Preliminary genetic assessment of New Zealand *Isoëtes* and *Nitella*, using DNA sequencing and RAPDs

D.E. Hofstra, C.E.C. Gemmill, and M.D. de Winton

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Cover: Swards of endemic *Isoëtes* growing within a clear, South Island lake.
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Preliminary genetic assessment of New Zealand *Isoëtes* and *Nitella*, using DNA sequencing and RAPDs

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ABSTRACT

A preliminary investigation on genetic variability in two groups of endemic New Zealand macrophytes—*Isoëtes* ('quillworts', Lycophyta) and *Nitella hookeri* (Charophyta)—was conducted by National Institute of Water & Atmospheric Research Ltd (NIWA). Molecular techniques of DNA sequencing and RAPDs (Random Amplified Polymorphic DNAs) were employed to give information at different scales of resolution. Comparisons were made with taxa of varying degrees of relatedness (e.g. GenBank accessions). The focus was on analysing morphologically and geographically different material to detect genetic distinctiveness; therefore, analyses were restricted to one or two individuals from a diverse number of lakes. DNA sequences in conjunction with RAPDs support the separation of *Isoëtes alpinus*, *Isoëtes kirkii* var. *flabellata* (Lake Omapere) and *Isoëtes kirkii* (Central North Island tetraploid) and agree with morphological and cytological findings reported in other studies. Analyses of *Nitella* in this study were initially complicated by contamination from epiphyte/endophyte DNA, and techniques were subsequently modified to reduce this risk. ITS (internal transcribed spacers) sequence data differentiated between the *Nitella hookeri* complex and a related species *N.* aff. *cristata*, and may be useful at resolving further differences within the group. As this is the first time that ITS region has been examined for this genus, it was not possible to compare our results with related taxa. This study provided genetic evidence in support of earlier workers' findings that describe distinct entities of *Isoëtes* and *Nitella* in New Zealand. It is recommended that population-level studies include within- and between-lake comparisons to determine genetic variation amongst taxa in more detail.

Keywords: macrophytes, quillworts, *Isoëtes* sp., Lycophyta, *Nitella hookeri*, (Charophyta), genetic variability, RAPDs, ITS, *rbc*L, *trn*L, *LFY*, *trn*I, New Zealand

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1. Introduction

Protection of native biodiversity increasingly recognises that important genetic character risk being masked by traditional approaches to taxonomy. One example is where cryptic species exist that are similar in morphology to, but genetically quite distinct from other, closely related taxa. In New Zealand, traditional taxonomic approaches based solely on morphological features have resulted in uncertain specific delineations and taxonomy of two endemic macrophyte complexes, *Isoëtes kirkii* A. Braun and *I. alpinus* Kirk (Isoëtaceae) and *Nitella hookeri* A Br. (Characeae). This has resulted in confusion over their status within New Zealand, with the result that important genetic differences between different populations may go unrecognised.

Populations of the endemic macrophyte, *Isoëtes* form a major community in many South Island lakes, while populations in North Island lakes are either extinct or under threat. Charophytes (including *N. hookeri*) are the most widespread group of macrophytes in New Zealand freshwaters and are critical to lake condition and ecological functioning, while their role in biodiversity and contribution to seed banks is key to lake vegetation resilience and restoration initiatives.

Suitable selection criteria for protection of lakes from different regions will be greatly enhanced if biogeographic scales of genetic variation can be identified for these endemic plants. Results will also identify implications for biodiversity protection and advance understanding of their biogeography at national and international scales.

1.1 *Isoëtes*

Isoëtes are spore-bearing vascular plants comprising approximately 200 species worldwide (Hoot & Taylor 2001), and are included in the plant division Pteridophyta, which includes both ferns and fern allies.

The common name of quillwort refers to the appearance of the upright linear leaves that arise from a short corm-like base. Sporangia may develop within the base of the leaves that contain either female megaspores or male microspores. Genetic recombination via spores produces new plants, but there is a suggestion that apomictic (see glossary, Appendix 1) reproduction can occur, i.e. germination of megaspores may proceed in the absence of microspores in some situations (Marsden 1976).

Speciation in *Isoëtes* appears to follow two pathways (Hoot & Taylor 2001), either occurring following isolation and genetic divergence, or following interspecific hybridization and chromosome doubling (autopolyploidy). Hybridization within *Isoëtes* species is suggested by overlapping distribution ranges, 1–2 spore types of different sizes and shapes within a sporangia (polymorphic spore formation) and evidence of polyploid sequences of chromosome number (Taylor et al. 1985).

