.

Aligning *D. geminata* sequences with diatom sequences from GenBank (Benson *et al.* 2008) provided clear boundaries between the 18S and ITS1, ITS1 and 5.8S, 5.8S and ITS2, and ITS2 and 28S regions (annotated ITS alignment in (Appendix 4, Table A.6). The closest relatives to *D. geminata* with ITS sequences in GenBank were *Nitzschia closterium f. minutissima* (Accession No: EF553459), *Pseudo-nitzschia delicatissima* (Accession No: AM118043.1), *Sellaphora pupula* (Accession No: AJ544651) and *Eunotia bilunaris* (Accession No: AM234654.1). The variability of the ITS regions of these species between the boundaries described above prevented their use as outgroups for the ITS *D. geminata* alignments. Pairwise distances for all alignments are provided in Tables A.8 - A.11)

Table 2.5: Statistics of the alignments generated to test phylogenetic relationships at different levels in this study.

DNA Region	Sequences (No.)	Alignment length (bp)	GC Content (%)	Pairwise Identity (%)	Identical Sites (%)
Diatom partial 18S	61	1111	45.8	90.0	55.9
D. geminata partial 18S-ITS	31	1154	40.5	98.7	81.5
D. geminata ITS	46	610	40.4	98.7	83.4
D. geminata ITS NZ Buller R. Population	7	597	40.3	99.3	97.8

Phylogeographic Investigation: The middle portion of the 18S-ITS2 alignment was removed due to missing data and the resulting alignment of 31 sequences was 1154 bp long with 98.7% pairwise identity (Table 2.5). Only the partial 18S region was available for the outgroup taxa *C. proxima* and *G. minuta* var. *cassiae* sequences. The proportion of identical sites, excluding the outgroup taxa of *C. proxima* and *G. minuta* var. *cassiae* due to their shorter length, was 81.5%. Of the 18S-ITS2 region, ITS1 had the highest GC content and ITS2 had the lowest GC content (44.1% and 36.7% respectively). Information of base frequencies for each alignment are given in Table A.13.

The ITS alignment was 610 bp in length and contained 83 polymorphic sites, of which 24 were parsimony informative (Table A.14). While the ITS1 and ITS2 regions contained a similar proportion of polymorphic sites (12.2% and 14.3% respectively), the ITS2 region contained almost twice as many variable characters as did ITS1. The 5.8S rDNA region contained 8.0% polymorphic sites. Pyramidine transversions were more common than purine transversions in all regions except the 5.8s rDNA (Table A.12, Appendix 4). The Poland sequence (P1) displayed the lowest pairwise similarity (Table A.8, Appendix 4) and was 605 bp long with three insertions and one deletion in the ITS1 region. ITS sequences from both

the Iran samples (HA2, HA6) contained an identical 3 bp insertion in ITS1 and were 600 bp long. The remaining 43 sequences were all 597 bp in length. With the exception of the ITS1 region of P1, all samples sequenced from the end of the 18S region aligned without ambiguity with all the sequences that included the partial 18S region (Appendix 4, Table A.6). As this region is not highly conserved among diatoms, the alignment confirms that these sequences belong to *D. geminata.*

As each sample potentially contains more than one individual of *D. geminata,* a population-level analysis of the ITS region was conducted on the New Zealand Buller River (40B) sample to investigate the level of ITS variation present within a single sample population. All seven transformants with the correct size insert were sequenced. The 597 bp long alignment had 99.3% pairwise similarity and 97.8% identical sites (Table 2.5). When these sequences were included with the other ITS sequences, 46 ITS sequences were aligned with a resulting alignment length of 610 bp, 98% pairwise identity and 83.4% identical sites.

Phylogenetic Analyses

Sufficient variation was found in the *D. geminata* 18S region for interspecies phylogenetic comparison and in the ITS region for intraspecies phylogeographic comparison. General trends were similar in Maximum Parsimony (MP) (Figs. A.10 - A.14) and Maximum Likelihood (ML) trees. ML trees are shown here as they were the most resolved trees (Figs. 2.5 - 2.8).

Taxonomic Investigation: The raphid pennate diatoms (Bacillariophyceae) formed a monophyletic clade based on the 18S region (Fig. 2.5), with the basal group being the designated outgroup of araphid pennate diatoms (Fragilariaceae). Species in the monophyletic Order Cymbellales grouped into three main clades (A, B and C) and appeared to be the most recently diverged of the raphid pennate diatoms. *D. geminata* samples formed a monophyletic group within Clade A, which contains

species in the Family Cymbellaceae (*Cymbella*, *Encyonema* and *Cymbopleura*) with 100% bootstrap support. *Cymbella proxima* is basal to the *D. geminata* clade. All species from the Family Gomphonemataceae (*Gomphonema* and *Gomphoneis*) formed the monophyletic Clade B, which is sister to Clade A. Clade C is sister to Clades A and B and contains other species from the Family Cymbellaceae (*Placoneis, Cymbella*) as well as *Neidium affine* from the Family Neidiaceae, Order Naviculales. Thus, according to the 18S data, the Family Cymbellaceae is paraphyletic.

Phylogeographic Investigation: The resolution of phylogenetic relationships for the 18S-ITS region of 29 *D. geminata* sequences, rooted with *Gomphoneis minuta* var *cassiae* and *Cymbella proxima* (Fig. 2.6) was limited by the presence of many short internal branches. Nevertheless, this rooted tree provided identification of more ancestral sequences from Europe and Iran (Clade ANC and Clade H), relative to more recently evolved sequences, mostly from USA and New Zealand. There was good bootstrap support (100%) for Clade H containing the Tar and Havir Rivers, Iran (HA2 and HA6 respectively) and low bootstrap support (51%) for Clade ANC containing the Atna River, Norway (N2) and the Matapedia River, Canada (MAL). This is particularly useful in providing an idea of a possible root for the ITS tree, as no sequences could be found in GenBank (Benson *et al.* 2008) to provide an outgroup to root the ITS tree. Three other clades (D, I1 and I2) were found within the most recently diverged sequences. Clade I1, containing sequences from Boulder Creek, USA (BC1, BC12), Lower Waiau River, New Zealand (LW1a, b) and Nidelva River, Norway (N1) had 69% bootstrap support. Clade I2 contained sequences from Buller and Oreti Rivers, New Zealand (40B.c14, OR3, OR2 and UO2) and Tisleia River, Norway (N6) and Clade D contained sequences from Vancouver Island, Canada (VI2, VI4) and Lee Vining Creek, USA (LV3).

The unrooted ITS tree (Fig. 2.7) showed two ancestral clades (ANC1 and ANC2), with ANC1 having 70% bootstrap support. Within ANC1, a clade containing Sandviksvassdraget River, Norway (N9) and Matapedia River, Canada (MAL) was strongly supported (94%). Within ANC2, a clade

containing Coquet River, UK (C1) and Vinstra River, Norway (N3) was strongly supported (92%). There was strong support (100%) for the Iran Clade (H). More recent sequences were separated from ancestral sequences with bootstrap support of 61%. As in the 18S-ITS tree, the two main clades I1 and I2 occurred among the more recently diverged sequences. Sequences belonging to Clade I1, with 63% bootstrap support, included those in the 18S-ITS tree as well as Rapid Creek, USA (RC2) and New Zealand Mararoa (GENCON.c1) and Buller River (40B.c3, 7, 8, 12, 16) sequences. Sequences belonging to Clade I2 included those in the 18S-ITS tree as well as USA rivers Prosser (PRS), Kootenai (KR1) and Virginia Lake Creek (VIR), Dokka River, Norway (N7), Vancouver Island, Canada (VI2) and New Zealand rivers Oreti (OR2), Waiau (LW2), Buller (40B.c11) and Mararoa (GENCON.c3). Furthermore, there is evidence for clustering within Clade I2, where there is 71% bootstrap support for OR3 and 40B.c11 basal to 40B.14, PRS and N7, which are grouped with 68% bootstrap support. Greater phylogenetic resolution was achieved using the entire ITS region rather than only ITS1, ITS2 or ITS1 and ITS2 concatenated. Removing P1 did not change phylogenetic inferences (Fig. A.15) and it was therefore included in further analyses.

The variation present within one *D. geminata* sample was examined using an unrooted ML tree (Fig. 2.8) of the ITS region for seven New Zealand Buller River sequences. This tree showed two distinct genotypes occurring within the sample population. Colonies 3, 7, 8, 12 and 16 formed one clade (G1) with strong bootstrap support (91%), while Colonies 11 and 14 formed another (G2). These two 40B genotypes clustered with the other New Zealand samples in the two main clades (Fig. 2.7), with G1 genotypes only being found in Clade I1 and G2 genotypes only being found in Clade I2. Furthermore, one of the Mararoa River colony sequences occurred in Clade I1 (GENCON.c1) and the other in Clade I2 (GENCON.c3).

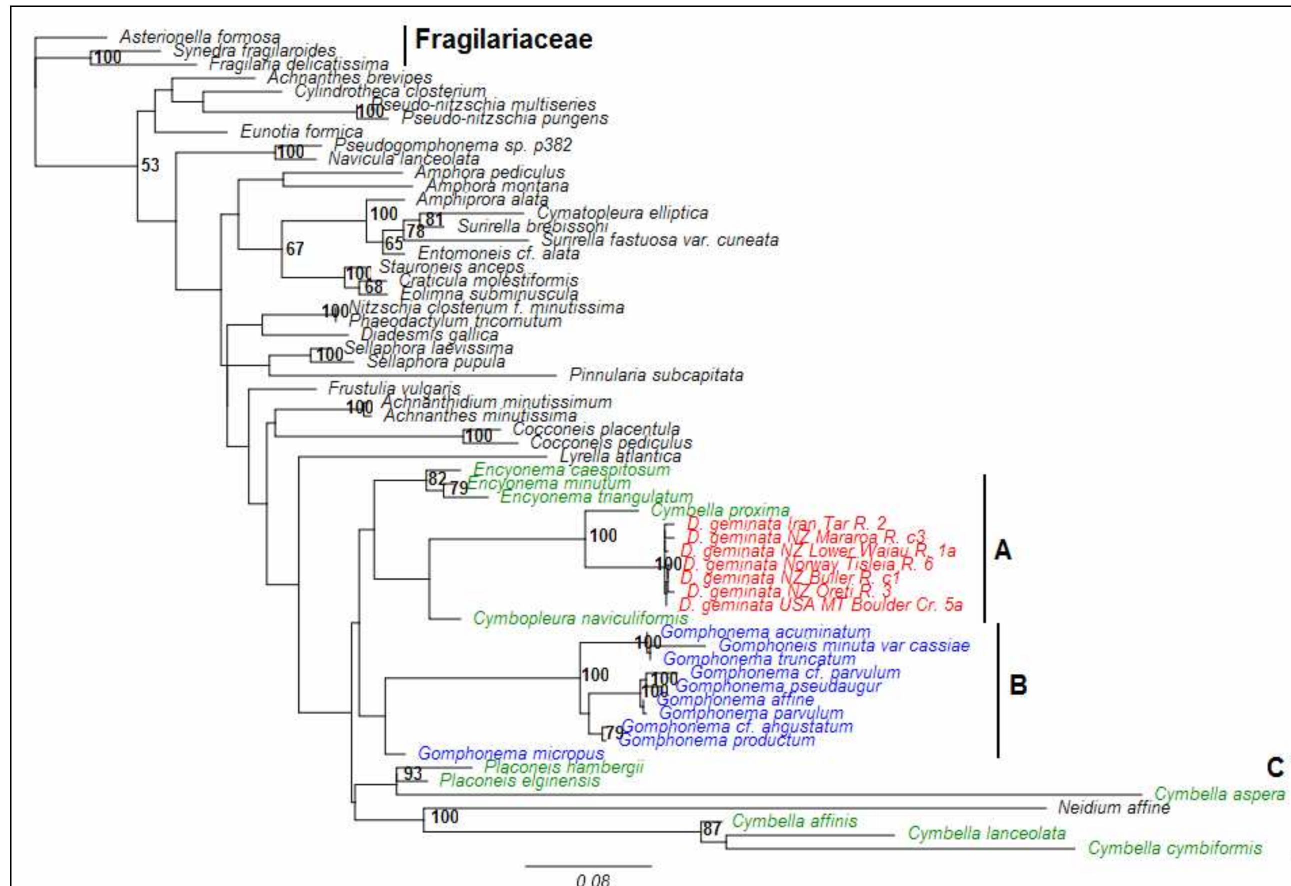


Figure 2.5 (14): Maximum Likelihood (ML) tree of the taxonomic placement of *D. geminata* based on 1111 bp long partial 18S region of 58 raphid pennate diatoms (Bacillariophyceae) rooted with 3 araphid pennate diatoms (Fragilariaceae) using TrN+I+G model of substitution. Numbers at nodes represent percentage boostrap support from 100 bootstrap replicates. Bootstrap values < 50% are not shown. Red = D. Geminata. Blue = Gomphonemataceae. Green = Cymbellaceae. A, B, C = clades within Order Cymbellales.

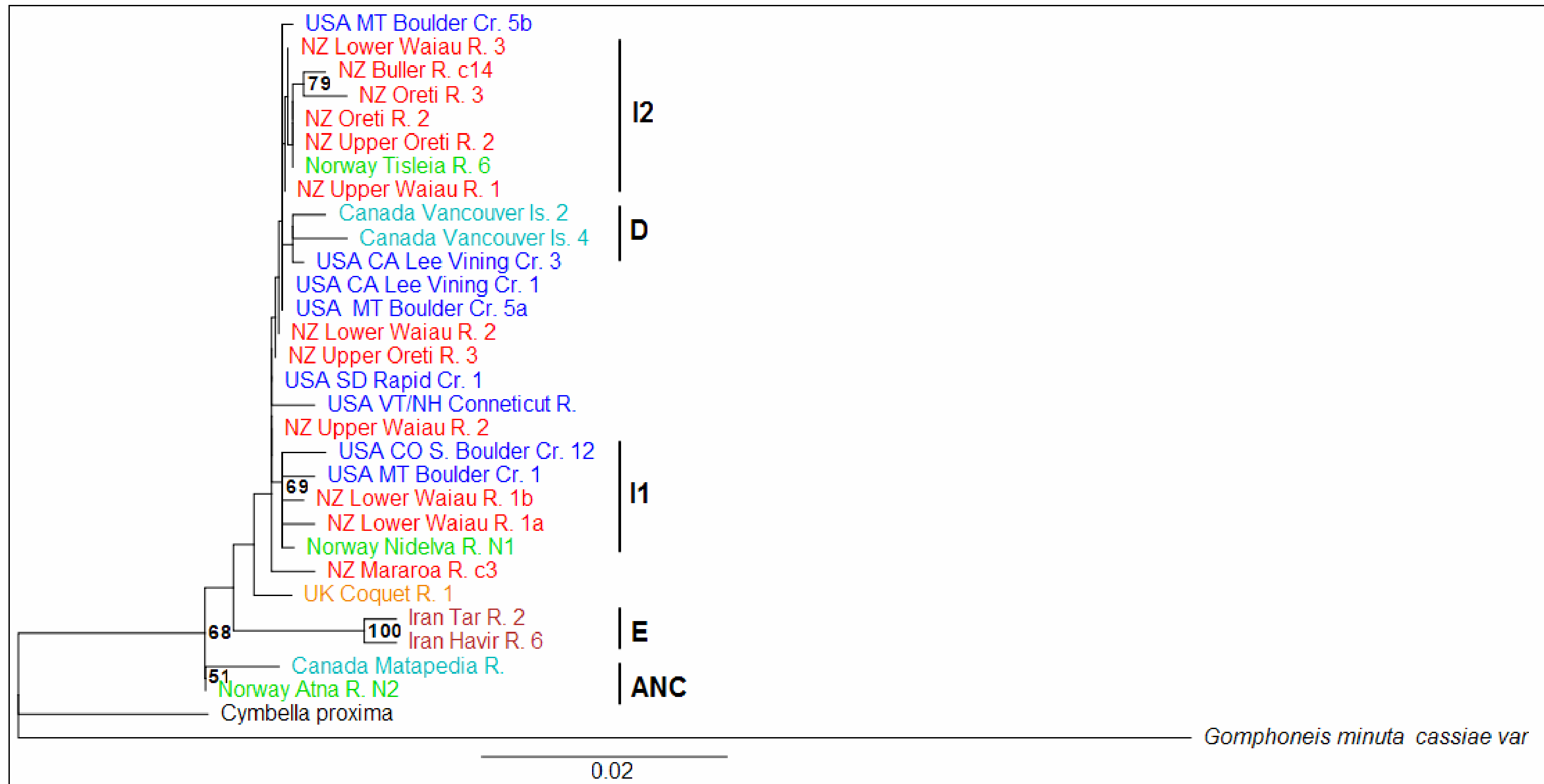


Figure 2.6 (15): Maximum Likelihood (ML) tree for the 1154bp long 18S-ITS region for 29 *D. geminata* sequences, rooted with *Gomphoneis minuta* var *cassiae* (Gomphonemataceae) and *Cymbella proxima* (Cymbellaceae) using the HKY+G model of substitution. Numbers at nodes represent percentage boostrap support from 100 bootstrap replicates. Bootstrap values < 50% are not shown. *D. geminata* sample colours: NZ=red, USA=blue, Norway=green, Canada=light blue, UK=orange, Iran=brown. I1 = Invasion Clade 1, I2 = Invasion Clade 2, E = Iran Clade, ANC = Ancestral Clade, D = Clade D.

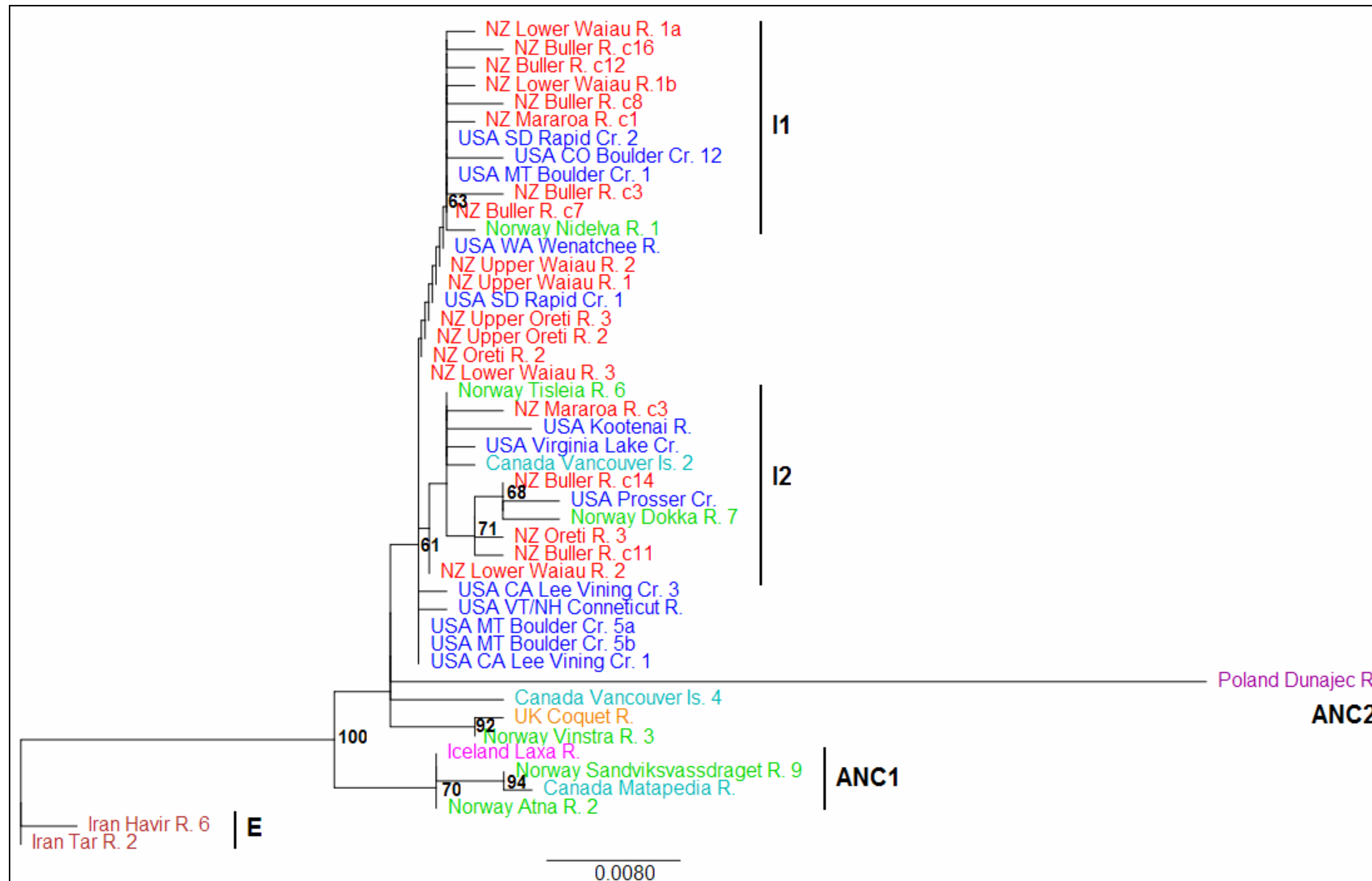


Figure 2.7 (16): Maximum Likelihood (ML) tree for the 610 bp long ITS region for 46 *D. geminata* sequences using the HKY model of substitution. Numbers at nodes represent percentage boostrap support from 100 bootstrap replicates. Bootstrap values < 50% are not shown. *D. geminata* sample colours: NZ=red, USA=blue, Norway=green, Canada=light blue, UK=orange, Iran=brown, Iceland=Pink, Poland=Purple. I1 = Invasion Clade 1, I2 = Invasion Clade 2, E = Iran Clade, ANC1 = Ancestral Clade 1, ANC2 = Ancestral Clade 2.

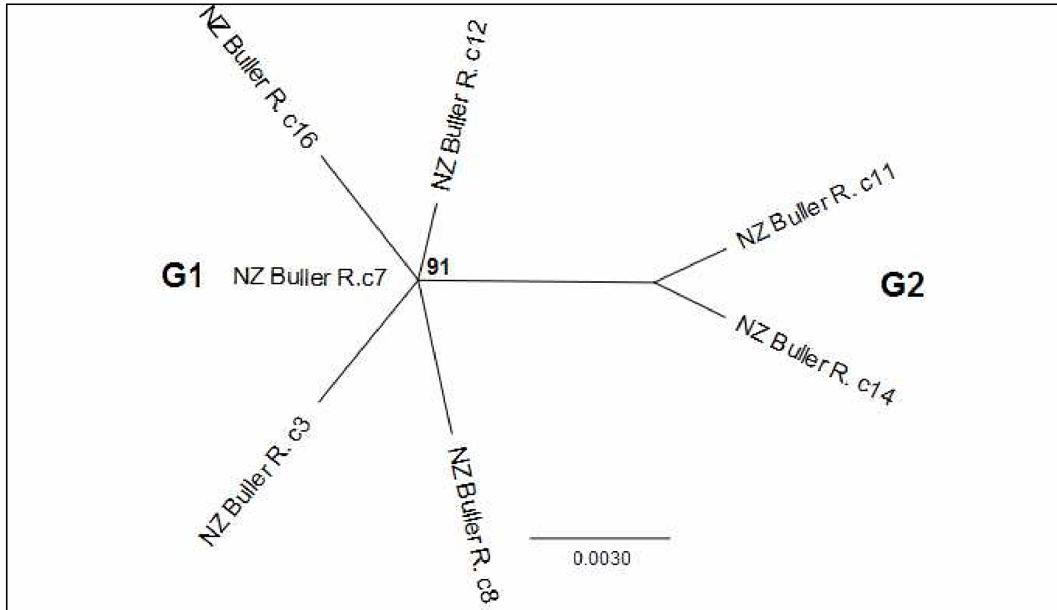


Figure 2.8 (17): Maximum Likelihood (ML) tree for the population study of the 597 bp long ITS region of seven cloned sequences from the New Zealand Buller River sample, using the HKY model. Numbers at nodes represent percentage boostrap support from 100 bootstrap replicates. Bootstrap values < 50% are not shown.

Although the 18S and ITS regions provide markers at different phylogenetic levels and although the 18S-ITS alignment did not include all the taxa present in the ITS alignment, the phylogenetic relationships were similar. The greatest incongruence between the 18S-ITS ML tree and the ITS ML tree is the position of VI4 (Vancouver Island, Canada) and GENCON.c3 (Mararoa, New Zealand). In the ITS tree, GENCON.c3 occurs in the I2 clade, rather than being basal to the I1 Clade as in the 18S-ITS tree. VI4 clustered with VI2 (Vancouver Island, Canada) and LV3 (Lee Vining Creek, USA) to form Clade D in the 18S-ITS tree. However, in the ITS tree Clade D disappears and VI4 clusters with the more ancestral sequences of Dunajec River, Poland (P1), Coquet River, UK (C1) and Vinstra River, Norway (N3). Apart from these differences, the ITS tree did not change the relationships inferred from the 18S tree.

2.5 Discussion

The genetic variation in the 18S-ITS region of *D. geminata* was sufficient to determine the likely position of this species taxonomically within the raphid pennate diatoms and to propose a likely route by which it arrived in New Zealand. Results suggest that *D. geminata* belongs to the family Cymbellaceae and that there may have been multiple introductions of *D. geminata* from Europe to northern North America and then from northern North America to New Zealand. Similar relationships obtained using both 18S-ITS and ITS regions suggest support for these results.

Identification

Cell shape was as expected for *D. geminata* for all samples except the Poland (P1) sample, which had a broader head-pole (Fig. 2.4). Recently, Mrozińska *et al.* (2006) described a new species, *Didymosphenia tatrensis*, in the Carpathian mountains of Poland, including the Dunajec River, from which P1 was sampled. This new species occurs in association with *D. geminata* and is described as having a broadly rounded head-pole, an almost rectangular foot-pole and striae becoming parallel at each pole. Stigmata are rarely more than one. The photograph of a P1 cell (Fig. 2.4) shows a broader head-pole than those from other *D. geminata* samples and the striae become parallel at the poles. However, it also has five stigmata rather than the one stigma characteristic of *D. tatrensis*. This sample was positively identified as *D. geminata* using the PCR-based detection method. Therefore, the identity of this sample is uncertain.

Morphologically, with the exception of P1 discussed above, variation was generally not diagnostic in distinguishing between samples during identification (Fig. 2.4). Cell size does not necessarily equate to genetic variation, as diatom cells decrease in size with each vegetative division (Mann 1999). Others have found that genetic diversity of diatoms is not reflected morphologically (Zechman *et al.* 1994) and some morphologically

delineated species may actually be cryptic species complexes (e.g. reproductively isolated groups in the *Cyclotella meneghiniana* complex cannot be distinguished morphologically but only genetically, Beszteri *et al.* 2005). Zechman *et al.* (1994) suggest that morphological features are under a different evolutionary rate to ITS sequences.

Taxonomic Investigation

The phylogenetic relationships reflected in this study incorporating the partial 18S region of *D. geminata* and other diatom sequences (Fig. 2.5) lend support to suggestions that *D. geminata* should be placed in the Family Cymbellaceae rather than Gomphonemataceae (Kociolek & Stoermer 1988; Moffat 1994). However, based on the 18S region, it appears that Cymbellaceae is paraphyletic and needs revision. Further investigation on the position of *Cymbella proxima,* basal to *D. geminata* in Fig. 2.5, would be of interest, as this species does not cluster with the other *Cymbella* species. Sequences from other *Didymosphenia* species would help to confirm the position of this genus within the pennate diatoms and shed light on the taxonomic placement of the Poland sample (P1) within *D. geminata* or *D. tatrensis*. As the *D. geminata*-specific primers have not been tested against other *Didymosphenia* species, it is possible that the Poland sample is *D. tatrensis*. Further testing of primers against other *Didymosphenia* species will be necessary to determine their specificity within the genus.

ITS features

The 597-605 bp ITS region of *D. geminata* is comparable in length to that of other species: the ITS region of the centric diatom *Thalassiosira weissflogii,* the dinoflagellate genus *Symbiodinium* and the angiosperms is 773-774 bp, 521-584 bp and less than 700 bp in length respectively (Baldwin *et al.* 1995; LaJeunesse 2002; Von Dassow *et al.* 2006). ITS1 (231-239 bp) is longer than ITS2 (203 bp), as in flowering plants (Baldwin

et al. 1995). This is in contrast to other diatom species, such as *Stephanodiscus* species and *Pseudo-nitzschia delicatissima* (Zechman *et al.* 1994; Orsini *et al.* 2004). However, unlike flowering plants, such as those of the Pacific genus *Pittosporum* (Gemmill *et al.* 2002), *D. geminata* ITS2 is more variable than ITS1. This trend is similar in the diatoms *Thalassiosira weissflogii*, *Navicula* species and *Skeletonema marinoi* Sarno et Zingone (Von Dassow *et al.* 2006; Creach *et al.* 2006; Godhe *et al.* 2006). Most *D. geminata* ITS sequences were identical in length. However, the Iran sequences (HA2, HA6) contained a 3 bp insert in ITS1 and the Poland sequence (P1) contained three inserts and one deletion within 100 bp of the Iran insert. Orsini *et al.* (2004) found a highly variable region of indels within the ITS1 region of *Pseudo-nitzschia delicatissima* sequences, which was excluded from alignments due to ambiguity. Thus, the poor alignment of P1 in this region may not indicate a different species. Interestingly, the sequences of the two P1 transformants sequenced were identical, reducing the possibility that we sequenced a rare ITS variant. When the P1 sequence was removed from the alignment, phylogenetic relationships did not change.

Phylogeography of D. geminata

Samples within rivers or from specific regions (e.g. Buller River New Zealand, USA, Norway) were not monophyletic, suggesting multiple introductions. Along with the presence of many short branches and the clustering of ITS types from one source into two main clades, this suggests a recent rapid spread of *D. geminata* from an unidentified source to new areas in Europe, northern North America and New Zealand, with mixing of populations from different sources in new locations. New Zealand's *D. geminata* is most closely related to samples from USA, western Canada and Norway. Multiple introductions from multiple sources into North America and then into New Zealand makes tracing the exact history of the *D. geminata* invasion difficult. Increased sampling, especially of source areas may enable clearer patterns to be resolved.

In the ITS phylogenetic analysis, New Zealand samples grouped into two main clades with samples from northern USA, Vancouver Island and three rivers in Norway (Fig. 2.7). It therefore appears that New Zealand's *D. geminata* may have arrived in at least two separate introductions, probably from northern North America. It may also have been transported out of New Zealand back to the USA and Norway at least once. This is based on the fact that the Prosser Creek (PRS) USA and Dokka River (N7) Norway samples formed a clade for which New Zealand samples (Oreti (OR2) and Mararoa (GENCON.c3) Rivers) were basal. Further sampling is needed to test this hypothesis.

There was poor resolution between many sequences from New Zealand samples, demonstrating the recentness of *D. geminata*'s arrival in New Zealand. The fact that different ITS sequences from the Mararoa (GENCON) and Buller (40B) Rivers fall within both Clade I1 and Clade I2 suggests that there may have been two main invasions of *D. geminata* into these New Zealand rivers, each with a distinct genotype. Clade I1 (Fig. 2.7) contains a number of recently diversified samples with unresolved relationships and short branch lengths. This clade and its basal sequences consist almost entirely of samples from USA and New Zealand, with the exception of that from Nidelva River, Norway (N1). The USA samples in this clade are from widespread sources in mid-western USA, including South Dakota (RC1,2), Montana (BC51) and Colorado (BC12). It therefore appears that there has been a recent, rapid spread and mixing of genotypes of *D. geminata* in these locations. In Clade I2, the Mararoa, Buller River and one Oreti River (OR3) ITS sequences showed significantly more variation and grouped with those from tUSA, Canada and Norway.

These results suggest that there have been several stages to the spread of *D. geminata* worldwide. Fossil evidence suggests *D. geminata* is native to Siberia, Finland and northern North America (Stoermer 1993; Sarmaja-Korjonen & Alhonen 1999; Polyakova & Stein 2004; Miettinen *et al.* 2005). As no samples from these locations have been included in this study, it is difficult to pinpoint the source of invasive *D. geminata* to new locations in

Europe. However, based on the 18S-ITS and ITS trees (Figs 2.6 and 2.7) showing mostly European (P1, N2, N3, N9, UK) and Eastern Canadian (MAL) sequences as being basal and having longer branch lengths, it appears that these locations were invaded by this species first. The 18S-ITS tree (Fig. 2.6) with its short internal branches for sequences form Norway, northern North America and New Zealand supports the recent arrival of *D. geminata* in these areas.

The molecular data obtained in this study correlate with reports of the spread of *D. geminata* worldwide. *D. geminata* was recorded as spreading and becoming invasive in Norway in the 1980s, Iceland and Vancouver Island, Western Canada, in the 1990s and in the USA and New Zealand in the last five to ten years (Spaulding & Elwell 2007; Kilroy 2008). The ITS tree (Fig. 2.7) suggests that *D. geminata* may have spread south from southwest Canada into the USA. Clade I2 may represent the first introduction of *D. geminata* to New Zealand as it has had more time to diverge, evident in its greater resolution and branch lengths compared to Clade I1. Clade I2 contains samples from Vancouver Island (VI2) and north-western USA (KR1, VIR) at its lowest level, suggesting that the first arrival of *D. geminata* in New Zealand may have come from this region. Of great interest is the position of two samples (Prosser River (PRS), USA and Dokka River (N7), Norway) within the New Zealand samples in Clade I2 (Fig. 2.7). *D. geminata* in the Dokka River, known to contain brown trout as in New Zealand rivers, was first observed in June 2006 (Lindstrøm & Skulberg 2008), which is after the initial discovery of *D. geminata* in New Zealand. This lends support to the molecular data that suggest that *D. geminata* in New Zealand may have been the source for new *D. geminata* introductions into other regions including Dokka River.

In summary, a potential sequence of invasion events (Fig. 2.9) could involve an introduction from an original source into new areas in Europe, followed by an introduction into north-western North America. From here it may have spread outwards across the United States and also to New

Zealand. From New Zealand, *D. geminata* may then have been dispersed again, this time back to the USA and Norway (Fig. 2.9). Iran's *D. geminata* most likely came from a source in Europe unrelated to USA or New Zealand *D. geminata*. However, based on the data available in this study, *D. geminata* introduced into Iran from its native range is also a possibility.

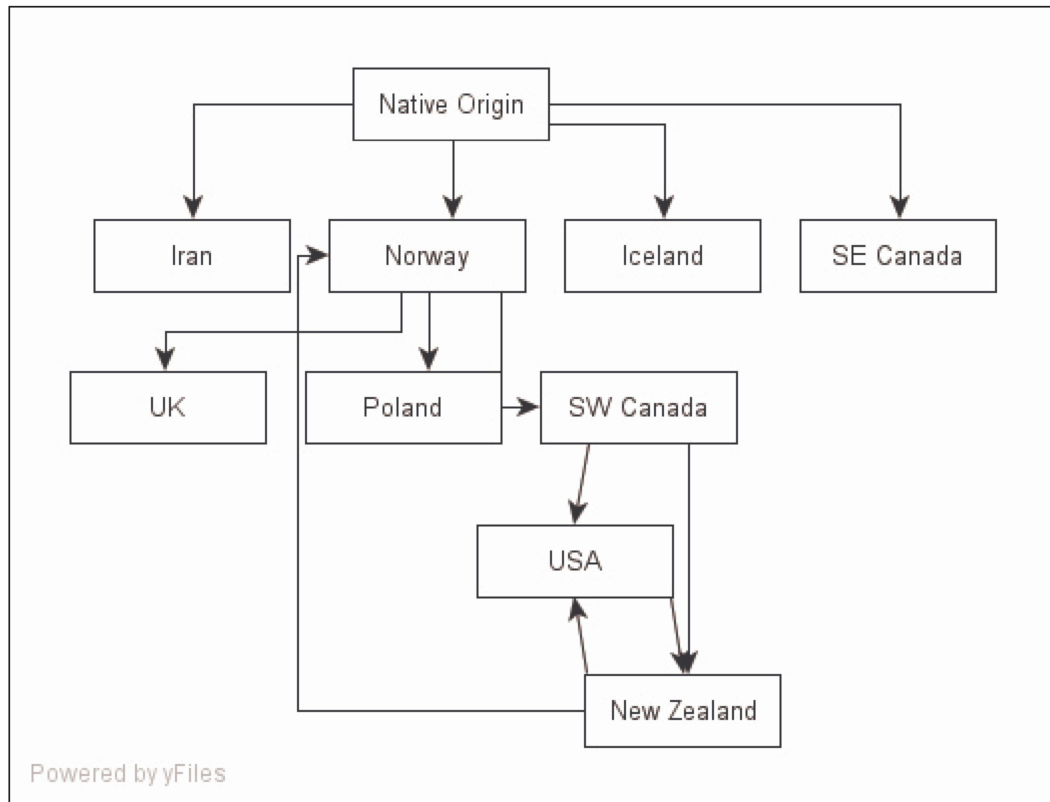


Figure 2.9 (18): Possible invasion succession of *D. geminata* from its origin through Europe and North America to New Zealand and back again.

ITS variability

As algal cells can contain multiple copies of rRNA genes under concerted evolution (Behnke *et al.* 2004; Créach *et al.* 2006), this region is ideal for unculturable diatoms and for environmental samples with low target cell abundance. However, environmental samples often require cloning, and while cloning efficiency of only the ITS region was high, cloning efficiency of the 18S-ITS region was low. Alverson & Kolnick (2005) also documented low cloning efficiency of the 18S rDNA region for the diatom genus *Skeletonema*. This may be purely due to length, or in part due to

interference of secondary folding during ligation. Heating purified PCR DNA at 65 ℃ for 10 min immediately prior to cloning and cloning immediately after amplification improved efficiency in this study.

Intragenomic ITS variants can pose significant problems for phylogenetic studies in some species. Harpke & Peterson (2007) caution using a single ITS sequence from a clone library to represent a sample in a phylogenetic analysis because intragenomic copies may be paraphyletic and a single sequence may give incorrect phylogenetic information. Many studies only examine one or a few cloned transformants from each population, as they are not intended for taxonomic study, which raises questions of how well they represent the population and how much variation exists within populations (Mann 1999). In cases with DNA from one organism or clonal line, direct sequencing enables detection of intragenomic variation, as more than one nucleotide state can be distinguished at each point in the sequence. However, this is not possible for environmental samples containing DNA from a number of individuals or regions where indels cause the sequences to shift out of alignment.

Several studies (Ki *et al.* 2004; Richlen & Barber 2005; Henrichs *et al.* 2008) have used DNA from single microalgal dinoflagellate cells to conduct phylogenetic investigations. Ideally intragenomic ITS variation from one cell would be assessed for *D. geminata.* However, no single cell extractions have been successfully performed for diatoms. Their heavily silicated shells appear to hinder successful extractions (Bérard *et al*. 2005). A limited examination of seven clones from the clone library of the Buller River, New Zealand (40B) sample showed that there is a low level of intrapopulational variation (2.2% polymorphic sites), with two main genotypes present (Table 2.5, Fig. 2.8). This variation was not sufficient to hinder elucidation of phylogenetic relationships, with 16.6% polymorphic sites occurring in the ITS region between all samples investigated. Multiple sequences from the clone libraries were obtained for several other samples (BC5, LW1, P1 and GENCON). GENCON (Mararoa River, New Zealand) showed similar variation to 40B (Mararoa River, New Zealand), while BC5 (Boulder Creek, USA) and LW1 (Waiau River, New Zealand)

did not differ enough to be placed on different branches in the ITS phylogenetic tree. The P1 (Dunajec River, Poland) cloned sequences were identical. Therefore, ITS variants do not appear be problematic for phylogenetic analyses in *D. geminata*.

As the DNA was from environmental samples, sequence data represent all the intrapopulational variation present. However, the proportion of intrapopulational variation is expected to be similar to the proportion of intragenomic variation if colonies are interbreeding (Kooistra *et al.* 2001). Thus, populations will be represented by distinct rDNA variants, some of which will occur within the genomes of individuals (Alverson & Kolnick 2005). Although currently no data are available on the rate of sexual reproduction for *D. geminata,* the intragenomic variation of *D. geminata* may be similar to that of the diatom genus *Skeletonema,* whose 18S rDNA genes contain 0.57-1.81% intragenomic polymorphic sites (Alverson & Kolnick 2005). This is based on the similar level of variation present in the Buller River (40B) population (2.2% polymorphic sites). As with *D. geminata*, indels in the ITS region were rare in *Skeletonema*. The presence of intragenomic variation in a genome suggests that the rate of mutation or speciation is exceeding the rate of concerted evolution or that concerted evolution has not yet proceeded to completion (Alverson & Kolnick 2005). This is highly possible considering the recent rapid spread of *D. geminata* in recent years to new locations, where it is under new selection pressures and has the potential to hybridize with *D. geminata* strains originating from many different locations. The elucidation of patterns of spread of *D. geminata* within the last three decades using the ITS region suggests that this ITS region is evolving rapidly, implying a high rate of mutation, short generation times and/or sexual reproduction.

Bidirectional sequencing was conducted for the ITS region to increase the likelihood that polymorphism was real and not a result of a sequencing artefact. *Taq*-mediated error or *E. coli* replication error may also induce some polymorphism. However, the estimated error rates for *Taq* polymerase and *E.coli* replication are only 1.1×10^{-4} and 1.0×10^{-9} errors nt^{-1} respectively. These methodical errors are unlikely to influence results

significantly (Alverson & Kolnick 2005). Furthermore, 15 of the 46 sequences were obtained by direct sequencing (WE1, UW1, UW2, UO2, UO3, RC1, OR2, OR3, C1, IC1, LV1, LW2, LW3, N2, N3: Table A.5, Appendix 4), showing that intragenomic variants were not a problem in these samples.

Future work

As the effects of natural selection on different genes may vary, it is important, where possible, to include more than one molecular marker in a phylogenetic study (Parker *et al.* 1998). Additional markers also provide more data from which to determine relationships. The greatest limitation of the ITS region is its small number of characters (Baldwin *et al.* 1995). Thus, incorporation of other markers would lend support to relationships found and may enhance tracing the spread of *D. geminata*. The external transcribed spacer (ETS) region, upstream of the 18s rDNA region, has been found to enhance the resolution of relationships when used together with data from the ITS region (Markos & Baldwin 2002). Other intergenic regions or anonymous DNA markers may contain enough variation to improve resolution of the recent invasion events of *D. geminata*. Chloroplast and mitochondrial DNA markers may also be helpful, as organellar DNA is inherited cytoplasmically and provides information on hybridization, introgression and founder effects (Parker *et al.* 1998). However, such markers would need to evolve rapidly enough to provide information on recent changes, without evolving so rapidly that meaningful patterns are obscured. The analysis presented here using the ITS region and supported at a lower level by the 18S rDNA region provides a good picture of the likely pattern of spread of *D. geminata*. This pattern can be further determined by incorporating other markers. For example, phylogenetic studies of the dinoflagellate genus *Symbiodinium,* performed using the nuclear ribosomal DNA large subunit 28S (nr28S), were supported by the chloroplast ribosomal large subunit 23S (cp23S), the chloroplast gene encoding the D1 protein of photosystem II (*psbA*) and the

mitochondrial cytochrome c-oxidase subunit 1 (*cox1*) gene (Pochon *et al.* 2006). A combination of cp23S and nr28S provided higher levels of statistical support and greater resolution of these relationships.

It would also be valuable to obtain more sampling coverage within rivers to determine the amount of variation present. This is especially important for rivers such as the Dokka River, Norway, as it could enable testing of the hypothesis that *D. geminata* from New Zealand may have been spread to other countries. Obtaining samples from regions where *D. geminata* is native may provide a clearer picture of the earlier events in the recent *D. geminata* worldwide invasion. As no genetic investigations have been conducted on other *Didymosphenia* species, it would be of great interest to investigate where these species are placed taxonomically in relation to *D. geminata*. This would also potentially provide an outgroup for rooting the *D. geminata* ITS trees. Sequencing the 18S region of these species would also provide insight into the specificity of the primers used in this study within the genus *Didymosphenia*.

Invasion implications

This study suggests that *D. geminata* has been introduced to New Zealand at least twice. The high level of genetic diversity in populations of *D. geminata* suggests that there have not been any significant founder effects such as bottlenecks, with no populations becoming fixed for a specific haplotype. Genetic variation is maximized if: 1) bottlenecks are brief, 2) there are multiple introductions, 3) the number of introduced individuals is large or 4) population growth is rapid (Suarez & Tsutsui 2008). All of these seem to have occurred for *D. geminata* in New Zealand. Large populations, such as those found in many of the areas invaded by *D. geminata*, are affected less by genetic drift and enable incorporation of diversity from multiple introductions through greater connectedness, enabling populations to attain the same or greater levels of variation that were present in the source populations (Suarez & Tsutsui 2008). Furthermore, as *D. geminata* can disperse up or down stream, admixture

of genotypes from two separate introductions to different sites in one river is likely to occur.

The coexistence of genotypes from multiple sources, as found within the Buller and Mararoa River samples, enables new recombinations and intraspecific hybridizations to occur, increasing polymorphisms (Kooistra *et al.* 2001). Recombination, hybridization and the movement of genotypes within and between invasive populations can increase the invasiveness of a species and improve its ability to adapt to new environments (Dlugosch & Parker 2008). This may help explain how *D. geminata* spread so rapidly through New Zealand since its first detection in the South Island in 2004 and more recently through the US, where it appears to be adapting to warmer temperatures (Spaulding & Elwell 2007; Kumar 2009).