Their conservative and simple anatomy means that taxonomic classification of *Isoëtes* based on morphology is problematic (Rydin & Wikström 2002). Nevertheless, a number of morphological characteristics are commonly used to differentiate species of *Isoëtes*, with the most widely used being ornamentation of the megaspores (Marsden 1976). Cytology is also used, with species specific sequences of chromosome number for *Isoëtes* based on the haploid number, n = 11 being recognised as arising from polyploidy and hybridization (Marsden 1976; Taylor et al. 1985; Hoot & Taylor 2001).

Isoëtes in New Zealand has been described as two species, *I. kirkii* A. Braun and *I. alpinus* T. Kirk (Allan 1961). However, unpublished work by Marsden (1979) described three varieties within one species, *I. kirkii* (*I. kirkii* var. *kirkii*, *I. kirkii* var. *alpina* (T. Kirk) Marsden & Chinnock, *I. kirkii* var. *flabellata*). Cytology for one population of each of those three varieties of *Isoëtes* established a diploid chromosome number of 2n = 22 (Marsden 1979). All taxa were considered endemic to this country and most closely related to Australian species (Marsden 1979).

Recently, D. Britton (University of Guelph, Canada) and D. Brunton (D. Brunton Consulting Services, Canada) investigated New Zealand *Isoëtes* using herbarium specimens, spore morphology, and cytology on fresh material supplied by National Institute of Water & Atmospheric Research Ltd (NIWA) from 11 lakes. Although inconclusive in resolving the taxonomy of the group, their study provided some indication of possible underlying genetic variability. They suggest New Zealand *Isoëtes* is best viewed as a single species with three morphologically similar subspecies, which exhibit reasonably distinctive geographical, cytological and ecological characteristics (D. Brunton Consulting Services, pers comm. 2003). Two of these subspecies were diploid and sexual: *Isoëtes kirkii* (s.s.) found at low elevation lakes, mostly on the North Island and currently a rare and declining population; and *I. kirkii* subsp. *alpina* found in higher elevation lakes on South Island and relatively common. Some entities on both Islands were difficult to assign to one or the other taxon. More difficult to interpret was the fact that several populations in Central North Island lakes were tetraploid, without microspores and apparently apomictic. These demonstrated virtually identical megaspore morphology, apart from a larger spore size, to that of *I. kirkii* subsp. *alpina* (D. Brunton Consulting Services, pers comm. 2003).

Preliminary analysis of the genetic composition of New Zealand *Isoëtes* was made using sequence variation of internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (Hofstra & Gemmill 1999). Plants from Lake Omapere, Northland, were compared with plants from two Central North Island lakes, one South Island lake and four species from abroad. The results suggested New Zealand taxa were genetically highly uniform and some were genetically indistinguishable from one Tasmanian species, *I. gunnii*, but all were readily distinguished from three North American species. Their conclusion was that the ITS region was not adequate to distinguish genetic differences within the New Zealand material given this limited sample.

New Zealand *Isoëtes* has been included in two genetic studies to determine intrageneric relationships within *Isoëtes*. Hoot & Taylor (2001) used ITS and *atp*B-*rbc*L spacer region data to place New Zealand *Isoëtes* (from Lake Brunner,

West Coast) into an Asian/Australian clade. Rydin & Wikström (2002) determined plastid *rbc*L sequences for New Zealand *Isoëtes* (from Lake Omapere, Northland), which showed a similar close association between the New Zealand and Australian taxa.

1.2 CHAROPHYTES

Charophytes are a group of distinctive green algae that superficially resemble higher submerged plants in that they have erect stems supporting regular whorls of branchlets and are anchored in the substrate by rhizoids. Both monoecious breeding systems (where male antheridia and female oogonia occur on the same plant) and dioecious breeding systems (where plants are of a single sex) are represented among the charophytes. Hardened sexual propagules, (oospores) develop from fertilised oogonia and are the units of dispersal (somewhat analogous to seeds). Other methods of reproduction and colonisation in charophytes include specialised and non-specialised vegetative propagules and starch bodies (bulbils), which form on the rhizoids.

Traditionally, charophytes were conservatively classified according to morphological characteristics such as cell development patterns, branching architecture and breeding system. However, there are now thought to be more functional species than previously recognised based on morphological criteria. Moreover, recent genetic studies have not fully supported the phylogeny and classification systems based on previous morphological criteria (Meiers et al. 1999).

New Zealand charophyte species were described by Wood & Mason (1977) who essentially found a subset of Australian species with no truly endemic species. Nevertheless, *Nitella hookeri* A. Br. was considered a virtual endemic in that it was abundant in New Zealand yet exceedingly rare in Australia and is recorded elsewhere only from remote Kerguelen Island in the South Indian Ocean. This monoecious species presented a confusing range in form and some forms were difficult to distinguish from the dioecious *N. cristata* A. Br., em R.D.W. unless bearing antheridia and/or oogonia (Wood & Mason 1977). The question whether *N. cristata* should be united with *N. hookeri* has been raised (Wood & Mason 1977).

Recently, morphological investigations into the *N. hookeri*/*N. cristata* complex have been carried out in collaboration with M. Casanova (Royal Botanic Gardens, Victoria, Australia). Results of that study indicate that New Zealand *N. hookeri* entities are very different from the Australian material and are likely to include one or two endemic entities (M. Casanova, Royal Botanic Gardens, pers comm. 2005). One change is the probable reinstatement of *N. tricellularis* (Nordst.), a species that was relegated to varietal status by Wood & Mason (1977). In addition, *N. cristata* in New Zealand does not conform to the typical concept for that species and is almost certainly a separate, closely related species that is also found in Australia (M. Casanova, Royal Botanic Gardens, pers. comm. 2005).

Nitella hookeri and *N.* aff. *cristata* from six locations each in New Zealand were included in genetic analyses undertaken by K. Karol (University of

Maryland, USA) based on *rbc*L sequencing. This technique is widely used to resolve phylogenetic relationships at the generic or specific level of charophytes, although it may not distinguish closely related taxa (Sakayama et al. 2002). Karol's results supported the separation of a *N*. aff. *cristata* taxon, but were too conservative to distinguish between entities of the *N. hookeri* complex (K. Karol, University of Maryland, pers. comm. 2003).

1.3 GENETIC TECHNIQUES

The use of PCR (polymerase chain reaction), DNA sequencing, and other techniques for comparing and visualising DNA differences and similarities (such as RAPDs; Random Amplified Polymorphic DNA) are used increasingly to elucidate the relationships between plants at all taxonomic levels.

Traditionally plant molecular systematics has relied on cpDNA (chloroplast DNA) coding regions such as *rbc*L to resolve higher taxonomic level relationships, but more recently investigators are turning to non-coding regions such as nuclear ribosomal (nr) DNA ITS (Soltis & Soltis 1998). In general cpDNA is considered more conserved, which can limit its applicability among closely related species and at the population level. However studies have 'successfully used cpDNA to examine population-level relationships and evolutionary processes within species' (Soltis & Soltis 1998).

In this study, based on results in published literature, personal communications with colleagues overseas, and authors' experiences, both coding and faster evolving non-coding regions of the cpDNA and nrDNA were examined for DNA sequence variation. The cpDNA regions including the *rbc*L (Manhart 1994; McCourt et al. 1996; Wolf et al. 1998; McCourt et al. 1999; K Karol, University of Maryland, pers. comm. 2003), *mat*K, *trn*L (UAA) intron (Taberlet et al. 1991), *trn*I intron (Chapman et al. 1998; Turmel et al. 2002) and cpDNA ITS (Samiguillin et al. 1998) have all been used to examine relationships in plants, including the related ferns, fern allies (Pryer et al. 2001) and green algae.

The ITS and ETS (External Transcribed Spacer) regions of the 18S and 26S nrDNA have been increasingly used to determine lower-level relationships among closely related plant species (Baldwin 1992; Baldwin et al. 1995; Markos & Baldwin 2002), including amongst *Isoëtes* (Hoot & Taylor 2001). More recently the LFY (second intron) has also been used to resolve relationships amongst *Isoëtes* (S. Hoot, University of Wisconsin, pers. comm. 1999).

In addition to DNA sequence data, RAPDs are valuable in assessing genetic variation. RAPDs have been used to investigate population level diversity, interspecific hybridisation and genetic distinctiveness amongst a wide variety of plant species (e.g. Martín et al. 1999; Holzapfel et al. 2002; Jiménez et al. 2005). RAPDs have the advantage of not requiring prior sequence knowledge of the target species and are known to produce comparable estimates to other techniques of AFLP (amplified fragment length polymorphism) and ISSR (intersimple sequence repeats) used to evaluate among and within-population diversity (Nybom 2004).