As most occurrences of *D. geminata* have been in popular trout and salmon fishing rivers, international anglers are likely vectors for long distance dispersal. Felt-soled waders having been proven to provide environments suitable for survival of viable *D. geminata* cells for months if kept damp and in cool conditions under 20 ℃ (Kilroy *et al.* 2006). This method of dispersal not only poses a threat to increasing the genetic diversity and thus the potential increase in invasiveness of *D. geminata* in New Zealand, but it also poses a threat to the countries the anglers return to, causing further dispersal and mixing of genotypes worldwide. For example, there is some evidence from the ITS data to suggest that New Zealand's *D. geminata* may have been taken back to the USA and Norway. It is therefore important for ongoing border control and campaigns, such as the MAF (Ministry of Agriculture and Forestry) Biosecurity New Zealand's "Check, Clean, Dry" campaign (Didymo Long-Term Management Programme 2007), to increase public awareness and responsibility in preventing the spread of *D. geminata* to new areas and the mixing of populations between areas.

CHAPTER 3: CONCLUSION

3.1 Summary

The results of the research presented in this thesis suggest that the invasive alga *Didymosphenia geminata* may have been introduced to northern North America through multiple introductions from Europe and then to New Zealand through multiple introductions from northern North America. It also appears possible that *D. geminata* from New Zealand may have been carried back to northern North America and Europe. There appear to be at least two main genotypes present in New Zealand rivers and this admixture of genotypes from different sources may enable *D. geminata* to adapt to new environments. It is therefore crucial to limit the spread and mixing of *D. geminata* from different populations and to prevent new genotypes being introduced to pre-existing populations.

The cause of the sudden explosive invasiveness of *D. geminata,* evident by its increased range and ecological tolerances in the last few decades, is currently unknown. Although it was originally described as a diatom native to cool oligotrophic habitats (Patrick & Reimer 1975), it has been found in warmer, mesotrophic habitats in recent years (Kawecka & Sanecki 2003; Szabo *et al.* 2005), although not always in massive blooms. It is therefore possible that *D. geminata* has existed for some time at low levels in a wider range of habitats than was previously realised. This is especially possible considering the fact that so little information was available about *D. geminata* until recently. Furthermore, *D. geminata* may not be as oligotrophic as was first expected (Kawecka & Sanecki 2003) as it is sometimes found in mesotrophic and not oligotrophic habitats (Noga 2003; Beltrami *et al.* 2008).

Another possibility is that it has adapted to new conditions. Diatom geographic ranges are limited by dispersal, environments and change of environments over time (Vanormelingen *et al.* 2008). The widespread long

distance dispersal of *D. geminata* by humans has reduced natural dispersal barriers and the potential adaptation of *D. geminata* to new environments may have reduced environmental barriers. It is possible that pre-adaptation of a new variant has increased its invasion success, enabling its rapid spread worldwide. New *D. geminata* populations have demonstrated that they are able to tolerate physical and chemical fluctuations (Bhatt *et al.* 2005; Oberholster *et al.* 2005; Szabo *et al.* 2005). As *D. geminata* has recently been found in habitats disturbed by human influence, including below dams and reservoirs (Kawecka & Sanecki 2003; Kolmakov *et al.* 2008), it may be highly competitive in disturbed environments. As such, it may become more of a problem as human influences on waterways increase. Its community-level impacts (Larned *et al.* 2007) increase concern of its effects on native flora and fauna.

3.2 Future Work

Results in this study are based on the phylogenetic analysis of the 18S-ITS region of nuclear ribosomal DNA (nrDNA) from 37 samples worldwide. Factors influencing the robustness of phylogenetic studies include sample size, sample frequency, quality of genetic material and resolution of the genetic markers used (Alverson & Kolnick 2005). Between one and five samples were analysed per river and sequences were either obtained directly following PCR or after cloning. Between one and seven clones from each clone library were sequenced. Low numbers of sequences from cloned material may not represent the genetic variation present in a population adequately. Increasing the number of clones sequenced per population may increase the resolution of the results, as would increasing the number of samples from each location.

The samples used in this investigation were mostly from areas where *D. geminata* has become invasive and there are no samples from areas where it is thought to be native. Sampling regions where *D. geminata* is considered to be native such as Lake Baikal, Russia (Metzeltin & Lange-

Bertalot 1995) would improve understanding of the earlier events in the *D. geminata* invasion, potentially including determining the original source of the invasion and the genetic variation present between invasive and native strains. Another region of interest from which to obtain samples is India, where *D. geminata* was first discovered at approximately the same time as in New Zealand (Bhatt *et al.* 2005) and which may have a different source of introduction as it is distant from the other invasion locations. Sampling populations over time would help to determine whether seasonal variants exist. Furthermore, it was observed that many cells from older samples were shattered, and that newly extracted DNA from fresh samples increased the success of PCR amplification and downstream processing. Thus, ideally, samples would be treated carefully to reduce breakage and analyses would be performed soon after sample collection.

To increase the resolution of relationships and to lend support to results presented in this thesis, it would be beneficial to investigate the phylogeography of *D. geminata* using additional molecular markers (Parker *et al.* 1998). This would require either development of *D. geminata*-specific primers for other nuclear, organellar or anonymous PCR-based markers discussed in Chapter 1, culturing of a single strain from a single isolated *D. geminata* cell, or DNA extraction from a single cell. Development of specific primers is a time-consuming, labour-intensive and potentially costly process. Culturing a single *D. geminata* strain requires strict precautions to prevent spread of this highly invasive species and is not possible for the majority of the samples, which are stored in ethanol and are no longer viable. Extracting DNA from a single cell may be the most cost and time effective method. While extraction from single *D. geminata* cells was met with some success (Appendix 4), this process needs optimization to increase the success rate. Furthermore, using DNA from a single cell as template for PCR requires costly whole genome amplification or the use of multi-copy genes, such as nuclear ribosomal DNA, as markers. As there is one chloroplast in *D. geminata*, only multicopy chloroplast RNA genes may provide enough template for PCR and these may not display enough useful genetic variation. Mitochondrial

markers may be more successful as there are potentially hundreds of mitochondria in cells (Avise 1998; Alverson 2008), but may not display enough variation for phylogeographic investigations.

One advantage of using DNA extracted from a single cell is that it enables analysis of intragenomic versus intrapopulation ITS variation. If there is intrapopulation polymorphism, there should also be intraindividual variation in a sexually reproducing population (Hillis & Davis 1988). However, there is no information on the rate of sexual reproduction of *D. geminata*, either in its natural or invaded habitats. While clonal reproduction is important for *D. geminata*, it can causes severe bottlenecks on introduction. The amount of variation present in the New Zealand *D. geminata* populations (Chapter 2) suggests that they are undergoing sexual reproduction. Alternatively, clonal populations may consist of variant complexes. Intragenomic ITS analysis may provide an estimate of the amount of sexual reproduction occurring within populations.

Looking to the future, with the recent development of new sequencing technologies, population studies may soon occur at the genome level, rather than the gene level. These new technologies enable thousands of small sequence reads at once and include 454 Life Sciences pyrosequencing, Solexa/Illumina 1G SBS (1 Gigabase 'sequencing by synthesis') sequencing and AB SOLiD (Applied Biosystems Sequencing by Oligonucleotide Ligation and Detection). Sequencing large amounts of whole populations, metagenomics, is rapidly becoming more feasible. Metagenomics is especially important in environmental genomics. From a molecular ecology viewpoint, genome sequencing will improve determination of population-level variation as well as polymorphisms associated with specific traits (Hudson 2008) such as sudden invasiveness or tolerance of an environmental variable. However, next generation sequencing technologies have drawbacks such as short sequence reads, which make *de novo* sequencing difficult. They also have less accuracy than Sanger sequencing, requiring greater coverage depth. Computational support for analysing such large data sets will be crucial. These new technologies will probably cause a shift from laboratory-

focussed research towards the bioinformatics of interpreting whole genome information (Hudson 2008).

3.3 Concluding Recommendations

Adaptive changes of native species in response to invasions can occur, which may explain why the effects of invasions can initially be extreme, only to be reduced over time since the first explosion of invasive spread (Strayer *et al.* 2006). Eventually, introduced species may become integrated into the native community. However, native populations may also undergo slow decline and loss in genetic variation, with potentially severe impacts on native organisms (Strauss *et al.* 2006a). Isolated endemic communities, such as those on islands like New Zealand, may not be able to adapt rapidly enough to prevent extinction, due to low genetic variation, small populations and limited habitat availability (Strauss *et al.* 2006a). This is especially the case for species that occupy similar niches to *D. geminata.* Alternatively, some communities may evolve so rapidly former functionality is restored quickly, masking genetic change through phenotypic maintenance. This can mask the potentially severe impacts of an invader, which become obvious in fragile, less robust communities. As *D. geminata* has been shown to shift the composition of species communities (Larned *et al.* 2007), limiting its spread is important for maintaining ecosystems in their natural state, especially when there are native and endemic species at risk. There are a number of endemic diatoms present in New Zealand (Kilroy *et al.* 2007) and as the full diversity of diatom communities are only starting to be recognised with the advent of molecular taxonomy, preserving these communities is important. New Zealand rivers also contain several endemic fish species, whose habitats may be severely altered by *D. geminata* (Larned *et al.* 2007).

Limiting the diversity of invasive *D. geminata* populations will be especially important if potential control mechanisms aim to include biological control. Biological control agents may be more suited to controlling species with

low diversity, because such species may possess less genetic potential to develop defence mechanisms (Dlugosch & Parker 2008). However, considering the large amount of diversity present in New Zealand populations, biological control agents may not be appropriate means of control, and there are currently no known natural enemies of *D. geminata*. Currently, the best means of control include regulating river flows and using Gemex^{TM}, a chelated copper formulation, to treat infected waterways (Larned *et al.* 2007; Shearer *et al.* 2008). The latter is only effective on small populations, highlighting the need for early detection of *D. geminata* in newly infected waterways.

The results of this study suggest that there is high genetic diversity of *D. geminata* in New Zealand, with admixture of variants from several locations in the Unites States, Canada and Norway. This increases the potential for new, more invasive variants to arise and for *D. geminata* to adapt to a wider range of environmental conditions. There is therefore a great need to continue the New Zealand Ministry of Agriculture and Forestry (MAF) publicity programme (Didymo Long-Term Management Programme 2007) and to enforce proper control to prevent new introductions of *D. geminata* variants, even in locations where it is already present. As *D. geminata* is found in popular trout fishing rivers, it is extremely important to ensure that anglers follow sufficient decontamination procedures (Kilroy *et al.* 2006) to prevent further mixing and spread of *D. geminata* variants. Banning the use of felt-soles waders in New Zealand from 1 October 2008 (Fish & Game New Zealand 2008) is therefore a step in the right direction. The fact that New Zealand rivers attract anglers from many overseas locations means that not only are New Zealand rivers in danger of receiving *D. geminata* cells from many locations, but also that it has a responsibility to help prevent *D. geminata* spreading to other locations worldwide.

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APPENIDCES

APPENDIX 1: *Didymosphenia geminata* Worldwide Distribution

Table A.1 (6): Description of international water bodies with *Didymosphenia geminata*. N=North. S = South. Mts = Mountains. R = River. L = Lake. Str = Stream.

Country	Region	Water Body	Survey Year	Dominant Genera	Water/Catchment Information	References
Austria	-	Enns R.	-	-	-	Acs et alin: Beltrami *et al.* 2008
Canada	-	Kooteni R.		*D. geminata*	Eutrophic	Holderman *et al.* (2004)
	N. Yukon Territory	Grande Caverne Glacée	1980s-1990s	*D. geminata*	Calcium-rich	Lauriol *et al.* (2006)
	Rocky Mts/Great Plains	Red Deer R.			Oligotrophic, 1 dam	Kirkwood *et al.* (2007)
	Rocky Mts/Great Plains	Upper Bow R.	1990s-2002	*D. geminata*	Nutrients, 5 dam	Kirkwood *et al.* (2007)
	St. Lawrence, Quebec	Matapedia	2006			Bothwell & Spaulding (2008)
	S.Saskatchewan, Alberta	Red Deer R. & Bow R.	2004-2005		Oligotrophic	Kirkwood *et al.* (2007)
	Vancouver Island	Heber River	1989			Bothwell *et al.* (2008)
China	Qinghai-Xizang Plateau (Qinghai Province)	Yangze R.	1906	*Gomphonemaceae, Cymbellaceae*	Unpolluted	Merechkowsky, cited in Li *et al.* (2003)
	Zhanjiakou region, Hebei (bordering Mongolia)	Shuiquan L., Huanggai L., Gonghui L.	1991		Saline (1.87-3.34 g L^{-1})	Wen & Zhi-Hui (1999)
Czech Republic	-	Moravka R.	-	-	Enriched, below Moravka R. reservoir	Gágyorová & Marvan (2002)
Finland	Lapland	Teno R.	-	*Tabellaria*, *Synedra*, *Achnanthes*, *Eunotia*, *Cymbella, Gomphonema, D. geminata.*	Oligotrophic	Komulaynen (2008).
Hungary, Ukraine, Romania	Danube Basin	Tisza R.	1991-2000	-	Eutrophic	Szabo *et al.* (2005)
Iceland	Borgarfjörður, W. Iceland	River Hvítá and others -	1990s	-	-	Jonsson *et al.*, cited in Spaulding & Elwell 2007

Country	Region	Water Body	Survey Year	Dominant Genera	Water/Catchment Information	References
India	Eastern and Western Himalayas	Kishanganga, Ravi, Teesta, Lachen Chhu & Lachung Chhu R.	2001-2003	*D. geminata* (Kishanganga & Ravi R.	Oligotrophic, Soft	Bhatt et al. (2008)
India	Jammu and Kashmir, Northwest Himalayas	Kishanganga R.	2001	*Cymbella*, *Achnanthes, Hannaea*	Unpolluted, glacial and snow fed.	Bhatt *et al.* (2005)
Iran	Central Alburz Mts.	-	2008	-	Limestone catchment -	Alimohammadian (2008)
Ireland	-	Many streams	-	-	-	Metzeltin & Lange-Bertalot 1995; Ní Chatháin *et al.* 2004.
Italy	-	Drava River/Rio Sesto Affluent	-	*Achnanthidium, Encyonema*	Oligotrophic	Beltrami *et al.* (2008)
	Alto Adige/Su¨ dtirol, Danube Basin	Drava River and Rio Sesto affluent	2006+2002	-	Oligotrophic	Beltrami *et al.* (2008)
	Piemonte, Lazio & Lombardia	-	1920s	-	-	Giaj-Levra & Abate, cited in Beltrami *et al.* (2008).
	Trentino	Several streams		-	Mesotrophic	Beltrami *et al.* 2007
Norway	-	Glama R.	1980s	*D. geminata*		Skulberg & Lillehammer, cited in Ellwood & Whitton 2007
	Trøndelag	Sokna R.		*D. geminata*	Oligotrophic	Biggs & Stokseth (1996)
	-	Tenojoki R.	2003	-	Olligotrophic	Miettinen (2006)
Poland	-	Vistula R.			Eutrophic, Saline	Kawecka & Sanecki (2003)
	Carpathian Mts.	San R.	1995	*Achnanthes, Cymbella, Gomphonema, Diatoma, Navicula*	Mesotrophic Hard – cal. & mag.	Kawecka & Sanecki (2003)
	Orawa	Czarna Orawa R.	-	*Navicula*, *Cymbella*, *Nitzschia*, *Cocconeis, Achnanthes*	Oligotrophic	Kawecka & Sanecki (2003)
	Orawa	Zubrzyca Str.	-	*Achnanthes, Cymbella, D. geminata*	Mesotrophic	Kawecka & Sanecki (2003)
Romania	Prut R. Basin	Prut R.	-	-	-	Cărăuş, 2003
	Siret R. Basin	Bistrita R., Bicaz Dam	-	-	-	Cărăuş, 2003
	Somes R. Basin	Doda Pilii R., Salauta R.	-	-	-	Cărăuş, 2003
Russia	Far East Primorsky	Dzhigitovka R.	1976-1982	*Gomphonema, Cymbella*	Oligotrophic	Medvedeva (2001)
	Far East Primorsky	Serebryanka R.	1976-1982	*Gomphonema, Cymbella*	Oligotrophic	Medvedeva (2001)
	Far East Primorsky	Sokhantinoe L.	1976-1982	*Gomphonema, Cymbella*	Oligotrophic	Medvedeva (2001)
	Republic of Karelia	Lizhma R.	-	-	-	Komulaynen (2006)
	Siberia	Baikal L.	1997-1998	*D. geminata on boulders near shore*	Only where currents resuspend sediments	Flower *et al.* (2004) Kravtsova *et al.* (2004)

Country	Region	Water Body	Survey Year	Dominant Genera	Water/Catchment Information	References
Russia	Siberia	Yenisei R. (Krasnoyarsk Hydroelectric Station)	2002-2006	*D. geminata*	High light +no riparian shade	Kolmakov *et al.* (2008)
	White Sea	Guba Chupa	2003	-	(in sub-ice water)	Ratkova & Wassman (2005)
Scotland	-	Moffat Water	-	-	-	Antoine & Benson-Evans (1984)
	-	Spittal of Shea	1915	-	-	Antoine & Benson-Evans (1984)
Serbia	Danube R. Basin	Danube R.	2004	-	-	Cadjo *et al.* 2004
Serbia & Montenegro		-	-	-	Slightly polluted	Szabo *et al.* (2005)
Switzerland (East)	Macun Lakes Region, N.Basin	Mezza-Gluna Str. inlet,	-	-	Groundwater & snowmelt	Robinson & Kawecka (2005)
	Macun Lakes Region, S.Basin	Sura Str. inlet, Inmez Str. inlet & outlet, Inmez L.	-	-	High Nitrogen, low Phosphorus, low organic matter. Glacier fed	Robinson & Kawecka (2005)
Turkey	Ardahan	Cildir L.	1991-1993	*Cyclotella, Aulacoseria*, *Navicula Melosira, Pinnularia*, *Gomphonema*, *Epithemia*, *Cymbella*, *Synedra, Fragilaria*	Oligotrophic, Lava-set	Akbulut & Yildiz (2002) (Didymo rare)
	Borçka-Artvin	Karagöl L.	2001-2002	*Cymbella*, *Navicula, Fragilaria*	Eutrophic	Kolayli & Şahin (2007)
	Sebinkarahisar-Giresun	Catal L.	2002	*Cymbella*, *Fragilaria*, *Navicula, Pinnularia*	Eutrophic	Şahin (2004)
	Trabzon	Sana R.	1995	*Cymbella*, *Didymosphenia*, *Gomphonema*, *Cocconeis*, *Navicula, Melosira*	Eutrophic	Kolayli *et al.* (1998)
	Trabzon	Yanbolu R.	2000-2001	*Melosira*, *Synedra*, *Cymbella*, *Ceratoneis, Amphora*	Polluted	Şahin (2003)
UK	-	Durley Beck	-	-	-	Antoine & Benson-Evans (1984)
	-	Haweswater	1963	--	-	Antoine & Benson-Evans (1984)
	-	Ulwin R.	1994	*Achnanthidium minutissimum, D. geminata*	-	Kelly (2003)
	N. Yorkshire	Stony Gill	-	-	Peat & limestone soils	Spaulding & Elwell (2007)
	Northumberland	Coquet R.	-	-	Peat & limestone soils-	Spaulding & Elwell (2007)
	River Tees catchment	-	-	-	-	Ellwood & Whitton (2007)
USA	Brooks Range	Blueberry Creek	1994	-	-	Edwardson *et al.* (2003)
	Idaho	Bonners Ferry	-	*D. geminata*, Cymbella	-	Holderman *et al.* (2004-2005)
	Intermontane Plateau, Alaskan interior	Tanana R.	1979-1983	-	-	LaPerriere (1989)
Wales	-	Afon Aran & Wye R.	1979-1981	-	-	Antoine & Benson-Evans (1984)

APPENDIX 2: New Zealand Surveys and Sample Collection

Site Selection

The surveys conducted from 2005-2007 in New Zealand aimed to determine the extent to which *D. geminata* is “present in sites where it is likely to have been introduced and become established”. Two assumptions underlying this question include that its principal means of spread has been via human vectors and that some sites are more suitable environments for the establishment of *D. geminata* than others (Duncan & Wilkins 2006). Rivers were sampled if they were visited by many people and if they had medium-high *D. geminata* habitat suitability (Duncan *et al.* 2005; Kilroy *et al.* 2005). Rivers were not sampled if *D. geminata* was established in them in order to reduce the chance of contaminating surveying equipment (Duncan 2006a).

From the May 2006 survey onwards (Duncan & Wilkins 2006), sites were chosen from a list compiled using information from the Ministry for the Environment, angling data and Kilroy *et al.*’s (2005) Likely Environments Map (LEM). Survey sites were selected using a ranking system based on several criteria, including number of visitors per year, water use activities, accessibility, likelihood of transfer, favorability of the environment, and social or cultural importance

Site Sampling

A reach of approximately 50 m, downstream from public access was surveyed at each site. Random samples of algae from rock scrapings were taken. Surveyors also collected site information, such as shade, water clarity, stream width, bed composition, water conductivity and a photograph of the reach sampled. Suspected *D. geminata* colonies were noted and sampled. If samples had to be stored they were placed in the fridge overnight but not frozen.

In August 2006, drift net sampling was included (Cary *et al.* 2006; Duncan 2006b). The drift net consisted of a large net made of 47 µm mesh. A 47 µm mesh netting square was spread over the sampling unit, which was screwed to the end of the net (Fig. A.1). A 500 µm and then a 250 µm pre-net filter was placed over the mouth of the net to reduce debris in the net. The net was attached to a pole and deployed in the upper half of the stream for 10 minutes in a fast flowing area, upstream of the benthic sampling region. The sampling unit was then unscrewed from the net and the sampled material and netting square were placed into a 110 mL Elkay sampling container. The container was tipped to one side and once the filtrate had settled, 2 mL of the concentrated suspended material was removed using a disposable pipette and placed into a 14 mL graduated falcon tube pre-filled with ethanol.

Figure A.1(19): Photograph of drift net used for sampling *D. geminata*. Taken from Cary *et al.* (2006).

Microscope Methods

Samples were concentrated by settling for up to 8 hours and then pouring or pipetting off excess water. 3 x 1 mL sub-samples were examined at x100 to x200 magnification using an inverted microscope. Cells containing

green chloroplasts were described as alive, while those lacking or with distorted chloroplasts were described as dead (Duncan & Wilkins 2006). A sample was positive if a subsample contained three live cells, or if the three subsamples examined contained at least five live cells. If most cells were dead or if only one or two cells were found then this was treated as a suspect positive and required re-sampling. A sample was defined as negative only if no cells were found in any subsamples (Duncan 2007).

Survey Detection Ability

The ability of these surveying methods to detect *D. geminata* cells was good. Very low cell levels were found, even when there were little algae visible at the site. At the Oreti River site, low levels of *D. geminata* were detected over 80 km downstream from any visible growth. The DNA detection method was found to be very sensitive and cost effective, enabling greater coverage of waterways (Duncan 2007).

Decontamination Methods

To prevent contamination of upstream regions, surveyors started upstream and worked downstream. Before moving to other water bodies, surveyors discarded disposable materials and washed all other equipment, including waders, in a 2% bleach solution for 1 min (Duncan *et al.* 2005). The drift net was deployed for 5-10 min before and after sampling to flush any cells out and before moving to a new site, nets were soaked in bleach for at least 1 min. When DNA detection methods were included, nets were soaked in at least 5% bleach for 15 min. In the summer 2006-2007 survey, further precautions were added to prevent cross-contamination. This included pre-labeling of sampling containers before going into the field, recording all drift net ID numbers on sample sheets for traceability and division of surveyors into two-man teams. One person performed “wet” duties, while a second performed “dry” duties (Duncan 2007).

Table A.2 (7): Summary of New Zealand South Island waterways positive for *Didymosphenia geminata* as at December 2007 (Duncan *et al.* 2007; Duncan 2006a; Duncan 2006b; Duncan 2007; Duncan & Wilkins 2006; Didymo National Delimiting Survey 2007). OPS=Ongoing Passive Surveillance (OPS).

Region	Waterbody	Month	Year	Detection Method
Canterbury	Waitaki River	January	2006	OPS+Survey
	Ahuriri River	February		OPS
	Ohau River	May		Survey
	Twizel River	May		Survey
	Omarama Stream	August		Survey
	Waihao River	November		Survey
	Tekapo River	February	2007	OPS
	Deep Creek	April		OPS
	Hurunui River	April		OPS
	North Opuha River	April		OPS
	Stoney Creek	April		OPS
	Tasman River	April		OPS
	Rangitata River	May		Survey
	Hakataramea	December		Survey
	Ohau	December		Survey
	Rakaia	December		Survey
Nelson	Buller River	September	2005	OPS
	Gowan River	May	2006	Survey
	Takaka River	January	2007	OPS
	Motueka River	February		Survey
	Matakitaki River	May		Survey
Otago	Clutha River	September	2005	OPS
	Hawea River	September		OPS
	Von River	October		Survey
	Lake Dunstan	November		OPS
	Fraser River	May	2006	OPS
	Matukituki River	August		Survey
	Lake Wakatipu	November		OPS
	Makarora River	November		OPS
	Motatapu River	November		OPS
	Wilkin River	November		OPS
	Diamond Creek	December		OPS
	Greenstone River	January	2007	Survey
	Kawarau River	January		Survey
	Lindis River	January		Survey
	Manuherikia	February		Survey
	Shotover River	April		OPS

Region	Waterbody	Month	Year	Detection Method
Otago	Cardrona River	May	2007	Survey
	Dart River	May		Survey
	Kakanui River	May		Survey
	Young River	May		Survey
	Hunter River	December		Survey
	Manuherikia	December		Survey
	Timaru River	December		Survey
Southland	Lower Waiau River	0ctober	2004	N/A
	Mararoa River	0ctober		N/A
	Oreti River	September	2005	OPS
	Upper Waiau River	September		OPS
	Lake Te Anau	November		OPS
	Lake Manapouri	November		Survey
	Aparima River	March	2006	OPS
	Upukerora River	May		Survey
	Princhester Creek	June		OPS
	Eglinton River	August		OPS
	Hamilton Burn	August		Survey
	Mataura River	August		Survey
	Hollyford River	November		OPS
	Whitestone River	December		OPS
	Snag Burn	January	2007	OPS
	Mystery Burn	January		OPS
	Ettrick River	January		OPS
	Point Burn	February		OPS
	Tunnel Burn	February		OPS
	Wairaurahiri River	February		OPS
	McIlvor Burn	March		OPS
	Loch Burn	March		OPS
Westland	Haast River	January	2007	Survey

Table A.3 (8): Survey details for New Zealand Surveys 2005-2007 (Duncan *et al.* 2007; Duncan 2006a; Duncan 2006b; Duncan 2007; Duncan & Wilkins 2006; Didymo National Delimiting Survey 2007).

Survey Date	No. Total Sites Selected	Sites Surveyed				No Sites Not sampled	Reasons not sampled	References
		North Island		South Island				
		No. of Sites	%	No. of Sites	%			
Oct 2005	492	386	78	106	22	20	dry, too deep, no access, unsuitable substrate	Duncan *et al.* (2005)
Jan 2006	119	38	30	87	70	6	remote	Duncan (2006a)
May 2006	400	139	35	261	65	14	river too high, wrong substrate, hard to access, *D. geminata* already identified, site duplication	Duncan & Wilkins (2006)
Aug 2006	108	53	48	55	51	1	River too high & substrate unsuitable	Duncan (2006b)
Nov 2006	130	65	41	65	59	21	Flooding of the Southern Alps	Duncan (2007)
Feb 2007	158	65	41	93	59	0	-	Duncan (2007)
May 2007	166	66	40	100	60	0	-	Duncan (2007)
Nov-Dec 2007	145	74	51	71	49	0	-	Didymo National Delimiting Survey (2007)

APPENDIX 3: Protocols

DNA Extraction

Sample preparation

1. Sample stored in 70% ethanol at -20 ℃ to preser ve cells.
2. Mix sample by vortexing tube 5 s.
3. Place 1 mL sample in 1.5 mL microcentrifuge tube using a disposable plastic pipette.
4. Centrifuge 10 000 rpm, 4 min.
5. Carefully discard alcohol supernatant using 1 mL pipette, without disturbing pellet.
6. Add 1 mL Milli-Q H_2O, Vortex to mix.
7. Centrifuge 10 000 rpm, 4 min.
8. Discard supernatant.

Kit Extraction

Invitrogen PureLink Genomic DNA Kit (Mammalian Tissue and Mouse/Rat Tail Lysate Protocol)

1. Add 180 mL digestion buffer. (Check digestion buffer for precipitation first. If precipitated warm up in 40 ℃ incubator for approx. 10 min).
2. Add 20 mL proteinase K and use pipette tip to mix sample. (vortexing is too harsh at this step).
3. Place in Eppendorf Thermomixer heating block 55 ℃ for 1-4 hrs (usually 3 hrs).
4. Centrifuge 13 200 rpm, 3 min.
5. Carefully remove supernatant. Discard tube in Virkon.
6. Add 20 mL RNAse A, vortex briefly, incubate 2 min.
7. Add 200 mL lysis/binding buffer and vortex.

8. Add 200 mL 100% ethanol and vortex 5 s.
9. Transfer lysate into a cartridge tube using 1 mL pipette.
10. Centrifuge 10 000 *g* (rcf) for 1 min.
11. Discard collection tube and place in new collection tubes.
12. Add 500 mL Wash Buffer 1.
13. Centrifuge 10 000 *g* 1 min.
14. Discard collection tube or discard Wash Buffer and reuse tube.
15. Add 500 mL Wash Buffer 2.
16. Centrifuge 13 200 rpm, 3 min.
17. Transfer cartridge to microcentrifuge tubes.
18. Add 45 mL elution buffer to centre of tube to elute DNA properly.
19. Close inside tube and incubate 1 min.
20. Centrifuge 13 200 rpm, 1 min (Elute only once!).
21. Store DNA at -20 ℃ .

Dr. Ray Curson's Non-Kit Based Extraction Method:

1. Add 350 µL lysis buffer and 10 µL Proteinase K (60 µg μL^{-1}).
2. Heat in thermoblock at 850 rpm and 55 ℃ for 2 h rs and/or 37 ℃ overnight.
3. Add 350 µL 5 M Lithium Chloride (LiCl).
4. Mix by vortexing 5 s.
5. Add 750 µL $CHCl_3$ (chloroform).
6. Mix 45 min on suspension mixer (rotating vertical wheel).
7. Centrifuge 13 200 rpm, 10 min.
8. Take aqueous layer and precipitate with equal volume isopropanol.

Stock Lysis Buffer	Volume	Final Conc.
7M Tris (pH 9)	5 mL	0.7 M
10% Sodium Laurel Sulphate (SDS)	5 mL	1%
0.5M EDTA (pH 8)	5 mL	0.05 M
5M NaCl	1 mL	0.1 M
Milli-Q H_2O	34 mL	-
Total	**50 mL**	**-**

	Volume	Final Conc.
10 µL x Proteinase K (60 µg μL^{-1})	350 µL	1.7 µg μL^{-1}
LiCl (5 M)	350 µL	1.2 M

PCR Conditions

Conventional PCR Master Mix (12.5, 25.0 or 50 µL reactions)

Master Mix	Initial Conc.	Volume (µL)	Final Conc.
PCR buffer(-$MgCl_2$)	10x	1.25	1 x
$MgCl_2$	25 mM	1.25	2.50 mM
dNTPs	1 mM	1.25	0.10 mM
BSA*	0.2 mg mL^{-1}	2.50	0.04 µg $µL^{-1}$
Forward primer	10 µM	0.25	0.20 µM
Reverse primer	10 µM	0.25	0.20 µM
Platinum *Taq*	1 u $µL^{-1}$	0.35	0.028 u
H_2O	-	4.40	-
Template DNA	1:1-1:1000**	1.00	-
Total		**12.5**	

*BSA = Bovine serum albumin

** Final DNA concentrations were diluted to approximately 1 ng $µL^{-1}$ (1:1 for band-stabbed nested PCRs, 1:10 for extractions, 1:1000 for nested PCRs).

QPCR Master Mix (12.5 µL reactions)

Master Mix	Initial Conc.	Volume (µL)	Final Conc.
PCR buffer(-$MgCl_2$)	10x	1.25	1 x
$MgCl_2$	50 mM	1.25	5.00 mM
dNTPs	2 mM	1.25	0.20 mM
BSA	0.2 mg mL^{-1}	5.0	0.08 µg $µL^{-1}$
Forward primer	6 µM	0.1	0.05 µM
Reverse primer	6 µM	0.63	0.30 µM
Probe	10 µM	0.4	0.32 µM
Platinum *Taq*	5 u $µL^{-1}$	0.1	0.04 u
H_2O	-	1.52	-
Template DNA	1:1-1:1000	1.0	-
Total		**12.5**	

PCR and Sequencing Primers – see Chapter 2.3 Materials and Methods

PCR Cycles

General DNA amplification cycle

	Step	Temp (℃)	Time
	Initial denaturation	94	3 min
40 cycles	Denaturation	94	30 s
	Annealing	60	30 s
	Extension	72	70-105 s
	Final Extension	72	5 min
		4	hold

Colony Screening PCR

	Step	Temp (℃)	Time
	Initial denaturation	94	2 min
36 cycles	Denaturation	94	45 s
	Annealing	50	20 s
	Extension	72	110 s
	Final Extension	72	7 min
		4	hold

Sequencing Cycle

	Step	Temp (℃)	Time
	Initial denaturation	96	1 min
35 cycles	Denaturation	96	10 s
	Annealing	50	10 s
	Extension	60	180 s
	Final Extension	72	10 min
		4	hold

QPCR Cycle

	Step	Temp (℃)	Time
	Initial denaturation	95	2 min
40 cycles	Denaturation	95	10 s
	Annealing	60	45 s
		4	hold

Touchdown PCR

Touchdown PCR started at 65 ℃ and was reduced by 1 ℃ after every two cycles, until 60 ℃ was reached, followed by 20 cycles at 60 ℃ .

	Step	Temp (℃)	Time
	Initial denaturation	94	4 min
2 cycles	Denaturation	94	30 s
	Annealing	65	30 s
	Extension	72	70 s
2 cycles	Denaturation	94	30 s
	Annealing	64	30 s
	Extension	72	70 s
2 cycles	Denaturation	94	30 s
	Annealing	63	30 s
	Extension	72	70 s
2 cycles	Denaturation	94	30 s
	Annealing	62	30 s
	Extension	72	70 s
2 cycles	Denaturation	94	30 s
	Annealing	61	30 s
	Extension	72	70 s
20 cycles	Denaturation	94	30 s
	Annealing	60	30 s
	Extension	72	70 s
	Final Extension	72	5 min
		4	hold

Temperature Gradient PCR

A different annealing temperature is programmed into the thermocycler for each tube column across the thermocycler.

PCR Cleanup Methods

ExoSAP-IT (USB Corporation, USA) cleanup

Per reaction:	0.1 µL SAP
	0.2 µL EXO
	2.7 µL Milli-Q H_2O
	10 µL PCR product
Thermocycler program:	37 ℃ 30 min
	80 ℃ 15 min
	4 ℃ hold

PCR kit cleanup

PCR cleanup using the column-based GenScript QuickClean 5M PCR Purification Kit was performed according to the manufacturer's instructions.

Isopropanol cleanup

Primers are not removed using ExoSAP-IT cleanup so this method can be used for further purification of PCR products.

1. Add 2 volumes of 70% isopropanol to 10 µL DNA.
2. Centrifuge 3000 rpm, 30 min.
3. Wash twice with 70% ethanol.
4. Centrifuge 3000 rpm, 5 min, for each wash.
5. Resuspend in 10 µL Milli-Q H_2O.
6. Incubate at room temperature 30 min.
7. Nanodrop or run on gel to check.

DNA and PCR Quality and Quantity Indicators

Nanodrop

1. Place 2 μL Milli-Q H_2O on lens and close. Click OK to BLANK.
2. Wipe.
3. Place Milli-Q H_2O/elution buffer on lens and close. Click REBLANK.
4. Wipe.
5. Place 2 μL sample on lens and close.
6. Enter sample name and click MEASURE.
7. After all samples done click SHOW REPORT, adjust as necessary.

Electrophoresis

Diagnostic gel:

- 1.5% agarose for DNA over 1000 bp.
- 1.0% agarose for DNA under 1000 bp.
- 3 μL Ladder.
- 5 μL DNA + 1.0 μL gel loading buffer (GLB).
- 56-90 V for 30-45 min.
- Ethidium Bromide (EtBr) stain (20 μL L^{-1}) 15 min.
- De-stain in water 5-10 min.
- UV Alpha Imager Capture.

Gel Extractions

Conventional Band Stabs

1. Run gel as above.
2. Place on UV box.
3. Cut tip of 100 µL pipette tip.
4. Turn on UV and stab target band with pipette tip.
5. Turn off light and remove tip from gel.
6. Expel gel from tip into 1.5 mL microcentrifuge tube.

Resuspending gel band stab:

7. Add 70 µL Milli-Q H_2O to tube.
8. Heat in heatblock at 65 ℃ , 350 rpm for 10 min, vortexing once.
9. Centrifuge 13 200 rpm, 5 min.
10. Transfer liquid to new tube.
11. Proceed to PCR.

Low Melting Point (LMP) Gel Band Stab (adapted from Ma & DiFazio 2008)

1. 10 µL PCR product loaded into 1.5% agarose gel.
2. Run 56V, 45 min.
3. EtBr Stain, 20 min.
4. De-stain in water, 20 min.
5. Cut square of gel out below gel loading buffer stain (GLB) and fill with 0.1% LMP agarose gel.
6. When agarose sets, run 56 V approx. 30 min, until GLB stain is at the bottom of the LMP gel square.
7. Place gel on UV box.
8. Cut bottom off 100 µL pipette tip.
9. Use pipette to suck up correct band and place in tube.
10. Run again on gel to check, Re-PCR or send directly to sequencing.

"Freeze and Squeeze' method" (Thuring *et al.* 1975)

Freeze and Squeeze gel:

1. Pool 3 x 50 µL PCR reactions + 22 µL Gel Loading Buffer.
2. Load into 0.7% gel.
3. 5 µL ladder.
4. Run 33-67 V for 1-1.5 hrs.
5. EtBr stain (20 µL L^{-1}) 20-30 min.
6. De-stain in water 15-20 min.
7. UV Alpha Imager Capture to check for position of bands (exposed to UV light for 2 sec).

DNA excision:

1. Place gel on UV base, put on protective headgear, switch off lights.
2. Use cover slip to slice band in gel (Keep UV light turned on for minimum amount of time to prevent DNA mutations).
3. Turn off UV light and complete cutting out gel slice.
4. Lift slice out of gel and place onto waiting, labelled parafilm rectangle .
5. Roll parafilm around slice lengthways.
6. Seal top end of rolled parafilm well.
7. Freeze at -20 °C for 30 min.
8. Place unsealed end in top of labelled 1.5 mL microcentrifuge tube.
9. Press thumb and forefinger together just under gel at the open end.
10. Squeeze gel from the top, folding down the parafilm as the gel shrinks.
11. The exudate collects in the tube.

Gel Extraction:

1. Add equal volumes chloroform 050 Amyl-alcohol 24:1 and phenol solution to total the amount of DNA solution squeezed from gel.
2. Vortex 15 s.
3. Centrifuge 13 200 rpm, 5-10 min.

4. Take supernatant and place in new tube (leaves behind protein and organic layer).
5. Add equal volume chloroform to DNA (to wash the phenol out).
6. Vortex 15 s.
7. Centrifuge 13 200 rpm, 5 min.
8. Take supernatant and place in new tube.
9. Repeat as necessary or spin in speed vacuum briefly to remove any traces of chloroform.
10. Weigh solution in tube.
11. Add 1/10 weight of 3 M Sodium Acetate and 0.6 times volume of Isopropanol.
12. Vortex 3 s.
13. To precipitate DNA, float in liquid nitrogen for 15 min or freeze at -20 ℃ overnight or until cleanup.

Gel Extraction Cleanup:

1. Pre-cool centrifuge to 4 ℃.
2. Centrifuge 13 200 rpm, 20-30 min.
3. Pour out supernatant.
4. Remove any remaining supernatant using pipette.
5. Wash in 1 mL ethanol pre-cooled at -20 ℃.
6. Centrifuge 13 200 rpm, 5 min at 4 ℃.
7. Pour out supernatant.
8. Centrifuge 5 s and remove remaining ethanol with pipette.
9. Leave to air dry or speed vacuum until remaining ethanol has evaporated.

Cloning

If using a large PCR product for cloning, secondary structure may inhibit efficiency. Therefore, the final PCR extension time was changed to 30 minutes to ensure extension proceeded to completion and the cleaned up gel extracted PCR product was heated at 65 ℃ for 10 min in a thermoblock or thermocycler. This was followed immediately by cloning. All samples were cleaned up directly before cloning, within 2 weeks of extraction.

Half reactions were used of TOPO plasmid and TOPO or Mach 1 cells (TOPO TA cloning Kit, Invitrogen, 2004).

Master Mix	Volume per half reaction (µL)
Salt Solution 1	0.5
TOPO Plasmid	0.5
Milli-Q H_2O	0.0
DNA (PCR product)	2.0
Total	**3.0**

1. Add PCR product to Master Mix. Incubate ROOM TEMPERATURE 30 min.
2. Set water bath to 42 ℃.
3. Put Luria–Bertani (LB)-kanamycin plates on bench to warm up.
4. In last 5 min of incubation, thaw cells ON ICE and flick to check thawed.
5. Add all ligation reaction to a half reaction of cells.
6. Flick by hand to mix.
7. Incubate ON ICE 30 min.
8. Heat shock by holding tubes in 42 ℃ water bath for exactly 30 s.
9. Place on ice immediately for 2 min.
10. Add 250 µL S.O.C. (Super Optimal broth with Catabolite repression) Medium.

11. Incubate 1 hr at 37 ℃ by taping horizontal on an orbital shaking incubator.
12. Plate whole reaction over 3 plates, spreading with a sterile, disposable plastic hockey stick.
13. Place in 37 ℃ incubator overnight (colonies should be visible after 12 hrs).

Re-spreading and Streaking

- Re-spreading: add approx. 500 mL NaCl buffer to each plate and transfer resulting colony suspension to new plates to test for plate quality (e.g. Kanamycin effectiveness).
- Streaking: growth of too many colonies for selecting single colonies for inoculation may require re-streaking. Scrape colonies with sterilised metal loop. Spread loop across new plate in 3 different directions, thereby diluting the concentration three times.

Screening colonies

- Use M13F & R or T7 & T3 PCR primers.
- Scrape part of a colony with a pipette tip. Place tip in a PCR tube containing standard master mix. Mix by pipetting up and down several times.
- Perform PCR using colony screening cycle conditions.

Innoculation

1. Add 20 µL of 50 mg mL^{-1} kanamycin to universal bottles containing 20 mL LB.
2. Grip one end of a sterile wooden scraper using sterilised tweezers.
3. Scrape the desired colony with the other end of the wooden scraper.
4. Drop scraper into LB broth in universal bottle.
5. Place in 37 ℃ shaking incubator, 180 rpm overnight.

6. Growth appears 12-24 hrs later.
7. Once showing sufficient optical density, do plasmid extraction.

Plasmid extraction

1. GenScript (GC) Quickclean 5M Miniprep Kit.
2. Place 1.5 mL overnight culture into a 1.5 mL microcentrifuge tube..
3. Centrifuge 12 000 rpm, 30 s.
4. Remove and discard supernatant (remove any remaining supernatant in tube by pipette).
5. Add 100 µL Solution 1 to pellet.
6. Cap tube and resuspend by scraping the base of the tube across the surface of the tube rack 5 times.
7. Add 200 µL Solution 2. Mix by inverting 4-6 times.
8. Add 300 µL Solution 3. Mix by inverting 4-6 times.
9. Centrifuge 12 000 rpm, 5 min.
10. Transfer supernatant to column.
11. Centrifuge 12 000 rpm, 5 min.
12. Remove and discard flow through.
13. Add 500 µL Wash Solution to the column.
14. Centrifuge 12 000 rpm, 5 min.
15. Remove and discard the flow through.
16. Repeat once.
17. Centrifuge 12 000 rpm, 30 s.
18. Transfer column to clean 1.5 mL microcentrifuge tube.
19. Place on clean paper towel to air dry for 5-10 min.
20. Add 50 µL Milli-Q H_2O to centre of column.
21. Let column stand for 2 min.
22. Centrifuge 13 200 rpm, 2 min.
23. Nanodrop.

NB: If concentration is too low for sequencing, place in speed vacuum on medium drying capacity 15-20 min.

Single Cell Extraction

Single Cell Isolation

1. Cells isolated with compound microscope by capillary action or mouth pipetting.
2. Hold a glass rod at the base of a Bunsen flame.
3. Use sterile tweezers to pull the end of the rod gently until the rod narrows to the desired width.
4. Remove from the flame.
5. Score one side of the glass rod with one stroke of a diamond pencil.
6. Break rod at this mark.
7. Hold rod end at the base of the flame to melt and smoothen the edges.
8. Attach rubber tubing to wide end of glass rod.
9. Place 20 µL sample in microscope slide well.
10. Draw cell and minimal liquid up glass rod through capillary action.
11. Blow cell into separate well containing sterile Milli-Q H_2O to rinse.
12. Repeat steps 10-11 to rinse a second time.
13. Blow cell into 0.6 mL microcentrifuge tube.
14. Examine inside of tube to confirm presence of cell.
15. Freeze -20 °C until use.

Immediately prior to PCR use one of the following extraction methods:

Freeze-Thaw (adapted from Henrichs *et al.* 2008)

1. Centrifuge 13 200 rpm, 30 s.
2. 3 cycles of: 1 min -80 °C freezer, 1 min 80 °C heat block.
3. 1 µL used directly as PCR template.
4. Remainder stored at -20 °C.