2. Methods

The sources of the *Isoëtes* used in these analyses are summarised in Table 1. Included is *I. kirkii* from Lake Omapere, which was considered as var. *flabellata* by Marsden (1979), unusually tall material from the Channel Islands in Lake Manapouri, and plants from tetraploid populations in Lakes Rotoiti and Taupo.

The source of the charophytes used in these analyses is summarised in Table 2 and includes cultured *Nitella hookeri* and *N.* aff. *cristata* also used in morphological and genetic investigations in the authors previous collaborations with M. Casanova and K. Karol.

2.1 DNA EXTRACTION

Fresh plant material was collected from the plants listed in Tables 1 and 2. Plants were visually checked and cleaned of any apparent contaminating material (e.g. epiphytic algae, snails) at the time of collection. Immediately prior to DNA extraction, plants were further examined under a stereomicroscope to ensure they were free of visible attached algae.

Approximately 50 mg (fresh weight) of plant material was ground in liquid nitrogen and scraped into a 1.5-mL microcentrifuge tube containing 600 µL of preheated (65°C) CTAB isolation buffer (Doyle & Doyle 1990). Samples were incubated at 65°C for an hour with occasional agitation, after which 800 µL of chloroform/isoamyl alcohol (24:1, volume/volume) was added and the contents mixed by repeated inversion. The tubes were then centrifuged at 13 000 rpm in a benchtop microcentrifuge for 5 min. The aqueous phase was removed using a sterile pipette tip and transferred to a new tube. The extraction step was

TABLE 1. SOURCE OF THE MATERIAL USED FOR GENETIC ANALYSES OF *Isoëtes*.

SPECIES (THIS STUDY)	LOCATION	COLLECTION DATE	NO. OF PLANTS	PREVIOUS DETERMINATION*	CODE
Isoëtes alpinus	Lake Te Anau, Fiordland	23 Apr 2002	1	*I. kirkii* var. *kirkii*	IaTa
I. alpinus	Lake Manapouri, Fiordland	24 Apr 2002	1	*I. kirkii* var. ?†	IaMa2
I. alpinus (tall growing)	Lake Manapouri (Channel Islands), Fiordland	24 Apr 2002	1	*I. kirkii* var. ?†	IaMa3
I. alpinus (unknown location)	Ruakura unknown culture		2	*I. kirkii* var. ?†	IaCu, IaCu4
I. kirkii var. *flabellata*	Lake Omapere, Northland	19 Mar 1998	1	*I. kirkii* var. *flabellata*	IkOmp5
I. aff. *kirkii* (tetraploid)	Lake Rotoiti (Gisborne point), Central NI	10 Jun 2003	3	*I. kirkii* var. *alpina*	IkRiti51, IkRiti52, IkRiti53
I. aff. *kirkii* (tetraploid)	Lake Taupo (Te Moenga Bay), Central NI	10 Jun 2003	4	*Is kirkii* var. *kirkii*‡	IkTpo61, IkTpo62, IkTpo63, IkTpo64

* Following Marsden (1979).
† Species from this location not determined by Marsden (1979).
‡ Species from this location determined as *I. alpinus* and *I. kirkii* in Allen (1961).

repeated. The nucleic acids of both *Isoëtes* and *Nitella* were precipitated by adding 400 µL of ice-cold isopropanol and incubated at –20°C for approximately 1 h, followed by centrifuging for 10 min at 13 000 rpm. The pellet was resuspended in 500 µL 1M NaCl at 37°C. The nucleic acids were again centrifuged (13 000 rpm) for 5 min, transferred to a fresh tube and incubated at 95°C for 30 min. The nucleic acids were then precipitated using 500 µL of ice-cold isopropanol, incubated at –20°C for 30 min and then centrifuged (13 000 rpm) for 15 min. The pellet was washed in 70% ethanol, followed by a second wash in 95% ethanol, and vacuum drying. The DNA was resuspended in 50 µL TE (Tris EDTA) and treated with RNAse A. DNA once extracted was visualised on a 1.5% agarose TBE gel, with DNA standards (New England Bio Labs, Beverly, Massachusetts, USA, www.neb.com) to estimate quantity.

Subsequent DNA extraction from *Nitella* used a modified CTAB protocol (Hills & van Staden 2002) that had additional CTAB and salt incubation steps, or the DNeasy® plant mini kit (Qiagen, Valencia, California, USA www1.qiagen.com/) resulting in increased DNA yield for the small preparations (less than 50 mg of young shoots) that were necessary to reduce the risk of contaminants as well as avoiding plant parts that were highly mucilaginous.

TABLE 2. SOURCE OF THE *Nitella* MATERIAL USED FOR GENETIC ANALYSES OF CHAROPHYTES.

SPECIES (THIS STUDY)	COLLECTION SITE	DATE	PREVIOUS DETERMINATION*	CODE
Nitella aff. *cristata*	Lake Rotoaira, Waikato-King Country	1 Mar 2001	*N. cristata*	NcRaira1
N. hookeri?	Lake Rotoaira, Waikato-King Country	1 Mar 2001	*N. hookeri*	NhRaira2
N. hookeri?	Lake Gunn, Fiordland	7 Mar 2001	*N. hookeri*	NhGunn3
N. aff. *cristata*	Lake Taupo, Waikato-King Country	28 Feb 2001	*N. cristata*	NcTau4
N. aff. *cristata*	Lake Taupo, Waikato-King Country	28 Feb 2001	*N. cristata*	NcTau5
N. hookeri?	Lake Te Anau, Fiordland	8 Mar 2001	*N. hookeri*	NhTeA6
N. hookeri?	Lake Ellery, West Coast	9 Mar 2001	*N. hookeri*	NtEllery7
N. aff. *cristata*	Lake Taupo, Waikato-King Country	28 Feb 2001	*N. cristata*	NcTau8
N. aff. *cristata*	Lake Taupo, Waikato-King Country	28 Feb 2001	*N. cristata*	NcTau9
N. aff. *cristata*	Lake Moeraki, West Coast	9 Mar 2001	*N. cristata*	NcMoe10
N. aff. *cristata*	Lake Brunner, West Coast	11 Mar 2001	*N. cristata*	NcBru12
N. hookeri?	Lake Pearson, Canterbury	11 Mar 2001	*N. hookeri*	NtPea13
N. hookeri?	Lake Lyndon, Canterbury	11 Mar 2001	*N. hookeri*	NtLyn14
N. hookeri?	Lake Wanaka, Otago	5 Mar 2001	*N. hookeri*	NtWan15
N. hookeri?	Lake Ohau, Canterbury	3 Mar 2001	*N. hookeri*	NhOhau16
N. hookeri?	Lake Wakatipu, Otago	6 Mar 2001	*N. hookeri*	NhWak17
N. hookeri?	Lake Hawea Otago	4 Mar 2001	*N. hookeri*	NhHaw18
N. aff. *cristata*	Lake Carrot, Northland	19 Apr 2001	*N. cristata*	NcCar19
N. aff. *cristata*	Lake Humuhumu Northland	2 May 2001	*N. cristata*	NcHum20
N. hookeri?	Lake Okataina, Bay of Plenty	16 Aug 2001	*N. hookeri*	NhOk
N. aff. *cristata*	Lake Managatawhiri, South Auckland	22 Feb 2002	*N. cristata*	NcMan22
N. hyalina	Lake Te Roto, Chatham Islands	27 Feb 2003	*N. hyalina*	NyTeP23
N. pseudoflabellata	Culture at Ruakura		*N. pseudoflabellata* var. *mucosa*	Np
Chara australis	Culture at Ruakura		*Chara australis*	CcCu

* Following Wood & Mason (1977).

2.2 SEQUENCED DNA REGIONS

The regions of *Isoëtes* DNA that were amplified for sequencing were ITS (nrDNA ITS-1 to ITS-2), *rbc*L gene, *trn*L intron and *LFY* intron and the second *LFY* intron (Table 3). The *Nitella* DNA was also amplified for the ITS region and the *rbc*L gene as well as a cpDNA region (including the *trn*I intron) (Table 3).

DNA amplification via the polymerase chain reaction (PCR) was carried out in 0.5-mL thin walled microcentrifuge PCR tubes using a reaction volume of 100 µL for all reactions except LFY. Each reaction volume contained 5 µL of template DNA (c. 50–80 ng), 100 µM of each dNTP, 0.2 µM of each primer, 2–3 units of Taq (Roche Diagnostics, Basel, Switzerland www.roche-diagnostics.com), PCR buffer (with optimised $MgCl_2$ concentration between 2.5 and 5 mM) and 5% DMSO. An Eppendorf Mastercycler Gradient thermocycler was used, with varied annealing temperatures and run times that were optimised for the primers and plants investigated during the course of this study (Table 3).