Proteinase K (2008adapted from Ki *et al.* 2004)

1. Add 1 µL 200 µg mL^{-1} Proteinase K.
2. Incubate in thermoblock 55 ℃ 50 min.
3. Incubate in thermoblock 95 ℃ 10 min to deactiva te Proteinase K.
4. Cool to 4 ℃ for PCR.

Chelex (adapted from Richlen & Barber 2005)

1. Add 100 µL of 10% Chelex suspension to tube containing single cell.
2. Vortex 5 s.
3. Centrifuge 13 200 rpm, 15 s.
4. Incubate 95 ℃, 20 min.
5. Vortex 5 s.
6. Centrifuge 13 200 rpm, 15 s.
7. Store at 2 ℃ until PCR.
8. Centrifuge 13 200 rpm, 15 s.
9. Use 1 µL supernatant for PCR.

Media Recipes

50 x TAE (Tris-acetate-EDTA) buffer (pH 8.3)

Milli-Q H_2O	500 mL
TRIS	242 g
Acetic Acid	57.1 mL
EDTA	18.5 g
Milli-Q H_2O	Adjust to 1000 mL

Electrophoresis buffer (1x TAE)

20 mL of 50 x TAE buffer in 980 mL H_2O

LB broth or agar

Trypticase Peptone	10 g
Yeast Extract	5 g
NaCl	10 g
(Agarose for agar)	(15 g)
Milli-Q H_2O	1 L

1L makes approximately 60 plates or 40 universals bottles.

Add kanamycin (50 µg mL^{-1} final concentration)

- Add 500 µL of 50 mg mL^{-1} kanamycin to 500 mL bottle of agar once cool enough to pour bottle.
- Add 20 µL of 50 mg mL^{-1} kanamycin to 20 mL LB broth in universal bottle prior to inoculation.

Agarose gel

1.5% = 1.5 g agarose in 100 mL TAE
1.0% = 1.0 g agarose in 100 mL TAE
0.7% = 0.7 g agarose in 100 mL TAE

Equipment

- Alpha Imager (Alpha Innotech Corporation)
- BioRad DNA Engine Peltier Thermal Cycler Model No:PTC-200
- BioRad gel electrophoresis chambers
- Corbett Life Science Rotor-Gene 6000
- DNA 120 SpeedVac Concentrator. Thermo Electron Corporation
- Eppendorf Centrifuge 5415D
- Eppendorf Centrifuge 5415R
- Eppendorf Mastercycler ep
- Eppendorf Thermomixer comfort (heat block)
- Heraeus Function Line Incubator (37 ℃)
- Microcentrifuge Model MC-210. The Griffin Group. Inc.
- NanoDrop Spectrophotometer ND-1000
- Nikon Digital Sight D5-UI
- Olympus System Microscope. Model No: BX51
- Olympus Japan SZ-ST stereo microscope
- Ratex Orbital Mixer Incubator
- Ratek suspension mixer
- ROSI 1000 Thermolyne Reciprocating/Orbital Shaking Incubator

Software

- Alpha Ease FC Software Version 4.1.0. Alpha Innotech Corporation
- Clustal W (Chenna *et al.* 2003)
- Geneious Pro v. 4.5 software package (Biomatters Ltd., New Zealand
    - http://www.geneious.com/.
- GIMP 2.6.4. GNU Image Manipulation Program.
    - http://www.gimp.org/.
- ImagePro Plus Version 5.1.2.59. 1993-2005 Media Cybernetics Inc.
- Inkscape v. 0.44.1
    - http://www.inkscape.org/.
- Model Test 3.7 (Posada & Crandall 1998)
- NanoDrop ND-1000 Version 3.5.2 produced by Coleman Technologies Inc for NanoDrop Technologies.
- PhyML (Guindon & Gascuel 2003).
    - Sever: http://atgc.lirmm.fr/phyml.
- PAUP* v. 4.0b10 (Swofford, 2003; Sinauer Associates)
- RNAalifold WebServer (Gruber et al. 2008)
- http://rna.tbi.univie.ac.at/cgi-bin/RNAalifold.cgi.
- WAR (Webserver for aligning structural RNAs) (Torarinsson & Lindgreen 2008
    - http://genome.ku.dk/resources/war/.
- yEd v. 2.4.2.2. Powered by yFiles Graph Visualisation Library. Copyright: yWorks GmbH, All Rights Reserved.
    - http://yWorks.com.

APPENDIX 4: Additional Results

PCR Optimization

Samples used in this project were collected from different environments and consist of a unique community of organisms and physical conditions. Thus, different factors, such as inhibitors, influence the extraction and amplification of DNA for each sample. Several techniques were trialled to improve amplification and to increase specificity. Techniques trialled included using a temperature gradient to determine the optimal PCR temperature, a touchdown PCR, PCR enhancers and adjusting concentrations of master mix components and template DNA concentrations. For all trials, negative and positive controls were used. The negative control consisted of Milli-Q H_2O in place of DNA and the positive control used sample DNA known to amplify well.

Identifying and Reducing Inhibition

Template DNA from samples amplifying poorly was spiked with DNA from a positive sample, which was known to amplify well. Amplification of the positive DNA using the 641F-ITS4R primer set was greatly reduced in the spiked samples compared to the positive sample and no amplification of the desired 1800 bp long product occurred (Fig. A.2). This demonstrates the presence of inhibitors in these samples. The effects of inhibition were less pronounced for the 150 bp long products and the correct sized amplification product was produced for all samples. Thus, the effects of inhibitors are greatest when amplifying large regions of DNA. Furthermore, some samples may have contained degraded DNA, as small regions amplified but large regions did not.

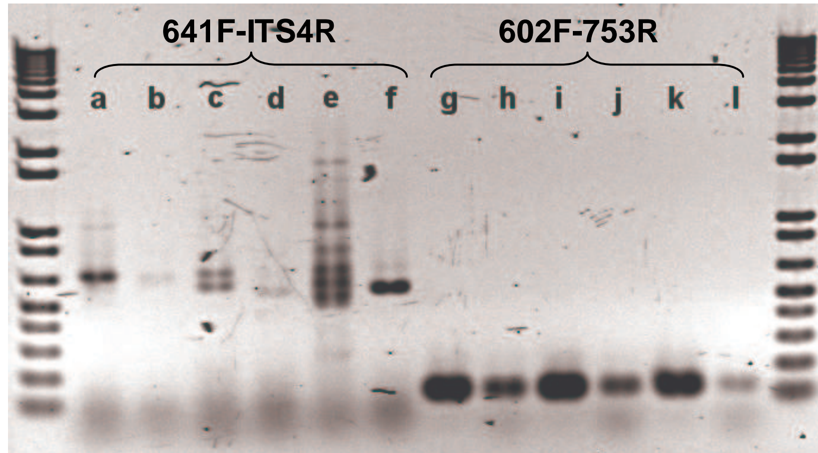

Figure A.2 (20): Gel comparing PCR products of small and large DNA regions using unspiked and spiked template DNA for two samples, VI4 and N1. (a, g) VI4, (b, h) N1, (c, i) VI4 spiked, (d, j) N1 spiked, (e, k) positive, (f, l) negative.

To reduce the effects of inhibition, samples were diluted 1:10 or 1:100 and the PCR enhancers DMSO, BSA and acetamide were trialled. Diluting DNA 1:10 or 1:100 (approx. 0.2-2.0 ng μL^{-1}) improved amplification. Adding acetamide (5% final concentration, Lange *et al.* 2002) to the master mix failed to produce amplification of all but the positive sample, as did adding DMSO (0-5% final concentration). Increasing BSA from a final concentration of 0.002 mg mL^{-1} to 0.04 mg mL^{-1} improved amplification, causing more samples to amplify and poorly amplifying samples to amplify better.

Reducing $MgCl_2$ from a final concentration of 5 mM to 2.5 mM improved PCR specificity slightly, as did reducing dNTPs from a final concentration of 5 mM to 2.5 mM and *Taq* from a final concentration of 0.04 U to 0.028 U. Final primer concentrations were reduced from 0.2 µM to 0.16 µM and 0.1 µM in an attempt to reduce the amount of primer dimers produced. However, this severely affected the amplification of the desired product (Fig. A.3) and thus the final primer concentrations were kept at 0.2 µM.

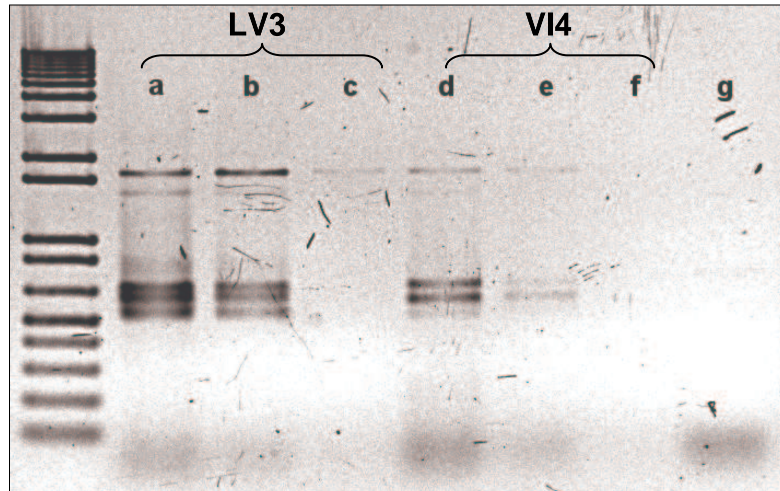

Figure A.3 (21): Gel showing effects of varying final PCR primer concentrations for two samples LV3 and VI4. (a, d) 0.2 µM μL^{-1} (b, e) 0.16 µM μL^{-1} (c, f) 0.10 µM μL^{-1} (g) negative 0.2 µM μL^{-1}

Four different *D. geminata*-specific forward primers (602F, 641F, 753F and 1659F) were trialled and some worked better than others did on different samples. The same PCR conditions and master mix were used for all primers, except for the extension stage, which was reduced from 105 s to 70 s for 1659F.

Temperature Optimization

PCR reactions were carried out across temperature gradients to find the optimal PCR annealing temperature for 602F, 753F and 1659F primers. An initial investigation using 602F at 50 ℃ and 60 ℃ showed better amplification at 60 ℃. A more detailed analysis showed that out of 58 ℃, 60 ℃ and 62 ℃, 60 ℃ produced the most amplification using 602F (Fig. A.4, a-f). 753F and 1659F primers were tested at 50 ℃, 55 ℃ and 60 ℃ and again 60 ℃ was found to produce the best amplification (Fig. A.4, g-r).

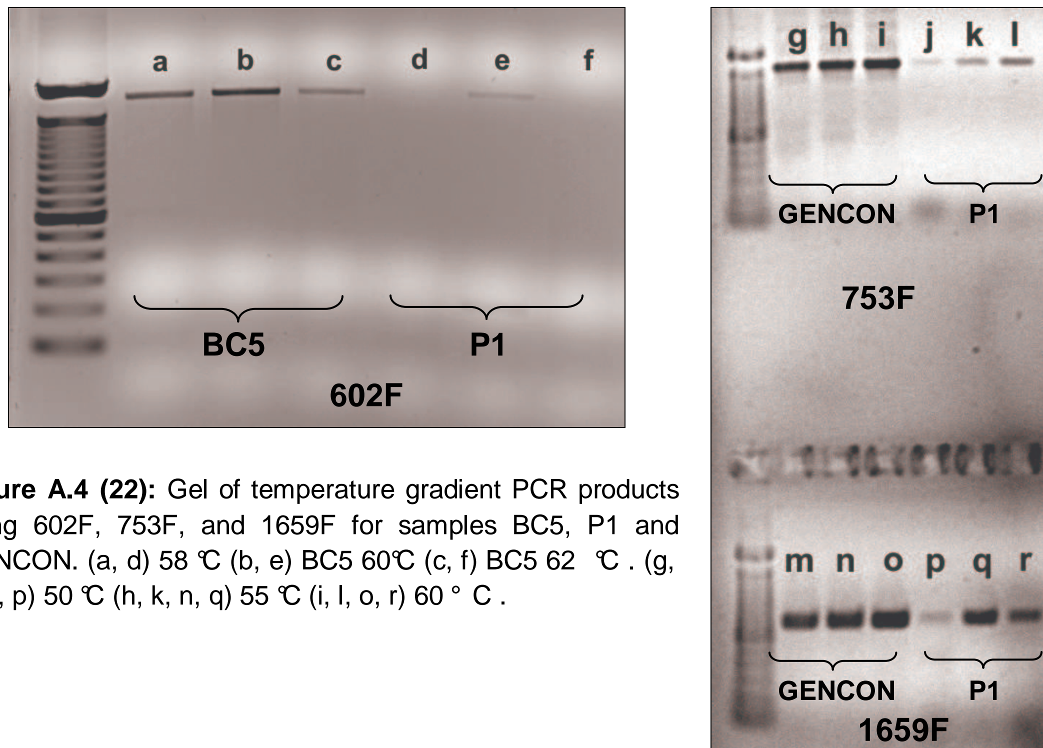

Figure A.4 (22): Gel of temperature gradient PCR products using 602F, 753F, and 1659F for samples BC5, P1 and GENCON. (a, d) 58 ℃ (b, e) BC5 60℃ (c, f) BC5 62 ℃ . (g, j, m, p) 50 ℃ (h, k, n, q) 55 ℃ (i, l, o, r) 60 ° C .

Touchdown PCR

PCR amplification produced multiple bands for many samples, even after temperature optimization. Obtaining a single PCR product was attempted using touchdown PCR (Appendix 3). This was met with limited success. Amplification of one sample produced a single band, a second sample showed two bands and other samples no longer amplified at all. Thus, touchdown PCR was not found to be an efficient method of obtaining PCR products for sequencing in this case.

Combination of Techniques Used to Obtain Sequences

Table A.4 (9): Table of methods and samples screened by Sarah Kelly with various forward primers and ITS4R, followed by cloning. ✓ = some amplification. ✔ = enough amplification for cloning. x = no amplification. **x** = enough amplification for cloning, but wrong PCR product. Y = gel DNA extraction performed.

Sample	PCR Primers					Nested PCR Primers				Gel DNA
ID	602	641	753	1659	EukA	602	641	753	1659	Extraction
40B	✔									Y
BC1	✓	✔		✓		✓				Y
BC12		✔								Y
CONN	✓	✔		✓		✓				Y
GENCON	✔	✓	✓	✓						Y
HA2	✔									
HA6	✔									
KR1		x			✓		x	✓	✔	Y
LV3	✓	✔	✓	✓				✓		Y
MAL		x			✓	✔				Y
N1	x	x		✓	✓	x	x	✔		Y
N6		y		✓		✔				Y
N7		x			x	x	x	x	✔	
N9		x		✔	✓	x	x	x		
P1		x	✓						✔	
PRS		x			✓	x	x	x	✔	Y
RC-2	✓				✓	✓	✓		✔	Y
V-2	✓	✔		✓						
VI4	x	✔		✓						Y
VIR		x		✔	x		x	x		
N4		x	x	x	✓	x	x	x	x	
ROB		X		✓	✓	x	x	x	**x**	

Table A.5 (10): Table of technician, extraction method, primers, presence of single or multiple PCR products and sequencing template used for each sample. CB=Catherine Bennett, SK=Sarah Kelly, TC=Tanya Chubb. Alt=Alternative non-kit-based DNA Extraction Method. F=Forward. C=cloned PCR product, P=direct PCR product.

Sample Code	Technician	DNA Extraction	Primers Used	PCR Products	Sequencing Template
40B	SK	Kit	602F-ITS4R	Multiple	C
BC1	SK	Kit	602F-ITS4R	Multiple	C
BC5	TC	Kit	602F-ITS4R	Single	C
BC12	SK	Alt	641F-ITS4R	Multiple	C
C1	TC	Kit	602F-ITS4R	Single	P
CONN	SK	Kit	641F-ITS4R	Multiple	C
GENCON	SK	Kit	602F-ITS4R	Multiple	C
HA2	SK	Alt	602F-ITS4R	Single	C
HA6	SK	Alt	602F-ITS4R	Single	C
IC1	CB	Kit	602F-1670R 1659F-ITS4R	Multiple	P
KR1	SK	Alt	EukA-ITS4R 1659F-ITS4R *(nested PCR)*	Multiple	C
LV1	CB	Kit	602F-1670R 1659F-ITS4R	Multiple	P
LV3	SK	Kit	641F-ITS4R	Multiple	C
LW1	TC	Kit	602F-ITS4R	Single	C
LW2	CB	Kit	602F-1670R 1659F-ITS4R	Multiple	P
LW3	CB	Kit	602F-1670R 1659F-ITS4R	Single	P
MAL	SK	Kit	EukA-ITS4R 602F-ITS4R *(nested PCR)*	Multiple	C
N1	SK	Alt	EukA-ITS4R 753F-ITS4R *(nested PCR)*	Multiple	C
N2	TC	Kit	602F-ITS4R	Single	P
N3	CB	Kit	602F-1670R 1659F-ITS4R	Multiple	P
N6	SK	Kit	EukA-ITS4R 602F-ITS4R *(nested PCR)*	Multiple	C

Sample Code	Technician	DNA Extraction	Primers Used	PCR Products	Sequencing Template
N7	SK	Alt	EukA-ITS4R 1659F-ITS4R *(nested PCR)*	Single	C
N9	SK	Alt	1659F-ITS4R	Single	C
OR2	CB	Kit	602F-1670R 1659F-ITS4R	Single	P
OR3	TC	Kit	602F-ITS4R	Single	P
P1	SK	Kit	EukA-ITS4R 1659F-ITS4R *(nested PCR)*	Single	C
PRS	SK	Alt	EukA-ITS4R 1659F-ITS4R *(nested PCR)*	Multiple	C
RC1	CB	Kit	602F-1670R 1659F-ITS4R	Multiple	P
RC2	SK	Alt	EukA-ITS4R 1659F-ITS4R *(nested PCR)*	Multiple	C
UO2	CB	Kit	602F-1670R 1659F-ITS4R	Single	P
UO3	CB	Kit	602F-1670R 1659F-ITS4R	Multiple	P
UW1	CB	Kit	602F-1670R 1659F-ITS4R	Single	P
UW2	CB	Kit	602F-1670R 1659F-ITS4R	Single	P
VI2	SK	Kit	641F-ITS4R	Single	C
VI4	SK	Kit	641F-ITS4R	Multiple	C
VIR	SK	Kit	1659F-ITS4R	Single	C
WE1	CB	Kit	602F-1670R 1659F-ITS4R	Multiple	P

To summarize Tables A.4 and A.5, of the 37 samples processed, 20, 12 and 5 samples were processed by Sarah Kelly, Catherine Barnett and Tanya Chubb respectively. 9 samples required using Dr. Ray Curson's extraction method rather than the kit-based method. 20 samples produced multiple amplification products. 22 samples were cloned as opposed to being sequenced directly. 24, 5, 1 and 7 samples were amplified using 602F, 641F, 753F and 1659F forward primers respectively.

Post-PCR DNA Processing

Most samples contained multiple PCR products of different sizes, which prevented direct sequencing (Table A.5). Where this occurred, the PCR product of the desired size was excised from electrophoresis gel and used for cloning. Eight samples required repeated amplification of PCR products directly or after band stabbing of the desired PCR product from the electrophoresis gel to obtain enough product for cloning (Table A.4, Nested PCR column).

Band Stabbing

It was expected that band stabbing the desired PCR product from an electrophoresis gel (Appendix 3) and reamplification of this DNA would provide a single product of the desired size that could be used for sequencing. However, the same banding patterns occurred when reamplifying the band stabbed DNA as had been present in the original PCR product. This means that the extra bands were probably caused from mispriming or polymerase skipping along the DNA template. Template skipping is especially likely to occur if strong secondary structure, such as that found in the ribosomal DNA used in this project, causes the *Taq* polymerase to shift to DNA on a paired strand. This skips out the loop section of DNA in between the two paired strands. Secondary folding can also cause the amplified DNA molecules to move differently through the gel and it inhibits cloning and sequencing reactions.

Some samples produced small amounts of amplified DNA, which could be due to very low levels of *D. geminata* DNA in the samples. In this case, the PCR product was run on a gel and the desired product was band stabbed. The DNA was extracted from the gel (Appendix 3) and reamplified using the original primers. Band-stabbed samples reamplified better when DMSO (7% final concentration) was used (Fig. A.5). Once again, 60 ℃ was the best annealing temperature for these reactions (Fig. A.5).

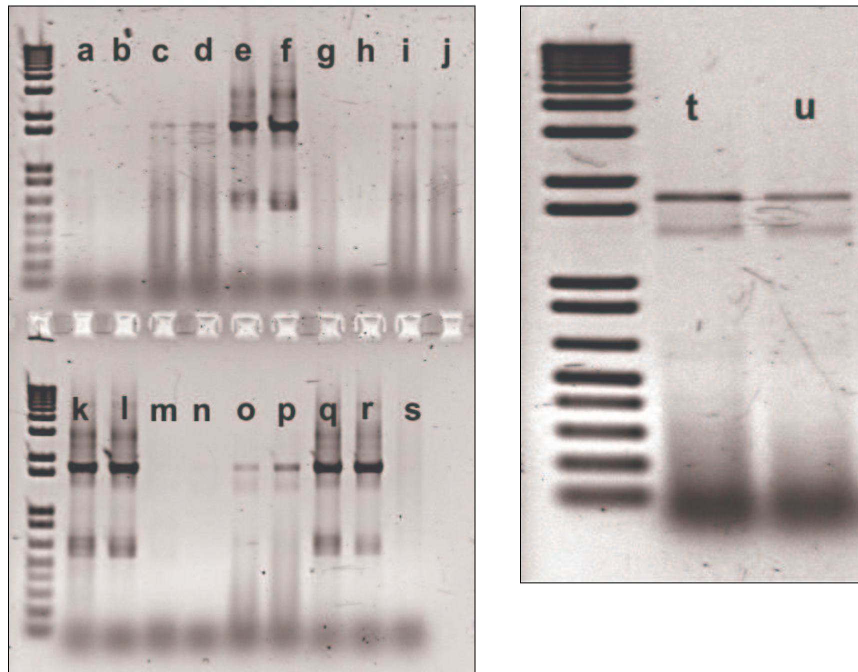

Figure A.5 (23): Gels showing effects of DMSO levels on PCR amplification for extracted BC-1 DNA (a, b, g, h, m, n), positive DNA (e, f, k, l, q, r) and band stabbed BC1 DNA (c, d, i, j, o, p, t, u) at 50 ℃ (a, c, e, g, i, k, m, o, q), 60 ℃ (b, d, f, h, j, l, n, p, r, t, u), 0% DM SO (a-f), 1% DMSO(g-l), 5% DMSO (m-s), 7% DMSO (t), 10% DMSO (u), (s) negative.

Nested PCRs

Other samples were amplified using ITS4R and the eukaryote primer EukA, found at the beginning of the 18S rDNA region. This increased the amount of DNA of the desired region, which was then amplified using *D. geminata*-specific primers to select for *D. geminata* rDNA. The template of the second PCR consisted of the first PCR product diluted 1:1000 or 1:10 000,

Gel Extraction for Cloning

In order to obtain DNA product of the desired size for cloning, large PCR reactions (3 x 50 µL) were performed and run on 0.7% agarose gels. The ”freeze and squeeze” gel extraction technique was performed to extract DNA from the gel (Appendix 3). DNA was concentrated and washed immediately before cloning. Heating the DNA at 65 ℃ for 10

minutes immediately before cloning improved cloning efficiency of the large products. Gel extraction using a kit (GenScript 5M DNA Gel Extraction Kit) was less efficient than using the “Freeze and Squeeze” method described above.

Two samples (RC2 and KR1) failed to clone successfully. When run on a gel it was apparent that the DNA had changed following gel extraction. Instead of two bands close together being visible as in the samples that cloned successfully (BC12 and BC1, Fig. A.6i c, d), only the two lower bands were visible (Fig. A.6i a, b). This may mean that secondary folding had proceeded to completion for these samples, completely inhibiting cloning.

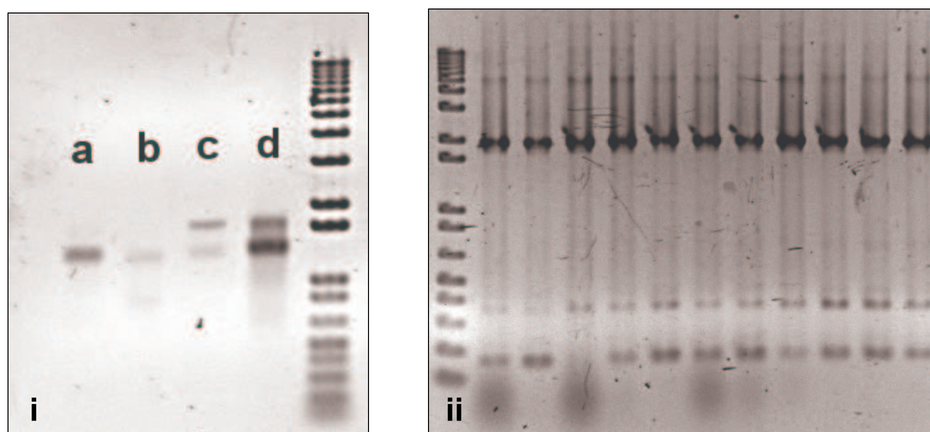

Figure A.6 (24): Gels showing post-gel extraction band migration of (i) the two lower bands only of RC2 (a) and KR1 (b) post-gel extraction as opposed to both bands of BC12 (c) and BC1 (d)) and (ii) results of screening all 11 colonies from cloning GENCON.

After cloning, 3-11 colonies were screened using M13F and M13R or the PCR primers. 100% of the screened colonies from most samples had the correct size insert (Fig. A.6ii). On average, 12 transformants were obtained for long (1800 bp) PCR products and thousands of transformants were obtained for short (800 bp) PCR products. Colonies with short inserts required re-streaking on new plates in dilution series to obtain single colonies for screening, inoculation and sequencing (Appendix 3). 90% of the sequenced PCR inserts were orientated in the forward direction in the plasmid.

Low melting point gel band stabs

A method of band stabbing DNA from low melting point (LMP) gel (Ma & DiFazio 2008) was trialled (Appendix 3). This method would enable sequencing or cloning of products separated by size on a gel and would be less time-consuming than the “Freeze and Squeeze” method. PCR products were separated on a 1.5% gel and stained with ethidium bromide (Fig. A.7 a). They were then allowed to run into LMP 0.1% gel and sucked up using a pipette (Fig. A.7 b). Some of the removed liquid was run on a gel and the correct band appeared. Reamplfication of this PCR product had limited success and direct sequencing was not successful. Successful amplification of the PCR product only worked within 24 hours of band stabbing and thus the DNA must degrade quickly. Thus, this method was only used to remove the small band from the 40B sample and reamplify it to determine its sequence.

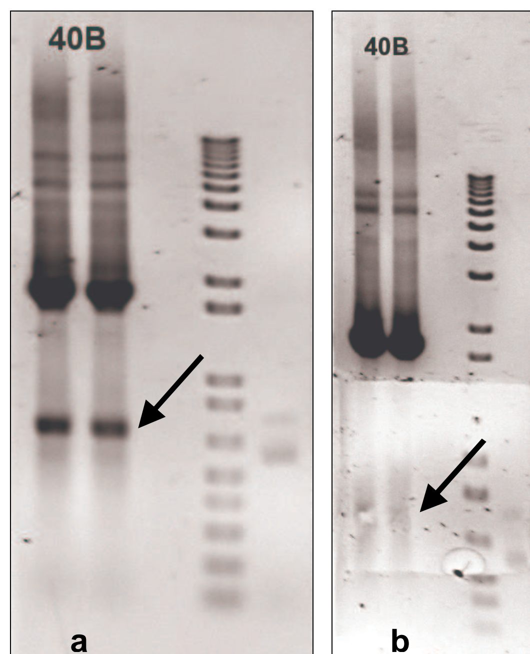

Figure A.7 (25): Low Melting Point DNA extraction gel. (a) 1.5% Gel with PCR product of a 40B plasmid extract amplified with 602F, arrow pointing to the EtBr stained smaller amplification product. (b) Gel with square cut out and filled with LMP (0.1%) gel and the smaller band removed by pipette.

Extra PCR Amplification Band Investigation

The most prominent extra band produced by PCR of the long 18S-ITS2 region using all three early 18S forward primers (602F, 641F and 753F) was approximately 650 bp. This band appeared in nearly all samples containing multiple bands. To investigate the nature of this PCR product, it was removed from LMP gel as described above and reamplified with 602F-ITS4R. This was successful but was not sequenced successfully due to using elution buffer rather than Milli-Q H_2O during kit cleanup. It was then reamplified using 1659F-ITS4R and the sequenced product was identical to the ITS region from the ITS sequence of the long PCR product of the same sample. However, the QPCR detection technique was also used on this amplified product and a positive identification occurred even though the QPCR primer and probe sites lie outside of the ITS region. Thus, it is still not clear exactly what this band is. It is most likely produced by polymerase jumping from the initial primer site to the beginning of the ITS region due to secondary folding of the 18S DNA region.

Testing Specificity of Primers

Specificity of the *D. geminata*-specific primers was tested on *Gomphoneis minuta* var *cassiae,* a native New Zealand species believed to be closely related to *D. geminata*. Very faint amplification of products occurred for all primers (Fig. A.8), but none of these products were the size expected for amplification of *D. geminata* DNA. In fact, these products were almost identical in size to those produced by amplification using the general ITS5F-ITS4R primers. Thus, it is unlikely that *D. geminata*-specific primers amplify *Gomphoneis* DNA causing it to be mistaken as *D. geminata* DNA. However, analyses in this project have revealed that *D. geminata* is probably more closely related to members of Cymbellaceae and it may be important to test these primers on DNA from *Cymbella* species.

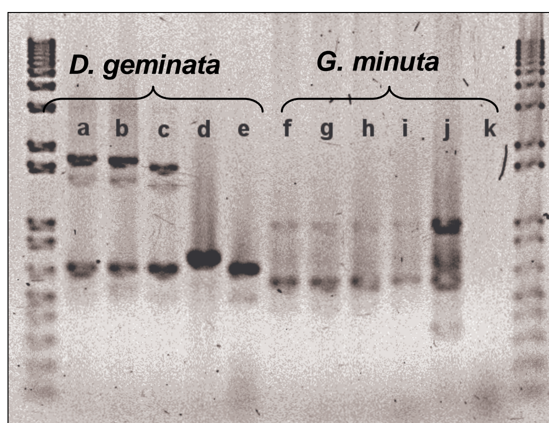

Figure A.8 (26): Gel showing tests of *D. geminata*-specific forward primers and ITS4R on a *D. geminata* sample (GENCON) and *Gomphoneis minuta* var. *cassiae*. The general forward primer ITS5 was included for comparison. (a, f) 602F (b, g) 753F (c, h) 641F (d, i) 1659F (e-j) ITS5F (k) negative.

Single Cell Extraction

It would be ideal to amplify DNA extracted from a single *D. geminata* cell. This may reduce the need for cloning when culturing a strain from a single cell is not possible, as DNA would come from a single individual. This would also enable identification of intragenomic ITS variants if they occur in *D. geminata*. No single cell extraction has been successfully employed for diatom cells as their silica shells make such extraction more difficult.

Several techniques (Appendix 3) developed for dinoflagellate species, which have polysaccharide shells, were tested on single *D. geminata* cells. The proteinase K protocol may have amplified the smaller but not the bigger PCR product (Fig. A.9 b), although the negative was contaminated by a PCR product of similar size. The Chelex protocol amplified the large product well (Fig. A.9 a), as did the freeze-thaw method (Fig. A.9 c). However, the quantity of PCR product produced was not enough for sequencing and reamplification was not attempted at this time. Later, amplification following single cell extraction was unsuccessful.

Thus, these techniques need further development as they could provide a powerful tool for genetic analysis of unculturable organisms. Pursuing a

Chelex protocol may be most successful in this case. Iwatani *et al.* (2005) used a similar protocol to amplify DNA from ten diatom *Thalassiosira nordenskioeldii* cells, with an additional incubation step at -20℃ prior to incubation at 95℃. Single cell extraction would remove the need for species-specific primers, enabling amplification of other markers (e.g. the mitochondrial cytochrome oxidase subunit 1 (*cox1*) gene) for *D. geminata*.

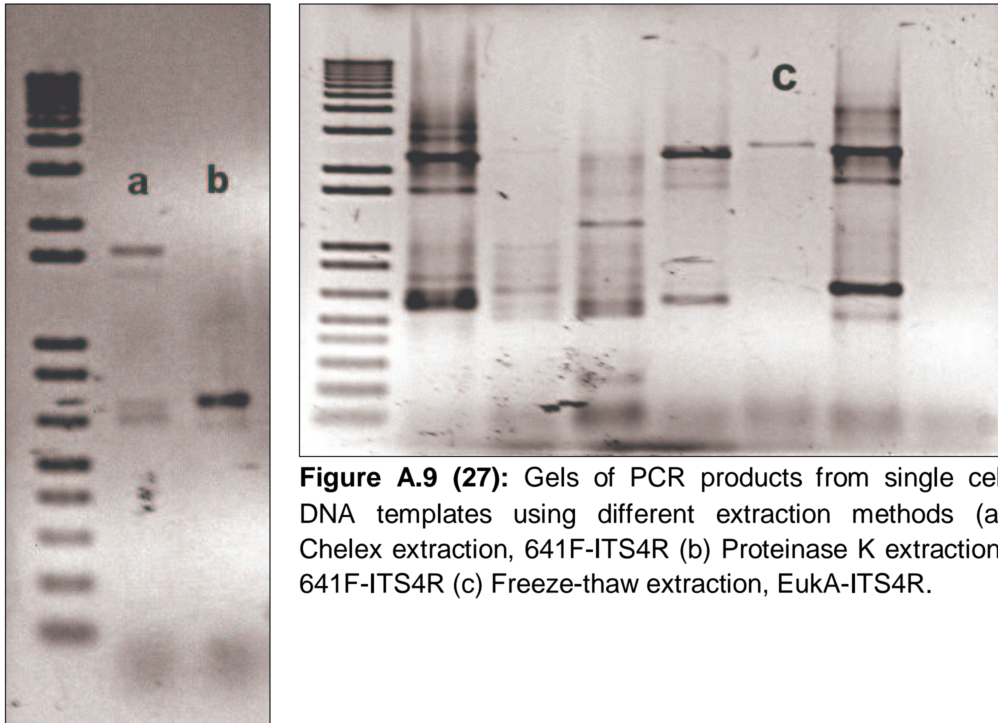

Figure A.9 (27): Gels of PCR products from single cell DNA templates using different extraction methods (a) Chelex extraction, 641F-ITS4R (b) Proteinase K extraction, 641F-ITS4R (c) Freeze-thaw extraction, EukA-ITS4R.

QPCR Detection of *D. geminata*

The *D. geminata* QPCR detection method was performed as described by Cary *et al.* (2007) on samples where amplification of the large 18S-ITS2 region was not working, in order to ensure that they were positive for *D. geminata*. Results showed that most samples, including P1, were positive for *D. geminata*. N4 and N9 were just below threshold. No N4 cells could be identified by microscopy and amplification with the 1659F primer was unsuccessful. However, N9 amplified with the 1659F primer and *D. geminata* cells were positively identified by microscopy. The 18S rDNA may have degraded for this sample.

18S and ITS Alignments

Table A.6(11): Output of 610 bp ITS sequence alignment of 46 *D. geminata* sequences. Dots = bases identical to the consensus sequence. Letters = differences in sequences to the consensus sequence. Dashes = gaps. ITS regions highlighted in grey.

	1 – 65
	ITS1
ITS consensus	**TTACCACACAATAATCCAAACCGCGCTTACATTAAACCCATAGGCTCTGGCC---CTGCTAACTA**
Canada Kootenai R. 1	..................G.................................---..........
Canada Matapedia R.	....---..........
Canada Vancouver Is. 2	....---..........
Canada Vancouver Is. 4	....---.A........
Iceland Laxa R. 1	....---..........
Iran Havir R. 6	....ATC.G........
Iran Tar R. 2	....ATC.G........
NZ Buller R. .c11	....---..........
NZ Buller R. .c12	....---..........
NZ Buller R. .c14	....---..........
NZ Buller R. .c16	....---..........
NZ Buller R. .c3	....---..........
NZ Buller R. .c7	....---..........
NZ Buller R. .c8	....A...........---..........
NZ L. Waiau R. 1a	....---..........
NZ L. Waiau R. 1b	....---..........
NZ L. Waiau R. 2	....---..........
NZ L. Waiau R. 3	....---..........
NZ Mararoa R. .c1	....---..........
NZ Mararoa R. .c3	....---..........
NZ Oreti R. 2	....---..........
NZ Oreti R. 3	....---..........
NZ U. Oreti R. 2	....---..........
NZ U. Oreti R. 3	....---..........
NZ U. Waiau R. 1	....---..........
NZ U. Waiau R. 2	....---..........
Norway Atna R. 2	....---..........
Norway Dokka R. 7	....---..........
Norway Nidelva R. 1	....---..........
Norway Sandvik. R. 9	....---..........
Norway Tisleia R. 6	....---..........
Norway Vinstra R. 3	....---..........
Poland Dunajec R.	.......................A.T....TCA..........-..G.T...---....C...AC
UK C-1	....---..........
USA Boulder Cr. 1	....---..........
USA Boulder Cr. 12	.....................................T..............---..........
USA Boulder Cr. 5a	....---..........
USA Boulder Cr. 5b	....---..........
USA Conneticut R.	....---..........
USA Lee Vining Cr. 1	....---..........
USA Lee Vining Cr. 3	....---..........
USA Prosser Cr.	....---..........
USA Rapid Cr. 1	....---..........
USA Rapid Cr. 2	....---..........
USA Vancouver Is. R	....---..........
USA Wenatchee R.	....---..........

	66 – 130
ITS consensus	**GTGGT--------AAACCCCTCTGTAACCCATCGAGTCTCTCTGGTGTTTACGA-CCTTATAAGA**
Canada Kootenai R. 1	.....--------...-..........
Canada Matapedia R.	.....--------...-..........
Canada Vancouver Is. 2	.....--------...-..........
Canada Vancouver Is. 4	.....--------...-..........
Iceland Laxa R. 1	.....--------...-..........
Iran Havir R. 6	.....--------G........C........................A......-..........
Iran Tar R. 2	.....--------G.................................A......-..........
NZ Buller R. .c11	.....--------.......................................A.-..........
NZ Buller R. .c12	.....--------...-..........
NZ Buller R. .c14	.....--------.......................................A.-..........
NZ Buller R. .c16	.....--------...-..........
NZ Buller R. .c3	.....--------...................T.....................-..........
NZ Buller R. .c7	.....--------...-......-...
NZ Buller R. .c8	.....--------...-..........
NZ L. Waiau R. 1a	.....--------...-..........
NZ L. Waiau R. 1b	.....--------...........C.............................-..........
NZ L. Waiau R. 2	.....--------.......................................R.-..........
NZ L. Waiau R. 3	.....--------.......................................R.-..........
NZ Mararoa R. .c1	.....--------...-..........

```
NZ Mararoa R. .c3       ....--------..........................................-..........
NZ Oreti R. 2           ....--------........................................R.-..........
NZ Oreti R. 3           ....--------........................................A.-..........
NZ U. Oreti R. 2        ....--------........................................R.-..........
NZ U. Oreti R. 3        ....--------........................................R.-..........
NZ U. Waiau R. 1        ....--------..........................................-..........
NZ U. Waiau R. 2        ....--------..........................................-..........
Norway Atna R. 2        ....--------..........................................-..........
Norway Dokka R. 7       ....--------........................................A.-..........
Norway Nidelva R. 1     ....--------..........................................-..........
Norway Sandvik. R. 9    ....--------..........................................-..........
Norway Tisleia R. 6     ....--------..........................................-..........
Norway Vinstra R. 3     ....--------..........................................-..........
Poland Dunajec R.       A...TAATAATT..TGAAT.A...............................T.C..........
UK C-1                  ....--------..........................................-..........
USA Boulder Cr. 1       ....--------..........................................-..........
USA Boulder Cr. 12      ....--------..........................................-..........
USA Boulder Cr. 5a      ....--------..........................................-..........
USA Boulder Cr. 5b      ....--------..........................................-..........
USA Conneticut R.       ....--------..........................................-..........
USA Lee Vining Cr. 1    ....--------..........................................-..........
USA Lee Vining Cr. 3    ....--------..........................................-..........
USA Prosser Cr.         ....--------........................................A.-..........
USA Rapid Cr. 1         ....--------........................................R.-..........
USA Rapid Cr. 2         ....--------..........................................-..........
USA Vancouver Is. R     ....--------..........................................-..........
USA Wenatchee R.        ....--------..........................................-..........

                        131                                                           195

ITS consensus           TCTCATTATGTGC-CACTGCCGTGATGATGCAACCTGGAGGCCAACGAGTACGATCCACCAATTT
Canada Kootenai R. 1    .............-...................................................
Canada Matapedia R.     .............-...................................................
Canada Vancouver Is. 2  .............-...................................................
Canada Vancouver Is. 4  .............-...................................................
Iceland  Laxa R. 1      .............-...................................................
Iran Havir R. 6         .............-..TA........A...........TA.........................
Iran Tar R. 2           .............-..TA....................TA.........................
NZ Buller R. .c11       .............-...................................................
NZ Buller R. .c12       .............-...................................................
NZ Buller R. .c14       .............-.................G.................................
NZ Buller R. .c16       .............-...................................................
NZ Buller R. .c3        .............-...................................................
NZ Buller R. .c7        .............-...................................................
NZ Buller R. .c8        .............-...................................................
NZ L. Waiau R. 1a       .............-...................................................
NZ L. Waiau R. 1b       .............-...................................................
NZ L. Waiau R. 2        .............-...................................................
NZ L. Waiau R. 3        .............-...................................................
NZ Mararoa R. .c1       .............-...................................................
NZ Mararoa R. .c3       .............-......T............................................
NZ Oreti R. 2           .............-...................................................
NZ Oreti R. 3           .............-...................................................
NZ U. Oreti R. 2        .............-...................................................
NZ U. Oreti R. 3        .............-...................................................
NZ U. Waiau R. 1        .............-...................................................
NZ U. Waiau R. 2        .............-...................................................
Norway Atna R. 2        .............-...................................................
Norway Dokka R. 7       ....G........-.................G.................................
Norway Nidelva R. 1     .............-...................................................
Norway Sandvik. R. 9    .............-...................................................
Norway Tisleia R. 6     .............-...................................................
Norway Vinstra R. 3     .............-...................................................
Poland Dunajec R.       .............C..T.......................-TG......................
UK C-1                  .............-...................................................
USA Boulder Cr. 1       .............-...................................................
USA Boulder Cr. 12      .............-...................................................
USA Boulder Cr. 5a      .............-...................................................
USA Boulder Cr. 5b      .............-...................................................
USA Conneticut R.       .............-...................................................
USA Lee Vining Cr. 1    .............-...................................................
USA Lee Vining Cr. 3    .............-...................................................
USA Prosser Cr.         .............-.................G.................................
USA Rapid Cr. 1         .............-...................................................
USA Rapid Cr. 2         .............-...................................................
USA Vancouver Is. R     .............-...................................................
USA Wenatchee R.        .............-...................................................

                        196                                                           260
                                                                    5.8S
ITS consensus           AAATTCAATCGACAGCTTGACGAGAGTCAAGTTTAAAAGTATAACATTCCAATACAACTTTCAGC
Canada Kootenai R. 1    .................................................................
Canada Matapedia R.     ...................G.................G...........................
Canada Vancouver Is. 2  .................................................................
Canada Vancouver Is. 4  .................................................................
Iceland  Laxa R. 1      ...................R.............................................
```

Sample	Sequence
Iran Havir R. 6	...................G...
Iran Tar R. 2	...................G...
NZ Buller R. .c11	....
NZ Buller R. .c12	....
NZ Buller R. .c14	....
NZ Buller R. .c16	...............................A.................................
NZ Buller R. .c3	....
NZ Buller R. .c7	....
NZ Buller R. .c8	....
NZ L. Waiau R. 1a	....
NZ L. Waiau R. 1b	....
NZ L. Waiau R. 2	....
NZ L. Waiau R. 3	....
NZ Mararoa R. .c1	....
NZ Mararoa R. .c3	....
NZ Oreti R. 2	....
NZ Oreti R. 3	....
NZ U. Oreti R. 2	....
NZ U. Oreti R. 3	....
NZ U. Waiau R. 1	....
NZ U. Waiau R. 2	....
Norway Atna R. 2	...................R...
Norway Dokka R. 7	....
Norway Nidelva R. 1	......................G..
Norway Sandvik. R. 9	...................G...
Norway Tisleia R. 6	....
Norway Vinstra R. 3	....
Poland Dunajec R.	....
UK C-1	........G..
USA Boulder Cr. 1	....
USA Boulder Cr. 12	....
USA Boulder Cr. 5a	....
USA Boulder Cr. 5b	....
USA Conneticut R.	....
USA Lee Vining Cr. 1	....
USA Lee Vining Cr. 3	....G....
USA Prosser Cr.	....
USA Rapid Cr. 1	....
USA Rapid Cr. 2	....
USA Vancouver Is. R	....T....................
USA Wenatchee R.	....