Following amplification 5 µL of each PCR product was loaded onto a 1.5% agarose gel with 1xTBE and ethidium bromide, DNA bands were visualised under UV light and photographed. The remaining PCR products were then cleaned using a Qiaquick® PCR purification kit (Qiagen) and sequenced at the University of Waikato DNA sequencing facility using DYEnamic™ ET Dye Terminator Cycle Sequencing Kit chemistry for cycle sequencing and run on a MegaBACE® DNA analysis system (Amersham Biosciences, GE Healthcare, Piscataway, New Jersey, USA www.amersham.com/index.html). Where multiple bands (more than one PCR product) were obtained, the desirable band was excised and cleaned for sequencing using the Qiaquick® gel extraction kit (Qiagen, USA).

TABLE 3. PRIMERS AND THERMOCYCLING CONDITIONS FOR SEQUENCED REGIONS OF *Isoëtes* AND *Nitella* DNA.

PLANT	PRIMERS	REFERENCE	THERMOCYCLING CONDITIONS
Isoëtes and *Nitella*	ITS5HP and ITS4 (for regions ITS-1- ITS-2)	White et al. 1990 (for ITS4) (Authors unpubl. data for ITS5HP)	5 min at 96°C, 30 cycles of (30 s at 95°C, 30 s at 55°C, 45 s at 72°C), 10 min at 72°C, hold at 4°C
Isoëtes	*trn*L intron (cpDNA, Tab C and Tab D)	Taberlet et al. 1991	5 min at 96°C, 30 cycles of (30 s at 95°C, 30 s at 55°C, 45 s at 72°C), 10 min at 72°C, hold at 4°C
Isoëtes	*rbc*L 1F and *rbc*L 1409R	Wikström & Kenrick 1997	3 min at 94°C, 30 cycles of (30 s at 94°C, 30 s at 45°C, 3 min at 72°C), hold at 4°C
Isoëtes	*LFY* Lftxr and *LFY* Lfsxl3	S. Hoot, University of Wisconsin, USA, pers. comm. 1999 (but see Hoot & Taylor 2001)	1st amplification using PCR beads: 5 min at 95°C, 35 cycles of (1 min at 95°C, 1 min at 45°C, 2 min at 72°C), 5 min at 72°C, hold at 4°C. 2nd amplification using excised band as template: 4 min at 94°C, 30 cycles of (30 s at 94°C, 30 s at 45°C, 2 min at 72°C) 10 min at 72°C, hold at 4°C
Nitella	*rbc*L RH1 and *rbc*L 1385R	Manhart 1994 (K. Karol, University of Maryland, pers. comm. 2003)	2 min at 94°C, 35 cycles of (10 s at 94°C, 10 s at 45°C, 45 s at 72°C), 2 min at 72°C, hold at 4°C
Nitella	*rbc*L chrF1 and *rbc*L chrR4	Sakayama et al. 2002	3 min at 94°C, 30 cycles of (30 s at 94°C, 30 s at 45°C, 3 min at 72°C), hold at 4°C
Nitella	*trn*I Tur1 and *trn*I Tur9	Turmel et al. 2002	3 min at 94°C, repeat 30 times (30 sec at 94°C, 30 s at 45°C, 3 min at 72°C), hold at 4°C

Amplification of LFY sequences was carried out in a 25 µL reaction volume using PuRETaq Ready-To-Go™ PCR Beads (Amersham Biosciences), with 5 µL of template DNA under the conditions described in Table 3. These amplification products were loaded onto a 2% LMT agarose gel (with 1H TBE and ethidium bromide) to separate the 1100 bp product from a 1050 bp product that was also amplified. The 1100 bp band was excised from the gel and used as template for a subsequent PCR in a 50 µL reaction volume. Each PCR reaction contained 1 to 10 µL of initial PCR product, 100 µM of each dNTP, 0.2 µM of each primer, up to 5 units of *Taq* (Roche), PCR buffer (with 5 mM $MgCl_2$) and 5% DMSO, and was cycled under the conditions outlined in Table 3. The second amplification products were loaded onto a 2% LMT agarose gel with 1xTBE and ethidium bromide, from which the 1100 bp band was excised and purified using a Qiaquick® gel extraction kit. Samples were then sequenced at the University of Waikato DNA sequencing facility (as described above).

Sequences were edited and aligned in Sequencher™ vers. 4.1 (Gene Codes Corporation, USA). Where possible, New Zealand *Isoëtes* were compared for the same region with different *Isoëtes* species (from GenBank) to confirm the sensitivity of these DNA regions in distinguishing taxa. Phylogenetic trees were generated via heuristic searches under the optimality criterion of parsimony with step-wise addition, TBR branch-swapping, ACCTRAN, and MulTrees options. We chose to present unrooted trees as the focus of this study was on the New Zealand samples and an assessment of the phylogenetic relationships of New Zealand *Isoëtes* to overseas species was beyond the scope of this study. We generated consensus trees (strict and 50% majority) in cases where we had more than one equally parsimonious tree. All analyses were performed using PAUP* version 4.1b10 (Swofford 2002). Aligned data matrices are available from the authors upon request.

Additional regions of *Isoëtes* DNA that were investigated for sequencing include *mat*K (Johnson & Soltis 1994) and ETS (Baldwin & Markos 1998). Methods and primers to amplify these regions were also investigated for *Nitella* DNA as well as a cpDNA intergenic transcribed spacer region (Samiguillin et al. 1998) and the *trn*L intron (Taberlet et al. 1991), however the protocols for these regions were not optimised due to time constraints.

2.3 RAPDS

RAPD PCR was undertaken on those samples for which clean (single band) products were obtained for ITS using universal primers. This included *Isoëtes* samples IaCu, IaMa3, IaTa, IkOmp5 and IkTpo62. Where more than one individual was available for a population, such as for Lake Taupo (four plants) and Lake Manapouri (two plants), only one individual was used in this preliminary study to find primers that would reveal higher level differences, i.e. those between populations (different lakes).

The primers that were initially screened for polymorphic band patterns for both *Isoëtes* and *Nitella* were: OPA7, OPA9, OPA10, OPA11, OPA12, OPA14, OPA15, OP16, OPA18, OPA19, OPA20, OPP1-OPP5, OPP7-OPP20 (by Operon Biotechnologies, Inc., Huntsville, Alabama, USA www.operon.com).

Primers used for subsequent PCR for the *Isoëtes* samples were OPP3, OPP4, OPP8, OPP9, OPP16, OPP17, OPP18 and OPP19. RAPD reactions were carried out in a 25 μL reaction volume in 0.2-mL PCR tubes, containing 2.5 μL PCR buffer, 100 μM of each dNTP, 1 unit of Taq, 0.25 μM of primer and 10 ng of plant DNA. Thermocycling conditions were 3 min at 94°C, 35 cycles of (15 s at 94°C, 15 s at 36°C, 1 min at 72°C), 10 min at 72°C with a final wait at 4°C in the Eppendorf Mastercycler Gradient thermocycler.

RAPDs were separated on a 1.5% agarose and 1xTBE gel with ethidium bromide. Bands were visualised under UV light and photographed for later scoring and analyses. All primer and DNA combinations were run in duplicate on the same day and at a later date to test the repeatability of RAPD profiles. Band presence/absence was recorded for all sample and primer combinations with repeatable polymorphic bands. UPGMA analyses were conducted for the RAPD data using Nei's (1972) genetic distance, with the software program TFPGA (Tools for Population Genetic Analysis, Version 1.3; Miller 1997).

3. Results

3.1 *Isoëtes*

Isoëtes DNA was obtained from all isolates in Table 1 and amplified with primers for six different regions of the DNA, three of which were optimised (Table 3), and one (the ITS) has been previously optimised (Hofstra & Gemmill 1999). Sequence data results are reported for ITS, *rbc*L, *trn*L intron and *LFY* regions, although not for all individuals, and in some cases data from only one strand has been incorporated in analyses either because of time constraints, or because the sequence was both of excellent quality and identical in sequence to other samples.

Amplification of the ITS region (ITS-1, ITS-2 and the intervening 5.8s gene) resulted in PCR products of c. 800 base pairs (bp). The parsimony analysis of edited sequences resulted in 255 equally parsimonious trees of 36 steps with a consistency index (CI) of 1. ITS data from Hofstra & Gemmill (1999) were also included in the parsimony analyses (Fig. 1). There were only 2 parsimony informative characters, with little difference between all samples from New Zealand and *I. gunnii* from Australia.