	261 ... 325
ITS consensus	**AATGGATGTCTTGGTTCCCAACACAATGAAGAACACAGCGAAATGTGATACGTAATACGAATTGC**
Canada Kootenai R. 1	....
Canada Matapedia R.	....
Canada Vancouver Is. 2	....
Canada Vancouver Is. 4	......................................T..........................
Iceland Laxa R. 1	....
Iran Havir R. 6	....
Iran Tar R. 2	....
NZ Buller R. .c11	....A.
NZ Buller R. .c12	.........................G.......................................
NZ Buller R. .c14	....
NZ Buller R. .c16	....
NZ Buller R. .c3	....A..
NZ Buller R. .c7	....
NZ Buller R. .c8	....
NZ L. Waiau R. 1a	....
NZ L. Waiau R. 1b	....
NZ L. Waiau R. 2	....
NZ L. Waiau R. 3	....
NZ Mararoa R. .c1	....
NZ Mararoa R. .c3	....
NZ Oreti R. 2	....
NZ Oreti R. 3	....
NZ U. Oreti R. 2	....
NZ U. Oreti R. 3	....
NZ U. Waiau R. 1	....
NZ U. Waiau R. 2	....
Norway Atna R. 2	....
Norway Dokka R. 7	....
Norway Nidelva R. 1	....
Norway Sandvik. R. 9	....
Norway Tisleia R. 6	....
Norway Vinstra R. 3	....
Poland Dunajec R.	....G....
UK C-1	....
USA Boulder Cr. 1	....
USA Boulder Cr. 12	....
USA Boulder Cr. 5a	....
USA Boulder Cr. 5b	....
USA Conneticut R.	....
USA Lee Vining Cr. 1	....
USA Lee Vining Cr. 3	....
USA Prosser Cr.	....

```
USA Rapid Cr. 1          .................................................................
USA Rapid Cr. 2          .................................................................
USA Vancouver Is. R      .................................................................
USA Wenatchee R.         .................................................................
```

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                         326                                                           390
ITS consensus            AAGACTTTGTGAATCATTGAATTTTTGAACGCATATTGCGCTGCCAGATTCTTTCTGGGAGCATA
Canada Kootenai R. 1     .................................................................
Canada Matapedia R.      ..................................................T..............
Canada Vancouver Is. 2   ...........................................T.....................
Canada Vancouver Is. 4   ..................................................T..............
Iceland  Laxa R. 1       ..................................................T..............
Iran Havir R. 6          ..................................................T..............
Iran Tar R. 2            ..................................................T..............
NZ Buller R. .c11        .................................................................
NZ Buller R. .c12        .................................................................
NZ Buller R. .c14        .................................................................
NZ Buller R. .c16        .................................................................
NZ Buller R. .c3         .................................................................
NZ Buller R. .c7         .................................................................
NZ Buller R. .c8         .............................T...................................
NZ L. Waiau R. 1a        .................................................................
NZ L. Waiau R. 1b        .................................................................
NZ L. Waiau R. 2         .................................................................
NZ L. Waiau R. 3         .................................................................
NZ Mararoa R. .c1        .................................................................
NZ Mararoa R. .c3        .................................................................
NZ Oreti R. 2            .................................................................
NZ Oreti R. 3            .................................................................
NZ U. Oreti R. 2         .................................................................
NZ U. Oreti R. 3         .................................................................
NZ U. Waiau R. 1         .................................................................
NZ U. Waiau R. 2         .................................................................
Norway Atna R. 2         ..................................................T..............
Norway Dokka R. 7        .................................................................
Norway Nidelva R. 1      .................................................................
Norway Sandvik. R. 9     ..................................................T..............
Norway Tisleia R. 6      .................................................................
Norway Vinstra R. 3      ..................................................T..............
Poland Dunajec R.        ........................A.........................T..............
UK C-1                   ..................................................T..............
USA Boulder Cr. 1        .................................................................
USA Boulder Cr. 12       .................................................................
USA Boulder Cr. 5a       .................................................................
USA Boulder Cr. 5b       .................................................................
USA Conneticut R.        .................................................................
USA Lee Vining Cr. 1     .................................................................
USA Lee Vining Cr. 3     .................................................................
USA Prosser Cr.          .................................................C...............
USA Rapid Cr. 1          .................................................................
USA Rapid Cr. 2          .................................................................
USA Vancouver Is. R      .................................................................
USA Wenatchee R.         .................................................................
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```
                         391                                                           455
                                         ITS2
ITS consensus            CACGACAGAGTATCATCAAACCCCAATCTATAACATTATTTGTTATGGGCAAGTGAAGTTGGCTT
Canada Kootenai R. 1     .............T...................................................
Canada Matapedia R.      ......................................CG.........................
Canada Vancouver Is. 2   .................................................................
Canada Vancouver Is. 4   .................................................................
Iceland  Laxa R. 1       .............................R........YK.........................
Iran Havir R. 6          ............................C.........CG.........................
Iran Tar R. 2            ............................C.........CG.........................
NZ Buller R. .c11        .................................................................
NZ Buller R. .c12        .................................................................
NZ Buller R. .c14        .................................................................
NZ Buller R. .c16        ...........T.....................................................
NZ Buller R. .c3         .................................................................
NZ Buller R. .c7         .................................................................
NZ Buller R. .c8         .................................................................
NZ L. Waiau R. 1a        .................................................................
NZ L. Waiau R. 1b        .................................................................
NZ L. Waiau R. 2         .................................................................
NZ L. Waiau R. 3         .................................................................
NZ Mararoa R. .c1        .................................................................
NZ Mararoa R. .c3        .................................................................
NZ Oreti R. 2            .................................................................
NZ Oreti R. 3            ...............................................................C.
NZ U. Oreti R. 2         .................................................................
NZ U. Oreti R. 3         .................................................................
NZ U. Waiau R. 1         .................................................................
NZ U. Waiau R. 2         .................................................................
Norway Atna R. 2         .............................R........C..........................
Norway Dokka R. 7        .................................................................
```

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Norway Nidelva R. 1         .................................................................
Norway Sandvik. R. 9        ......................................CG.........................
Norway Tisleia R. 6         .................................................................
Norway Vinstra R. 3         .............................G...................................
Poland Dunajec R.           ...............................G.......G...C.....................
UK C-1                      .............................G...................................
USA Boulder Cr. 1           .................................................................
USA Boulder Cr. 12          .................................................................
USA Boulder Cr. 5a          .................................................................
USA Boulder Cr. 5b          .................................................................
USA Conneticut R.           .................................................................
USA Lee Vining Cr. 1        .................................................................
USA Lee Vining Cr. 3        .................................................................
USA Prosser Cr.             .................................................................
USA Rapid Cr. 1             .................................................................
USA Rapid Cr. 2             .................................................................
USA Vancouver Is. R         .................................................................
USA Wenatchee R.            .................................................................

                            456                                                           520

ITS consensus               TAAACAAAGCACTCCTTCTAAGTTATTTAGTGCAGAGGCATTTTCACATTGTAGGAAACTCAGTG
Canada Kootenai R. 1        ......................................T..........................
Canada Matapedia R.         ...T.................................A...........................
Canada Vancouver Is. 2      ......................................T..........................
Canada Vancouver Is. 4      ...........................................A.....................
Iceland  Laxa R. 1          ...W.................................AY..........................
Iran Havir R. 6             .T...............................................................
Iran Tar R. 2               .T...............................................................
NZ Buller R. .c11           ......................................T..........................
NZ Buller R. .c12           .................................................................
NZ Buller R. .c14           ......................................T..........................
NZ Buller R. .c16           .................................................................
NZ Buller R. .c3            .................................................................
NZ Buller R. .c7            .................................................................
NZ Buller R. .c8            .................................................................
NZ L. Waiau R. 1a           .................................................................
NZ L. Waiau R. 1b           .................................................................
NZ L. Waiau R. 2            ......................................Y..........................
NZ L. Waiau R. 3            ......................................Y..........................
NZ Mararoa R. .c1           .................T...............................................
NZ Mararoa R. .c3           ......................................T..........................
NZ Oreti R. 2               ......................................Y..........................
NZ Oreti R. 3               ......................................T..........................
NZ U. Oreti R. 2            ......................................Y..........................
NZ U. Oreti R. 3            ......................................Y..........................
NZ U. Waiau R. 1            ......................................Y..........................
NZ U. Waiau R. 2            ......................................Y..........................
Norway Atna R. 2            ...W.................................A...........................
Norway Dokka R. 7           ...........................C..........T..........................
Norway Nidelva R. 1         ................................................................R
Norway Sandvik. R. 9        ...T.................................A...........................
Norway Tisleia R. 6         ......................................T..........................
Norway Vinstra R. 3         .....................................AT..........................
Poland Dunajec R.           ..........................C.........T............................
UK C-1                      .....................................AT..........................
USA Boulder Cr. 1           .................................................................
USA Boulder Cr. 12          ...............................................G.................
USA Boulder Cr. 5a          .................................................................
USA Boulder Cr. 5b          .................................................................
USA Conneticut R.           C................................................................
USA Lee Vining Cr. 1        .................................................................
USA Lee Vining Cr. 3        .................................................................
USA Prosser Cr.             ......................................T..........................
USA Rapid Cr. 1             ......................................Y..........................
USA Rapid Cr. 2             .................................................................
USA Vancouver Is. R         ......................................T..........................
USA Wenatchee R.            ......................................Y..........................

                            521                                                           585

ITS consensus               TATTTGTTTCAACGTGCTATTGGTCAAAATTCACTTTTTGACTGTCTTCTTAACTGATCTCTGGC
Canada Kootenai R. 1        ..............................................................A..
Canada Matapedia R.         ...........................G...T........G........................
Canada Vancouver Is. 2      .................................................................
Canada Vancouver Is. 4      ............................G....................................
Iceland  Laxa R. 1          ...........................R...Y........R........................
Iran Havir R. 6             ........................T........................................
Iran Tar R. 2               ........................T........................................
NZ Buller R. .c11           .................................................................
NZ Buller R. .c12           ...............................................................A.
NZ Buller R. .c14           .................................................................
NZ Buller R. .c16           ...............................................................A.
NZ Buller R. .c3            ...............................................................A.
NZ Buller R. .c7            ...............................................................A.
NZ Buller R. .c8            ...............................................................A.
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NZ L. Waiau R. 1a      ..............................................C................A.
NZ L. Waiau R. 1b      ...............................................................A.
NZ L. Waiau R. 2       ...............................................................R.
NZ L. Waiau R. 3       ...............................................................R.
NZ Mararoa R. .c1      ...............................................................A.
NZ Mararoa R. .c3      ........................................................G........
NZ Oreti R. 2          ...............................................................R.
NZ Oreti R. 3          .................................................................
NZ U. Oreti R. 2       ...............................................................R.
NZ U. Oreti R. 3       ...............................................................R.
NZ U. Waiau R. 1       ...............................................................R.
NZ U. Waiau R. 2       ...............................................................R.
Norway Atna R. 2       ...........................G..T..................................
Norway Dokka R. 7      .................................................................
Norway Nidelva R. 1    .......................................................R.......A.
Norway Sandvik. R. 9   ...........................G..T........G.........................
Norway Tisleia R. 6    .................................................................
Norway Vinstra R. 3    .................................................................
Poland Dunajec R.      .................................................................
UK C-1                 .................................................................
USA Boulder Cr. 1      ...............................................................A.
USA Boulder Cr. 12     ...............................................................A.
USA Boulder Cr. 5a     .................................................................
USA Boulder Cr. 5b     .................................................................
USA Conneticut R.      .................................................................
USA Lee Vining Cr. 1   .................................................................
USA Lee Vining Cr. 3   .................................................................
USA Prosser Cr.        ............................C....................................
USA Rapid Cr. 1        ...............................................................R.
USA Rapid Cr. 2        ...............................................................A.
USA Vancouver Is. R    .................................................................
USA Wenatchee R.       ...............................................................R.

                       586                   610

ITS consensus          TGTGTAGGACTACCCGCTGAATTTA
Canada Kootenai R. 1   .........................
Canada Matapedia R.    .........................
Canada Vancouver Is. 2 .........................
Canada Vancouver Is. 4 .........................
Iceland  Laxa R. 1     .........................
Iran Havir R. 6        .........................
Iran Tar R. 2          .........................
NZ Buller R. .c11      .........................
NZ Buller R. .c12      .........................
NZ Buller R. .c14      .........................
NZ Buller R. .c16      .........................
NZ Buller R. .c3       .........................
NZ Buller R. .c7       .........................
NZ Buller R. .c8       .........................
NZ L. Waiau R. 1a      .........................
NZ L. Waiau R. 1b      .........................
NZ L. Waiau R. 2       .........................
NZ L. Waiau R. 3       .........................
NZ Mararoa R. .c1      .........................
NZ Mararoa R. .c3      .........................
NZ Oreti R. 2          .........................
NZ Oreti R. 3          .........................
NZ U. Oreti R. 2       .........................
NZ U. Oreti R. 3       .........................
NZ U. Waiau R. 1       .........................
NZ U. Waiau R. 2       .........................
Norway Atna R. 2       .........................
Norway Dokka R. 7      .........................
Norway Nidelva R. 1    .........................
Norway Sandvik. R. 9   .........................
Norway Tisleia R. 6    .........................
Norway Vinstra R. 3    .........................
Poland Dunajec R.      .........................
UK C-1                 .........................
USA Boulder Cr. 1      .........................
USA Boulder Cr. 12     .........................
USA Boulder Cr. 5a     .........................
USA Boulder Cr. 5b     .........................
USA Conneticut R.      .........................
USA Lee Vining Cr. 1   .........................
USA Lee Vining Cr. 3   .........................
USA Prosser Cr.        .........................
USA Rapid Cr. 1        .........................
USA Rapid Cr. 2        .........................
USA Vancouver Is. R    .........................
USA Wenatchee R.       .........................
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Table A.7 (12): Output of 1113 bp alignment of 61 diatom sequences. Letters = differences in sequences to the consensus sequence. Dashes = gaps. Grey shading= *D. geminata* sequences. Yellow shading = *D. geminata*-specific primer sites. Red shading = primer names.

	1 … 60
Primers	***602F:*** **GTGATGGAATTTGAA** ***641:*** **TCAGAA-ACTGTCATCC**
18S Consensus	**GTGGTCGTATTTGACTGCC- ------TTTTTTGGTGT--- --TTCTGGGGACTGCCATCC**
Achnanthes brevipes	....CT..CG.G.G.G...- TAGCAC.CAG.GCCA.CGCT --C.GCCC.CGTC.......
Achnanthes minutissima	.....GAC.G...TGGT..A -------.......AC--- --.AAC.ATTGT........
Achnanthidium minutissimum	.....GAC.G...TGGT..- ------A.......AC--- --.AAC.ATTGT........
Amphiprora alata	...C.T..TCAC.-A..T.C TT------...G-..ACG-- --...GT..ATG...-....
Amphora montana	.....-.GTCCCTGAG.T.C GAT--T----CC...AC--- --..T...............
Amphora pediculus	....AT.-..C.TGAG.T.C ACG--T...G.....AC--- --.AT..A.ATTGT......
Asterionella formosa	.....T.G.GCAACG.-G.C CTTCAC..G.G.T.GTGCTT --G.T..TCTC.GT......
Cocconeis pediculus	.....T.ATCGCTGGAT..T CT-----..AGA..C..CC- --G.G...TTG--A......
Cocconeis placentula	....AT.CTCACTTGGT..T C-------..GA..C.CCA- --G.G......--T......
Craticula molestiformis	.....GCGC..GCGGC.T.C AT---T.AC..G-..TCT-- --GC.GT..TCGC.......
Cylindrotheca closterium	.....GTAC....G.G...- -CG-TCAC.A.GT.ATGGAG C-..GCT.AAGTC.......
Cymatopleura elliptica	...CAT..CCC..TA..T.C AT--AT.G...G-...CC-- --...AA...CG...-....
Cymbella affinis	.....A..T...AGA.TT.- ---------..C..A----- --....TT.A.........T
Cymbella aspera	.....G.A.....CT..T.- -------...CATAGA.G-- --AG..TCA-T..A....G.
Cymbella cymbiformis	...--GTA.C..TCAGC--- GG---TCA....T.AC---- --....CTA.GTAA.....T
Cymbella lanceolata	A...AACG.AC..CGGT..- --------....T.G.---- --...AAT.AGT.-T...TT
Cymbella proxima	...A.G.A..C...A....- ---------....A.GC.A-- --..TG.AC-..C.T....T
Cymbopleura naviculiformis	.....G...CC...A....- ------A.....T.GT.C-- --AGTCA..TG...T....T
D. geminata Canada Matapedia R.	...A.G.A......A.A.-- --------....AAA..G-- -T...A.AA-....T....T
D. geminata Canada Vancouver Is. 2	..A.AA-....T.....
D. geminata Canada Vancouver Is. 4	..A.AA-....T.....
D. geminata Iran Havir R. 6	...A.G.A......A.A.-- --------....AAA..G-- -T...A.AA-....T....T
D. geminata Iran Tar R. 2	...A.G.A......A.A.-- --------....AAA..G-- -T...A.AA-....T....T
D. geminata NZ Buller R. .c14	...A.G.A......A.A.-- --------....AAA..G-- -T...A.AA-....T....T
D. geminata NZ L. Waiau R. 2	...T....T
D. geminata NZ L. Waiau R. 3	...A.AA-....T....T
D. geminata NZ Lee Vining Cr. 3	..A.AA-....T.....
D. geminata NZ Mararoa R. .c3	...A.G.A......A.A.-- --------....AAA..G-- -T...A.AA-....T....T
D. geminata NZ Oreti R. 2	....T....T
D. geminata NZ U. Oreti R. 2	....T....T
D. geminata NZ U. Oreti R. 3	AA-....T....T
D. geminata NZ U. Waiau R. 1	...T....T
D. geminata NZ U. Waiau R. 2	....T....T
D. geminata Norway Atna R. 2	
D. geminata Norway Nidelva R. 1	
D. geminata Norway Tisleia R. 6	...A.G.A......A.A.-- --------....AAA..G-- -T...A.AA-....T....T
D. geminata UK C 1	
D. geminata USA Boulder Cr. 1	...A.G.A......A.A.-- --------....AAA..G-- -T...A.AA-....T....T
D. geminata USA Boulder Cr. 5a	...A.G.A......A.A.-- --------....AAA..G-- -T...A.AA-....T....T
D. geminata USA Conneticut R.	...A.AA-....T.....
D. geminata USA Lee Vining Cr. 1	...A.AA-....T....T
D. geminata USA Rapid Cr. 1	....T....T
D. geminata USA-Boulder Cr. 12	..A.AA-....T.....
D. geminata NZ L. Waiau R. 1a	...A.G.A......A.A.-- --------....AAA..G-- -T...A.AA-....T....T
D. geminata NZ Oreti R. 3	...A.G.A......A.A.-- --------....AAA..G-- -T...A.AA-....T....T
Diadesmis gallica	....CG..TC.CATGGT..C AA-----....G....CC-- ---GT....AG.A.......
Encyonema caespitosum	.....G.-...GTGG..T.C ------.C.......C.--- --CG.CAC.AT.........
Encyonema minutum	....CG.-...GTGGC.T.C ------.C.......TC--- --.GTCAC.AT.........
Encyonema triangulatum	.....T.-...G..GA.T.C ------.C.......AC--- --..TCAT.AT.A.......
Entomoneis cf. alata	...TGTACCCC..-A..T.C TT------...G-..ACG-- --..TG....TG...-....
Eolimna subminuscula	.....GCG.C.GCGGC.T.C AT---T.A...G-..TCT-- --GC.GT..TTG........
Eunotia formica	....CA.GCG.C.G.G...- CGACAC.G.G.GT.G..GCC --.GT...C.C.-.......
Fragilaria delicatissima	...---.ATGGGC.G.-.TG GTTCGCCC......GCGGTA CT.GGGCA.CT.GT......
Frustulia vulgaris	....CG.CG-CCTGG..T.C A---AT..A..G-..ACTA- --.--C...TG.........
Gomphoneis minuta var cassiae	...A.GA.GC...GGA...- ------.C......AT.--- --CCT.TA-C.T..T....T
Gomphonema acuminatum	....CT..GC...GGA...A ---------....T.GA.T-- --CCT.AA.C..G......T
Gomphonema affine	....CA..GC.C..G....- --------.....T.GACG-- --C....A.C..A.......
Gomphonema cf. angustatum	....C...G..CA.G....- -------A....T.GA.G-- --C.TC.AAC..A.......
Gomphonema cf. parvulum	....CA..GC.C..G....- --------.....T.GACA-- --C....A.C..A.......
Gomphonema micropus	.....G.-T..CTG.G...C ------.C......C.C--- --.ATCA..A..........
Gomphonema parvulum	....CA..GC.C..G....- --------.....T.GACG-- --C....A.C..A.......
Gomphonema productum	....C...G..CA.G....- -------A....T.GA.G-- --C.TC.AAC..A.......
Gomphonema pseudaugur	....CA..GC.C..G....- --------.....T.GACA-- --C....A.C..A.......
Gomphonema truncatum	....CT..GC...GGA...- -------A....T.GA.T-- --CCT.AA.C.TG......T
Lyrella atlantica	C..A.GCC.C....AGT..A --------.....T..AC--- --.A..A..TGT..T.....
Navicula lanceolata	....CGTACGG..TG.T..G GG---CAC..G.T..C.GAG -TAA.CT.CTGT........
Neidium affine	T..A.TAC.G.CTGAGCT.- -------CC.....G..TG- --C.TG.TAAGTGAT.T.T.
Nitzschia closterium f. minutissima	.....G.GCCCG.--..G.T CGGCCT.AG..G.CC..TGC -TG.T.T...T.C.......
Phaeodactylum tricornutum	.....G.GCCCG.--..G.T CGGCCT.AG..G.CC..TGC T-G.T.T...T.C.......
Pinnularia subcapitata	.....-.CTGCCTG.A.T.C AAT--TAGC.......C--- --.AGC...TGG.......T
Placoneis elginensis	....GG..GCC.-CTG.T.- ------CA........C--- --CGTGA..A..C.......
Placoneis hambergii	....CG.CCCCG.CTG...C A-------........C--- --.GG.C...GG........
Pseudo-nitzschia multiseries	.....GTGTCCA.T.G...T TTGCTC...GAGT.AT.GCG C-.GTACT..T.......GT
Pseudo-nitzschia pungens	.....GTGTCCA.T.G...T TTGCTC...GAGT.AT.GCG C-.GTACT..T.......GT
Pseudogomphonema sp. p 382	....CGTGTGGC..GCT..G GG---CAC...GT..C.GAG C-AGT.C.CCG.........
Sellaphora laevissima	....CG.C..G..CGGT..A AA--ATG....G-...CTG- ----TC.C.TG.A.......
Sellaphora pupula	....NNNC.GG..CGGCT.C GAA-ATG....G-..TCTG- --CA.GT-T.CA.-......
Stauroneis anceps	....CG-.G.GCC.TG..GT CCAT---..A..T.-..AC- -TGC.GT..CCG........
Surirella brebissoni	...CAT..CCC.AGG..T.C AT--AT.G...G-..ACG-- --....A...CG...-....

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Surirella fastuosa var                 ...CA..CCCCAAGA..T.C ATA--T.GC..G-..ACA-- --.G....T.TG...-....
Synedra fragilaroides                  ...----G....CG.G.TTC GGTCGCAC.AA.T....GTA CA.GAGTC.TGAGT......

                                       61                                                        120
Primers                                GTGGGTG
18S Consensus                          TTGGGTGGAA-YCTGTGTGG CATTAGGTTGTCGTGCAGGG GATGCCCATCGTTTACTGTG
Achnanthes brevipes                    ..........-C........ .................... ..........T.........
Achnanthes minutissima                 ..........-C........ .....AC............. ....................
Achnanthidium minutissimum             ..........-C........ .....AC............. ....................
Amphiprora alata                       ..........-C........ .................... .....T..............
Amphora montana                        .........T-C........ .............C...... ....................
Amphora pediculus                      ..........-T........ .................... ....................
Asterionella formosa                   ..........-C........ .....A.............. ..........T.........
Cocconeis pediculus                    .........T-GT..C.... ....C........C...... ....................
Cocconeis placentula                   .........T-G........ ....C........C.....T ....................
Craticula molestiformis                .........T-G........ .............C....T. ....................
Cylindrotheca closterium               .........T-C..A..... .....A.........T.... ....................
Cymatopleura elliptica                 .......A..-C........ ...............T.... A....T..............
Cymbella affinis                       .......A..-C.A.C.... ......T........TT..T ...T......A.........
Cymbella aspera                        CA...CT...-TT......A ......T....T.....AA. .........-CA........
Cymbella cymbiformis                   .......A..-C.AA..... ......T.....CC.TT..T ...T......A.........
Cymbella lanceolata                    ...T..AA..-C.A.C.... ......T....T....T..T ...ATG....A.........
Cymbella proxima                       ..A.......-TT..CT... ............AG....A. ......T...C.........
Cymbopleura naviculiformis             .......A..-T........ ............A.....A. ......T...A.........
D. geminata Canada Matapedia R.        G.........-TT...T... ............AG....A. ......T...C.........
D. geminata Canada Vancouver Is. 2     G.........-TT...T... ............AGA...A. ......T...C.........
D. geminata Canada Vancouver Is. 4     G.........-TT...T... ...........NAGA...A. ......T...C.........
D. geminata Iran Havir R. 6            G.........-TT...T... ............AG....A. ......T...C.........
D. geminata Iran Tar R. 2              G.........-TT...T... ........C...AG....A. ......T...C.........
D. geminata NZ Buller R. .c14          G.........-TT...T... ............AGA...A. ......T...C.........
D. geminata NZ L. Waiau R. 2           G.........-TT...T... ............AGR...A. ......T...C.........
D. geminata NZ L. Waiau R. 3           G.........-TT...T... ............AGR...A. ......T...C.........
D. geminata NZ Lee Vining Cr. 3        G.........-TT...T... ............AKA...A. ......T...C.........
D. geminata NZ Mararoa R. .c3          G.........-TT...T... ............AG....A. ......T..AC.........
D. geminata NZ Oreti R. 2              G.........-TT...T... ............AGR...A. ......T...C.........
D. geminata NZ U. Oreti R. 2           G.........-TT...T... ............AGA...A. ......T...C.........
D. geminata NZ U. Oreti R. 3           G.........-TT...T... ............AGR...A. ......T...C.........
D. geminata NZ U. Waiau R. 1           G.........-TT...T... ............AGA...A. ......T...C.........
D. geminata NZ U. Waiau R. 2           G.........-TT...T... ............AGR...A. ......T...C.........
D. geminata Norway Atna R. 2
D. geminata Norway Nidelva R. 1
D. geminata Norway Tisleia R. 6        G.........-TT...T... ............AGA...A. ......T...C.........
D. geminata UK C 1                          .....-TT...T... ............AG....A. ......T...C.........
D. geminata USA Boulder Cr. 1          G....Y....-TT...T... ............AG....A. ......T...C.........
D. geminata USA Boulder Cr. 5a         G.........-TT...T... ............AGR...A. ......T...C.........
D. geminata USA Conneticut R.          G.........-TT...T... ............AG....A. ......T...C.........
D. geminata USA Lee Vining Cr. 1       G.........-TT...T... ............AGA...A. ......T...C.........
D. geminata USA Rapid Cr. 1            G.........-TT...T... ............AGR...A. ......T...C.........
D. geminata USA-Boulder Cr. 12         G.........-TT...T... ............AG....A. ..N.N.N...C.........
D. geminata NZ L. Waiau R. 1a          G.........-TT...T... ............AG....A. ......T...C.........
D. geminata NZ Oreti R. 3              G.........-TT...T... ............AGA...A. ......T...C.........
Diadesmis gallica                      ..........-C........ .................... ....................
Encyonema caespitosum                  ..........-C...C.... ...............T.... ........C.A.........
Encyonema minutum                      ..........-C........ .................... .....T....A.........
Encyonema triangulatum                 ..........AC........ ................T... .....T....A.........
Entomoneis cf. alata                   ..........-C........ .............G...... .....T..............
Eolimna subminuscula                   .........T-T........ ....TA.............. ..C-................
Eunotia formica                        ..........-C........ .................... ....................
Fragilaria delicatissima               ..........-T........ .................... ..........T.........
Frustulia vulgaris                     ..........-C........ .................... .....T..............
Gomphoneis minuta var cassiae          .....A...T-TT....... ......C....T.CAT.... ...A.T..A...........
Gomphonema acuminatum                  .....A....-TTC...... ......C....T.CATG... ...A.T..A...........
Gomphonema affine                      .....A....-TTC...... ...........T.CATG... ...A.T..A.A.........
Gomphonema cf. angustatum              .....A....-TT....... ...........T.CATG... ...A.T..A...........
Gomphonema cf. parvulum                .....A....-TTC...... ...........T.CATG... ...A.T..A.T.........
Gomphonema micropus                    ..........-T........ .............C...... ..........A.........
Gomphonema parvulum                    .....A....-TTC...... ...........T.CATG... ...A.T..A.A.........
Gomphonema productum                   .....A....-TT....... ...........T.CATG... ...A.T..A.A.........
Gomphonema pseudaugur                  .....A....-TTC...... ...........T.CATG... ...A.T..A.T.........
Gomphonema truncatum                   .....A....-TTC...... ......C....T.CATG... ...A.T..A...........
Lyrella atlantica                      ......A..G-CT....... .................... ...A..T...A....T....
Navicula lanceolata                    ..........-C........ .................... ..........A.........
Neidium affine                         .C....T..G-C.GA..... ...........T.CATT..T .C..Y..G..C.........
Nitzschia closterium f. minutissima    ..........-T.A...... .....A.......C..T... ....................
Phaeodactylum tricornutum              ..........-T.A...... .....A.......C..T... ....................
Pinnularia subcapitata                 ..........-C.....G.. ......C....TT....... ..AAT...............
Placoneis elginensis                   ..........-C........ .................... .....T....A.........
Placoneis hambergii                    .........T-C........ .............C...... ..........A.........
Pseudo-nitzschia multiseries           ..........-T........ .....A.............. ....................
Pseudo-nitzschia pungens               ..........-T........ .....A........A.T.A. .G..................
Pseudogomphonema sp. p 382             ..........-C........ .................... ....................
Sellaphora laevissima                  ..........-C...C.... .................... ....................
Sellaphora pupula                      ..........-T........ .................... ....................
Stauroneis anceps                      .........T-C........ .................... ...A................
Surirella brebissoni                   ..........-C........ .................... .....T..............
Surirella fastuosa var                 ..........-CT....... .................... ...A.T..............
Synedra fragilaroides                  ..........-T........ .................... ....................
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                                    121                                                      180
Primers                                                753: GACTTACGTCGATGAAT GTATT
18S Consensus                       AAAAAATCAGAGCGTTCAAA GCAGGCTTATGCCGTTGAAT GTATTAGCATGGAATAATAA
Achnanthes brevipes                 .......T....T....... .................... A.................G.
Achnanthes minutissima              ..........C......... .................... ..C.................
Achnanthidium minutissimum          ..........C......... .................... ..C.................
Amphiprora alata                    .......T....T....... .................... A...................
Amphora montana                     .......T....T....... .........C.......... A.................G.
Amphora pediculus                   .......T....T....... ..............C..... A.................G.
Asterionella formosa                .......T....T....... .........C.......... A.................G.
Cocconeis pediculus                 ..........C......C.. .................... ..................G.
Cocconeis placentula                ..........C......... .................... ..................G.
Craticula molestiformis             .......T....T....... .................... A...................
Cylindrotheca closterium            .......T....T....... .................... A...................
Cymatopleura elliptica              .......T....T....... .................... A.......C...........
Cymbella affinis                    ..........T......... .............A...... ...................T
Cymbella aspera                     .........AT......... .............AC..... ..G.................
Cymbella cymbiformis                ..........T......... .............A...... ...................T
Cymbella lanceolata                 ..........T......... .............A...... ...................T
Cymbella proxima                    ..........T......... .................... ....................
Cymbopleura naviculiformis          ..........C......... .............A...... ....................
D. geminata Canada Matapedia R.     ..........T......... ....A....C.T..A..... ....................
D. geminata Canada Vancouver Is. 2  ..........T......... ....A....C.T..A..... ....................
D. geminata Canada Vancouver Is. 4  ..........T......... ....A....C.T..A..... ....................
D. geminata Iran Havir R. 6         ..........T......... ....A....C.T..A..... ....................
D. geminata Iran Tar R. 2           ..........T......... ....A....C.T..A..... ....................
D. geminata NZ Buller R. .c14       ..........T......... ....A....C.T..A..... ....................
D. geminata NZ L. Waiau R. 2        ..........T......... ....A....C.T..A..... ....................
D. geminata NZ L. Waiau R. 3        ..........T......... ....A....C.T..A..... ....................
D. geminata NZ Lee Vining Cr. 3     ..........T......... ....A....C.T..A..... ....................
D. geminata NZ Mararoa R. .c3       ..........T......... ....A....C.T..A..... ....................
D. geminata NZ Oreti R. 2           ..........T......... ....A....C.T..A..... ....................
D. geminata NZ U. Oreti R. 2        ..........T......... ....A....C.T..A..... ....................
D. geminata NZ U. Oreti R. 3        ..........T......... ....A....C.T..A..... ....................
D. geminata NZ U. Waiau R. 1        ..........T......... ....A....C.T..A..... ....................
D. geminata NZ U. Waiau R. 2        ..........T......... ....A....C.T..A..... ....................
D. geminata Norway Atna R. 2                                         ..A..... .....RR.............
D. geminata Norway Nidelva R. 1                             .A....C.T..A..... ...................R
D. geminata Norway Tisleia R. 6     ..........T......... ....A....C.T..A..... ....................
D. geminata UK C 1                  ..........T......... ....A....C.T..A..... ....................
D. geminata USA Boulder Cr. 1       ..........T......... ....A....C.T..A..... ....................
D. geminata USA Boulder Cr. 5a      ..........T......... ....A....C.T..A..... ....................
D. geminata USA Conneticut R.       ..........T......... ....A...GC.T..A..... ....................
D. geminata USA Lee Vining Cr. 1    ..........T......... ....A....C.T..A..... ....................
D. geminata USA Rapid Cr. 1         ..........T......... ....A....C.T..A..... ....................
D. geminata USA-Boulder Cr. 12      ..........T.....Y... .N..A....C.T..A..... ....................
D. geminata NZ L. Waiau R. 1a       G.........T......... ....A....C.T..A..... ....................
D. geminata NZ Oreti R. 3           ..........T......... ....A....C.T..A..... ....................
Diadesmis gallica                   .......T....T....... .........C.......... A...................
Encyonema caespitosum               ..........C......... .................... ....................
Encyonema minutum                   ..........C......... .................... ....................
Encyonema triangulatum              ..........C......... .................... ...A................
Entomoneis cf. alata                .......T....T....... .........A.......... A...................
Eolimna subminuscula                .......T....T....... .................... A...................
Eunotia formica                     .......T....T....... .................... A.................G.
Fragilaria delicatissima            .......T....T....... .................... A.................G.
Frustulia vulgaris                  .......T..C......... ...A........T....... A.................G.
Gomphoneis minuta var cassiae       ..........C......... ...A........T..-.... ....................
Gomphonema acuminatum               ..........C......... ...A........T..-.... ....................
Gomphonema affine                   ..........C......... ...A........TC...... ....................
Gomphonema cf. angustatum           ..........C......... ...A........TC...... ....................
Gomphonema cf. parvulum             ..........C......... ...A........TC...... ....................
Gomphonema micropus                 ..........C......... .................... ....................
Gomphonema parvulum                 ..........C......... ...A........TC...... ....................
Gomphonema productum                ..........C......... ...A........TC...... ....................
Gomphonema pseudaugur               ..........C......... ...A........TC...... ....................
Gomphonema truncatum                ..........C......... ...A........T..-.... ....................
Lyrella atlantica                   ..........T......... ...A........T....... ....................
Navicula lanceolata                 .......T....T....... .................... A.................G.
Neidium affine                      .......T....T....... ......A.T....AC..... A..A........G.......
Nitzschia closterium f. minutissima .......T....T....... .........C.......... A.................G.
Phaeodactylum tricornutum           .......T....T....... .........C.......... A.................G.
Pinnularia subcapitata              .......T....T....... .............AA..... A..C..............G.
Placoneis elginensis                ..........C......... .............A...... ....................
Placoneis hambergii                 ..........C......... .........C...A...... ....................
Pseudo-nitzschia multiseries        .......T....T....... .................... A.................G.
Pseudo-nitzschia pungens            .......T....T....... .................... A.................G.
Pseudogomphonema sp. p 382          .......T....T....... .................... A.................G.
Sellaphora laevissima               .......T....T....... .................... A.................G.
Sellaphora pupula                   .......T....T....... .........C.......... A.................G.
Stauroneis anceps                   .......T....T....... .................... A...................
Surirella brebissoni                .......T....T....... .................... A...................
Surirella fastuosa var              .......T....T....... .................... A...................
Synedra fragilaroides               .......T....T....... .................... A...................
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                                    181                                                            240

18S Consensus                       GATAGGACCTTGGTACTATT TTGTTGGTTTGCGCACCGAG GTAATGATTAATAGGGACAG
Achnanthes brevipes                 .................... .................... ....................
Achnanthes minutissima              ..............G..... .............T...... ....................
Achnanthidium minutissimum          ..............G..... .............T...... ....................
Amphiprora alata                    .................... .................... ...........C........
Amphora montana                     ..........C......... ................T.G. ....................
Amphora pediculus                   ........T.G...G..... .................TT. ....................
Asterionella formosa                ..........G......... .................... ....................
Cocconeis pediculus                 ..........C......... ................T.G. ....................
Cocconeis placentula                ..........C......... ................T.G. ....................
Craticula molestiformis             ......T.T.....C..... .............GG..AGA .A.........C........
Cylindrotheca closterium            .................... .................A.. ....................
Cymatopleura elliptica              .....A.............. .............A..T.G. ...........C...A...A
Cymbella affinis                    ..........C......... ...........T.....A.. .................T..
Cymbella aspera                     ............T.G..... ..........AT...TA-.. ..................T.
Cymbella cymbiformis                ..........C......... ...........T.....A.. .................T..
Cymbella lanceolata                 ....T.....C...T..... ...........T.....A.. .................T..
Cymbella proxima                    ...........T........ ...........T.T..TC.. ...........A...A....
Cymbopleura naviculiformis          .................... ...........T........ ....................
D. geminata Canada Matapedia R.     ...........T.C...... ...........T.T.TA... ...........A...A....
D. geminata Canada Vancouver Is. 2  ...........T........ ...........T.T.TA... ...........A...A....
D. geminata Canada Vancouver Is. 4  ...........T........ ...........T.T.TA... ...........A...A....
D. geminata Iran Havir R. 6         ...........T........ ...........T.T.TA... ...........A...A....
D. geminata Iran Tar R. 2           ...........T........ ...........T.T.TA... ...........A...A....
D. geminata NZ Buller R. .c14       ...........T........ ...........T.T.TA... ...........A...A....
D. geminata NZ L. Waiau R. 2        ...........T........ ...........T.T.TA... ...........A...A....
D. geminata NZ L. Waiau R. 3        ...........T........ ...........T.T.TA... ...........A...A....
D. geminata NZ Lee Vining Cr. 3     ...........T........ ...........T.T.TA... ...........A...A....
D. geminata NZ Mararoa R. .c3       ...........T........ ...........T.T.TA... ...........A...A....
D. geminata NZ Oreti R. 2           ...........T........ ...........T.T.TA... ...........A...A....
D. geminata NZ U. Oreti R. 2        ...........T........ ...........T.T.TA... ...........A...A....
D. geminata NZ U. Oreti R. 3        ...........T........ ...........T.T.TA... ...........A...A....
D. geminata NZ U. Waiau R. 1        ...........T........ ...........T.T.TA... ...........A...A....
D. geminata NZ U. Waiau R. 2        ...........T........ ...........T.T.TA... ...........A...A....
D. geminata Norway Atna R. 2        ...........T........ ...........T.NNNN... .K.........A...A....
D. geminata Norway Nidelva R. 1     ..W........T........ ...........T.T.TA... ...........A...A....
D. geminata Norway Tisleia R. 6     ...........T........ ...........T.T.TA... ...........A...A....
D. geminata UK C 1                  ...........T........ ...........T.T.TA... ...........A...A....
D. geminata USA Boulder Cr. 1       ...........T........ ...........T.T.TA... ...........A...A....
D. geminata USA Boulder Cr. 5a      ...........T........ ...........T.T.TA... ...........A...A....
D. geminata USA Conneticut R.       ...........T........ ...........T.T.TA... ...........A...A....
D. geminata USA Lee Vining Cr. 1    ...........T........ ...........T.T.TA... ...........A...A....
D. geminata USA Rapid Cr. 1         ...........T........ ...........T.T.TA... ...........A...A....
D. geminata USA-Boulder Cr. 12      ...........T...Y.... ...........T.T.TA... ...........A...A....
D. geminata NZ L. Waiau R. 1a       ...........T........ ...........T.T.TA... ...........A...A....
D. geminata NZ Oreti R. 3           ...........T........ ...........T.T.TA... ...........A...A....
Diadesmis gallica                   .................... .................... ....................
Encyonema caespitosum               .................... ...........T........ ....................
Encyonema minutum                   .................... ...........T........ ....................
Encyonema triangulatum              .................... ...........T........ ....................
Entomoneis cf. alata                .................... .................... ...........C........
Eolimna subminuscula                ......T.T.....C..... .............G...AGA .A.........C........
Eunotia formica                     .................... .................A.. ....................
Fragilaria delicatissima            .................... .................... ....................
Frustulia vulgaris                  .................... .................... ....................
Gomphoneis minuta var cassiae       .................... ...........T........ .................T..
Gomphonema acuminatum               .................... ...........T........ ....................
Gomphonema affine                   ........T..A........ .............T.TTA.. .................T..
Gomphonema cf. angustatum           .................... .................... ....................
Gomphonema cf. parvulum             ........A..A........ .............T..AA.T ..........G.........
Gomphonema micropus                 .................... .................... .................T..
Gomphonema parvulum                 ........T..A........ .............T.TTA.. .................T..
Gomphonema productum                .................... .................... ....................
Gomphonema pseudaugur               .........G.A........ .............T.TTTC. ......G..........T..
Gomphonema truncatum                .................... ...........T........ ....................
Lyrella atlantica                   T.......T........... ..........AT....TA.. ....................
Navicula lanceolata                 ........T..TT.G..... ...............AA... ....................
Neidium affine                      ..A.....T........... ..A........T...T.A.. ......G.............
Nitzschia closterium f. minutissima .................... .................... ....................
Phaeodactylum tricornutum           .................... .................... ....................
Pinnularia subcapitata              A.......T........... ..............G..A.A ....................
Placoneis elginensis                .................... .................... ....................
Placoneis hambergii                 .................... .................... ....................
Pseudo-nitzschia multiseries        T................... ................TA.. ...........G........
Pseudo-nitzschia pungens            T................... ................TA.. ...........G........
Pseudogomphonema sp. p 382          ........T..TTCG..... ..............GAA... ....................
Sellaphora laevissima               .................... .................... ....................
Sellaphora pupula                   .................... .................... ....................
Stauroneis anceps                   ......T.T.....C..... .............GG...GA .A.........C........
Surirella brebissoni                .................... ..............G..... ...........C........
Surirella fastuosa var              .................... ................T... ...........C.....T..
Synedra fragilaroides               .................... .................... ....................
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	241		300
18S Consensus	**TTGGGGTATTC-GTATTCC**	**ATTGTCAGAGGTGAAATTCT**	**TGGATTTTTGGAAGACGAAC**
Achnanthes brevipes	...........-.......	....	....
Achnanthes minutissima	...........-.......	....	.......C............
Achnanthidium minutissimum	...........-.......	....	.......C............
Amphiprora alata	...........-.......	....	....
Amphora montana	...T.......-.......	....	.......C............
Amphora pediculus	...........-.......	....	.......C............
Asterionella formosa	...........-.......	....	....
Cocconeis pediculus	...........-.....G.	....	.......C..C.........
Cocconeis placentula	...........-.....G.	....	.......C..C.........
Craticula molestiformis	...........-.......	....	.......C............
Cylindrotheca closterium	...........-.....T.	....	..........A.........
Cymatopleura elliptica	...........-.......	....	....
Cymbella affinis	..........T-.......	....	................A...
Cymbella aspera	.C.......CT-.......	G.C.CG..............	.A..CCGA........A...
Cymbella cymbiformis	..........T-.......	....	................A...
Cymbella lanceolata	..........T-.......	....	................A...
Cymbella proxima	..........T-.......	....	................A...
Cymbopleura naviculiformis	...........-.......	....	....
D. geminata Canada Matapedia R.	..........T-.......	....	........-.......A...
D. geminata Canada Vancouver Is. 2	..........T-.......	....	................A...
D. geminata Canada Vancouver Is. 4	..........T-.......	....	................A...
D. geminata Iran Havir R. 6	..........T-.......	....	................A...
D. geminata Iran Tar R. 2	..........T-.......	....	................A...
D. geminata NZ Buller R. .c14	..........T-.......	....	................A...
D. geminata NZ L. Waiau R. 2	..........T-.......	....	................A...
D. geminata NZ L. Waiau R. 3	..........T-.......	....	................A...
D. geminata NZ Lee Vining Cr. 3	..........T-.......	....	................A...
D. geminata NZ Mararoa R. .c3	..........T-.......	....	................A...
D. geminata NZ Oreti R. 2	..........T-.......	....	................A...
D. geminata NZ U. Oreti R. 2	..........T-.......	....	................A...
D. geminata NZ U. Oreti R. 3	..........T-.......	....	................A...
D. geminata NZ U. Waiau R. 1	..........T-.......	....	................A...
D. geminata NZ U. Waiau R. 2	..........T-.......	....	................A...
D. geminata Norway Atna R. 2	..........T-.......	...........Y........	..R..........R..A...
D. geminata Norway Nidelva R. 1	..........T-.......	....	................A...
D. geminata Norway Tisleia R. 6	..........T-.......	....	................A...
D. geminata UK C 1	..........T-.......	....	................A...
D. geminata USA Boulder Cr. 1	.C........T-.......	....	................A...
D. geminata USA Boulder Cr. 5a	..........T-.......	....	................A...
D. geminata USA Conneticut R.	..........T-.......	....	................A...
D. geminata USA Lee Vining Cr. 1	..........T-.......	....	................A...
D. geminata USA Rapid Cr. 1	..........T-.......	....	................A...
D. geminata USA-Boulder Cr. 12	..........T-.......	................C...	................A...
D. geminata NZ L. Waiau R. 1a	..........T-.......	....	................A...
D. geminata NZ Oreti R. 3	..........T-.......	....	................A...
Diadesmis gallica	...........-.......	....	.......C............
Encyonema caespitosum	...........-.......	....	.......C............
Encyonema minutum	...........-.......	....	.......C............
Encyonema triangulatum	...........-.......	....	....
Entomoneis cf. alata	...........-.......	....	....
Eolimna subminuscula	...........-.......	....	.......C............
Eunotia formica	...........-.......	....	....
Fragilaria delicatissima	...........-.......	....	....
Frustulia vulgaris	...........-.......	....	....
Gomphoneis minuta var cassiae	...........-.......	....	....
Gomphonema acuminatum	...........-.......	....	....
Gomphonema affine	...........-.......	....	....
Gomphonema cf. angustatum	...........-.......	....	.......C............
Gomphonema cf. parvulum	........T..-.......	....	.......C............
Gomphonema micropus	...........-.......	....	....
Gomphonema parvulum	...........-.......	....	....
Gomphonema productum	...........-.......	....	....
Gomphonema pseudaugur	...........-.......	....	....
Gomphonema truncatum	...........-.......	....	....
Lyrella atlantica	..........T-.......	.........A..........	................AG..
Navicula lanceolata	...........-.......	....	....
Neidium affine	..........T-.......	....	.......C........A...
Nitzschia closterium f. minutissima	...........-.......	....	.......C............
Phaeodactylum tricornutum	...........-.......	....	.......C............
Pinnularia subcapitata	...........-.......	..A.................	....
Placoneis elginensis	...........-.......	....	.......C............
Placoneis hambergii	...........-.......	....	.......C............
Pseudo-nitzschia multiseries	..........T-.......	....	................A...
Pseudo-nitzschia pungens	..........T-.......	....	................A...
Pseudogomphonema sp. p 382	...........-.......	....	....
Sellaphora laevissima	...........-.......	....	....
Sellaphora pupula	.........C.-.......	....	....
Stauroneis anceps	...........-.......	....	.......C............
Surirella brebissoni	...........-.......	....	.......C............
Surirella fastuosa var	...........C.......	....	....
Synedra fragilaroides	...........-.......	....	....

	301		360
18S Consensus	**TACTGCGAAAGCATTTACCA**	**AGGAT-GTTTTCATTAATCA**	**AGAACGAAAGTT-AGGGGA-**
Achnanthes brevipes	....	.....-..............	............-......-
Achnanthes minutissima	....	.....-..............	............-......-
Achnanthidium minutissimum	....	.....-..............	............-......-
Amphiprora alata	....	.....-..............	............-......-
Amphora montana	..A.............T...	.....-......G.......	............-......-
Amphora pediculus	....	.....-..............	............-......-
Asterionella formosa	....	.....-..............	............-......-
Cocconeis pediculus	....	.....-..............	............-......-
Cocconeis placentula	....	.....-..............	............-......-
Craticula molestiformis	....	.....-..............	............-......-
Cylindrotheca closterium	....	.....-..............	............-......-
Cymatopleura elliptica	....	.....-..............	............-......-
Cymbella affinis	....	.....-..............	............-......-
Cymbella aspera	A...................	.....-..............	............G....-.-
Cymbella cymbiformis	....	.....T..............	............-......-
Cymbella lanceolata	..............C.....	.....-..............	............-......-
Cymbella proxima	....	.....-..............	............-......-
Cymbopleura naviculiformis	....	.....-..............	............-......-
D. geminata Canada Matapedia R.	....	.....-..............	............-......-
D. geminata Canada Vancouver Is. 2	....	.....-..............	............-......-
D. geminata Canada Vancouver Is. 4	....	.....-..............	............-......-
D. geminata Iran Havir R. 6	....	.....-..............	............-......-
D. geminata Iran Tar R. 2	....	.....-..............	............AG.....-
D. geminata NZ Buller R. .c14	....	.....-..............	............-......-
D. geminata NZ L. Waiau R. 2	....	.....-..............	............-......-
D. geminata NZ L. Waiau R. 3	....	.....-..............	............-......-
D. geminata NZ Lee Vining Cr. 3	....	.....-..............	............-......-
D. geminata NZ Mararoa R. .c3	....	.....-..............	............-......-
D. geminata NZ Oreti R. 2	....	.....-..............	............-......-
D. geminata NZ U. Oreti R. 2	....	.....-..............	............-......-
D. geminata NZ U. Oreti R. 3	....	.....-..............	............-......-
D. geminata NZ U. Waiau R. 1	....	.....-..............	............-......-
D. geminata NZ U. Waiau R. 2	....	.....-..............	............-......-
D. geminata Norway Atna R. 2	...K................	.....-..............	........RK.Y-......-
D. geminata Norway Nidelva R. 1	....	.....-..............	............-......-
D. geminata Norway Tisleia R. 6	....	.....-..............	............-......-
D. geminata UK C 1	....	.....-..............	............-......-
D. geminata USA Boulder Cr. 1	....	.....-..............	............-......A
D. geminata USA Boulder Cr. 5a	....	.....-..............	............-......-
D. geminata USA Conneticut R.	....	.....-..............	............-......-
D. geminata USA Lee Vining Cr. 1	....	.....-..............	............-......-
D. geminata USA Rapid Cr. 1	....	.....-..............	............-......-
D. geminata USA-Boulder Cr. 12	....	.....-..............	............-......-
D. geminata NZ L. Waiau R. 1a	....	.....-..............	............-......-
D. geminata NZ Oreti R. 3	....	.....-..............	............-......-
Diadesmis gallica	....	.....-..............	............-......-
Encyonema caespitosum	....	.....-..............	............-......-
Encyonema minutum	....	.....-..............	............-......-
Encyonema triangulatum	....	.....T..............	............-......-
Entomoneis cf. alata	....	.....-..............	............-......-
Eolimna subminuscula	....	.....-..............	............-......-
Eunotia formica	....	.....-..............	............-......-
Fragilaria delicatissima	....	.....-..............	............-......-
Frustulia vulgaris	....	.....-..............	............-......-
Gomphoneis minuta var cassiae	....	.....-..............	............-......-
Gomphonema acuminatum	....	.....-..............	............-......-
Gomphonema affine	....	.....-..............	............-......-
Gomphonema cf. angustatum	....	.....-..............	............-......-
Gomphonema cf. parvulum	...............A....	.....-..............	............-......-
Gomphonema micropus	....	.....-..............	............-......-
Gomphonema parvulum	....	.....-..............	............-......-
Gomphonema productum	....	.....-..............	............-......-
Gomphonema pseudaugur	....	.....-..............	............T......-
Gomphonema truncatum	....	.....-..............	............-......-
Lyrella atlantica	....	.....-..............	............-......-
Navicula lanceolata	....	.....-..............	............-......-
Neidium affine	A...................	.....-..............	............-......-
Nitzschia closterium f. minutissima	....	.....-..............	............-......-
Phaeodactylum tricornutum	....	.....-..............	............-......-
Pinnularia subcapitata	....	.....-..............	............-......-
Placoneis elginensis	G...................	.....-..............	............-......-
Placoneis hambergii	....	.....-..............	............-......-
Pseudo-nitzschia multiseries	....	.....-..............	............-......-
Pseudo-nitzschia pungens	....	.....-..............	............-......-
Pseudogomphonema sp. p 382	....	.....-..............	............-......-
Sellaphora laevissima	....	.....-..............	............-......-
Sellaphora pupula	....	.....-..............	............-......-
Stauroneis anceps	....	.....-..............	............-......-
Surirella brebissoni	....	.....-..............	............-......-
Surirella fastuosa var	....	.....-..............	............-......-
Synedra fragilaroides	....	.....-..............	............-......-

	361		420
18S Consensus	**TCGAAGATGATTAGATACCA**	**TCGTAGTCTTAACCATAAAC**	**TATGCCGACAAGGGATTGGT**
Achnanthes brevipes	....	....	C
Achnanthes minutissima	....	....	....
Achnanthidium minutissimum	....	....	....
Amphiprora alata	....	....	......A............C
Amphora montana	....	....	............A......G
Amphora pediculus	....	....	C
Asterionella formosa	....	....	C
Cocconeis pediculus	....	....	....
Cocconeis placentula	....	....	....
Craticula molestiformis	....	....	C
Cylindrotheca closterium	....	....	....
Cymatopleura elliptica	....	....	......A.............
Cymbella affinis	....	....	......A.............
Cymbella aspera	....	.........C..........	................A...
Cymbella cymbiformis	....	....	......A.............
Cymbella lanceolata	....	....	......A.............
Cymbella proxima	....	....	......A.............
Cymbopleura naviculiformis	....	....	....
D. geminata Canada Matapedia R.	....	....	......A............A
D. geminata Canada Vancouver Is. 2	....	....	......A.............
D. geminata Canada Vancouver Is. 4	....	....	......A.............
D. geminata Iran Havir R. 6	....	....	......A.............
D. geminata Iran Tar R. 2	....	....	......A.............
D. geminata NZ Buller R. .c14	....	....	......A.............
D. geminata NZ L. Waiau R. 2	....	....	......A.............
D. geminata NZ L. Waiau R. 3	....	....	......A.............
D. geminata NZ Lee Vining Cr. 3	....	....	......A.............
D. geminata NZ Mararoa R. .c3	....	....	......A.............
D. geminata NZ Oreti R. 2	....	....	......A.............
D. geminata NZ U. Oreti R. 2	....	....	......A.............
D. geminata NZ U. Oreti R. 3	....	....	......A.............
D. geminata NZ U. Waiau R. 1	....	....	......A.............
D. geminata NZ U. Waiau R. 2	....	....	......A.............
D. geminata Norway Atna R. 2	........N...NN.....N	Y.DNNNN........WW...	.NN...A...R...N....K
D. geminata Norway Nidelva R. 1	....	....	......A.............
D. geminata Norway Tisleia R. 6	....	....	......A.............
D. geminata UK C 1	....	....	......A.............
D. geminata USA Boulder Cr. 1	....	....	......A.............
D. geminata USA Boulder Cr. 5a	....	....	......A.............
D. geminata USA Conneticut R.	....	.....A..............	......A.............
D. geminata USA Lee Vining Cr. 1	....	....	......A.............
D. geminata USA Rapid Cr. 1	....	....	......A.............
D. geminata USA-Boulder Cr. 12	....	....	......A.............
D. geminata NZ L. Waiau R. 1a	....	....	......A.............
D. geminata NZ Oreti R. 3	.......C............	....	......A.............
Diadesmis gallica	....	....	....
Encyonema caespitosum	....	....	....
Encyonema minutum	....	....	....
Encyonema triangulatum	....	....	....
Entomoneis cf. alata	....	....	......A............C
Eolimna subminuscula	....	....	C
Eunotia formica	....	....	C
Fragilaria delicatissima	....	....	C
Frustulia vulgaris	....	....	C
Gomphoneis minuta var cassiae	....	....	....
Gomphonema acuminatum	....	....	....
Gomphonema affine	....	....	....
Gomphonema cf. angustatum	....	....	....
Gomphonema cf. parvulum	....	....	....
Gomphonema micropus	....	....	....
Gomphonema parvulum	....	....	....
Gomphonema productum	....	....	....
Gomphonema pseudaugur	....	....	....
Gomphonema truncatum	....	....	....
Lyrella atlantica	....	....	......A.............
Navicula lanceolata	....	....	....
Neidium affine	....	....	......A.........C..C
Nitzschia closterium f. minutissima	....	....	C
Phaeodactylum tricornutum	....	....	C
Pinnularia subcapitata	....	....	......AGTT.........C
Placoneis elginensis	....	....	....
Placoneis hambergii	....	....	....
Pseudo-nitzschia multiseries	....	....	....
Pseudo-nitzschia pungens	....	....	C
Pseudogomphonema sp. p 382	....	....	....
Sellaphora laevissima	....	....	.......GTT.........C
Sellaphora pupula	....	....	.......GTT.........C
Stauroneis anceps	....	....	C
Surirella brebissoni	....	....	......A............C
Surirella fastuosa var	....	....	......A............C
Synedra fragilaroides	....	....	C

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                                    421                                                        480

18S Consensus                       GGGG-TTTCGTAATGTCTCC ATCAGCACCTTATGAGAAAT CAC-AAGTTTTTGGGTTCCG
Achnanthes brevipes                 ..A.-......TT....... G................... ...-....C...........
Achnanthes minutissima              ....-......T.C...... .................... ...-................
Achnanthidium minutissimum          ....-......T.C...... .................... ...-................
Amphiprora alata                    ....T......T.C...... G................... ...-................
Amphora montana                     AAA.-......T.C...... C...........G....... ..T-................
Amphora pediculus                   ....-.C....T.C...... G.T................. ...-................
Asterionella formosa                ..T--CG.T...T..A.C.. G................... ...-....C...........
Cocconeis pediculus                 ....-.G..A.TTG...... .................... ...-................
Cocconeis placentula                ....-.G....T.C...... .................... ...-................
Craticula molestiformis             ....-......T.C...... G................... ...-................
Cylindrotheca closterium            ..A.-......T........ .................... ...-....C...........
Cymatopleura elliptica              ....C......T.C.....T G................... ..T-................
Cymbella affinis                    ....-CC........AGC.. ............A....... ...-....C...........
Cymbella aspera                     AAA.-..C....G.....TT ...T.......CGAG....C .-A-....C...........
Cymbella cymbiformis                ....-CC........AGC.. ............A....... ..T-....C...........
Cymbella lanceolata                 ....-.C........AGCT. ............A....... ...-....C...........
Cymbella proxima                    ....-..C.........C.. ............A....... .-A-................
Cymbopleura naviculiformis          ....-C.............. .................... ..T-................
D. geminata Canada Matapedia R.     ....-............C.. ............G....... ..A-................
D. geminata Canada Vancouver Is. 2  ....-............C.. ............G....... ..A-................
D. geminata Canada Vancouver Is. 4  ....-............C.. ............G....... ..A-................
D. geminata Iran Havir R. 6         ....-............C.. ............G....... ..A-................
D. geminata Iran Tar R. 2           ....-............C.. ............G....... .GA-................
D. geminata NZ Buller R. .c14       ....-............C.. ............G....... ..A-................
D. geminata NZ L. Waiau R. 2        ....-............C.. ............G....... ..A-................
D. geminata NZ L. Waiau R. 3        ....-............C.. ............G....... ..A-................
D. geminata NZ Lee Vining Cr. 3     ....-............C.. ............G....... ..A-................
D. geminata NZ Mararoa R. .c3       ....-............C.. ............G....... ..A-................
D. geminata NZ Oreti R. 2           ....-............C.. ............G....... ..A-................
D. geminata NZ U. Oreti R. 2        ....-............C.. ............G....... ..A-................
D. geminata NZ U. Oreti R. 3        ....-............C.. ............G....... ..A-................
D. geminata NZ U. Waiau R. 1        ....-............C.. ............G....... ..A-................
D. geminata NZ U. Waiau R. 2        ....-............C.. ............G....... ..A-................
D. geminata Norway Atna R. 2        ....-....NN..NNN.C.N NNNNNNN..D..G....... ..A-................
D. geminata Norway Nidelva R. 1     ....-............C.. ............G....... ..A-................
D. geminata Norway Tisleia R. 6     ....-............C.. ............G....... ..A-................
D. geminata UK C 1                  ....-............C.. ............G....... ..A-................
D. geminata USA Boulder Cr. 1       ....-..........C.C.. ............G....... ..A-................
D. geminata USA Boulder Cr. 5a      ....-............C.. ............G....... ..A-................
D. geminata USA Conneticut R.       ....-............C.. ............G....... ..A-................
D. geminata USA Lee Vining Cr. 1    ....-............C.. ............G....... ..A-................
D. geminata USA Rapid Cr. 1         ....-............C.. ............G....... ..A-................
D. geminata USA-Boulder Cr. 12      ....-............C.. ............G....... ..A-................
D. geminata NZ L. Waiau R. 1a       ....-............C.. ............G....... ..A-................
D. geminata NZ Oreti R. 3           ....-............C.. G...........G....... ..A-................
Diadesmis gallica                   ....-......TCC...... .................... ...-................
Encyonema caespitosum               ..A.-..C............ .................... ...-................
Encyonema minutum                   ..A.-............... .................... ...-................
Encyonema triangulatum              ..A.-............... .................... ...-................
Entomoneis cf. alata                ....T......T.C...... G................... ...-................
Eolimna subminuscula                ....-.C....T.C...... G................... ...-................
Eunotia formica                     ....-....T.TT....... G................... ...-....C...........
Fragilaria delicatissima            ..T--CG.T...T..A.... G................... ...-....C...........
Frustulia vulgaris                  ....-............... G................... ...-....C...........
Gomphoneis minuta var cassiae       A.A.-..............T ............G....... ..T-....C...........
Gomphonema acuminatum               A.A.-..............T ............G....... ..T-....C...........
Gomphonema affine                   ....-............... ............G....... ..T-....C...........
Gomphonema cf. angustatum           ....-............... ............G....... ..T-....C...........
Gomphonema cf. parvulum             ....-............... ............G....... ..T-....C...........
Gomphonema micropus                 ....-............... .................... ...-................
Gomphonema parvulum                 ....-............... ............G....... ..T-....C...........
Gomphonema productum                ....-............... ............G....... ..T-....C...........
Gomphonema pseudaugur               ....-............... ............G....... ..T-....C...........
Gomphonema truncatum                A.A.-..............T ............G....... ..T-....C...........
Lyrella atlantica                   ....-.C....T.C...... .................... ...-................
Navicula lanceolata                 ..A.-.C....T........ .................... ...-....C...........
Neidium affine                      A.T.-..A...T.....G.T GC.G................ ...-....C...........
Nitzschia closterium f. minutissima ....-......T.C...... G................... ...-....C...........
Phaeodactylum tricornutum           ....-......T.C...... G................... ...-....C...........
Pinnularia subcapitata              A...-.A....T.C...C.T G................... ..TT..AC............
Placoneis elginensis                ....-..C............ .................... ...-................
Placoneis hambergii                 ....-..C............ .................... ...-................
Pseudo-nitzschia multiseries        ..A.-......TTC...... ...........G........ ..T-....C...........
Pseudo-nitzschia pungens            ..A.-.C....TTC...... G..........G........ ..T-....C...........
Pseudogomphonema sp. p 382          ..A.-.C....T.C...... .................... ...-....C...T..G....
Sellaphora laevissima               A...-C.....T.C.....T G................... ..TT..AC............
Sellaphora pupula                   A...-C.....T.C.....T G...........C....... ..TT..AC.......C....
Stauroneis anceps                   ....-......T.C...... G................... ...-................
Surirella brebissoni                ....C......T.C...... G................... ..A-................
Surirella fastuosa var              A...C......T.C.....T G...........G....... ...-................
Synedra fragilaroides               ..T--CG.T...T..A.C.. G................... ...-....C...........
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                                       481                                                        540

18S Consensus                          GGGGGAGTATGGTCGCAAGG CTGAAACTTAAAGAAATTGA CGGAAGGGCACCACCAGGAG
Achnanthes brevipes                    .................... .................... ....................
Achnanthes minutissima                 .................... .................... ....................
Achnanthidium minutissimum             .................... .................... ....................
Amphiprora alata                       .................... .................... ....................
Amphora montana                        .................... .................... ....................
Amphora pediculus                      .................... .................... ....................
Asterionella formosa                   .................... .................... ....................
Cocconeis pediculus                    .................... .................... ....................
Cocconeis placentula                   .................... .................... ....................
Craticula molestiformis                .................... .................... ....................
Cylindrotheca closterium               .................... .................... ....................
Cymatopleura elliptica                 .................... .................... ....................
Cymbella affinis                       ..............A..... .................... ....................
Cymbella aspera                        ...................T .................... ....................
Cymbella cymbiformis                   ..............A..... ...............-...- ....................
Cymbella lanceolata                    ..............A..... .................... ....................
Cymbella proxima                       .................... .................... ....................
Cymbopleura naviculiformis             .................... .................... ....................
D. geminata Canada Matapedia R.        .................... Y................... ......NNNNNNNNNNNNNN
D. geminata Canada Vancouver Is. 2     .................... ......T............. ....................
D. geminata Canada Vancouver Is. 4     .................... .................... ....................
D. geminata Iran Havir R. 6            .................... .................... ....................
D. geminata Iran Tar R. 2              .................... .................... ....................
D. geminata NZ Buller R. .c14          .................... .................... ....................
D. geminata NZ L. Waiau R. 2           .................... .................... ....................
D. geminata NZ L. Waiau R. 3           .................... .................... ....................
D. geminata NZ Lee Vining Cr. 3        .................... .................... ....................
D. geminata NZ Mararoa R. .c3          .................... .................... ....................
D. geminata NZ Oreti R. 2              .................... .................... ....................
D. geminata NZ U. Oreti R. 2           .................... .................... ....................
D. geminata NZ U. Oreti R. 3           .................... .................... ....................
D. geminata NZ U. Waiau R. 1           .................... .................... ....................
D. geminata NZ U. Waiau R. 2           .................... .................... ....................
D. geminata Norway Atna R. 2           .................... .................... ....................
D. geminata Norway Nidelva R. 1        .................... .................... ....................
D. geminata Norway Tisleia R. 6        .................... .................... ....................
D. geminata UK C 1                     .................... .................... ....................
D. geminata USA Boulder Cr. 1          .................... .................... ....................
D. geminata USA Boulder Cr. 5a         .................... .................... ....................
D. geminata USA Conneticut R.          .................... .................... ....................
D. geminata USA Lee Vining Cr. 1       .................... .................... ....................
D. geminata USA Rapid Cr. 1            .................... .................... ....................
D. geminata USA-Boulder Cr. 12         .................... .................... ....................
D. geminata NZ L. Waiau R. 1a          .................... .................... ....................
D. geminata NZ Oreti R. 3              .................... .................... ....................
Diadesmis gallica                      .................... .................... ....................
Encyonema caespitosum                  .................... .................... ....................
Encyonema minutum                      .................... .................... ....................
Encyonema triangulatum                 .................... .................... ....................
Entomoneis cf. alata                   .................... .................... ....................
Eolimna subminuscula                   .................... .................... ....................
Eunotia formica                        .................... .................... ....................
Fragilaria delicatissima               .................... .................... ....................
Frustulia vulgaris                     .................... .................... ....................
Gomphoneis minuta var cassiae          .................... .................... ....................
Gomphonema acuminatum                  .................... .................... ....................
Gomphonema affine                      .................... .................... ....................
Gomphonema cf. angustatum              .................... .................... ....................
Gomphonema cf. parvulum                .................... .................... ....................
Gomphonema micropus                    .................... .................... ....................
Gomphonema parvulum                    .................... .................... ....................
Gomphonema productum                   .................... .................... ....................
Gomphonema pseudaugur                  .................... .................... ....................
Gomphonema truncatum                   .................... .................... ....................
Lyrella atlantica                      .................... .................... ....................
Navicula lanceolata                    .................... .................... ....................
Neidium affine                         .................... .................... ....................
Nitzschia closterium f. minutissima    .................... .................... ....................
Phaeodactylum tricornutum              .................... .................... ....................
Pinnularia subcapitata                 .................... .................... ....................
Placoneis elginensis                   .................... .................... ....................
Placoneis hambergii                    .................... .................... ....................
Pseudo-nitzschia multiseries           .................... .................... ....................
Pseudo-nitzschia pungens               .................... .................... ....................
Pseudogomphonema sp. p 382             ..........T......... .................... ....................
Sellaphora laevissima                  ..............A..... .................... ....................
Sellaphora pupula                      ..............A..... .................... ....................
Stauroneis anceps                      .................... .................... ....................
Surirella brebissoni                   .................... .................... ....................
Surirella fastuosa var                 .................... .................... ....................
Synedra fragilaroides                  .................... .................... ....................
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	541		600
18S Consensus	**TGGAGCC-TGCGGCTTAATT**	**TGACTCAACACGGG-AAAAC**	**TTACCAGGTCCAGACATAGT**
Achnanthes brevipes	.......-............	..............-.....	....
Achnanthes minutissima	.......-............	..............-.....	....
Achnanthidium minutissimum	.......-............	..............-.....	....
Amphiprora alata	.......-............	..............G.....	....
Amphora montana	.......-............	..............-.....	................A...
Amphora pediculus	.......-............	..............-.....	....
Asterionella formosa	.......-............	..............-...G.	....
Cocconeis pediculus	.......-............	..............-G....	....
Cocconeis placentula	.......-............	..............-.....	....
Craticula molestiformis	.......-............	..............-.....	....
Cylindrotheca closterium	.......-............	..............-.....	....
Cymatopleura elliptica	.......-............	..............-.....	.................G..
Cymbella affinis	.......-............	..............-.....	....
Cymbella aspera	.......-............	..............-...T.	C
Cymbella cymbiformis	......--.A..........	..............-.....	....
Cymbella lanceolata	.......-............	..............-.....	....
Cymbella proxima	.......-............	..............-G....	....
Cymbopleura naviculiformis	.......-............	..............-G....	....
D. geminata Canada Matapedia R.	NNNNNNN-NNNNNNNNNNNN	NNNNNNNNNNNNNNNNNN-N	NNNNNNNNNNNNNNNNNNNN
D. geminata Canada Vancouver Is. 2	.......-............	..............G...-.	....
D. geminata Canada Vancouver Is. 4	.......-............	..............G...-.	....
D. geminata Iran Havir R. 6	.......-............	..............G...-.	....
D. geminata Iran Tar R. 2	.......-............	..............-G....	....
D. geminata NZ Buller R. .c14	.......-............	..............-G....	....
D. geminata NZ L. Waiau R. 2	.......-............	..............G...-.	....
D. geminata NZ L. Waiau R. 3	.......-............	..............G...-.	....
D. geminata NZ Lee Vining Cr. 3	.......-............	..............G...-.	....
D. geminata NZ Mararoa R. .c3	.......-............	..............-G....	....
D. geminata NZ Oreti R. 2	.......-............	..............G...-.	....
D. geminata NZ U. Oreti R. 2	.......-............	..............G...-.	....
D. geminata NZ U. Oreti R. 3	.......-............	..............G...-.	....
D. geminata NZ U. Waiau R. 1	.......-............	..............G...-.	....
D. geminata NZ U. Waiau R. 2	.......-............	..............G...-.	....
D. geminata Norway Atna R. 2	.......-............	..............G...-.	....
D. geminata Norway Nidelva R. 1	.......-............	..............G...-.	....
D. geminata Norway Tisleia R. 6	.......-............	..............-G....	....
D. geminata UK C 1	.......-............	..............G...-.	....
D. geminata USA Boulder Cr. 1	.......-...........C	..............G...-.	....
D. geminata USA Boulder Cr. 5a	.......-............	..............-G....	....
D. geminata USA Conneticut R.	.......-............	..............G...-.	....
D. geminata USA Lee Vining Cr. 1	.......-............	..............G...-.	....
D. geminata USA Rapid Cr. 1	.......-............	..............G...-.	....
D. geminata USA-Boulder Cr. 12	.......-............	..............G...-.	....
D. geminata NZ L. Waiau R. 1a	.......-............	..............-G....	....
D. geminata NZ Oreti R. 3	.......-............	..............-G....	....
Diadesmis gallica	.......-............	..............-.....	....
Encyonema caespitosum	.......-............	..............-.....	....
Encyonema minutum	.......-............	..............-.....	....
Encyonema triangulatum	.......-............	..............-.....	....
Entomoneis cf. alata	.......-............	..............-.....	....
Eolimna subminuscula	.......-............	..............-.....	....
Eunotia formica	.......-............	..............-.....	....
Fragilaria delicatissima	......T-............	..............-.....	....
Frustulia vulgaris	.......-............	..............-.....	...............G....
Gomphoneis minuta var cassiae	.......-............	..............-G....	....
Gomphonema acuminatum	.......-............	..............-G....	....
Gomphonema affine	.......-............	..............-G....	....
Gomphonema cf. angustatum	.......-............	..............-.....	....
Gomphonema cf. parvulum	.......-............	..............-G....	....
Gomphonema micropus	.......-............	..............-.....	....
Gomphonema parvulum	.......-............	..............-G....	....
Gomphonema productum	.......-............	..............-.....	....
Gomphonema pseudaugur	.......-............	..............-G....	....
Gomphonema truncatum	.......-............	..............-G....	....
Lyrella atlantica	.......-............	..............-.....	....
Navicula lanceolata	.......-............	..............-.....	....
Neidium affine	.......-............	..............-G....	....
Nitzschia closterium f. minutissima	......T-............	..............-.....	....
Phaeodactylum tricornutum	......T-............	..............-.....	....
Pinnularia subcapitata	.......-............	..............-G....	....
Placoneis elginensis	.......-............	..............-.....	....
Placoneis hambergii	.......-............	..............-.....	....
Pseudo-nitzschia multiseries	.......-............	..............-.....	....
Pseudo-nitzschia pungens	.......-............	..............-.....	....
Pseudogomphonema sp. p 382	.......-............	..............-.....	....
Sellaphora laevissima	.......-............	..............-.....	....
Sellaphora pupula	.......-............	..............-.....	....
Stauroneis anceps	.......-............	..............-G....	....
Surirella brebissoni	.......-............	..............-.....	....
Surirella fastuosa var	...C...A............	..............-.....	....
Synedra fragilaroides	.......-............	..............-.....	....

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                                         601                                                   660

18S Consensus                            GAGG-ATTGACAGATTGA-G AGC---TCTTTCTT--GATT -CTATGGG-TGGTGGTGCAT
Achnanthes brevipes                      ....-.............-. ...---........--.... -.......-...........
Achnanthes minutissima                   ....-.............-. ...---.......---.... -.......-...........
Achnanthidium minutissimum               ....-.............-. ...---........--.... -.......-...........
Amphiprora alata                         ....-.............-. ...---........--.... -.......-...........
Amphora montana                          ....-.............-. ...---........--.... -..T....-...........
Amphora pediculus                        ....-.............-. ...---........--.... -.......-...........
Asterionella formosa                     ....-.............-. ...---........--.... T.......G...........
Cocconeis pediculus                      ....-.............-. ...---........--.... -......T-...........
Cocconeis placentula                     ....-.............-. ...---........--.... -......T-...........
Craticula molestiformis                  ....-.............-. ...---........--.... -.......-...........
Cylindrotheca closterium                 ....-.............-. ...---........--.... -.......-...........
Cymatopleura elliptica                   ....-.............-. ..T---........--.... -.......-...........
Cymbella affinis                         T...-.............-. ...---........--.... -T......-...........
Cymbella aspera                          T.A.-.............-T ...---........--.... -.......-...........
Cymbella cymbiformis                     T.A.-G..T...T.G.T.-. .AGACG........--TG.. -T...T..G.......-...
Cymbella lanceolata                      T...-.............-. ...---........--.... -T......-...........
Cymbella proxima                         ....-.............-. ...---........--.... -.......-...........
Cymbopleura naviculiformis               ....-.............-. ...---........--.... -.......-...........
D. geminata Canada Matapedia R.          NNNN-NNNNNNNNNNNNN-N NNN---NNNNNNNN--NNNN -NNNNNNN-NNNNNNNNNNN
D. geminata Canada Vancouver Is. 2       ....-.............-. ...---........--.... -.......-...........
D. geminata Canada Vancouver Is. 4       ....-.............-. ...---........--.... -.......-...........
D. geminata Iran Havir R. 6              ....-.............-. ...---........--.... -.......-...........
D. geminata Iran Tar R. 2                ....-.............-. ...---........--.... -.......-...........
D. geminata NZ Buller R. .c14            ....-.............-. ...---........--.... -.......-...........
D. geminata NZ L. Waiau R. 2             ....-.............-. ...---........--.... -.......-...........
D. geminata NZ L. Waiau R. 3             ....-.............-. ...---........--.... -.......-...........
D. geminata NZ Lee Vining Cr. 3          ....-.............-. ...---........--.... -.......-...........
D. geminata NZ Mararoa R. .c3            ....-.............-. ...---........--.... -.......-...........
D. geminata NZ Oreti R. 2                ....-.............-. ...---........--.... -.......-...........
D. geminata NZ U. Oreti R. 2             ....-.............-. ...---........--.... -.......-...........
D. geminata NZ U. Oreti R. 3             ....-.............-. ...---........--.... -.......-...........
D. geminata NZ U. Waiau R. 1             ....-.............-. ...---........--.... -.......-...........
D. geminata NZ U. Waiau R. 2             ....-.............-. ...---........--.... -.......-...........
D. geminata Norway Atna R. 2             ....-.............-. ...---........--.... -.......-...........
D. geminata Norway Nidelva R. 1          ....-.............-. ...---........--.... -.......-...A.......
D. geminata Norway Tisleia R. 6          ....-.............-. ...---........--.... -.......-...........
D. geminata UK C 1                       ....-.............-. ...---........--.... -.......-...........
D. geminata USA Boulder Cr. 1            ....-.............-. ...---........--.... -.......-...........
D. geminata USA Boulder Cr. 5a           ....-.............-. ...---........--.... -.......-...........
D. geminata USA Conneticut R.            ....-.............-. ...---........--.... -.......-...........
D. geminata USA Lee Vining Cr. 1         ....-.............-. ...---........--.... -.......-...........
D. geminata USA Rapid Cr. 1              ....-.............-. ...---........--.... -.......-...........
D. geminata USA-Boulder Cr. 12           ....-.............-. ...---........--.... -.......-...........
D. geminata NZ L. Waiau R. 1a            ....-.............-. ...---........--.... -.......-...........
D. geminata NZ Oreti R. 3                ....-.............-. ...---........--.... -.......-...........
Diadesmis gallica                        ....-.............-. ...---........--.... -.......-...........
Encyonema caespitosum                    ....-.............-. ...---........--.... -.......-...........
Encyonema minutum                        ....-.............-. ...---........--.... -.......-...........
Encyonema triangulatum                   ....-.............-. ...---........--.... -.......-...........
Entomoneis cf. alata                     ....-.............-. ...---........--.... -.......-...........
Eolimna subminuscula                     ....-.............-. ...---........--.... -.......-...........
Eunotia formica                          ....-.............-. ...---........--.... -.......-...........
Fragilaria delicatissima                 ....-.............-. ...---.-......--.... T.......-...........
Frustulia vulgaris                       ....-.............-. ...---........T-.... -.......-...........
Gomphoneis minuta var cassiae            ....-.............-. ...---........--.... -.......-...........
Gomphonema acuminatum                    ....-.............-. ...---........--.... -.......-...........
Gomphonema affine                        ....-.............-. ...---........--.... -.......-...........
Gomphonema cf. angustatum                ....-.............-. ...---........--.... -.......-...........
Gomphonema cf. parvulum                  ....-.............-. ...---........--.... -.......-...........
Gomphonema micropus                      ....-.............-. ...---........--.... -.......-...........
Gomphonema parvulum                      ....-.............-. ...---........--.... -.......-...........
Gomphonema productum                     ....-..-..........-. ...---........--.... -.......-...........
Gomphonema pseudaugur                    ....-.............-. ...---........--.... -.......-...........
Gomphonema truncatum                     ....G.............-. ...---........--.... -.......-...........
Lyrella atlantica                        ....-.............-. ...---........--..W. -.Y.....-...........
Navicula lanceolata                      ....-.............-. ...---........--.... -.......-...........
Neidium affine                           ....-........G....-. ...---........--.... -.......-...........
Nitzschia closterium f. minutissima      ....-.............-. ...---........--.... -.......-...........
Phaeodactylum tricornutum                ....-.............-. ...---........--.... -.......-...........
Pinnularia subcapitata                   ....-........G....-. ...---........--.... -.......-...........
Placoneis elginensis                     ....-.............-. ...---........--.... -.......-...........
Placoneis hambergii                      ....-.............-. ...---........--.... -.......-...........
Pseudo-nitzschia multiseries             ....-.............-. ...---........--.... -.......-...........
Pseudo-nitzschia pungens                 ....-.............-. ...---........--.... -.......-...........
Pseudogomphonema sp. p 382               ....-.............-. ...---........--.... -.......-...........
Sellaphora laevissima                    ....-.............-. ...---........--.... -.......-...........
Sellaphora pupula                        ....-.............-. ...---........--.... -.......-...........
Stauroneis anceps                        ....-.............-. ...---........--.... -.......-...........
Surirella brebissoni                     ....-.............-. ...---........--.... -.......-...........
Surirella fastuosa var                   ...A-.............C. ...---........--.... -.......-...........
Synedra fragilaroides                    ....-.............-. ...---........--.... -.......-...........
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	661		720
18S Consensus	**GGCCGTTCTTA-GTTGGTGG**	**AGTGATTTG-TCTGGTTAAT**	**TCCGTTAACGA--ACGAGAC**
Achnanthes brevipes	...........-.......A	.........-..........	...........--.......
Achnanthes minutissima	...........-........	.........-..........	...........--.......
Achnanthidium minutissimum	...........-........	.........-..........	...........--.......
Amphiprora alata	...........-........	.........-..........	...........--.......
Amphora montana	...........-........	.........-..........	...........--.......
Amphora pediculus	...........-........	.........-..........	...........--.......
Asterionella formosa	...........-........	.........-..........	...........--.......
Cocconeis pediculus	...........-........	.........-..........	...........--.......
Cocconeis placentula	...........-........	.........-..........	...........--.......
Craticula molestiformis	...........-........	.........-..........	...........--.......
Cylindrotheca closterium	...........-........	.........-..........	...........--.......
Cymatopleura elliptica	...........-........	.........-..........	...........--.......
Cymbella affinis	...........-........	.........-..........	...........--.......
Cymbella aspera	...........-........	.........-..........	...........--.......
Cymbella cymbiformis	...........T...T....	T...--...-.....--...	C..TG......C----....
Cymbella lanceolata	...........-........	.........-........-.	......-....--.......
Cymbella proxima	...........-........	.........-..........	...........--.......
Cymbopleura naviculiformis	...........-........	.........-..........	...........--.......
D. geminata Canada Matapedia R.	NNNNNNNNNNN-NNNNNNNN	NNNNNNNNN-NNNNNNNNNN	NNNNNNNNNNN--NNNNNNN
D. geminata Canada Vancouver Is. 2	...........-........	.........T-.........	...........--.......
D. geminata Canada Vancouver Is. 4	...........-........	.........T-.........	...........--.......
D. geminata Iran Havir R. 6	...........-......NN	NNNNNNNNN-NNNNNNNNNN	NNNNNNNNNNN--NNNNNNN
D. geminata Iran Tar R. 2	...........-........	.........-..........	...........--.......
D. geminata NZ Buller R. .c14	...........-........	.........-..........	...........--.......
D. geminata NZ L. Waiau R. 2	...........-........	.........T-.........	...........--.......
D. geminata NZ L. Waiau R. 3	...........-........	.........T-.........	...........--.......
D. geminata NZ Lee Vining Cr. 3	...........-........	.........T-.........	...........--.......
D. geminata NZ Mararoa R. .c3	...........-........	.........-....A.....	...........--.......
D. geminata NZ Oreti R. 2	...........-........	.........T-.........	...........--.......
D. geminata NZ U. Oreti R. 2	...........-........	.........T-.........	...........--.......
D. geminata NZ U. Oreti R. 3	...........-........	.........T-.........	...........--.......
D. geminata NZ U. Waiau R. 1	...........-........	.........T-.........	...........--.......
D. geminata NZ U. Waiau R. 2	...........-........	.........T-.........	...........--.......
D. geminata Norway Atna R. 2	...........-........	.........T-.........	...........--....R..
D. geminata Norway Nidelva R. 1	...........-........	.........T-.........	...........--.......
D. geminata Norway Tisleia R. 6	...........-........	.........-..........	...........--.......
D. geminata UK C 1	...........-........	.........T-.........	...........--.......
D. geminata USA Boulder Cr. 1	...........-........	.........T-.........	.B.........--.......
D. geminata USA Boulder Cr. 5a	...........-........	.........-..........	...........--.......
D. geminata USA Conneticut R.	...........-........	.........T-.........	...........--.......
D. geminata USA Lee Vining Cr. 1	...........-........	.........T-.........	...........--.......
D. geminata USA Rapid Cr. 1	...........-........	.........T-.........	...........--.......
D. geminata USA-Boulder Cr. 12	...........-........	.........T-.........	...........--.......
D. geminata NZ L. Waiau R. 1a	...........-........	.........-..........	...........--.......
D. geminata NZ Oreti R. 3	...........-........	.........-..........	...........--.......
Diadesmis gallica	...........-........	.........-..........	...........--.......
Encyonema caespitosum	...........-........	.........-..........	...........--.......
Encyonema minutum	...........-........	.........-..........	...........--.......
Encyonema triangulatum	...........-........	.........-..........	...........--.......
Entomoneis cf. alata	...........-........	.........-..........	...........--.......
Eolimna subminuscula	...........-........	.........-..........	...........--.......
Eunotia formica	...........-........	.........-..........	...........--.......
Fragilaria delicatissima	......CT----A...TG..	........AAC.........	...........--.......
Frustulia vulgaris	...........-........	.........-..........	...........--.......
Gomphoneis minuta var cassiae	...........-........	.........-..........	...........--.......
Gomphonema acuminatum	...........-........	.........-..........	...........--.......
Gomphonema affine	...........-........	.........-..........	...........--.......
Gomphonema cf. angustatum	...........-........	.........-..........	...........--.......
Gomphonema cf. parvulum	...........-........	.........-..........	...........--.......
Gomphonema micropus	...........-........	.........-..........	...........--.......
Gomphonema parvulum	...........-........	.........-..........	...........--.......
Gomphonema productum	...........-........	.........-..........	...........--.......
Gomphonema pseudaugur	...........-........	.........-..........	...........--.......
Gomphonema truncatum	...........-........	.........-..........	...........--.......
Lyrella atlantica	...........-........	.........TCTC.......	...........--.......
Navicula lanceolata	...........-........	.........-..........	...........--.......
Neidium affine	...........-........	.........-..........	...........--.......
Nitzschia closterium f. minutissima	...........-........	.........-..........	...........--.......
Phaeodactylum tricornutum	...........-........	.........-..........	...........--.......
Pinnularia subcapitata	...........-........	.........-..........	...........--.......
Placoneis elginensis	...........-........	.........-..........	...........--.......
Placoneis hambergii	...........-........	.........-..........	...........--.......
Pseudo-nitzschia multiseries	...........-........	.........-..........	...........--.......
Pseudo-nitzschia pungens	...........-........	.........-..........	...........--.......
Pseudogomphonema sp. p 382	...........-........	.........-..........	...........--.......
Sellaphora laevissima	...........-........	.........-..........	...........--.......
Sellaphora pupula	...........-........	.........-..........	...........--.......
Stauroneis anceps	...........-........	.........-........-.	.......-...--.......
Surirella brebissoni	...........-........	.........-..........	...........--.......
Surirella fastuosa var	...........-........	.........-..........	...........CC.......
Synedra fragilaroides	...........-........	.........-..........	...........--.......

	721		780
18S Consensus	**CGCTGCCTGCTAAATAGTCC**	**AGTGAGTGATTT-TCAC-TG**	**ACTAGGG--CTTCTTAGAGG**
Achnanthes brevipes	.C...............CTT	G.C.........T....-..	G..GAA.--...........
Achnanthes minutissima	....	.........A..-....-..	..G..-.A-...........
Achnanthidium minutissimum	....	.........A..-....-..	..G..-.A-...........
Amphiprora alata	.C................T.	..C.........-....-..	G.C....--...........
Amphora montana	.C..................	TT.......-..T....-..	.T.T..A--...........
Amphora pediculus	.C..................	..C......-..T....-.A	G..C..T--...........
Asterionella formosa	.C................TG	G....A...A..T...T-..	....CA.--...........
Cocconeis pediculus	....	C.C......-..T....-..	G.G...T--...........
Cocconeis placentula	....	C.C......-..C....-..	G.G...T--...........
Craticula molestiformis	.C..................	C........A..-....-..	.TGC..A--...........
Cylindrotheca closterium	.C...............GTT	GCCT.....A..-....-..	GG.GA-AT-...........
Cymatopleura elliptica	.C................GT	G.........C.-A...-..	..C.T..--...........
Cymbella affinis	..................T.	TT.A...C..C-GCG..T.A	G--TT.AT-...........
Cymbella aspera	..................T.	.T.T........-....-..	.A.T.A.--...........
Cymbella cymbiformis	..................T.	TT.A...T....GCA..C.T	...TT.AT-...........
Cymbella lanceolata	..................TT	TCAA...C.A.--GA..T.C	GT..TA---...........
Cymbella proxima	.................ATT	.A.C........-....-..	.TC..AT--...........
Cymbopleura naviculiformis	...................T	..GT........-....-..	T.C....--...........
D. geminata Canada Matapedia R.	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNN-NNNN-N	NNNNNNN--NNNNNNNNNNN
D. geminata Canada Vancouver Is. 2	.................A.T	...T........T-...T-.	.TC...T--...........
D. geminata Canada Vancouver Is. 4	.................A.T	...T........T-...T-.	.TC...T--...........
D. geminata Iran Havir R. 6	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNN-NNNN-N	NNNNNNN--NNNNNNNNNNN
D. geminata Iran Tar R. 2	.................A.T	...T........-....-..	.TC...T--...........
D. geminata NZ Buller R. .c14	.................A.T	...T........-....-..	.TC...T--...........
D. geminata NZ L. Waiau R. 2	.................A.T	...T........T-...T-.	.TC...T--...........
D. geminata NZ L. Waiau R. 3	.................A.T	...T........T-...T-.	.TC...T--...........
D. geminata NZ Lee Vining Cr. 3	.................A.T	...T........T-...T-.	.TC...T--.........NN
D. geminata NZ Mararoa R. .c3	................AA.T	...T........-....-..	.TC...T--...........
D. geminata NZ Oreti R. 2	.................A.T	...T........T-...T-.	.TC...T--...........
D. geminata NZ U. Oreti R. 2	.................A.T	...T........T-...T-.	.TC...T--...........
D. geminata NZ U. Oreti R. 3	.................A.T	...T........T-...T-.	.TC...T--...........
D. geminata NZ U. Waiau R. 1	.................A.T	...T........T-...T-.	.TC...T--...........
D. geminata NZ U. Waiau R. 2	.................A.T	...T........T-...T-.	.TC...T--...........
D. geminata Norway Atna R. 2	.................A.T	...T........T-...T-.	.TC...T--...........
D. geminata Norway Nidelva R. 1	.................A.T	...T........T-...T-.	.TC...T--...........
D. geminata Norway Tisleia R. 6	.................A.T	...T........-....-..	.TC...T--...........
D. geminata UK C 1	.................A.T	...T........T-...T-.	.TC...T--...........
D. geminata USA Boulder Cr. 1	...........NNNNNNNNN	NNNNNNNNNNNNN-NNNN-N	NNNNNNN--NNNNNNNNNNN
D. geminata USA Boulder Cr. 5a	.................A.T	...T........-....-..	.TC...T--...........
D. geminata USA Conneticut R.	.................A.T	...T........T-...T-.	.TC...T--...........
D. geminata USA Lee Vining Cr. 1	.................A.T	...T........T-...T-.	.TC...T--...........
D. geminata USA Rapid Cr. 1	.................A.T	...T........T-...T-.	.TC...T--...........
D. geminata USA-Boulder Cr. 12	.................A.T	...T........T-...T-.	.TC...T--...........
D. geminata NZ L. Waiau R. 1a	.................A.T	...T........-....-..	.TC...T--...........
D. geminata NZ Oreti R. 3	.................A.T	...T........-....-..	.TC...T--...........
Diadesmis gallica	.C..................	G........A.-T....-..	.T.G.-.A-...........
Encyonema caespitosum	....	C..T........-....-..	.T.G...--...........
Encyonema minutum	.................CG.	...T........-....-..	G..C.A.--...........
Encyonema triangulatum	..................G.	...T........-....-..	...C.A.--...........
Entomoneis cf. alata	.C..................	G.C-......C.-....-..	G......--...........
Eolimna subminuscula	.C................T.	C........A..-....-..	..G..AA--...........
Eunotia formica	.C................TG	G.C........AG....-..	G..GCA.--...........
Fragilaria delicatissima	...................G	G..T.A......T...T-..	G...C.T--...........
Frustulia vulgaris	....	.........A..-....-..	..C...A--...........
Gomphoneis minuta var cassiae	.................CT.	G.CT........-....-..	G..T.A.--...........
Gomphonema acuminatum	.................CT.	G..T........-....-..	G..T.A.--...........
Gomphonema affine	.................CT.	G...........-....-..	...T.A.--...........
Gomphonema cf. angustatum	.................CT.	G..T........-....-..	G..T.A.--...........
Gomphonema cf. parvulum	.................CT.	G...........-....-..	...T.A.--...........
Gomphonema micropus	.................C..	G...........-....-..	.......--...........
Gomphonema parvulum	.................CT.	G...........-....-..	...T.A.--...........
Gomphonema productum	.................CT.	G..T........-....-..	G..T.A.--...........
Gomphonema pseudaugur	.................CT.	G...........-....-..	...T.A.--...........
Gomphonema truncatum	.................CT.	G..T........-....-..	G..T.A.--...........
Lyrella atlantica	..................A.	.ACC.....A..-....-..	CTAT..A--...........
Navicula lanceolata	.A...............C..	.........A.CT....-..	...GCT.G-...........
Neidium affine	.C..C....T.......CTT	TCC.........-....-..	GG..A.---...........
Nitzschia closterium f. minutissima	.C...............C..	.........A..-....-..	..C..-.G-...........
Phaeodactylum tricornutum	.C...............C..	.........A..-....-..	..C..-.G-...........
Pinnularia subcapitata	....A.T...........T.	...T.....-..T....-..	G..C.AA--.........A.
Placoneis elginensis	....	..CT........-....-..	G.CC...--...........
Placoneis hambergii	..................TT	G.CC........-....-..	GTGCAA.--...........
Pseudo-nitzschia multiseries	.C...............CA.	GCAT....-...A....-..	TG...-TG-...........
Pseudo-nitzschia pungens	.C...............CAA	.CAT....-...A....-..	TG..T-TG-...........
Pseudogomphonema sp. p 382	.A...............C..	.........A..T....-..	...GCT.G-...........
Sellaphora laevissima	.C.................T	G........A..-....-..	.T.G..A--...........
Sellaphora pupula	.C..................	.........A..-....-..	.TGG..A--...........
Stauroneis anceps	.C..................	C........A..-....-..	..G...A--...........
Surirella brebissoni	.C.................T	G.........C.-....-..	..C.T..--...........
Surirella fastuosa var	.C................TT	G.C........C-....-..	G..TT.AA-...........
Synedra fragilaroides	.C................TT	T.CT.A......T...T-..	GTATC.TAA...........

	781		840
18S Consensus	**GACGTGCATTCTATTAGATG**	**CAGGAAGATAGCGGCAATAA**	**CAGGTCTGTGATGCCCTTAG**
Achnanthes brevipes	.......G..........C.	...........G........	....
Achnanthes minutissima	.......G..........C.	....	....
Achnanthidium minutissimum	.......G..........C.	....	....
Amphiprora alata	...........G.C...T..	...........G........	....
Amphora montana	....	...........G........	....
Amphora pediculus	.......G.-...C....C.	...........G........	....
Asterionella formosa	.......G..........C.	...........G........	....
Cocconeis pediculus	...............C....	...............G....	....
Cocconeis placentula	...............C....	...............G....	....
Craticula molestiformis	....	...........G........	....
Cylindrotheca closterium	....	...........G........	....
Cymatopleura elliptica	.............C...T..	.....G.....G........	....
Cymbella affinis	..........T.-.......	....	....
Cymbella aspera	..........T.T.C.....	....	....
Cymbella cymbiformis	..........T.-.......	...T...G............	....
Cymbella lanceolata	..........T.-.......	....	....
Cymbella proxima	..........T.T.......	....	....
Cymbopleura naviculiformis	..........T.T.......	....	....
D. geminata Canada Matapedia R.	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNNNN
D. geminata Canada Vancouver Is. 2	..........T.........	....	....
D. geminata Canada Vancouver Is. 4	..........T.........	....	....
D. geminata Iran Havir R. 6	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNNNN
D. geminata Iran Tar R. 2	..........T.........	....	....
D. geminata NZ Buller R. .c14	..........T.........	....	....
D. geminata NZ L. Waiau R. 2	..........T.........	....	....
D. geminata NZ L. Waiau R. 3	..........T.........	....	....
D. geminata NZ Lee Vining Cr. 3	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNNNN
D. geminata NZ Mararoa R. .c3	..........T.........	....	....
D. geminata NZ Oreti R. 2	..........T.........	....	....
D. geminata NZ U. Oreti R. 2	..........T.........	....	....
D. geminata NZ U. Oreti R. 3	..........T.........	....	....
D. geminata NZ U. Waiau R. 1	..........T.........	....	....
D. geminata NZ U. Waiau R. 2	..........T.........	....	....
D. geminata Norway Atna R. 2	..........T.......NN	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNNNN
D. geminata Norway Nidelva R. 1	..........T.........	....	....
D. geminata Norway Tisleia R. 6	..........T.........	....	....
D. geminata UK C 1	..........T.........	....	....
D. geminata USA Boulder Cr. 1	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNNNN
D. geminata USA Boulder Cr. 5a	..........T.........	....	....
D. geminata USA Conneticut R.	..........T.........	....	....
D. geminata USA Lee Vining Cr. 1	..........T.........	....	....
D. geminata USA Rapid Cr. 1	..........T.........	....	....
D. geminata USA-Boulder Cr. 12	..........T.........	....	....
D. geminata NZ L. Waiau R. 1a	..........T.........	....	....
D. geminata NZ Oreti R. 3	..........T.........	....	....
Diadesmis gallica	.......G....N.....C.	........G..G........	....
Encyonema caespitosum	..........T.T.......	....	....
Encyonema minutum	..........T.T.......	....	....
Encyonema triangulatum	..........T.T.......	....	....
Entomoneis cf. alata	...........G.C...T..	...........G........	....
Eolimna subminuscula	....	...........G........	....
Eunotia formica	....	...........G........	....
Fragilaria delicatissima	.......G......A...C.	.....G..............	....
Frustulia vulgaris	....	....	....
Gomphoneis minuta var cassiae	.........AA.T...A...	....	....
Gomphonema acuminatum	.........AA.T...A...	....	....
Gomphonema affine	..........T.T.......	....	....
Gomphonema cf. angustatum	..........T.T.......	....	....
Gomphonema cf. parvulum	..........T.T.......	....	....
Gomphonema micropus	..........T.T.......	....	....
Gomphonema parvulum	..........T.T.......	....	....
Gomphonema productum	..........T.T.......	....	....
Gomphonema pseudaugur	..........T.T.......	....	....
Gomphonema truncatum	.........AA.T...A...	....	....
Lyrella atlantica	....	....	....
Navicula lanceolata	..............C.....	.....G.....T...T....	....
Neidium affine	..........T..C......	...........G........	....
Nitzschia closterium f. minutissima	.......G..........C.	...........G........	....
Phaeodactylum tricornutum	.......G..........C.	...........G........	....
Pinnularia subcapitata	....	.....G..............	....
Placoneis elginensis	..........T.........	....	....
Placoneis hambergii	..........T.........	....	....
Pseudo-nitzschia multiseries	.......G..........C.	...........G........	....
Pseudo-nitzschia pungens	.......G..........C.	...........G........	....
Pseudogomphonema sp. p 382	..............C.....	.....G.....T...T....	....
Sellaphora laevissima	....	...........G........	....
Sellaphora pupula	....	...........G........	....
Stauroneis anceps	....	...........G........	....
Surirella brebissoni	...........G.C...T..	...........G........	....
Surirella fastuosa var	...........G.C...T..	...........G........	....
Synedra fragilaroides	.......G..........C.	...........G........	....

	841		900
18S Consensus	**ATGTTCTGGGCCGCACGCGC**	**GCTACACTGATGCATTCAAC**	**--GAGT--TTTTCCTTGGCT**
Achnanthes brevipes	....C...............	....	--....-T-C.A....T..C
Achnanthes minutissima	....	....	--....---C.........C
Achnanthidium minutissimum	....	....	--....---C.........C
Amphiprora alata	....	....	--....---G.A.......C
Amphora montana	....	..............A.....	--....----.A.......C
Amphora pediculus	....	....	--....---G.A.......C
Asterionella formosa	....C...............	....	--....---C.A.......C
Cocconeis pediculus	....	....	--....---.-........C
Cocconeis placentula	....	....	--....---.-........C
Craticula molestiformis	....	....	--....--...A.......C
Cylindrotheca closterium	....C...............	....	--....--.C.A.......C
Cymatopleura elliptica	....	....	--....--G--A........
Cymbella affinis	....	............TG......	--A...----.......A..
Cymbella aspera	....	.........T...T......	--A..A--G..-........
Cymbella cymbiformis	....	............TG......	--A...----.......A..
Cymbella lanceolata	...........T........	............TG......	--A...----.......A..
Cymbella proxima	....	............T.C.....	--....----..........
Cymbopleura naviculiformis	....	............T.C.....	--....--G...........
D. geminata Canada Matapedia R.	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNN--	-NNNNN--NNNNNNNNNNNN
D. geminata Canada Vancouver Is. 2	...A................	............T.C....-	-C....--............
D. geminata Canada Vancouver Is. 4	...A................	............T.C....-	-C....--............
D. geminata Iran Havir R. 6	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNNN-	-NNNNN--NNNNNNNNNNNN
D. geminata Iran Tar R. 2	...A................	............T.C.....	--....--............
D. geminata NZ Buller R. .c14	...A................	............T.C.....	--....--............
D. geminata NZ L. Waiau R. 2	...A................	............T.C....-	-C....--............
D. geminata NZ L. Waiau R. 3	...A................	............T.C....-	-C....--............
D. geminata NZ Lee Vining Cr. 3	NNNNNNNNNNNNNNNNNNN.	............T.C....-	-C....--............
D. geminata NZ Mararoa R. .c3	...A................	............T.C.....	--....--............
D. geminata NZ Oreti R. 2	...A................	............T.C....-	-C....--............
D. geminata NZ U. Oreti R. 2	...A................	............T.C....-	-C....--............
D. geminata NZ U. Oreti R. 3	...A................	............T.C....-	-C....--............
D. geminata NZ U. Waiau R. 1	...A................	............T.C....-	-C....--............
D. geminata NZ U. Waiau R. 2	...A................	............T.C....-	-C....--............
D. geminata Norway Atna R. 2	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNNN-	-NNNNN--NNNNNNNNNNNN
D. geminata Norway Nidelva R. 1	...A................	............T.C....-	-C....--............
D. geminata Norway Tisleia R. 6	...A................	............T.C.....	--....--............
D. geminata UK C 1	...A................	............T.C....-	-C....--............
D. geminata USA Boulder Cr. 1	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNNN-	-NNNNN--NNNNNNNNNNNN
D. geminata USA Boulder Cr. 5a	...A..........--....	............T.C.....	--....--............
D. geminata USA Conneticut R.	...A................	............T.C....-	-C....--............
D. geminata USA Lee Vining Cr. 1	...A................	............T.C....-	-C....--............
D. geminata USA Rapid Cr. 1	...A................	............T.C....-	-C....--............
D. geminata USA-Boulder Cr. 12	...A................	............T.C....-	-C....--............
D. geminata NZ L. Waiau R. 1a	...A................	............T.C.....	--....--............
D. geminata NZ Oreti R. 3	...A................	............T.C.....	--....--............
Diadesmis gallica	....	....	--....GT...........C
Encyonema caespitosum	....	....	--....--.C..........
Encyonema minutum	....	....	--....--.C.A........
Encyonema triangulatum	....	....	--....--CC.A........
Entomoneis cf. alata	....	....	--....--G.-A.......C
Eolimna subminuscula	....	....	--....--...A.......C
Eunotia formica	....C...............	...............C....	--....-AA..........C
Fragilaria delicatissima	....C...............	...............G....	--....---G.A.......C
Frustulia vulgaris	....	..............A.....	--....-----........C
Gomphoneis minuta var cassiae	....	............TG.....-	AC....--............
Gomphonema acuminatum	....	............TG......	--....--............
Gomphonema affine	....	............TG......	--....--............
Gomphonema cf. angustatum	....	............TG......	--....--............
Gomphonema cf. parvulum	....	............TG......	--....--............
Gomphonema micropus	....	............T.......	--....--G...........
Gomphonema parvulum	....	............TG......	--....--............
Gomphonema productum	....	............TG......	--....--............
Gomphonema pseudaugur	....	............TG......	--....--............
Gomphonema truncatum	....	............TG......	--....--............
Lyrella atlantica	...........T........	....	--A...--.A......A...
Navicula lanceolata	....C...............	..............G.....	--....--...A.......C
Neidium affine	....	..................G.	--A...---G..........
Nitzschia closterium f. minutissima	....	....	--....GT...........C
Phaeodactylum tricornutum	....	....	--....GT...........C
Pinnularia subcapitata	....	....	--....----.........C
Placoneis elginensis	....	............T.......	--....--G...........
Placoneis hambergii	....	............T.C.....	--....--G...........
Pseudo-nitzschia multiseries	....	....	--....--.C.A.......C
Pseudo-nitzschia pungens	....	....	--....--.C.A.......C
Pseudogomphonema sp. p 382	....C...............	....	--....--...A.......C
Sellaphora laevissima	....	............T.......	--....--...C.......C
Sellaphora pupula	....	............T.......	--....--..CA.......C
Stauroneis anceps	....	....	--....--..AA.......C
Surirella brebissoni	....	....	--....---..A.......C
Surirella fastuosa var	.............G......	....	--A...--G.-A.......C
Synedra fragilaroides	....C...............	...............G....	AC....---G.A.......C

18S Consensus	901 GAGAGGCCTGGGCAATCTTT	TGAACGTGCATCGTGATAGG	960 GATAGATTATTGCAATTATT
Achnanthes brevipes	....	.....T..............	....
Achnanthes minutissima	C	-...................	....
Achnanthidium minutissimum	C	-...................	....
Amphiprora alata	....	....	....
Amphora montana	....	.....C..............	....
Amphora pediculus	.......T............	....	....
Asterionella formosa	............A......G	.T...C..............	....
Cocconeis pediculus	....	....	........C...........
Cocconeis placentula	....	....	........C...........
Craticula molestiformis	....	....	....
Cylindrotheca closterium	....	.....C..............	....
Cymatopleura elliptica	...........AT.......	....	....
Cymbella affinis	....A.T.....T......A	....A.CT............	....
Cymbella aspera	..A.A..........A...G	....T....G.......T..	........C...A....G..
Cymbella cymbiformis	....A.T............C	....ACCC.TG....T....	.................C..
Cymbella lanceolata	....A.T.....T......A	....A.CAT...........	....
Cymbella proxima	....A.......T......C	.A...T..............	....
Cymbopleura naviculiformis	....A...............	....T...............	....
D. geminata Canada Matapedia R.	NNNNNNNNNNNNNNNNNN..	.A...T..............	..............G.....
D. geminata Canada Vancouver Is. 2	..NNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNN..	....
D. geminata Canada Vancouver Is. 4	....A.......T.......	.A...T..............	....
D. geminata Iran Havir R. 6	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNNNN
D. geminata Iran Tar R. 2	....A.......T.......	.A...T..............	....
D. geminata NZ Buller R. .c14	....A.......T.......	.A...T..............	....
D. geminata NZ L. Waiau R. 2	....A.......T.......	.A...T..............	....
D. geminata NZ L. Waiau R. 3	....A.......T.......	.A...T..............	....
D. geminata NZ Lee Vining Cr. 3	.G..A.......T.......	.A...T..............	....
D. geminata NZ Mararoa R. .c3	....A.......T.......	.A...T..............	....
D. geminata NZ Oreti R. 2	....A.......T.......	.A...T..............	....
D. geminata NZ U. Oreti R. 2	....A.......T.......	.A...T..............	....
D. geminata NZ U. Oreti R. 3	....A.......T.......	.A...T..............	....
D. geminata NZ U. Waiau R. 1	....A.......T.......	.A...T..............	....
D. geminata NZ U. Waiau R. 2	....A.......T.......	.A...T..............	....
D. geminata Norway Atna R. 2	NNN.A.......T.......	.A...T..............	....
D. geminata Norway Nidelva R. 1	....A.......T.......	.A...T..............	....
D. geminata Norway Tisleia R. 6	....A.......T.......	.A...T..............	.....G..............
D. geminata UK C 1	....A.......T.......	.A...T..............	....
D. geminata USA Boulder Cr. 1	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNNNN
D. geminata USA Boulder Cr. 5a	....A.......T.......	.A...T..............	....
D. geminata USA Conneticut R.	....A.......T.......	.A...T..............	....
D. geminata USA Lee Vining Cr. 1	....A.......T.......	.A...T..............	....
D. geminata USA Rapid Cr. 1	....A.......T.......	.A...T..............	....
D. geminata USA-Boulder Cr. 12	....A.......T.......	.A...T..............	....
D. geminata NZ L. Waiau R. 1a	....A.......T.......	.A...T..............	....
D. geminata NZ Oreti R. 3	....A.......T.......	.A...T..............	....
Diadesmis gallica	....	.A..................	....
Encyonema caespitosum	....A...............	....T...............	....
Encyonema minutum	....A...............	....T...............	....
Encyonema triangulatum	....A...............	....T...............	....
Entomoneis cf. alata	....	....	....
Eolimna subminuscula	....	....	....
Eunotia formica	....	-...................	....
Fragilaria delicatissima	............A.....-G	....	....
Frustulia vulgaris	....	.....C..............	....
Gomphoneis minuta var cassiae	....A...............	.....TCA............	....
Gomphonema acuminatum	....A...............	.....TC.............	....
Gomphonema affine	....A..............G	.....TC.............	....
Gomphonema cf. angustatum	....A...............	.....TC.............	....
Gomphonema cf. parvulum	....A..............A	.....TC.............	....
Gomphonema micropus	....A...............	....	....
Gomphonema parvulum	....A..............G	.....TC.............	....
Gomphonema productum	....A...............	.....TC.............	....
Gomphonema pseudaugur	....A..............G	.....TC.............	....
Gomphonema truncatum	....A...............	.....TC.............	....
Lyrella atlantica	......T.....T.......	....TA..............	....
Navicula lanceolata	....	.A...C..............	....
Neidium affine	....T.......T.......	.T..T...............	.......C....A.......
Nitzschia closterium f. minutissima	....	....	....
Phaeodactylum tricornutum	....	....	....
Pinnularia subcapitata	...................G	...G................	....
Placoneis elginensis	..A.A...............	....	....
Placoneis hambergii	..A.A...............	.....A..............	....
Pseudo-nitzschia multiseries	....	.....T..............	....
Pseudo-nitzschia pungens	....	.....C..............	....
Pseudogomphonema sp. p 382	....	.A..................	....
Sellaphora laevissima	....	....	....
Sellaphora pupula	.......-............	....	....
Stauroneis anceps	....	....	....
Surirella brebissoni	.......TC...........	....	....
Surirella fastuosa var	.....-..............	.....T..............	....
Synedra fragilaroides	.......-....A.....-G	....	....

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                                    961                                                      1020

18S Consensus                       AATCTTGAACGAGGAATTCC TAGTAAACGCAG-ATCATCA ATCTGCATTGATTACGTCCC
Achnanthes brevipes                 .................... ............-....... ....................
Achnanthes minutissima              .................... ............-....... ....................
Achnanthidium minutissimum          .................... ............-....... ....................
Amphiprora alata                    .................... ............-....... ....................
Amphora montana                     .................... ............-....... ....................
Amphora pediculus                   .................... ............-....... ....................
Asterionella formosa                .................... ..........G.-G...... .C.C................
Cocconeis pediculus                 .................... ............-....... ....................
Cocconeis placentula                .................... ............-....... ....................
Craticula molestiformis             .................... ............-....... ....................
Cylindrotheca closterium            .................... ............-....... ....................
Cymatopleura elliptica              .................... ............-....... .C..................
Cymbella affinis                    .................... ...........A-....... G.T.................
Cymbella aspera                     GG....A............. .......T....-....... G...................
Cymbella cymbiformis                ...T.......G..G..... .C..CCC...GCC.....AC G.T.................
Cymbella lanceolata                 ......A............. ...........A-....... G.T.................
Cymbella proxima                    .................... ............-....... G...................
Cymbopleura naviculiformis          .................... ............-....... G...................
D. geminata Canada Matapedia R.     .................... ............-....... G...................
D. geminata Canada Vancouver Is. 2  .................... ............-....... G...................
D. geminata Canada Vancouver Is. 4  .................... ............-.....T. G...................
D. geminata Iran Havir R. 6         NNNNNNNNNNNNNNNNN... ............-....... G...................
D. geminata Iran Tar R. 2           .................... ............-....... G...................
D. geminata NZ Buller R. .c14       .................... ............-....... G...................
D. geminata NZ L. Waiau R. 2        .................... ............-....... G...................
D. geminata NZ L. Waiau R. 3        .................... ............-....... G...................
D. geminata NZ Lee Vining Cr. 3     .................... ............-....... G...................
D. geminata NZ Mararoa R. .c3       .................... ............-....... G...................
D. geminata NZ Oreti R. 2           .................... ............-....... G...................
D. geminata NZ U. Oreti R. 2        .................... ............-....... G...................
D. geminata NZ U. Oreti R. 3        .................... ............-....... G...................
D. geminata NZ U. Waiau R. 1        .................... ............-....... G...................
D. geminata NZ U. Waiau R. 2        .................... ............-....... G...................
D. geminata Norway Atna R. 2        .................... ............-....... G...................
D. geminata Norway Nidelva R. 1     .................... ............-....... G...................
D. geminata Norway Tisleia R. 6     .................... ............-....... G...................
D. geminata UK C 1                  .................... ............-....... G...................
D. geminata USA Boulder Cr. 1       NNNNNNNNNNNNNNNNNNNN NNNNNNNNNNNN-NNNNNNN NNNNNNNNNNNNNNNNNNNN
D. geminata USA Boulder Cr. 5a      .................... ............-....... G...................
D. geminata USA Conneticut R.       .................... ............-....... G...................
D. geminata USA Lee Vining Cr. 1    .................... ............-....... G...................
D. geminata USA Rapid Cr. 1         .................... ............-....... G...................
D. geminata USA-Boulder Cr. 12      .................... .....NNNNNNN-NNNNNNN NNNNNNNNNNNNNNNNNNNN
D. geminata NZ L. Waiau R. 1a       .................... ............-....... G...................
D. geminata NZ Oreti R. 3           .................... ............-....... G...................
Diadesmis gallica                   .................... ............-....... ....................
Encyonema caespitosum               .................... ............-....... ....................
Encyonema minutum                   .................... ............-....... ....................
Encyonema triangulatum              .................... ............-....... ....................
Entomoneis cf. alata                .................... ............-....... ....-...............
Eolimna subminuscula                .................... ............-....... ....................
Eunotia formica                     ......C............. ............-....... ....................
Fragilaria delicatissima            .................... ............-....... G...................
Frustulia vulgaris                  .................... ............-....... ....................
Gomphoneis minuta var cassiae       G................... .....G.....A-....... ..T...G.C...........
Gomphonema acuminatum               G................... .....G.....A-....... ..T...G.C...........
Gomphonema affine                   G................... .....G......-....... ......G.C...........
Gomphonema cf. angustatum           G................... .....G......-....... ......G.C...........
Gomphonema cf. parvulum             G................... .....G......-....... ......G.C...........
Gomphonema micropus                 .................... ............-....... ....................
Gomphonema parvulum                 G................... .....G......-....... ......G.C...........
Gomphonema productum                G................... .....G...T..-....... ......G.C...........
Gomphonema pseudaugur               G................... .....G......-....... ......G.C...........
Gomphonema truncatum                G................... .....G.....A-....... ..T...G.C...........
Lyrella atlantica                   .................... .......T....-....... ....................
Navicula lanceolata                 .................... ...........A-....... ..T.................
Neidium affine                      G.....C............. ......G....A-....... ..T....C............
Nitzschia closterium f. minutissima .................... ............-....... ....................
Phaeodactylum tricornutum           .................... ............-....... ....................
Pinnularia subcapitata              G................... ......G.....-T...... .A....GC............
Placoneis elginensis                .................... ............-....... ....................
Placoneis hambergii                 .................... ............-....... ....................
Pseudo-nitzschia multiseries        .................... ............-....... ....................
Pseudo-nitzschia pungens            .................... ............-....... ....................
Pseudogomphonema sp. p 382          .................... ...........A-....... ..T.................
Sellaphora laevissima               .................... ............-T...... .A..................
Sellaphora pupula                   .................... ............-T...... .A..................
Stauroneis anceps                   .................... ............-....... ....................
Surirella brebissoni                .................... ............-....... ....................
Surirella fastuosa var              .................... .......T....-....... ....................
Synedra fragilaroides               .................... ............-....... G...................
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	1021		1080
Primers			1659F GCTGGGG
18S Consensus	**TGCCCTTTGTACACACCGCC**	**CGTCGCACCTACCGATTGAA**	**TGGTCCGGTGAAGCCTCGGG**
Achnanthes brevipes	....	....	....
Achnanthes minutissima	....	....	....
Achnanthidium minutissimum	....	....	....
Amphiprora alata	....	..................G.	....
Amphora montana	....	..................G.	....
Amphora pediculus	....	..................G.	....
Asterionella formosa	....	....	....
Cocconeis pediculus	....	....	..................A.
Cocconeis placentula	....	....	..................A.
Craticula molestiformis	....	....	....
Cylindrotheca closterium	....	....	....
Cymatopleura elliptica	....	..................G.	..................A.
Cymbella affinis	....	....	....
Cymbella aspera	....	....	...........G.T..T...
Cymbella cymbiformis	....	-...TG.....AA.......	.....A.......GG.T...
Cymbella lanceolata	....	....	..................A.
Cymbella proxima	....	....	.............A......
Cymbopleura naviculiformis	....	....	....
D. geminata Canada Matapedia R.	....	...............C....	.............G......
D. geminata Canada Vancouver Is. 2	....	....	.............G......
D. geminata Canada Vancouver Is. 4	....	....	.............G..Y...
D. geminata Iran Havir R. 6	....	....	.............G......
D. geminata Iran Tar R. 2	....	....	.............G......
D. geminata NZ Buller R. .c14	....	....	.............G......
D. geminata NZ L. Waiau R. 2	....	....	.........NNG.-......
D. geminata NZ L. Waiau R. 3	....	....	.NNNNNNNNNNN.G......
D. geminata NZ Lee Vining Cr. 3	....	....	.............G......
D. geminata NZ Mararoa R. .c3	....	....	.............G......
D. geminata NZ Oreti R. 2	....	....	.............G......
D. geminata NZ U. Oreti R. 2	....	................NNNN	NNNNNNNNNNNNNNNNNNNN
D. geminata NZ U. Oreti R. 3	....	....	.....NNNNNNNNG......
D. geminata NZ U. Waiau R. 1	....	....	.............G....NN
D. geminata NZ U. Waiau R. 2	....	................NNNN	NNNNNNNNNNNNNNNNNNNN
D. geminata Norway Atna R. 2	..............W.....	....	.............G..N...
D. geminata Norway Nidelva R. 1	....	....	.............G......
D. geminata Norway Tisleia R. 6	....	....	.............G......
D. geminata UK C 1	....	....	.............G......
D. geminata USA Boulder Cr. 1	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNNNN	NNNNNNNNNNNNNNNNNNNN
D. geminata USA Boulder Cr. 5a	....	....	.............G......
D. geminata USA Conneticut R.	....	..........N....W....	.............G......
D. geminata USA Lee Vining Cr. 1	....	....	.............GN...N.
D. geminata USA Rapid Cr. 1	....	....	......NNNNNNNNNNNNNN
D. geminata USA-Boulder Cr. 12	NN..................	....	.............G...N..
D. geminata NZ L. Waiau R. 1a	....	....	.............G......
D. geminata NZ Oreti R. 3	....	....	.............G......
Diadesmis gallica	....	....	....
Encyonema caespitosum	....	....	....
Encyonema minutum	....	....	....
Encyonema triangulatum	....	....	....
Entomoneis cf. alata	....	..................G.	....
Eolimna subminuscula	....	....	....
Eunotia formica	....	....	....
Fragilaria delicatissima	....	....	....
Frustulia vulgaris	....	....	....
Gomphoneis minuta var cassiae	....	....	.............A......
Gomphonema acuminatum	....	....	.............A......
Gomphonema affine	....	....	.............A......
Gomphonema cf. angustatum	....	....	.............A......
Gomphonema cf. parvulum	....	....	.............A......
Gomphonema micropus	....	....	....
Gomphonema parvulum	..................T.	....	.............A......
Gomphonema productum	....	....	.............A......
Gomphonema pseudaugur	....	....	.............A......
Gomphonema truncatum	....	....	.............A......
Lyrella atlantica	....	....	...................A
Navicula lanceolata	....	....	....
Neidium affine	....	....	.............AT.....
Nitzschia closterium f. minutissima	....	..................G.	....
Phaeodactylum tricornutum	....	..................G.	....
Pinnularia subcapitata	....	....	....
Placoneis elginensis	....	....	....
Placoneis hambergii	....	....	....
Pseudo-nitzschia multiseries	....	....	....
Pseudo-nitzschia pungens	....	....	....
Pseudogomphonema sp. p 382	....	....	...........G........
Sellaphora laevissima	....	....	....
Sellaphora pupula	....	....	....
Stauroneis anceps	....	....	....
Surirella brebissoni	....	..................G.	..................A.
Surirella fastuosa var	................-...	G.CT........A.....G.	....
Synedra fragilaroides	....	....	....

	1081 1113
Primers	**ATTGCAGCTA**
18S Consensus	**ATTGTGACTAG-TGCCTTTA CTGGT-GTTGGTT**
Achnanthes brevipes	......G.AT.C.C....C. T...GAT.GT.AC
Achnanthes minutissima	....C..TG.A-........ T....-.......
Achnanthidium minutissimum	....C..TC..-........ T....-.C.....
Amphiprora alata	......G...T-......C. T....-.....C.
Amphora montana	......G.C..TG-...... .....-.......
Amphora pediculus	.........G.TG-...... T....-......C
Asterionella formosa	.......TG..T.T...... T...GA...T..C
Cocconeis pediculus	.......TGT.TG-...CGG TGCA.-----...
Cocconeis placentula	........GT.GG-...CGG ..CAC-----.GC
Craticula molestiformis	......G.CG.-........ .....-.CC....
Cylindrotheca closterium	......G.G..T.C...... T...GA...T..C
Cymatopleura elliptica	.........GT-.......G .....-..C..C.
Cymbella affinis	...C.AG.C..-........ T....-.A.....
Cymbella aspera	...T...TC..-..T...C. .....-AC.....
Cymbella cymbiformis	...T.A..A..-..TG.... T....-.A.....
Cymbella lanceolata	...C.AG.C..-........ T....-.A....C
Cymbella proxima	......G..GA-......C. .....-......C
Cymbopleura naviculiformis	...A....C..-......C. .....-.......
D. geminata Canada Matapedia R.	....CAG...AT-....... .....-.......
D. geminata Canada Vancouver Is. 2	....CAG...AT-....... .....-.......
D. geminata Canada Vancouver Is. 4	....CAG...AT-....... .....-.......
D. geminata Iran Havir R. 6	....CAG...AT-....... .....-.......
D. geminata Iran Tar R. 2	....CAG...A-........ .....-.......
D. geminata NZ Buller R. .c14	....CAG...A-........ .....-.......
D. geminata NZ L. Waiau R. 2	....CAG...AT-....... .....-.......
D. geminata NZ L. Waiau R. 3	....CAG...AT-....... .....-.......
D. geminata NZ Lee Vining Cr. 3	....CAG...AC-....... .....-.......
D. geminata NZ Mararoa R. .c3	....CAG...A-........ .....-.......
D. geminata NZ Oreti R. 2	....CAG...AT-....... .....-.......
D. geminata NZ U. Oreti R. 2	NNNNNAG...AT-....... .....-.......
D. geminata NZ U. Oreti R. 3	....CAG...AT-....... .....-.......
D. geminata NZ U. Waiau R. 1	NNNNNNN...AT-....... .....-.......
D. geminata NZ U. Waiau R. 2	NNNNCAG...AT-....... .....-.......
D. geminata Norway Atna R. 2	....CAGB..AT-....... .....-.......
D. geminata Norway Nidelva R. 1	....CAG..GAT-....... .....-.......
D. geminata Norway Tisleia R. 6	....CAG...A-........ .....-.......
D. geminata UK C 1	....CAG...AT-....... .....-.......
D. geminata USA Boulder Cr. 1	NNNNNNNNNNNN-NNNNNNN NNNNN-NNNNNNN
D. geminata USA Boulder Cr. 5a	....CAG...A-........ .....-.......
D. geminata USA Conneticut R.	....CAG...AT-....... .....-.......
D. geminata USA Lee Vining Cr. 1	.N..CAG...AT-....... .....-.......
D. geminata USA Rapid Cr. 1	NNNNNNNNNNNN-NNNNNNN NNNNN-NNN....
D. geminata USA-Boulder Cr. 12	....CAG...AT-....... .....-.......
D. geminata NZ L. Waiau R. 1a	....CAG...A-........ .....-.......
D. geminata NZ Oreti R. 3	....CAG...A-........ .....-.......
Diadesmis gallica	......G.C..-........ .....-.....C.
Encyonema caespitosum	........C..-........ .....-.....C.
Encyonema minutum	........C..-........ .....-.....C.
Encyonema triangulatum	.......TC..-........ .....-.C...C.
Entomoneis cf. alata	.........GT-........ T....-.GC....
Eolimna subminuscula	......G.CG.-........ .....-.CC....
Eunotia formica	........GGCT.C....C. ....GA.GCT...
Fragilaria delicatissima	.......T.T.TCT...... T...GGA..T..C
Frustulia vulgaris	......G.C..-........ .....-......C
Gomphoneis minuta var cassiae	.......G.GT-.......G .....-.A..T..
Gomphonema acuminatum	.......G.GT-......C. .....-.A..T..
Gomphonema affine	.......G.GT-........ .....-.A..T..
Gomphonema cf. angustatum	.......G.GT-......C. .....-.A..T..
Gomphonema cf. parvulum	.......G.GT-........ .....-.A..T..
Gomphonema micropus	.......GC..-........ .....-....T..
Gomphonema parvulum	.......G.GT-........ .....-.A..T..
Gomphonema productum	.......G.GT-......C. .....-.A..T..
Gomphonema pseudaugur	.......G.GT-......C. .....-.A..T..
Gomphonema truncatum	.......G.GT-......C. .....-.A..T..
Lyrella atlantica	.......TC..-........ .....-AC....A
Navicula lanceolata	......G.C..T.CA..... T...GA.....C.
Neidium affine	....CAG.C.T-...T..CG --...-.G...C.
Nitzschia closterium f. minutissima	........C..-........ T....-.......
Phaeodactylum tricornutum	........C..-........ T....-.......
Pinnularia subcapitata	....C..T...TG-...... .....-.C.....
Placoneis elginensis	......G..T.-......C. .....-.C.....
Placoneis hambergii	......G.C..-......C. .....-.C...C.
Pseudo-nitzschia multiseries	.......T...T.T...... T...AA...T..C
Pseudo-nitzschia pungens	.......T...T.T...... T...AA...T..C
Pseudogomphonema sp. p 382	........C..T.CA...C. T..TGA.......
Sellaphora laevissima	........C..-........ .....-.......
Sellaphora pupula	........C..-........ .....-.......
Stauroneis anceps	......G.CG.-........ .....-.CC....
Surirella brebissoni	.........GT-C....... T....-.GC....
Surirella fastuosa var	.........GC-.A...... .....-AA.A...
Synedra fragilaroides	.......TG..T.T...... T...GGA..T..C

Pairwise Distances for Chapter 2 Alignments

Table A.8 (13): Pairwise distances of *D. geminata* ITS alignment (Paup* output of uncorrected ("p") distance matrix)

	1	2	3	4	5	6	7	8
1 Canada KR1	-							
2 Canada MAL	0.02345	-						
3 Canada VI2	0.00670	0.02010	-					
4 Canada VI4	0.01508	0.02178	0.01173	-				
5 Iceland _IC1	0.00845	0.00172	0.00506	0.00840	-			
6 Iran HA2	0.03014	0.02680	0.02679	0.02679	0.01855	-		
7 Iran HA6	0.03349	0.03015	0.03015	0.03014	0.02197	0.00333	-	
8 Norway N9	0.02178	0.00168	0.01843	0.02010	0.00000	0.02512	0.02847	-
9 Norway N7	0.01173	0.02513	0.00838	0.01675	0.01011	0.03181	0.03516	0.02345
10 Norway N6	0.00503	0.01843	0.00168	0.01005	0.00336	0.02511	0.02847	0.01675
11 Norway N3	0.01005	0.01675	0.00670	0.01173	0.00000	0.02678	0.03014	0.01508
12 Norway N2	0.01511	0.00506	0.01174	0.01344	0.00000	0.02350	0.02687	0.00337
13 Norway N1	0.01011	0.02025	0.00673	0.01179	0.00680	0.02687	0.03023	0.01855
14 NZ_40B.c14	0.00838	0.02178	0.00503	0.01340	0.00675	0.02846	0.03181	0.02010
15 NZ_40B.c16	0.01173	0.02178	0.00838	0.01340	0.00845	0.02845	0.03181	0.02010
16 NZ_40B.c3	0.01173	0.02178	0.00838	0.01340	0.00849	0.02845	0.03180	0.02010
17 NZ_40B.c8	0.01173	0.02178	0.00838	0.01340	0.00846	0.02846	0.03181	0.02010
18 NZ_40B.c11	0.00838	0.02178	0.00503	0.01340	0.00679	0.02845	0.03180	0.02010
19 NZ_40B.c7	0.00838	0.01844	0.00503	0.01007	0.00510	0.02513	0.02849	0.01676
20 NZ_40B.c12	0.01005	0.02010	0.00670	0.01173	0.00676	0.02678	0.03013	0.01843
21 NZ_Gencon.c1	0.01005	0.02010	0.00670	0.01173	0.00677	0.02678	0.03014	0.01843
22 NZ_Gencon.c3	0.00838	0.02178	0.00503	0.01340	0.00673	0.02847	0.03182	0.02010
23 NZ LW1a	0.01005	0.02010	0.00670	0.01173	0.00679	0.02678	0.03013	0.01843

24 NZ LW1b	0.01005	0.02010	0.00670	0.01173	0.00679	0.02678	0.03013	0.01843
25 NZ LW2	0.00507	0.01689	0.00168	0.00841	0.00337	0.02352	0.02689	0.01518
26 NZ LW3	0.00507	0.01689	0.00168	0.00841	0.00337	0.02352	0.02689	0.01518
27 NZ OR2	0.00507	0.01689	0.00168	0.00841	0.00337	0.02352	0.02689	0.01518
28 NZ OR3	0.00838	0.02178	0.00503	0.01340	0.00676	0.02846	0.03181	0.02010
29 NZ UO2	0.00507	0.01689	0.00168	0.00841	0.00337	0.02352	0.02689	0.01518
30 NZ UO3	0.00507	0.01689	0.00168	0.00841	0.00337	0.02352	0.02689	0.01518
31 NZ UW1	0.00505	0.01682	0.00168	0.00839	0.00337	0.02349	0.02686	0.01513
32 NZ UW2	0.00505	0.01682	0.00168	0.00839	0.00337	0.02349	0.02686	0.01513
33 Poland P1	0.05540	0.05890	0.05204	0.05376	0.04723	0.06225	0.06559	0.05719
34 UK C1	0.01173	0.01843	0.00838	0.01340	0.00168	0.02846	0.03181	0.01675
35 USA BC1	0.00838	0.01843	0.00503	0.01005	0.00509	0.02510	0.02846	0.01675
36 USA BC5a	0.00670	0.01675	0.00335	0.00838	0.00338	0.02343	0.02679	0.01508
37 USA BC5b	0.00670	0.01675	0.00335	0.00838	0.00338	0.02343	0.02679	0.01508
38 USA BC12	0.01173	0.02178	0.00838	0.01340	0.00845	0.02846	0.03181	0.02010
39 USA CONN	0.00838	0.01843	0.00503	0.01005	0.00508	0.02511	0.02847	0.01675
40 USA LV1	0.00670	0.01675	0.00335	0.00838	0.00338	0.02343	0.02679	0.01508
41 USA LV3	0.00838	0.01843	0.00503	0.01005	0.00505	0.02511	0.02847	0.01675
42 USA PRS	0.01173	0.02513	0.00838	0.01508	0.01011	0.03181	0.03516	0.02345
43 USA RC1	0.00507	0.01689	0.00168	0.00841	0.00337	0.02352	0.02689	0.01518
44 USA RC2	0.00838	0.01843	0.00503	0.01005	0.00509	0.02510	0.02846	0.01675
45 USA VIR	0.00670	0.02010	0.00335	0.01173	0.00506	0.02679	0.03015	0.01843
46 USA WE	0.00505	0.01682	0.00168	0.00839	0.00337	0.02349	0.02686	0.01513

	9	10	11	12	13	14	15	16
9 Norway N7	-							
10 Norway N6	0.00670	-						
11 Norway N3	0.01173	0.00503	-					
12 Norway N2	0.01679	0.01007	0.00671	-				
13 Norway N1	0.01182	0.00506	0.01011	0.01183	-			
14 NZ_40B.c14	0.00335	0.00335	0.00838	0.01344	0.00844	-		
15 NZ_40B.c16	0.01340	0.00670	0.01173	0.01345	0.00503	0.01005	-	
16 NZ_40B.c3	0.01340	0.00670	0.01173	0.01346	0.00503	0.01005	0.00670	-

17 NZ_40B.c8	0.01340	0.00670	0.01173	0.01345	0.00503	0.01005	0.00670	0.00670
18 NZ_40B.c11	0.00670	0.00335	0.00838	0.01346	0.00841	0.00335	0.01005	0.01005
19 NZ_40B.c7	0.01006	0.00335	0.00838	0.01010	0.00168	0.00671	0.00336	0.00336
20 NZ_40B.c12	0.01173	0.00503	0.01005	0.01176	0.00338	0.00838	0.00503	0.00503
21 NZ_Gencon.c1	0.01173	0.00503	0.01005	0.01176	0.00335	0.00838	0.00503	0.00503
22 NZ_Gencon.c3	0.01005	0.00335	0.00838	0.01342	0.00844	0.00670	0.01005	0.01005
23 NZ LW1a	0.01173	0.00503	0.01005	0.01176	0.00335	0.00838	0.00503	0.00503
24 NZ LW1b	0.01173	0.00503	0.01005	0.01176	0.00335	0.00838	0.00503	0.00503
25 NZ LW2	0.00506	0.00000	0.00507	0.00844	0.00169	0.00169	0.00335	0.00336
26 NZ LW3	0.00506	0.00000	0.00507	0.00844	0.00169	0.00169	0.00335	0.00336
27 NZ OR2	0.00506	0.00000	0.00507	0.00844	0.00169	0.00169	0.00335	0.00336
28 NZ OR3	0.00670	0.00335	0.00838	0.01344	0.00841	0.00335	0.01005	0.01005
29 NZ UO2	0.00506	0.00000	0.00507	0.00844	0.00169	0.00169	0.00335	0.00336
30 NZ UO3	0.00506	0.00000	0.00507	0.00844	0.00169	0.00169	0.00335	0.00336
31 NZ UW1	0.00673	0.00000	0.00505	0.00842	0.00168	0.00337	0.00335	0.00336
32 NZ UW2	0.00673	0.00000	0.00505	0.00842	0.00168	0.00337	0.00335	0.00336
33 Poland P1	0.05544	0.05036	0.05204	0.05380	0.05215	0.05205	0.05371	0.05366
34 UK C1	0.01340	0.00670	0.00168	0.00838	0.01179	0.01005	0.01340	0.01340
35 USA BC1	0.01005	0.00335	0.00838	0.01009	0.00168	0.00670	0.00335	0.00335
36 USA BC5a	0.00838	0.00168	0.00670	0.00839	0.00338	0.00503	0.00503	0.00503
37 USA BC5b	0.00838	0.00168	0.00670	0.00839	0.00338	0.00503	0.00503	0.00503
38 USA BC12	0.01340	0.00670	0.01173	0.01344	0.00506	0.01005	0.00670	0.00670
39 USA CONN	0.01005	0.00335	0.00838	0.01007	0.00506	0.00670	0.00670	0.00670
40 USA LV1	0.00838	0.00168	0.00670	0.00839	0.00338	0.00503	0.00503	0.00503
41 USA LV3	0.01005	0.00335	0.00838	0.01007	0.00506	0.00670	0.00670	0.00670
42 USA PRS	0.00670	0.00670	0.01173	0.01679	0.01179	0.00335	0.01340	0.01340
43 USA RC1	0.00506	0.00000	0.00507	0.00844	0.00169	0.00169	0.00335	0.00336
44 USA RC2	0.01005	0.00335	0.00838	0.01009	0.00168	0.00670	0.00335	0.00335
45 USA VIR	0.00838	0.00168	0.00670	0.01174	0.00673	0.00503	0.00838	0.00838
46 USA WE	0.00673	0.00000	0.00505	0.00842	0.00168	0.00337	0.00335	0.00336

	17	18	19	20	21	22	23	24
17 NZ_40B.c8	-							
18 NZ_40B.c11	0.01005	-						
19 NZ_40B.c7	0.00336	0.00672	-					
20 NZ_40B.c12	0.00503	0.00838	0.00168	-				
21 NZ_Gencon.c1	0.00503	0.00838	0.00168	0.00335	-			
22 NZ_Gencon.c3	0.01005	0.00670	0.00670	0.00838	0.00838	-		
23 NZ LW1a	0.00503	0.00838	0.00168	0.00335	0.00335	0.00838	-	
24 NZ LW1b	0.00503	0.00838	0.00168	0.00335	0.00335	0.00838	0.00335	-
25 NZ LW2	0.00335	0.00168	0.00000	0.00169	0.00168	0.00339	0.00169	0.00169
26 NZ LW3	0.00335	0.00168	0.00000	0.00169	0.00168	0.00339	0.00169	0.00169
27 NZ OR2	0.00335	0.00168	0.00000	0.00169	0.00168	0.00339	0.00169	0.00169
28 NZ OR3	0.01005	0.00335	0.00671	0.00838	0.00838	0.00670	0.00838	0.00838
29 NZ UO2	0.00335	0.00168	0.00000	0.00169	0.00168	0.00339	0.00169	0.00169
30 NZ UO3	0.00335	0.00168	0.00000	0.00169	0.00168	0.00339	0.00169	0.00169
31 NZ UW1	0.00335	0.00335	0.00000	0.00168	0.00168	0.00337	0.00169	0.00169
32 NZ UW2	0.00335	0.00335	0.00000	0.00168	0.00168	0.00337	0.00169	0.00169
33 Poland P1	0.05370	0.05198	0.05041	0.05205	0.05201	0.05375	0.05202	0.05202
34 UK C1	0.01340	0.01005	0.01006	0.01173	0.01173	0.01005	0.01173	0.01173
35 USA BC1	0.00335	0.00670	0.00000	0.00168	0.00168	0.00670	0.00168	0.00168
36 USA BC5a	0.00503	0.00503	0.00168	0.00335	0.00335	0.00503	0.00335	0.00335
37 USA BC5b	0.00503	0.00503	0.00168	0.00335	0.00335	0.00503	0.00335	0.00335
38 USA BC12	0.00670	0.01005	0.00335	0.00503	0.00503	0.01005	0.00503	0.00503
39 USA CONN	0.00670	0.00670	0.00335	0.00503	0.00503	0.00670	0.00503	0.00503
40 USA LV1	0.00503	0.00503	0.00168	0.00335	0.00335	0.00503	0.00335	0.00335
41 USA LV3	0.00670	0.00670	0.00335	0.00503	0.00503	0.00670	0.00503	0.00503
42 USA PRS	0.01340	0.00670	0.01006	0.01173	0.01173	0.01005	0.01173	0.01173
43 USA RC1	0.00335	0.00168	0.00000	0.00169	0.00168	0.00339	0.00169	0.00169
44 USA RC2	0.00335	0.00670	0.00000	0.00168	0.00168	0.00670	0.00168	0.00168
45 USA VIR	0.00838	0.00503	0.00503	0.00670	0.00670	0.00503	0.00670	0.00670
46 USA WE	0.00335	0.00335	0.00000	0.00168	0.00168	0.00337	0.00169	0.00169

	25	26	27	28	29	30	31	32
25 NZ LW2	-							
26 NZ LW3	0.00000	-						
27 NZ OR2	0.00000	0.00000	-					
28 NZ OR3	0.00168	0.00168	0.00168	-				
29 NZ UO2	0.00000	0.00000	0.00000	0.00168	-			
30 NZ UO3	0.00000	0.00000	0.00000	0.00168	0.00000	-		
31 NZ UW1	0.00000	0.00000	0.00000	0.00335	0.00000	0.00000	-	
32 NZ UW2	0.00000	0.00000	0.00000	0.00335	0.00000	0.00000	0.00000	-
33 Poland P1	0.04871	0.04871	0.04871	0.05202	0.04871	0.04871	0.04878	0.04878
34 UK C1	0.00674	0.00674	0.00674	0.01005	0.00674	0.00674	0.00673	0.00673
35 USA BC1	0.00000	0.00000	0.00000	0.00670	0.00000	0.00000	0.00000	0.00000
36 USA BC5a	0.00000	0.00000	0.00000	0.00503	0.00000	0.00000	0.00000	0.00000
37 USA BC5b	0.00000	0.00000	0.00000	0.00503	0.00000	0.00000	0.00000	0.00000
38 USA BC12	0.00337	0.00337	0.00337	0.01005	0.00337	0.00337	0.00335	0.00335
39 USA CONN	0.00169	0.00169	0.00169	0.00670	0.00169	0.00169	0.00169	0.00169
40 USA LV1	0.00000	0.00000	0.00000	0.00503	0.00000	0.00000	0.00000	0.00000
41 USA LV3	0.00168	0.00168	0.00168	0.00670	0.00168	0.00168	0.00168	0.00168
42 USA PRS	0.00504	0.00504	0.00504	0.00670	0.00504	0.00504	0.00672	0.00672
43 USA RC1	0.00000	0.00000	0.00000	0.00168	0.00000	0.00000	0.00000	0.00000
44 USA RC2	0.00000	0.00000	0.00000	0.00670	0.00000	0.00000	0.00000	0.00000
45 USA VIR	0.00168	0.00168	0.00168	0.00503	0.00168	0.00168	0.00168	0.00168
46 USA WE	0.00000	0.00000	0.00000	0.00335	0.00000	0.00000	0.00000	0.00000

	33	34	35	36	37	38	39	40
33 Poland P1	-							
34 UK C1	0.05375	-						
35 USA BC1	0.05034	0.01005	-					
36 USA BC5a	0.04869	0.00838	0.00168	-				
37 USA BC5b	0.04869	0.00838	0.00168	0.00000	-			
38 USA BC12	0.05372	0.01340	0.00335	0.00503	0.00503	-		
39 USA CONN	0.05037	0.01005	0.00335	0.00168	0.00168	0.00670	-	
40 USA LV1	0.04869	0.00838	0.00168	0.00000	0.00000	0.00503	0.00168	-

41 USA LV3	0.05040	0.01005	0.00335	0.00168	0.00168	0.00670	0.00335	0.00168
42 USA PRS	0.05541	0.01340	0.01005	0.00838	0.00838	0.01340	0.01005	0.00838
43 USA RC1	0.04871	0.00674	0.00000	0.00000	0.00000	0.00337	0.00169	0.00000
44 USA RC2	0.05034	0.01005	0.00000	0.00168	0.00168	0.00335	0.00335	0.00168
45 USA VIR	0.05204	0.00838	0.00503	0.00335	0.00335	0.00838	0.00503	0.00335
46 USA WE	0.04878	0.00673	0.00000	0.00000	0.00000	0.00335	0.00169	0.00000

	41	42	43	44	45	46
41 USA LV3	-					
42 USA PRS	0.01005	-				
43 USA RC1	0.00168	0.00504	-			
44 USA RC2	0.00335	0.01005	0.00000	-		
45 USA VIR	0.00503	0.00838	0.00168	0.00503	-	
46 USA WE	0.00168	0.00672	0.00000	0.00000	0.00168	-

Table A.9 (14): Pairwise distances of *D. geminata* 18S-ITS alignment (Paup* output of uncorrected ("p") distance matrix).

		1	2	3	4	5	6	7
1	Gomphoneis minut	-						
2	Cymbella proxima	0.08555	-					
3	NZ OR3	0.09002	0.03373	-				
4	Norway N6	0.08551	0.02912	0.00435	-			
5	Canada VI4	0.08554	0.03207	0.01047	0.00610	-		
6	Canada VI2	0.09039	0.03409	0.00696	0.00261	0.00697	-	
7	NZ UW2	0.08565	0.02702	0.00436	0.00000	0.00524	0.00261	-
8	NZ UW1	0.08554	0.02911	0.00435	0.00000	0.00524	0.00261	0.00000
9	NZ UO3	0.08566	0.02701	0.00349	0.00000	0.00525	0.00261	0.00000
10	NZ UO2	0.08554	0.02911	0.00349	0.00000	0.00525	0.00261	0.00000
11	UK C1	0.08544	0.02208	0.00881	0.00440	0.00792	0.00616	0.00353
12	NZ OR2	0.08566	0.02701	0.00349	0.00000	0.00525	0.00261	0.00000
13	USA RC1	0.08566	0.02701	0.00349	0.00000	0.00525	0.00261	0.00000
14	Norway N2	0.04186	0.01137	0.00984	0.00592	0.00794	0.00792	0.00493
15	Norway N1	0.05941	0.02419	0.00760	0.00285	0.00665	0.00475	0.00093
16	Canada MAL	0.09286	0.03221	0.01743	0.01307	0.01572	0.01569	0.01137
17	NZ LW3	0.08566	0.02701	0.00349	0.00000	0.00525	0.00261	0.00000
18	NZ LW2	0.08566	0.02701	0.00349	0.00000	0.00525	0.00261	0.00000
19	NZ LW1a	0.08928	0.02873	0.00957	0.00522	0.00959	0.00783	0.00262
20	NZ LW1b	0.09010	0.02980	0.00870	0.00435	0.00873	0.00696	0.00175
21	USA LV3	0.08729	0.03197	0.00696	0.00261	0.00523	0.00348	0.00174
22	USA LV1	0.08557	0.02910	0.00522	0.00087	0.00523	0.00348	0.00000
23	Iran HA6	0.08741	0.02697	0.02086	0.01652	0.01829	0.01913	0.01481

24 Iran HA2	0.09358	0.03338	0.02086	0.01651	0.01828	0.01912	0.01481
25 NZ_Gencon.c3	0.08960	0.02914	0.00783	0.00348	0.00958	0.00609	0.00262
26 USA CONN	0.09402	0.03373	0.00957	0.00522	0.00785	0.00609	0.00349
27 USA BC5b	0.08819	0.03194	0.00609	0.00174	0.00611	0.00435	0.00087
28 USA BC5a	0.08569	0.02701	0.00522	0.00087	0.00523	0.00348	0.00000
29 USA BC12	0.09267	0.03340	0.01050	0.00612	0.00875	0.00699	0.00352
30 USA BC1	0.09140	0.03264	0.00957	0.00522	0.00960	0.00783	0.00262
31 NZ_40B.c14	0.08550	0.02913	0.00522	0.00261	0.00871	0.00522	0.00262

	8	9	10	11	12	13	14
8 NZ UW1	-						
9 NZ UO3	0.00000	-					
10 NZ UO2	0.00000	0.00000	-				
11 UK C1	0.00441	0.00354	0.00441	-			
12 NZ OR2	0.00000	0.00000	0.00000	0.00354	-		
13 USA RC1	0.00000	0.00000	0.00000	0.00354	0.00000	-	
14 Norway N2	0.00493	0.00494	0.00493	0.00494	0.00494	0.00494	-
15 Norway N1	0.00093	0.00094	0.00094	0.00667	0.00094	0.00094	0.00694
16 Canada MAL	0.01223	0.01138	0.01225	0.01238	0.01138	0.01138	0.00585
17 NZ LW3	0.00000	0.00000	0.00000	0.00354	0.00000	0.00000	0.00494
18 NZ LW2	0.00000	0.00000	0.00000	0.00354	0.00000	0.00000	0.00494
19 NZ LW1a	0.00348	0.00263	0.00349	0.00793	0.00263	0.00263	0.00788
20 NZ LW1b	0.00262	0.00175	0.00262	0.00704	0.00175	0.00175	0.00782
21 USA LV3	0.00174	0.00174	0.00174	0.00617	0.00174	0.00174	0.00593
22 USA LV1	0.00000	0.00000	0.00000	0.00528	0.00000	0.00000	0.00492
23 Iran HA6	0.01567	0.01482	0.01569	0.01762	0.01482	0.01482	0.01684
24 Iran HA2	0.01567	0.01483	0.01568	0.01763	0.01483	0.01483	0.01591
25 NZ_Gencon.c3	0.00349	0.00262	0.00349	0.00617	0.00262	0.00262	0.00793
26 USA CONN	0.00436	0.00350	0.00437	0.00706	0.00350	0.00350	0.00589

27 USA BC5b	0.00087	0.00087	0.00087	0.00616	0.00087	0.00087	0.00579
28 USA BC5a	0.00000	0.00000	0.00000	0.00441	0.00000	0.00000	0.00492
29 USA BC12	0.00438	0.00352	0.00439	0.00797	0.00352	0.00352	0.00889
30 USA BC1	0.00349	0.00263	0.00350	0.00794	0.00263	0.00263	0.00789
31 NZ_40B.c14	0.00262	0.00175	0.00175	0.00704	0.00175	0.00175	0.00893

	15	16	17	18	19	20	21
15 Norway N1	-						
16 Canada MAL	0.01431	-					
17 NZ LW3	0.00094	0.01138	-				
18 NZ LW2	0.00094	0.01138	0.00000	-			
19 NZ LW1a	0.00284	0.01482	0.00263	0.00263	-		
20 NZ LW1b	0.00280	0.01395	0.00175	0.00175	0.00435	-	
21 USA LV3	0.00286	0.01395	0.00174	0.00174	0.00609	0.00522	-
22 USA LV1	0.00190	0.01220	0.00000	0.00000	0.00435	0.00348	0.00174
23 Iran HA6	0.01804	0.01918	0.01482	0.01482	0.01826	0.01739	0.01739
24 Iran HA2	0.01712	0.01918	0.01483	0.01483	0.01825	0.01738	0.01738
25 NZ_Gencon.c3	0.00478	0.01482	0.00262	0.00262	0.00696	0.00609	0.00609
26 USA CONN	0.00475	0.01482	0.00350	0.00350	0.00696	0.00609	0.00435
27 USA BC5b	0.00284	0.01307	0.00087	0.00087	0.00522	0.00435	0.00261
28 USA BC5a	0.00190	0.01134	0.00000	0.00000	0.00349	0.00261	0.00174
29 USA BC12	0.00380	0.01578	0.00352	0.00352	0.00613	0.00528	0.00524
30 USA BC1	0.00378	0.01483	0.00263	0.00263	0.00522	0.00348	0.00609
31 NZ_40B.c14	0.00573	0.01569	0.00175	0.00175	0.00783	0.00696	0.00522

	22	23	24	25	26	27	28
22 USA LV1	-						
23 Iran HA6	0.01565	-					
24 Iran HA2	0.01564	0.00520	-				
25 NZ_Gencon.c3	0.00435	0.01826	0.01826	-			
26 USA CONN	0.00435	0.01826	0.01825	0.00696	-		
27 USA BC5b	0.00087	0.01652	0.01651	0.00522	0.00522	-	
28 USA BC5a	0.00000	0.01479	0.01479	0.00348	0.00348	0.00087	-
29 USA BC12	0.00524	0.01923	0.01922	0.00786	0.00613	0.00613	0.00438
30 USA BC1	0.00435	0.01827	0.01825	0.00697	0.00696	0.00522	0.00349
31 NZ_40B.c14	0.00348	0.01912	0.01912	0.00609	0.00783	0.00435	0.00348

	29	30	31
29 USA BC12	-		
30 USA BC1	0.00615	-	
31 NZ_40B.c14	0.00873	0.00783	-

Table A.10 (15): Pairwise distances of 18S diatom alignment (Paup* output of uncorrected ("p") distance matrix). L=Lower Waiau River, O=Oreti River, B=Buller River, M=Mararoa River.

		1	2	3	4	5	6	7
1	Achnanthes brevi	-						
2	Achnanthes minut	0.07153	-					
3	Achnanthidium mi	0.06962	0.00375	-				
4	Amphiprora alata	0.05461	0.05378	0.05377	-			
5	Amphora pediculu	0.07520	0.06844	0.06843	0.05811	-		
6	Amphora pediculu	0.06938	0.06163	0.06163	0.05311	0.05742	-	
7	Asterionella for	0.06603	0.07539	0.07442	0.07641	0.07885	0.06463	-
8	Cocconeis pedicu	0.08559	0.06289	0.06295	0.07641	0.07733	0.07060	0.08838
9	Cocconeis placen	0.08198	0.06407	0.06410	0.07001	0.07266	0.06419	0.08857
10	Craticula molest	0.07127	0.06110	0.05922	0.05354	0.05873	0.06053	0.08262
11	Cylindrotheca cl	0.04644	0.06210	0.06480	0.06026	0.06783	0.06673	0.06528
12	Cymatopleura ell	0.08175	0.07633	0.07633	0.04505	0.07765	0.08039	0.09584
13	Cymbella affinis	0.11172	0.09402	0.09112	0.09234	0.10472	0.10745	0.10955
14	Cymbella aspera	0.14470	0.13005	0.12526	0.13289	0.13729	0.14431	0.14925
15	Cymbella cymbifo	0.16109	0.14531	0.14232	0.14839	0.15536	0.15607	0.15671
16	Cymbella lanceol	0.12816	0.11398	0.11202	0.11795	0.12961	0.12369	0.12950
17	Cymbella proxima	0.09734	0.08530	0.08619	0.08434	0.09132	0.09201	0.10182
18	Cymbopleura navi	0.08470	0.06702	0.06507	0.06806	0.07953	0.07740	0.09108
19	Diadesmis gallic	0.05720	0.04527	0.04529	0.05005	0.05411	0.06237	0.07145
20	D. geminata Iran	0.10957	0.08327	0.08507	0.08609	0.09145	0.09570	0.11015
21	D. geminata Norw	0.10862	0.08231	0.08410	0.08512	0.09047	0.09471	0.10919
22	D. geminata NZ L	0.10768	0.08137	0.08316	0.08417	0.08953	0.09378	0.10825
23	D. geminata NZ O	0.10769	0.08326	0.08504	0.08420	0.09049	0.09378	0.10828
24	D. geminata NZ B	0.10769	0.08137	0.08315	0.08418	0.08954	0.09376	0.10826
25	D. geminata NZ M	0.10958	0.08325	0.08503	0.08607	0.09144	0.09565	0.11013
26	D. geminata USA	0.10705	0.08065	0.08245	0.08346	0.08884	0.09307	0.10760
27	Encyonema caespi	0.07449	0.05580	0.05389	0.06162	0.07121	0.07285	0.08658
28	Encyonema minutu	0.06790	0.05481	0.05197	0.05590	0.07114	0.06716	0.08091
29	Encyonema triang	0.07070	0.05766	0.05291	0.05863	0.07208	0.06905	0.07897

30	Entomoneis cf. a	0.05856	0.05676	0.05580	0.02160	0.05828	0.05333	0.07759
31	Eolimna subminus	0.06852	0.05736	0.05453	0.04887	0.06349	0.06150	0.07893
32	Eonotia formica	0.04268	0.07046	0.06948	0.05838	0.07628	0.06299	0.06245
33	Fragilaria delic	0.07840	0.08899	0.08992	0.08712	0.09425	0.07329	0.05783
34	Frustulia vulgar	0.05827	0.04336	0.04337	0.05004	0.06166	0.06066	0.06869
35	Gomphoneis minut	0.10374	0.09075	0.08880	0.09542	0.09775	0.09840	0.11663
36	Gomphonema acumi	0.09709	0.09082	0.08887	0.09270	0.09485	0.09654	0.11101
37	Gomphonema affin	0.09892	0.08594	0.08401	0.09070	0.09194	0.09174	0.10621
38	Gomphonema cf. a	0.09041	0.07936	0.07743	0.08127	0.08533	0.08609	0.10436
39	Gomphonema cf. p	0.10169	0.08689	0.08497	0.09354	0.09474	0.09457	0.10996
40	Gomphonema micro	0.07253	0.05100	0.04906	0.05869	0.06257	0.06427	0.07993
41	Gomphonema parvu	0.09987	0.08689	0.08495	0.09164	0.09289	0.09268	0.10715
42	Gomphonema produ	0.09049	0.08227	0.08033	0.08225	0.08825	0.08901	0.10444
43	Gomphonema pseud	0.09984	0.08881	0.08686	0.09167	0.09482	0.09557	0.10906
44	Gomphonema trunc	0.09803	0.08985	0.08791	0.09268	0.09486	0.09654	0.11193
45	Lyrella atlantic	0.10371	0.08023	0.07824	0.08598	0.08910	0.08406	0.10706
46	Navicula lanceol	0.06324	0.06293	0.06102	0.06580	0.07432	0.07392	0.07629
47	Neidium affine	0.12255	0.12300	0.12199	0.10326	0.11487	0.11092	0.12601
48	Nitzschia closte	0.05958	0.05000	0.04897	0.04813	0.05949	0.05642	0.07098
49	Phaeodactylum tr	0.05958	0.05000	0.04897	0.04813	0.05949	0.05642	0.07098
50	Pinnularia subca	0.09929	0.08521	0.08333	0.08346	0.08630	0.08159	0.09269
51	Placoneis elgine	0.06883	0.05958	0.05673	0.05403	0.07025	0.06436	0.08567
52	Placoneis hamber	0.06784	0.06618	0.06143	0.06354	0.06636	0.07281	0.08658
53	Pseudo-nitzschia	0.05945	0.06874	0.06955	0.06307	0.07805	0.06839	0.07429
54	Pseudo-nitzschia	0.06410	0.07625	0.07704	0.06683	0.08367	0.07025	0.07616
55	Pseudogomphonema	0.06695	0.07143	0.06950	0.07337	0.07999	0.07488	0.08097
56	Sellaphora laevi	0.06562	0.05931	0.05930	0.05943	0.06336	0.06409	0.07496
57	Sellaphora pupul	0.06961	0.06136	0.06134	0.05966	0.06254	0.06048	0.07710
58	Stauroneis ancep	0.07127	0.05546	0.05360	0.04510	0.06077	0.06243	0.08268
59	Surirella brebis	0.06939	0.06113	0.06019	0.03090	0.06919	0.06325	0.08537
60	Surirella fastuo	0.07335	0.07932	0.08018	0.04798	0.07513	0.07674	0.09691
61	Synedra fragilar	0.06334	0.07747	0.07834	0.06899	0.08291	0.06195	0.05121

		8	9	10	11	12	13	14
8	Cocconeis pedicu	-						
9	Cocconeis placen	0.02543	-					
10	Craticula molest	0.07414	0.06769	-				
11	Cylindrotheca cl	0.08303	0.07572	0.06466	-			
12	Cymatopleura ell	0.09615	0.08686	0.07118	0.08256	-		
13	Cymbella affinis	0.10906	0.10363	0.10501	0.09845	0.10895	-	
14	Cymbella aspera	0.14246	0.14377	0.14267	0.14169	0.15535	0.12943	-
15	Cymbella cymbifo	0.15615	0.15192	0.16199	0.14570	0.16322	0.08100	0.17158
16	Cymbella lanceol	0.12539	0.11926	0.12982	0.11280	0.13393	0.05204	0.15152
17	Cymbella proxima	0.09547	0.09398	0.09354	0.09436	0.09657	0.08832	0.12555
18	Cymbopleura navi	0.07988	0.07736	0.07904	0.08355	0.08393	0.08421	0.11294
19	Diadesmis gallic	0.07412	0.06861	0.05259	0.05632	0.07046	0.09780	0.13670
20	D. geminata Iran	0.09943	0.09886	0.09715	0.09880	0.09826	0.09299	0.12709
21	D. geminata Norw	0.09844	0.09789	0.09618	0.09784	0.09729	0.09204	0.12627
22	D. geminata NZ L	0.09749	0.09694	0.09524	0.09690	0.09635	0.09110	0.12533
23	D. geminata NZ O	0.09940	0.09884	0.09525	0.09878	0.09637	0.09299	0.12721
24	D. geminata NZ B	0.09750	0.09694	0.09524	0.09690	0.09636	0.09110	0.12533
25	D. geminata NZ M	0.09940	0.09886	0.09713	0.09877	0.09824	0.09298	0.12632
26	D. geminata USA	0.09680	0.09624	0.09457	0.09622	0.09568	0.09038	0.12470
27	Encyonema caespi	0.07427	0.06981	0.06970	0.06675	0.07934	0.07683	0.10838
28	Encyonema minutu	0.07521	0.06885	0.07347	0.06577	0.07645	0.08065	0.10929
29	Encyonema triang	0.07712	0.07268	0.07534	0.06949	0.07739	0.07768	0.11023
30	Entomoneis cf. a	0.07475	0.06735	0.05173	0.06317	0.04414	0.09640	0.13799
31	Eolimna subminus	0.07709	0.07062	0.01587	0.06180	0.06934	0.10125	0.13894
32	Eonotia formica	0.07737	0.07278	0.06373	0.04741	0.07980	0.10605	0.14279
33	Fragilaria delic	0.09343	0.09273	0.09618	0.08992	0.10667	0.12071	0.15289
34	Frustulia vulgar	0.06947	0.06491	0.05654	0.05923	0.07444	0.09390	0.13224
35	Gomphoneis minut	0.09449	0.09490	0.10357	0.09578	0.10943	0.09849	0.13110
36	Gomphonema acumi	0.09530	0.09469	0.10361	0.09494	0.10472	0.09475	0.12816
37	Gomphonema affin	0.09143	0.08892	0.09596	0.09390	0.09994	0.09193	0.12707
38	Gomphonema cf. a	0.08565	0.08217	0.09503	0.08735	0.09803	0.09374	0.12334
39	Gomphonema cf. p	0.09423	0.09170	0.09689	0.09769	0.10367	0.09659	0.13180

40 Gomphonema micro	0.06759	0.06024	0.06871	0.07239	0.07458	0.07691	0.11765
41 Gomphonema parvu	0.09238	0.08988	0.09691	0.09484	0.10089	0.09288	0.12801
42 Gomphonema produ	0.08858	0.08511	0.09794	0.08835	0.09906	0.09292	0.12439
43 Gomphonema pseud	0.09335	0.09085	0.10069	0.09862	0.10182	0.09657	0.12982
44 Gomphonema trunc	0.09533	0.09473	0.10360	0.09587	0.10471	0.09573	0.12910
45 Lyrella atlantic	0.09050	0.08614	0.08658	0.09106	0.10456	0.10025	0.13411
46 Navicula lanceol	0.08944	0.08770	0.07015	0.06323	0.08796	0.10401	0.13669
47 Neidium affine	0.12971	0.12714	0.11710	0.11115	0.12661	0.12254	0.15725
48 Nitzschia closte	0.07929	0.07098	0.05810	0.06244	0.07506	0.09961	0.14180
49 Phaeodactylum tr	0.07929	0.07098	0.05810	0.06244	0.07506	0.09961	0.14180
50 Pinnularia subca	0.10453	0.09907	0.07929	0.09382	0.10479	0.12036	0.15086
51 Placoneis elgine	0.06854	0.06018	0.06405	0.06875	0.08024	0.08351	0.11582
52 Placoneis hamber	0.07042	0.06105	0.06966	0.06580	0.08583	0.08911	0.12144
53 Pseudo-nitzschia	0.08571	0.08216	0.07675	0.05574	0.08534	0.10398	0.13973
54 Pseudo-nitzschia	0.09323	0.08969	0.08049	0.06224	0.09003	0.10867	0.14538
55 Pseudogomphonema	0.08754	0.08299	0.07389	0.06690	0.09551	0.11353	0.13770
56 Sellaphora laevi	0.08053	0.07892	0.06012	0.06270	0.07421	0.10307	0.14293
57 Sellaphora pupul	0.08265	0.08004	0.06026	0.06947	0.07819	0.11145	0.14102
58 Stauroneis ancep	0.07420	0.06865	0.01866	0.06471	0.06189	0.10418	0.14404
59 Surirella brebis	0.08273	0.07629	0.05242	0.06938	0.03547	0.10312	0.14579
60 Surirella fastuo	0.09258	0.08798	0.07132	0.07605	0.06088	0.10936	0.14248
61 Synedra fragilar	0.08685	0.08237	0.08005	0.06719	0.09701	0.10492	0.14184

	15	16	17	18	19	20	21
15 Cymbella cymbifo	-						
16 Cymbella lanceol	0.09550	-					
17 Cymbella proxima	0.14149	0.10937	-				
18 Cymbopleura navi	0.12864	0.10049	0.05548	-			
19 Diadesmis gallic	0.15387	0.11891	0.08912	0.07173	-		
20 D. geminata Iran	0.14415	0.11778	0.03670	0.06194	0.08886	-	
21 D. geminata Norw	0.14317	0.11682	0.03671	0.06097	0.08789	0.00469	-
22 D. geminata NZ L	0.14222	0.11588	0.03576	0.06003	0.08695	0.00375	0.00281
23 D. geminata NZ O	0.14413	0.11777	0.03764	0.06191	0.08885	0.00563	0.00281
24 D. geminata NZ B	0.14222	0.11587	0.03577	0.06003	0.08695	0.00375	0.00094

25	D. geminata NZ M	0.14412	0.11777	0.03765	0.06191	0.08883	0.00562	0.00469
26	D. geminata USA	0.14163	0.11523	0.03493	0.05925	0.08624	0.00282	0.00094
27	Encyonema caespi	0.13070	0.09776	0.07159	0.04496	0.05303	0.07424	0.07328
28	Encyonema minutu	0.13262	0.09780	0.07253	0.04308	0.05391	0.07799	0.07703
29	Encyonema triang	0.12874	0.09770	0.07255	0.04589	0.05960	0.07891	0.07794
30	Entomoneis cf. a	0.15079	0.12318	0.08750	0.07111	0.05109	0.08822	0.08725
31	Eolimna subminus	0.16016	0.12505	0.09537	0.07807	0.05639	0.09897	0.09800
32	Eonotia formica	0.15342	0.12335	0.09528	0.08000	0.05438	0.10748	0.10653
33	Fragilaria delic	0.16939	0.13795	0.10717	0.08967	0.09065	0.11555	0.11458
34	Frustulia vulgar	0.14320	0.10639	0.08060	0.05487	0.04542	0.08251	0.08154
35	Gomphoneis minut	0.14207	0.11384	0.08768	0.07504	0.10104	0.09499	0.09215
36	Gomphonema acumi	0.14207	0.11583	0.08569	0.07216	0.09447	0.09679	0.09395
37	Gomphonema affin	0.13547	0.11204	0.08000	0.07490	0.09062	0.08826	0.08542
38	Gomphonema cf. a	0.13536	0.11477	0.07810	0.06835	0.08308	0.08923	0.08639
39	Gomphonema cf. p	0.14109	0.11669	0.08282	0.07865	0.09155	0.09202	0.08918
40	Gomphonema micro	0.12988	0.10072	0.06677	0.03745	0.05481	0.07134	0.07037
41	Gomphonema parvu	0.13643	0.11301	0.08094	0.07583	0.09157	0.08920	0.08636
42	Gomphonema produ	0.13458	0.11397	0.07817	0.06748	0.08598	0.08931	0.08647
43	Gomphonema pseud	0.14012	0.11668	0.07999	0.07678	0.09349	0.09190	0.08824
44	Gomphonema trunc	0.14114	0.11490	0.08664	0.07309	0.09540	0.09773	0.09489
45	Lyrella atlantic	0.14668	0.10415	0.08783	0.07608	0.08587	0.09320	0.09224
46	Navicula lanceol	0.15294	0.11748	0.09788	0.08058	0.05889	0.10140	0.10044
47	Neidium affine	0.16832	0.12830	0.12211	0.11866	0.11441	0.12378	0.12093
48	Nitzschia closte	0.14380	0.11787	0.09174	0.07322	0.04219	0.09340	0.09245
49	Phaeodactylum tr	0.14380	0.11787	0.09174	0.07322	0.04219	0.09340	0.09245
50	Pinnularia subca	0.16891	0.13762	0.11178	0.09340	0.08980	0.11268	0.11170
51	Placoneis elgine	0.13658	0.10170	0.06861	0.04028	0.06147	0.07880	0.07783
52	Placoneis hamber	0.13359	0.10256	0.07246	0.04775	0.06048	0.08173	0.08076
53	Pseudo-nitzschia	0.15407	0.12041	0.09066	0.08445	0.06464	0.09698	0.09604
54	Pseudo-nitzschia	0.15591	0.12323	0.09630	0.08913	0.07403	0.10260	0.09978
55	Pseudogomphonema	0.15875	0.12419	0.10370	0.08533	0.05982	0.11004	0.10910
56	Sellaphora laevi	0.15323	0.11935	0.09271	0.06588	0.04333	0.09529	0.09432
57	Sellaphora pupul	0.15397	0.12574	0.09835	0.07059	0.05201	0.09617	0.09519
58	Stauroneis ancep	0.16216	0.12536	0.09848	0.07722	0.05358	0.09644	0.09549

59 Surirella brebis	0.15447	0.12616	0.09652	0.08005	0.05825	0.09351	0.09255
60 Surirella fastuo	0.16002	0.13255	0.10369	0.09655	0.07064	0.10529	0.10433
61 Synedra fragilar	0.15235	0.12331	0.09813	0.08460	0.07255	0.10550	0.10452

	22	23	24	25	26	27	28
22 D. geminata NZ L	-						
23 D. geminata NZ O	0.00375	-					
24 D. geminata NZ B	0.00188	0.00188	-				
25 D. geminata NZ M	0.00375	0.00563	0.00375	-			
26 D. geminata USA	0.00094	0.00188	0.00000	0.00282	-		
27 Encyonema caespi	0.07234	0.07422	0.07234	0.07422	0.07161	-	
28 Encyonema minutu	0.07609	0.07797	0.07609	0.07797	0.07537	0.01779	-
29 Encyonema triang	0.07701	0.07888	0.07700	0.07888	0.07628	0.02623	0.01593
30 Entomoneis cf. a	0.08631	0.08632	0.08631	0.08820	0.08560	0.06648	0.05976
31 Eolimna subminus	0.09706	0.09706	0.09706	0.09894	0.09639	0.07061	0.07061
32 Eonotia formica	0.10558	0.10558	0.10559	0.10748	0.10492	0.07630	0.07160
33 Fragilaria delic	0.11364	0.11366	0.11364	0.11552	0.11300	0.08795	0.08604
34 Frustulia vulgar	0.08060	0.08061	0.08060	0.08249	0.07990	0.05121	0.04736
35 Gomphoneis minut	0.09309	0.09308	0.09121	0.09497	0.09142	0.07236	0.06860
36 Gomphonema acumi	0.09489	0.09489	0.09301	0.09677	0.09323	0.07043	0.06479
37 Gomphonema affin	0.08636	0.08636	0.08448	0.08824	0.08467	0.06942	0.06941
38 Gomphonema cf. a	0.08733	0.08733	0.08545	0.08921	0.08566	0.06283	0.05720
39 Gomphonema cf. p	0.09012	0.09012	0.08825	0.09200	0.08845	0.07127	0.07126
40 Gomphonema micro	0.06943	0.07131	0.06943	0.07131	0.06869	0.03566	0.03282
41 Gomphonema parvu	0.08729	0.08729	0.08542	0.08918	0.08561	0.07036	0.07035
42 Gomphonema produ	0.08740	0.08740	0.08553	0.08929	0.08574	0.06385	0.05820
43 Gomphonema pseud	0.08918	0.08918	0.08731	0.09106	0.08750	0.07317	0.07316
44 Gomphonema trunc	0.09583	0.09583	0.09396	0.09771	0.09418	0.07135	0.06571
45 Lyrella atlantic	0.09131	0.09319	0.09131	0.09318	0.09062	0.07610	0.07806
46 Navicula lanceol	0.09950	0.10138	0.09950	0.10137	0.09883	0.07131	0.06755
47 Neidium affine	0.12187	0.12000	0.11999	0.12375	0.12027	0.11507	0.11311
48 Nitzschia closte	0.09150	0.09152	0.09152	0.09341	0.09084	0.06388	0.05921
49 Phaeodactylum tr	0.09150	0.09152	0.09152	0.09341	0.09084	0.06388	0.05921
50 Pinnularia subca	0.11076	0.11078	0.11076	0.11264	0.11012	0.09923	0.09356

51 Placoneis elgine	0.07690	0.07877	0.07689	0.07876	0.07617	0.04038	0.03661
52 Placoneis hamber	0.07982	0.08170	0.07983	0.08171	0.07913	0.04786	0.04409
53 Pseudo-nitzschia	0.09509	0.09699	0.09511	0.09700	0.09441	0.07696	0.07132
54 Pseudo-nitzschia	0.10071	0.09883	0.09884	0.10262	0.09907	0.08539	0.07881
55 Pseudogomphonema	0.10815	0.11004	0.10816	0.11005	0.10751	0.07888	0.07327
56 Sellaphora laevi	0.09338	0.09338	0.09338	0.09526	0.09269	0.06034	0.06220
57 Sellaphora pupul	0.09425	0.09426	0.09425	0.09612	0.09357	0.06889	0.06887
58 Stauroneis ancep	0.09454	0.09454	0.09455	0.09645	0.09387	0.06974	0.07069
59 Surirella brebis	0.09162	0.09163	0.09162	0.09350	0.09093	0.07362	0.07075
60 Surirella fastuo	0.10340	0.10340	0.10339	0.10530	0.10274	0.08994	0.08421
61 Synedra fragilar	0.10359	0.10359	0.10357	0.10544	0.10289	0.08199	0.07821

	29	30	31	32	33	34	35
29 Encyonema triang	-						
30 Entomoneis cf. a	0.06160	-					
31 Eolimna subminus	0.07340	0.05083	-				
32 Eonotia formica	0.07348	0.05098	0.06288	-			
33 Fragilaria delic	0.08883	0.08653	0.09721	0.08042	-		
34 Frustulia vulgar	0.04922	0.05486	0.05467	0.05352	0.08224	-	
35 Gomphoneis minut	0.07327	0.09285	0.09978	0.09797	0.12023	0.08147	-
36 Gomphonema acumi	0.06760	0.08913	0.10173	0.09228	0.11449	0.07772	0.01688
37 Gomphonema affin	0.06753	0.08806	0.09312	0.09128	0.11532	0.07479	0.04127
38 Gomphonema cf. a	0.06095	0.07864	0.09314	0.08375	0.10681	0.06721	0.03657
39 Gomphonema cf. p	0.07127	0.09089	0.09407	0.09503	0.11906	0.07763	0.04595
40 Gomphonema micro	0.03470	0.05689	0.06870	0.06781	0.07943	0.04354	0.06285
41 Gomphonema parvu	0.06847	0.08901	0.09406	0.09223	0.11627	0.07574	0.04221
42 Gomphonema produ	0.06008	0.07963	0.09605	0.08475	0.10690	0.06820	0.03756
43 Gomphonema pseud	0.07129	0.09096	0.09784	0.09412	0.11817	0.07766	0.04408
44 Gomphonema trunc	0.06852	0.08911	0.10171	0.09322	0.11449	0.07866	0.01595
45 Lyrella atlantic	0.07610	0.08989	0.08466	0.09610	0.11516	0.07110	0.09866
46 Navicula lanceol	0.07504	0.07436	0.06831	0.06786	0.08968	0.05171	0.10133
47 Neidium affine	0.11124	0.10921	0.11899	0.11414	0.14202	0.11671	0.12452
48 Nitzschia closte	0.06397	0.04916	0.05532	0.05592	0.08446	0.04617	0.09394
49 Phaeodactylum tr	0.06397	0.04916	0.05532	0.05592	0.08446	0.04617	0.09394

50 Pinnularia subca	0.09059	0.08373	0.07646	0.08725	0.10797	0.08408	0.10497
51 Placoneis elgine	0.04126	0.05787	0.06877	0.06312	0.08421	0.04738	0.07508
52 Placoneis hamber	0.04974	0.06554	0.07249	0.06586	0.09455	0.05784	0.08065
53 Pseudo-nitzschia	0.07509	0.06315	0.07396	0.06501	0.08777	0.06865	0.10052
54 Pseudo-nitzschia	0.08071	0.06879	0.07677	0.06873	0.09057	0.07146	0.10895
55 Pseudogomphonema	0.08169	0.07723	0.07581	0.06321	0.09439	0.06299	0.10612
56 Sellaphora laevi	0.06502	0.05763	0.06199	0.05616	0.09413	0.04502	0.09613
57 Sellaphora pupul	0.07261	0.06159	0.06117	0.06481	0.09437	0.05178	0.09983
58 Stauroneis ancep	0.07446	0.04612	0.01871	0.06561	0.09722	0.05278	0.09989
59 Surirella brebis	0.07264	0.02439	0.05338	0.06098	0.09331	0.06128	0.10377
60 Surirella fastuo	0.08889	0.04422	0.06949	0.06866	0.10688	0.07558	0.10050
61 Synedra fragilar	0.08189	0.07208	0.08197	0.06439	0.04576	0.07079	0.10540

	36	37	38	39	40	41	42
36 Gomphonema acumi	-						
37 Gomphonema affin	0.03280	-					
38 Gomphonema cf. a	0.02530	0.02154	-				
39 Gomphonema cf. p	0.03561	0.01124	0.02341	-			
40 Gomphonema micro	0.06279	0.05712	0.05431	0.06273	-		
41 Gomphonema parvu	0.03374	0.00094	0.02247	0.01217	0.05805	-	
42 Gomphonema produ	0.02626	0.02062	0.00281	0.02530	0.05342	0.02156	-
43 Gomphonema pseud	0.03374	0.00749	0.02341	0.01405	0.06087	0.00843	0.02343
44 Gomphonema trunc	0.00094	0.03373	0.02623	0.03654	0.06371	0.03467	0.02720
45 Lyrella atlantic	0.10345	0.09492	0.10056	0.10051	0.07621	0.09586	0.09973
46 Navicula lanceol	0.10135	0.09751	0.09566	0.10315	0.06660	0.09845	0.09479
47 Neidium affine	0.12554	0.12643	0.12356	0.13017	0.11413	0.12738	0.12558
48 Nitzschia closte	0.08840	0.08648	0.07898	0.08742	0.05263	0.08742	0.08186
49 Phaeodactylum tr	0.08840	0.08648	0.07898	0.08742	0.05263	0.08742	0.08186
50 Pinnularia subca	0.10014	0.10295	0.10202	0.10767	0.09068	0.10389	0.10304
51 Placoneis elgine	0.06939	0.07121	0.05998	0.07308	0.03466	0.07215	0.06095
52 Placoneis hamber	0.07871	0.07678	0.06929	0.07865	0.04213	0.07772	0.07027
53 Pseudo-nitzschia	0.09681	0.09764	0.09201	0.10141	0.07421	0.09858	0.09305
54 Pseudo-nitzschia	0.10432	0.10514	0.09951	0.10891	0.08358	0.10607	0.10055
55 Pseudogomphonema	0.10143	0.10132	0.09664	0.10601	0.07413	0.10226	0.09766

56 Sellaphora laevi	0.09052	0.09042	0.08475	0.09325	0.05845	0.09136	0.08578
57 Sellaphora pupul	0.09711	0.09512	0.09137	0.09795	0.06504	0.09606	0.09238
58 Stauroneis ancep	0.09902	0.09133	0.09229	0.09229	0.06877	0.09226	0.09519
59 Surirella brebis	0.09909	0.09523	0.08953	0.09613	0.06793	0.09618	0.09245
60 Surirella fastuo	0.10143	0.09760	0.09470	0.10315	0.08332	0.09858	0.09575
61 Synedra fragilar	0.10264	0.10351	0.09506	0.10820	0.07715	0.10445	0.09607

	43	44	45	46	47	48	49
43 Gomphonema pseud	-						
44 Gomphonema trunc	0.03467	-					
45 Lyrella atlantic	0.10145	0.10252	-				
46 Navicula lanceol	0.10411	0.10040	0.09279	-			
47 Neidium affine	0.12834	0.12458	0.12635	0.12315	-		
48 Nitzschia closte	0.08931	0.08932	0.09216	0.06797	0.11501	-	
49 Phaeodactylum tr	0.08931	0.08932	0.09216	0.06797	0.11501	0.00000	-
50 Pinnularia subca	0.10960	0.10016	0.10204	0.10026	0.11728	0.09119	0.09119
51 Placoneis elgine	0.07311	0.07031	0.08186	0.07325	0.11428	0.06213	0.06213
52 Placoneis hamber	0.07868	0.07869	0.07901	0.07788	0.11314	0.06488	0.06488
53 Pseudo-nitzschia	0.10138	0.09775	0.09203	0.08251	0.12620	0.05851	0.05851
54 Pseudo-nitzschia	0.10888	0.10526	0.09764	0.08623	0.12430	0.06037	0.06037
55 Pseudogomphonema	0.10509	0.10236	0.09949	0.02685	0.13072	0.06701	0.06701
56 Sellaphora laevi	0.09325	0.09144	0.09045	0.06921	0.12094	0.05335	0.05335
57 Sellaphora pupul	0.09796	0.09803	0.09320	0.07043	0.12961	0.05161	0.05161
58 Stauroneis ancep	0.09511	0.09899	0.08858	0.07021	0.11902	0.05531	0.05531
59 Surirella brebis	0.09806	0.09908	0.09886	0.07952	0.12070	0.05536	0.05536
60 Surirella fastuo	0.10036	0.10142	0.09645	0.09178	0.12893	0.07984	0.07984
61 Synedra fragilar	0.10732	0.10263	0.09689	0.08383	0.12559	0.07578	0.07578

	50	51	52	53	54	55	56
50 Pinnularia subca	-						
51 Placoneis elgine	0.08510	-					
52 Placoneis hamber	0.09263	0.02905	-				
53 Pseudo-nitzschia	0.09551	0.08086	0.08262	-			
54 Pseudo-nitzschia	0.09830	0.08837	0.09011	0.01018	-		

55 Pseudogomphonema	0.09934	0.07701	0.08258	0.07601	0.08065	-	
56 Sellaphora laevi	0.07522	0.06690	0.06974	0.07200	0.07668	0.07107	-
57 Sellaphora pupul	0.07918	0.06987	0.07264	0.07597	0.07970	0.07231	0.01874
58 Stauroneis ancep	0.07947	0.06603	0.07257	0.07595	0.08065	0.07301	0.06015
59 Surirella brebis	0.09350	0.06888	0.07640	0.07403	0.07683	0.08142	0.06483
60 Surirella fastuo	0.09851	0.08319	0.08506	0.08453	0.08824	0.09747	0.07533
61 Synedra fragilar	0.09853	0.08011	0.07907	0.07357	0.07543	0.08479	0.08272

Table A.11 (16): Pairwise distances of population-level ITS alignment of 40B sample (Paup* output of uncorrected ("p") distance matrix).

	1	2	3	4	5	6	7
1 NZ 40B.c12	-						
2 NZ 40B.c14	0.00838	-					
3 NZ 40B.c16	0.00503	0.01005	-				
4 NZ 40B.c3	0.00503	0.01005	0.00670	-			
5 NZ 40B.c8	0.00503	0.01005	0.00670	0.00670	-		
6 NZ 40B.c11	0.00838	0.00335	0.01005	0.01005	0.01005	-	
7 NZ 40B.c7	0.00168	0.00671	0.00336	0.00336	0.00336	0.00672	-

Alignment and Tree Building Statistics

Table A.12 (17): Model Test Statistics. Hierarchical Likelihood Ratio Tests (hLRTs) for alignments in Chapter 2

DNA Region	Substitution Model	Bootstrap Replicates	Transition/ Transversion ratio	Proportion of Invariable Sites	No. of Substituion Rate Categories	Gamma Distribution Parameter
Diatom partial 18S	TrN+I+G	100	Estimated	0.4115	4	0.3733
D. geminata partial 18S-ITS	HKY+G	100	4.1936	0	4	0.2134
D. geminata ITS	HKY	100	1.8354	0	-	Equal
D. geminata NZ Buller R. ITS	HKY	100	3.0044	0	-	Equal

Table A.13 (18): PhyML Maximum Likelihood Statistics for alignments in Chapter 2

DNA Region	Likelihood (loglk)	GC Content (%)	Nucleotide Frequencies			
			A	C	G	T
Diatom partial 18S	-9956.71401	48.5	0.25179	0.18730	0.27042	0.29050
D. geminata partial 18S-ITS	-2363.65590	40.5	0.29994	0.18406	0.22062	0.29539
D. geminata ITS	-1451.75506	40.4	0.29708	0.22155	0.18232	0.29905
D. geminata NZ Buller R. ITS	-904.91527	40.3	0.29823	0.22188	0.18095	0.29895

Table A.14 (19): Maximum Parsimony Statistics for alignments in Chapter 2

DNA Region	Total Characters (No.)	Constant Characters (No.)	Variable Characters	
			Parsimony-Uninformative (No.)	Parsimony-Informative (No.)
Diatom partial 18S	1,111	672	163	276
D. geminata partial 18S-ITS	1,154	1042	85	27
D. geminata ITS	610	527	59	24
D. geminata NZ Buller R. ITS	597	585	9	3

Additional Phylogenetic Trees

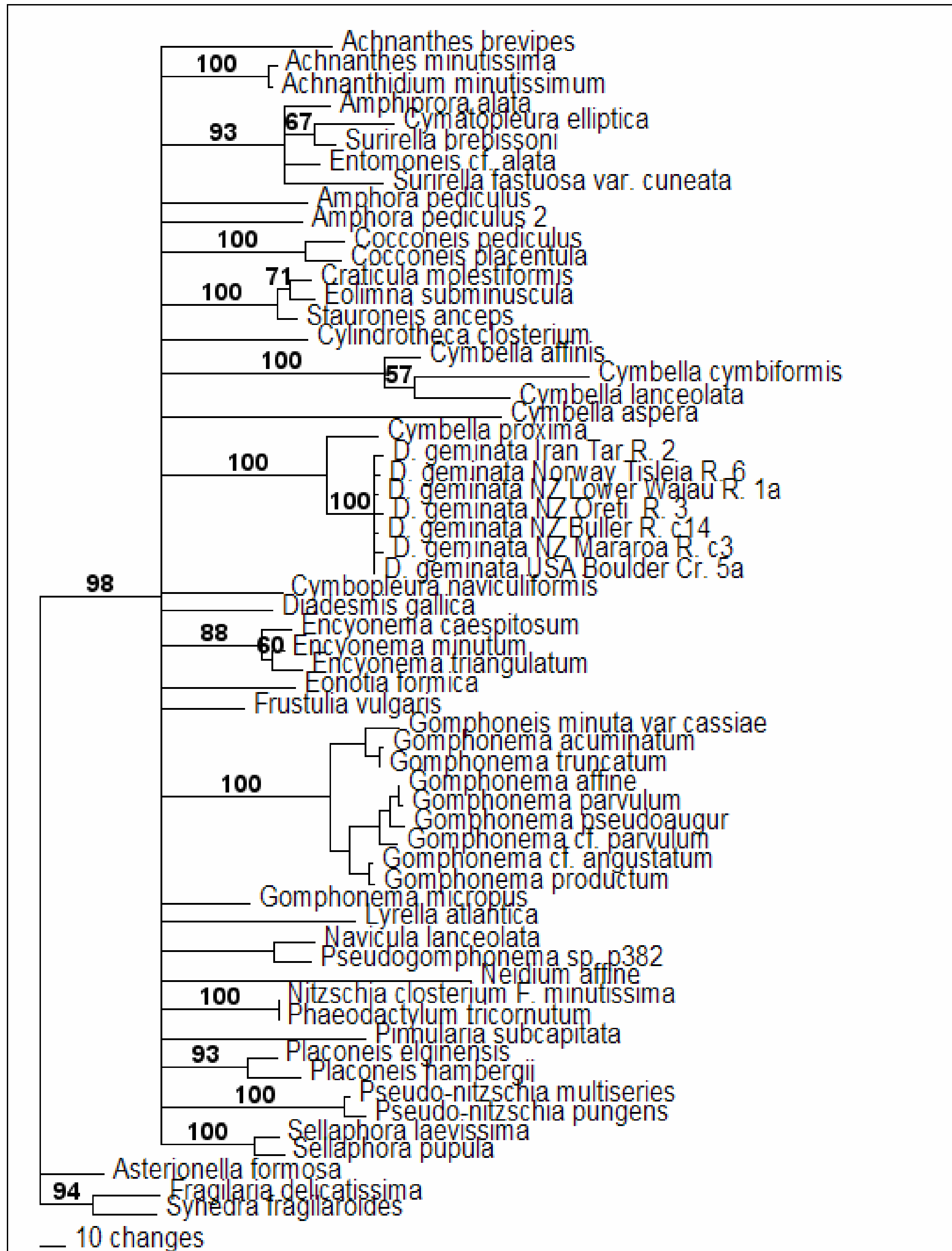

Figure A.10 (28): Strict consensus Maximum Parsimony (MP) tree of the taxonomic placement of *D. geminata* based on 1111 bp long partial 18S region of 58 raphid pennate diatoms (Bacillariophyceae) rooted with 3 araphid pennate diatoms (Fragilariophyceae). Numbers represent percentage boostrap support from 1000 bootstrap replicates. Bootstrap values < 50% are not shown.

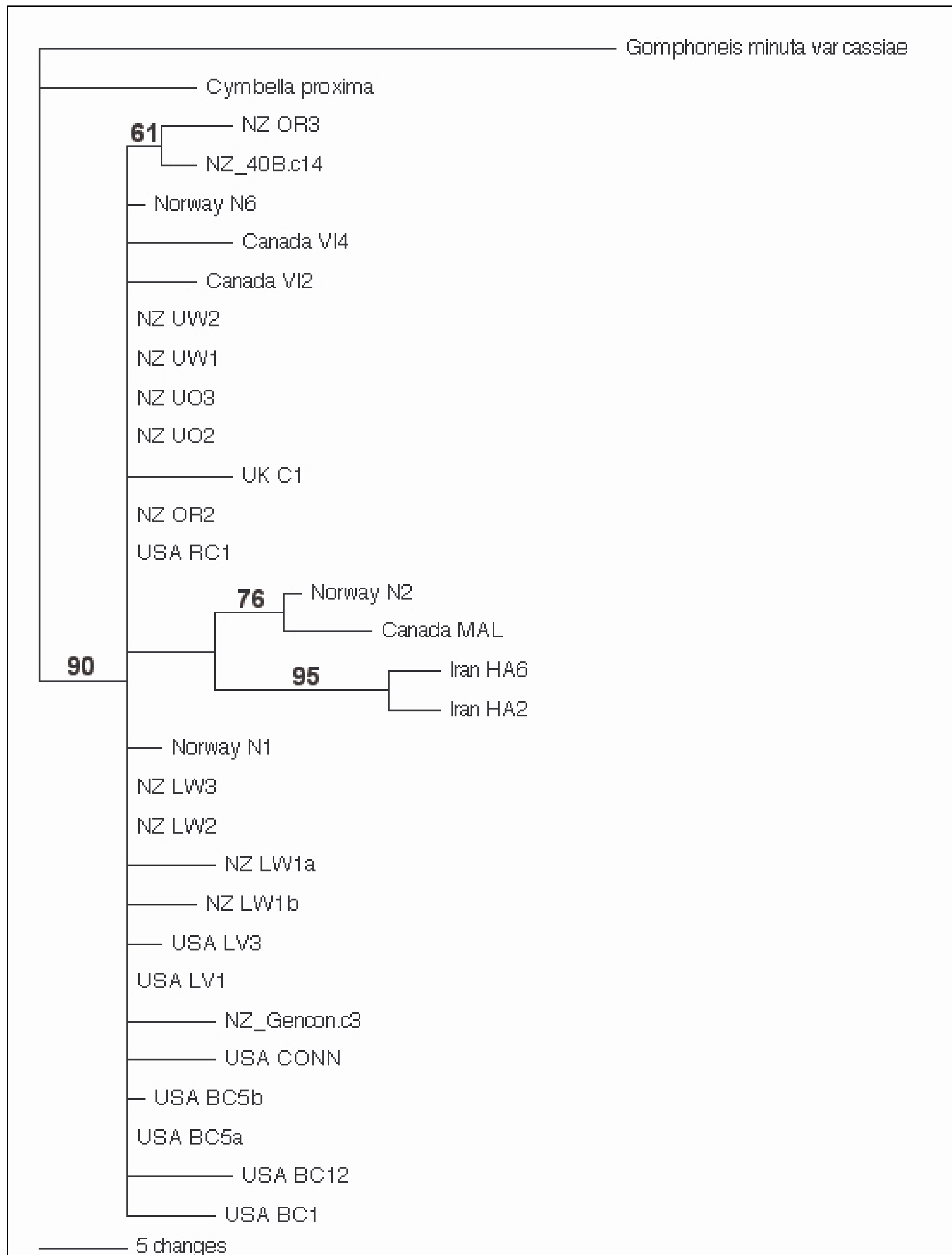

Figure A.11 (29): Strict consensus Maximum Parsimony (MP) tree for the 1154 bp long 18S-ITS region for 29 *D. geminata* sequences, rooted with *Gomphoneis minuta* var *cassiae* (Gomphonemataceae) and *Cymbella proxima* (Cymbellaceae). Numbers represent percentage boostrap support from 1000 bootstrap replicates. Bootstrap values < 50% are not shown.

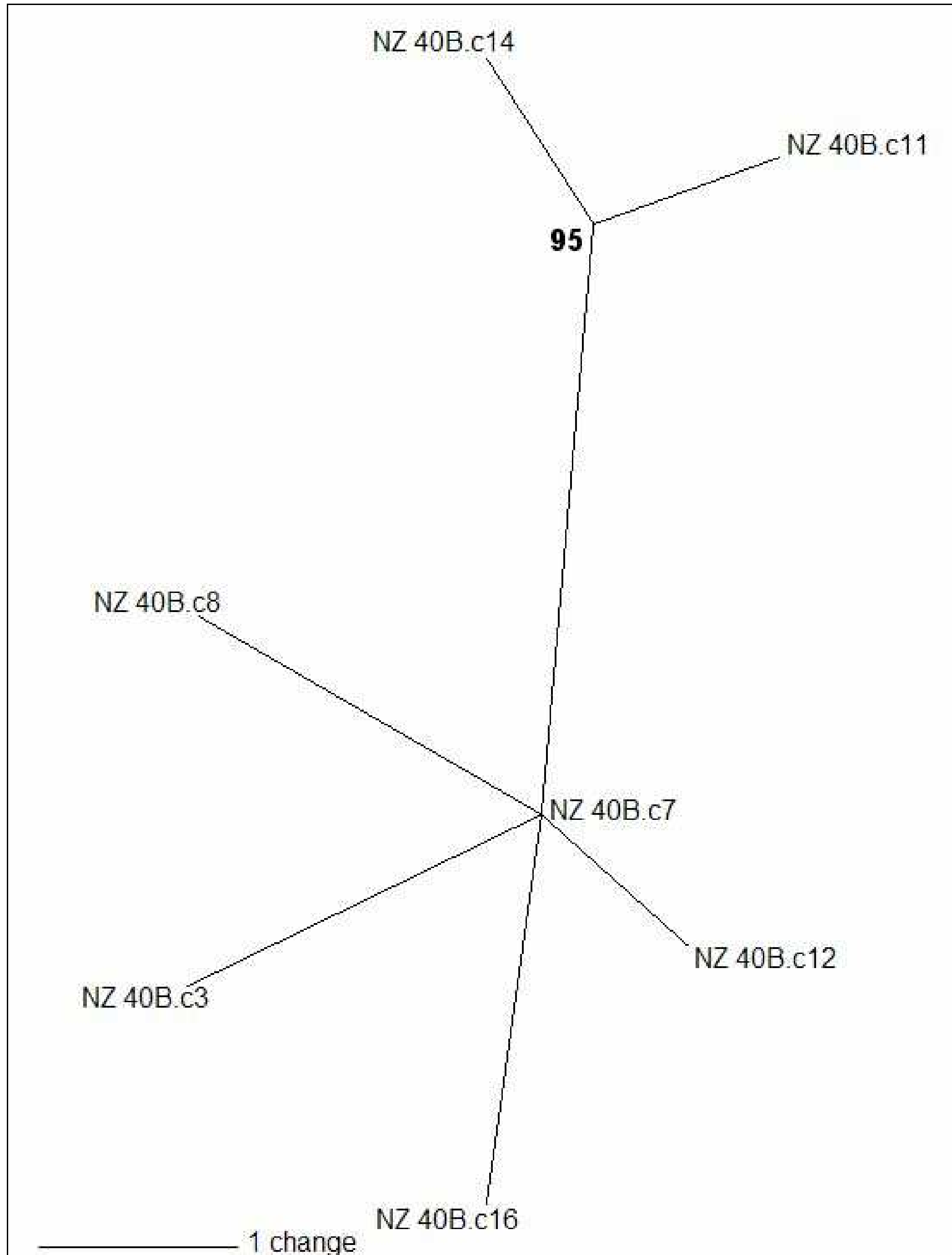

Figure A.12 (30): Unrooted strict consensus Maximum Parsimony (MP) tree for the 597 bp long ITS region of seven cloned sequences from the New Zealand Buller River sample. Numbers represent percentage boostrap support from 1000 bootstrap replicates. Bootstrap values < 50% are not shown.

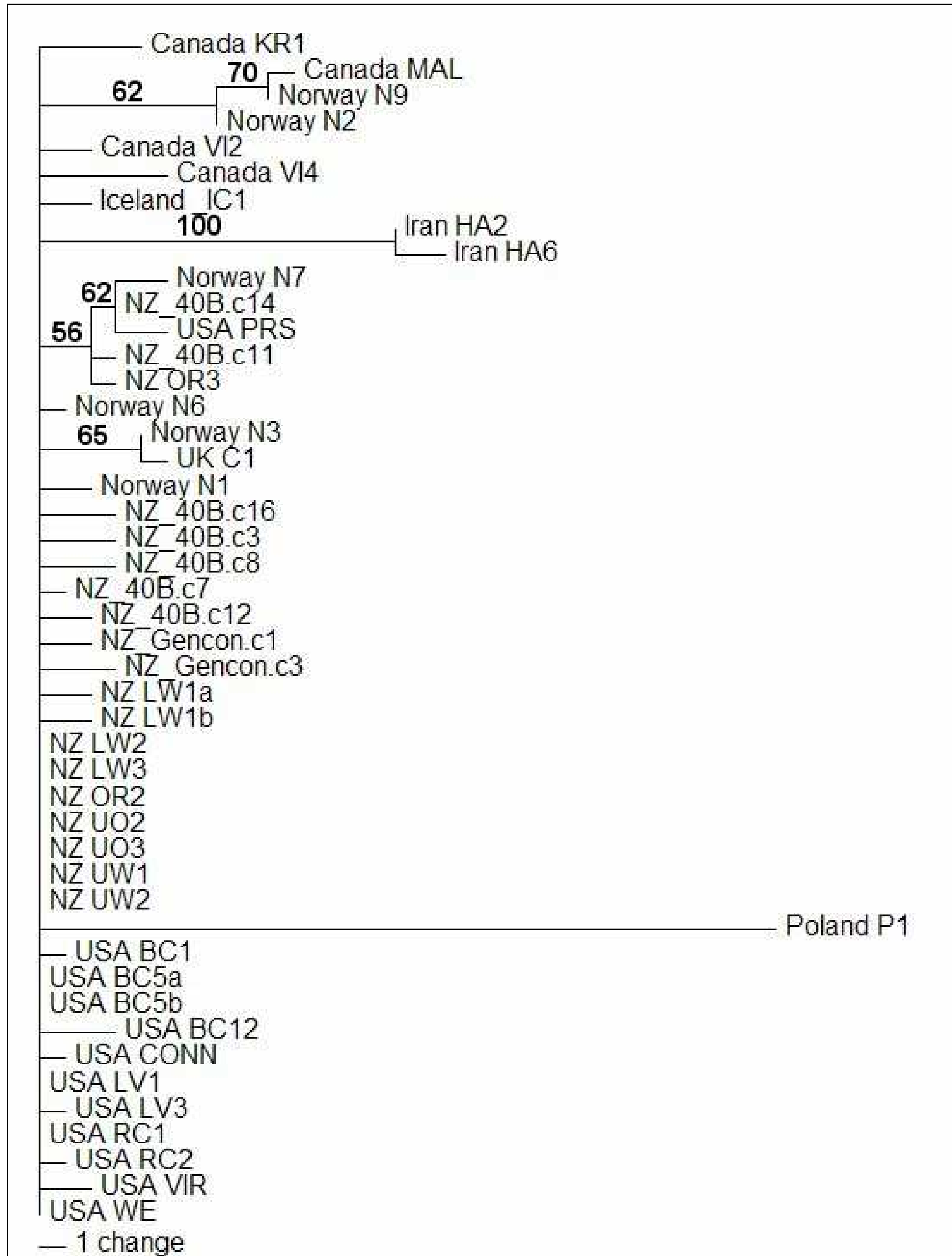

Figure A.13 (31): Unrooted strict consensus Maximum Parsimony (MP) tree for the 610 bp long ITS region for 46 *D. geminata* sequences. Numbers represent percentage boostrap support from 1000 bootstrap replicates. Bootstrap values < 50% are not shown.

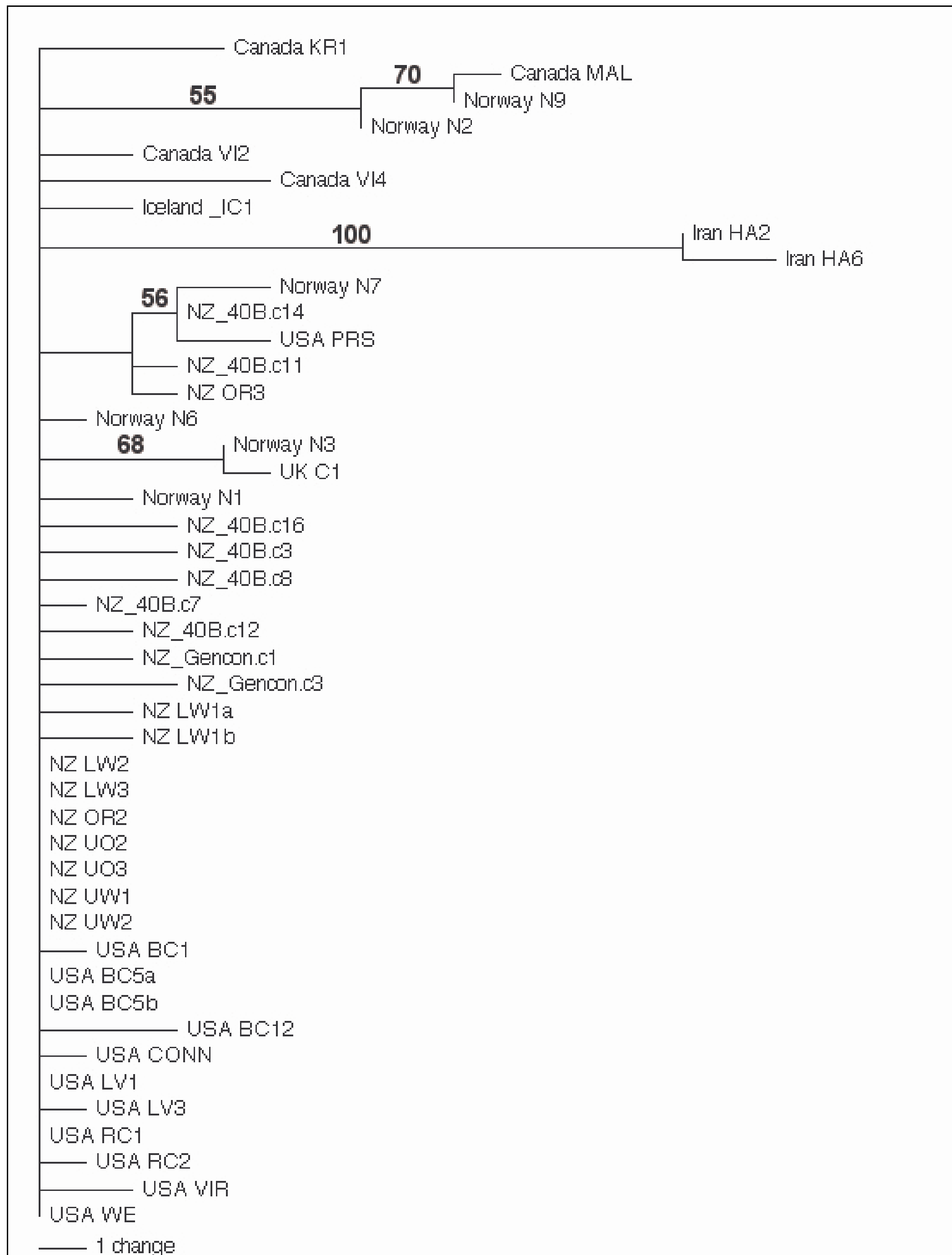

Figure A.14 (32): Unrooted strict consensus Maximum Parsimony (MP) tree for the 600 bp long ITS region without P1 for 45 *D. geminata* sequences. Numbers represent percentage boostrap support from 1000 bootstrap replicates. Bootstrap values < 50% are not shown.

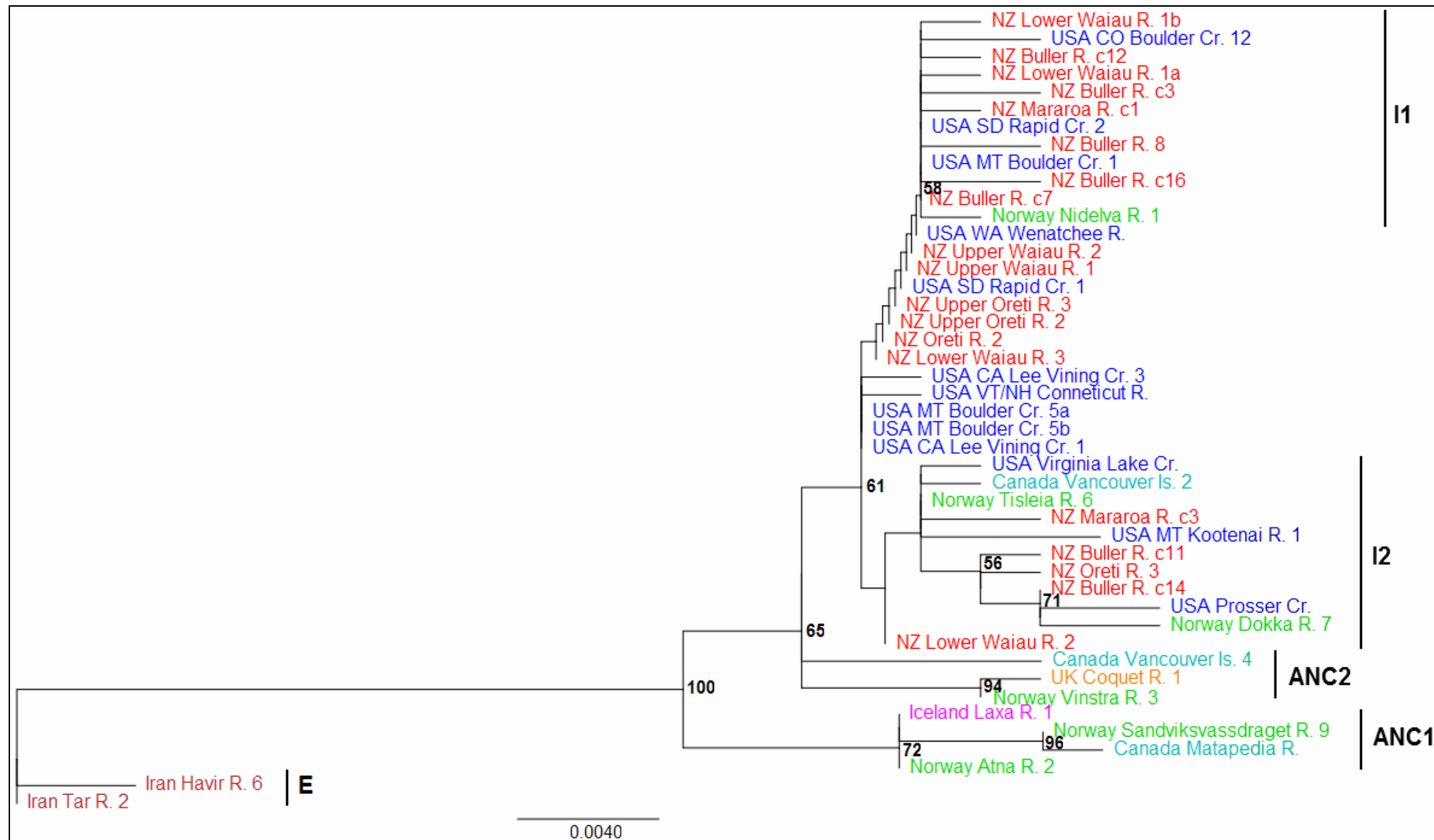

Figure A.15 (33): Maximum Likelihood (ML) tree for the 600 bp long ITS region withouth P1 for 45 *D. geminata* sequences. Numbers at nodes represent percentage boostrap support from 100 bootstrap replicates. Bootstrap values < 50% are not shown. *D. geminata* sample colours: NZ=red, USA=blue, Norway=green, Canada=light blue, UK=orange, Iran=brown, Iceland=Pink, Poland=Purple. I1 = Invasion Clade 1, I2 = Invasion Clade 2, H = Iran Clade, ANC1 = Ancestral Clade 1, ANC2 = Ancestral Clade 2.

rRNA Secondary Folding Structures

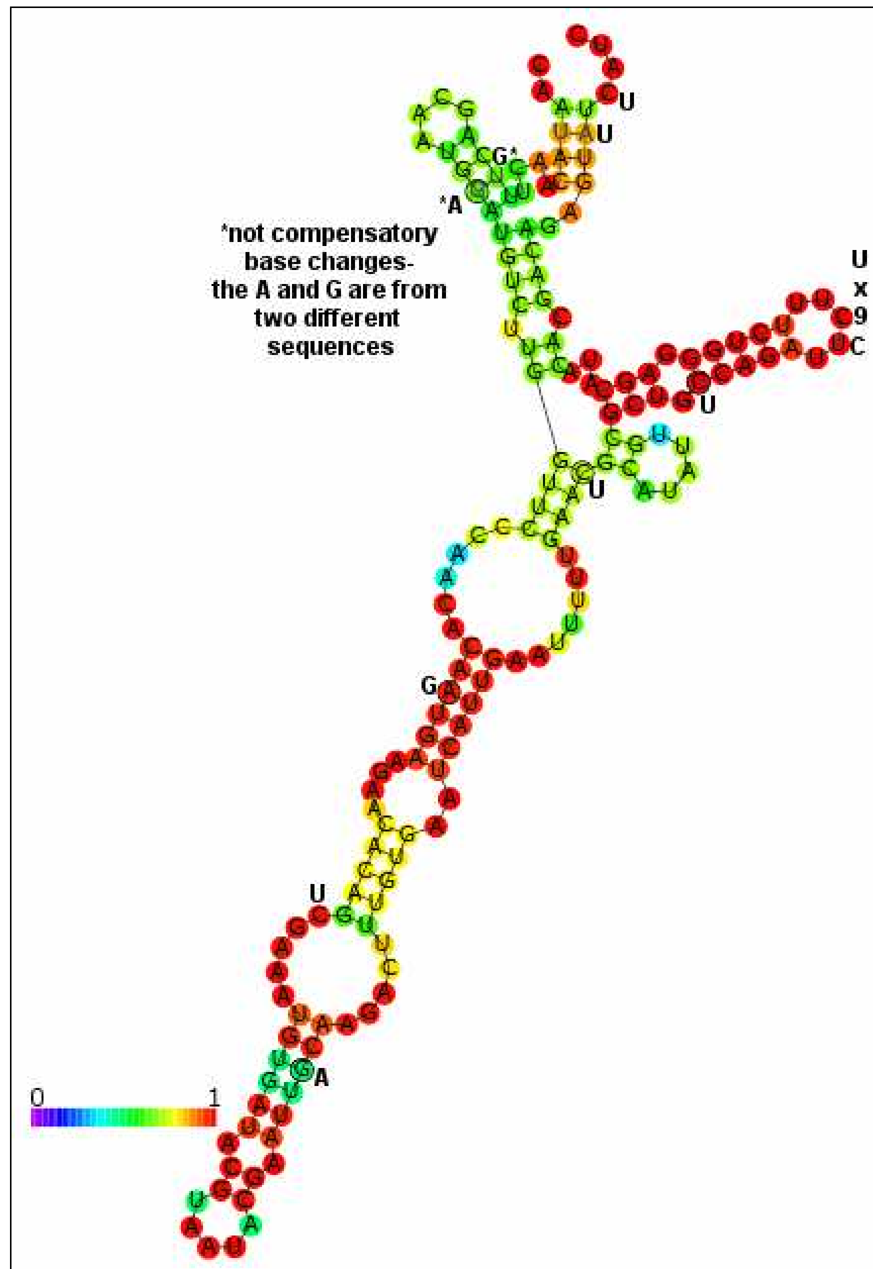

Figure A.16 (34): Secondary structure of 5.8S folded using the 5.8S alignment of 45 *D. geminata* sequences and the new RNAalifold with RIBOSUM scoring. Shading: base pair probabilities increase closer to 1.

The 5.8S, ITS1 and ITS2 alignments of all 45 *D. geminata* sequences were analysed using the RNAalifold WebServer (Gruber et al. 2008). The Poland sample (P1) was excluded based on its ambiguous taxonomic position. Regions coloured in red were most highly conserved across several folding programs accessed through the Webserver for Aligning

non-coding RNAs (WAR) (Torarinsson & Lindgreen 2008). The secondary structures produced using the RNAalifold WebServer (Figs. A.16-A.18) were examined for the presence of any compensatory base changes (CBCs) present. Only one potential CBC site was found in 5.8S and one in ITS1 and these were not true CBC sites as the base mutations were each found in difference sequences. Therefore, the characters used in the ITS alignments in chapter two are all likely to represent original base changes uninfluenced by previous base changes in the region.

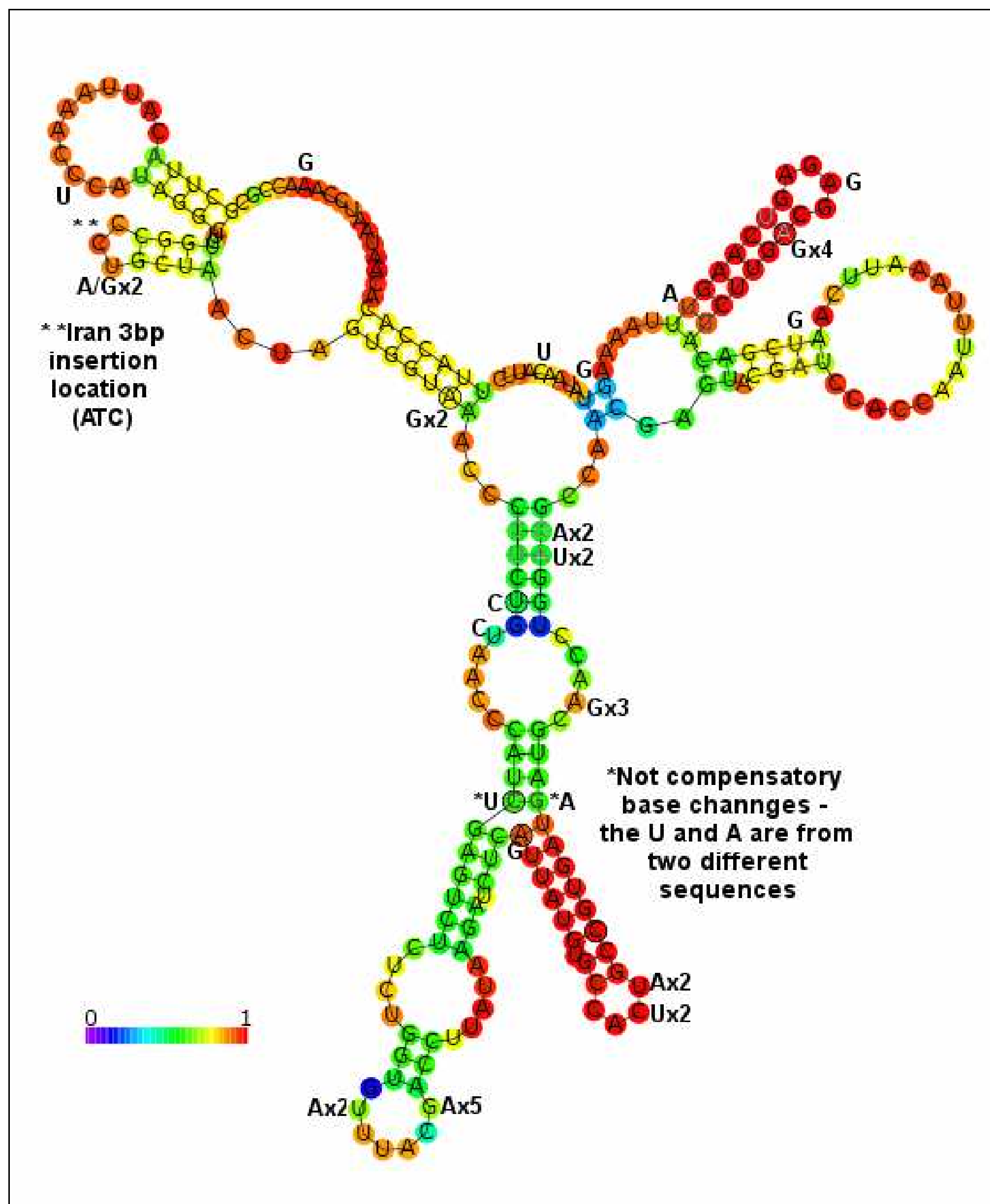

Figure A.17 (35): Secondary structure of ITS1 folded using the ITS1 alignment of 45 D. geminata sequences, excluding indels, and the new RNAalifold with RIBOSUM scoring. Shading: base pair probabilities increase closer to 1.

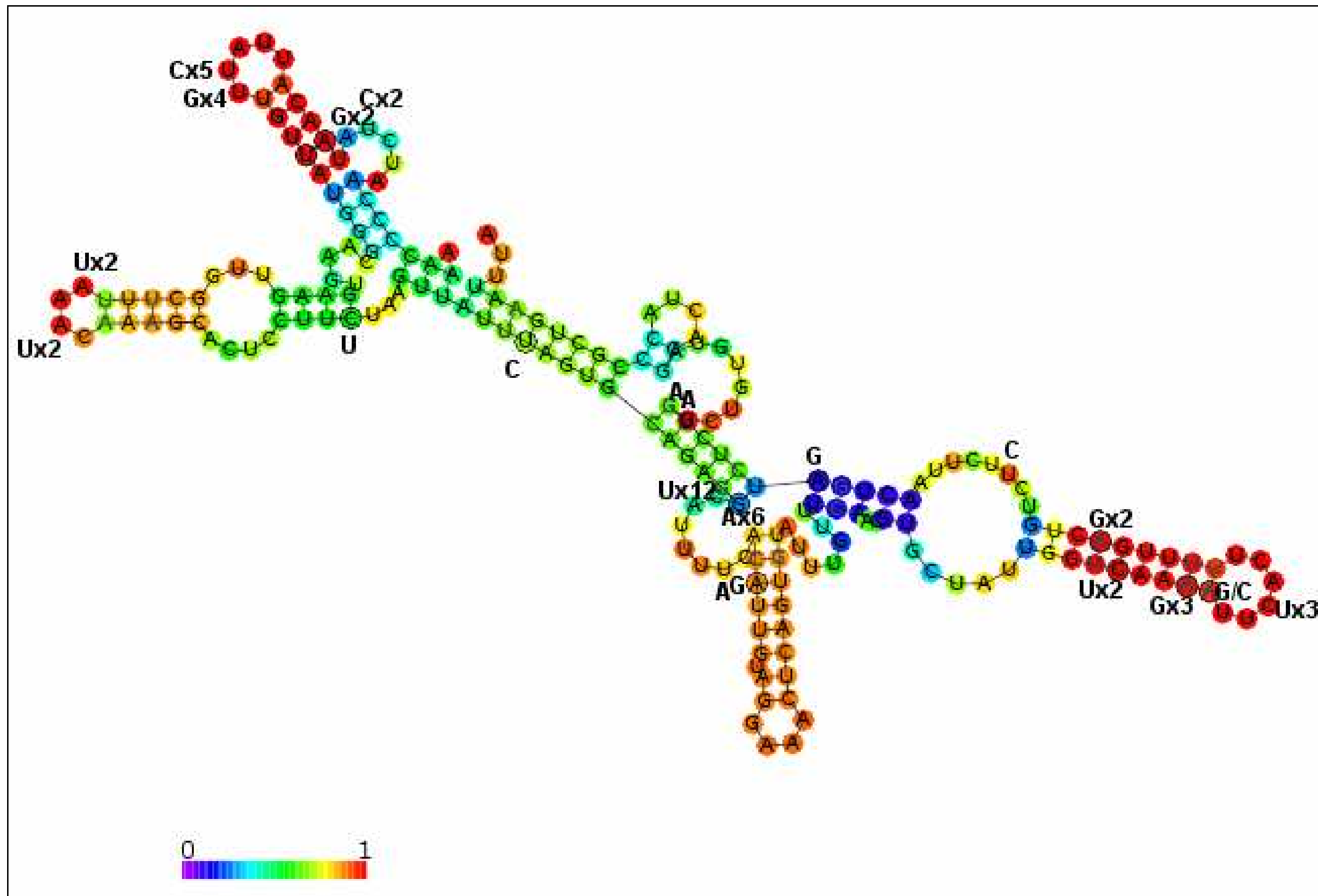

Figure A. 18 (36): Secondary structure of ITS2 folded using the ITS2 alignment of 45 *D. geminata* sequences and the new RNAalifold with RIBOSUM scoring. Shading: base pair probabilities increase closer to 1.

APPENDIX 5: Glossary

Stramenopiles: Protists with a distinct type of flagella that is modified or completely lost in some lineages

Algae: Unicellular or simple multicellular photosynthetic eukaryotes that do not have multicellular sex organs

Allelic richness: The number of alleles present per locus in a population

Allopatric: Populations in geographically overlapping regions that are separated by some other factor, e.g.) seasons

Anthropogenic: The effects of human activities and influences as opposed to natural processes

Asexual reproduction: Reproduction through mitosis (cell division) rather than meiosis (which involves the fusion of gametes), thus involving only one parent

Bootstrapping methods: A statistical method involving creation of pseudoreplicate data sets by random sampling of the character matrix to determine sampling variance

Bottleneck: An extreme loss of variation in a population due to genetic drift associated with reduced population size

Clade: A monophyletic group of taxa arising from a single common ancestor

Cluster analyses: Analyses classifying objects such as taxa into groups, based on shared characteristics

Cryptic species: Species that cannot be distinguished based on morphological characters, but are clearly different at the genetic level

Epilithic: Attaching to stones and rocks

Epipelic: Attaching to fine sediments

Epiphytic: Attaching to plants

Endemic: Taxa restricted to a specific geographic unit

Eutrophic: Bodies of water rich in nutrients and primary productivity

Genetic drift: A completely random shift in allele frequencies over time, especially evident in small populations

Heterokonts: Ochrophyte algae belonging to the stramenopiles and containing chlorophyll *a* and *c* and the brown pigment fucoxanthin.

Heterozygosity: The proportion of loci possessing differing alleles in an individual

Hybridization: The crossing of individuals with different genetic compositions, for example from genetically unique populations or species

Inbreeding depression: The reduced fitness of a population due to inbreeding of individuals of a small population, leading to accumulation of harmful mutations and loss of genetic variation

Indigenous: A taxon native to a region or ecosystem

Introduced species: Species introduced to a region by anthropogenic intervention

Invasion: The spread of a species to new areas, where it has a negative effect on the species and communities already in place. While it is possible to have invasive and non-invasive natives and non-natives, most of the invasive species described in this thesis are invasive non-natives.

Lentic: Still bodies of water, such as lakes and ponds

Lotic: Flowing bodies of water, such as rivers and streams

Maximum Likelihood: A statistical method that uses a model of evolution to determine the most likely hypothesis of phylogenetic relationships given the parameters of the model and the sequences observed

Maximum Parsimony: A statistical method that selects the phylogenetic tree that requires the least number of substitutions to explain the sequence data observed

Mesotrophic: Bodies of water with moderate quantities of nutrients

Metapopulation: A group of spatially divided subpopulations, between which gene flow is restricted.

Monophyletic: A group of taxa consisting entirely of all the species arising from a single common ancestor

Native species: Species that occur in an area naturally, without human introduction

Oligotrophic: Bodies of water that are low in nutrients and organic matter

Orthologous genes: Genes that are formed by speciation events and represent direct species relationships

Outcrossing: The crossing of genotypes of unrelated individuals through outbreeding

Panmictic: Metapopulations where subpopulations are so well linked that mating is random throughout the whole metapopulation.

Paralogous genes: Genes that arise through gene duplication, and which may not represent relationships accurately. They may be copies of regions of DNA found at different loci in the genome.

Population (genetic): A group of individuals of a species, living close enough so that any member can mate with any other member.

Population (ecological): A group of interacting individuals of a species occupying the same space and time.

Phylogenetics: The study of evolutionary relatedness among organisms

Phylogeography: The study of the geographic distributions of genealogical lineages of taxa in time and space

Sympatric: Populations in geographically separated regions