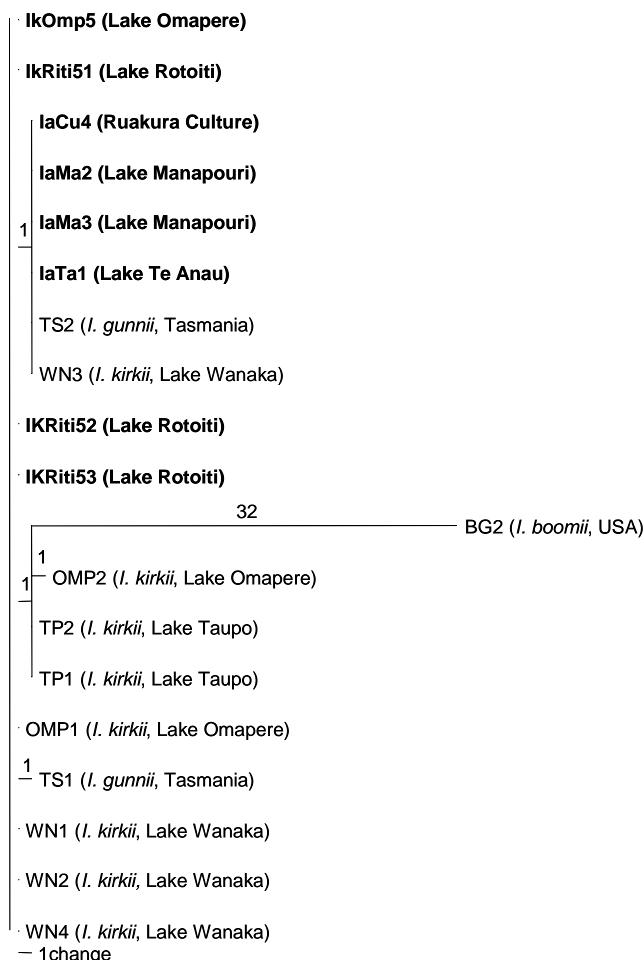
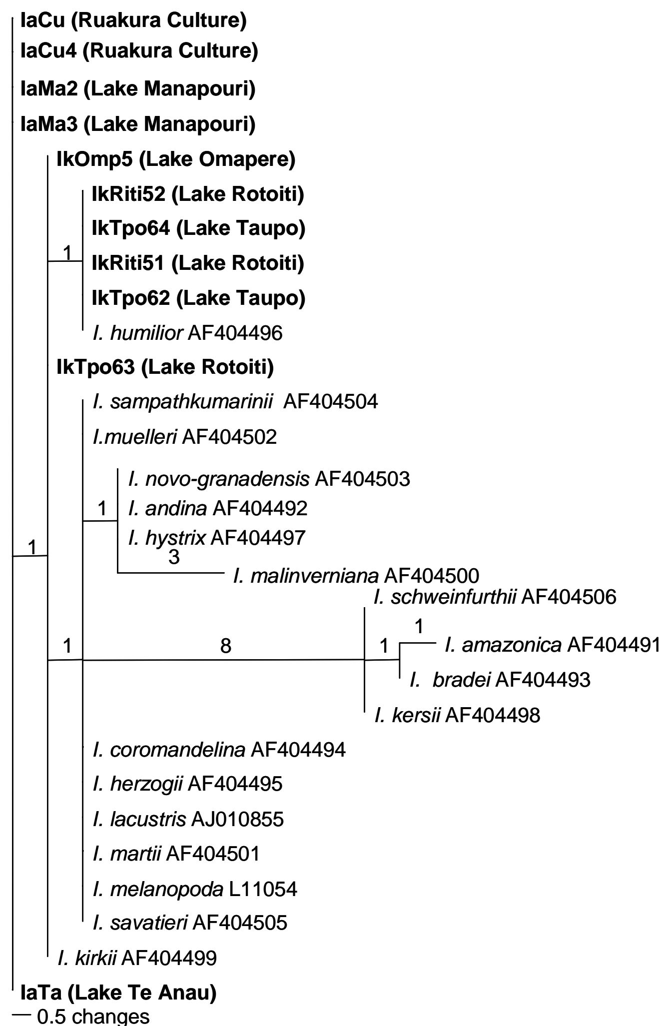


Figure 1. One of the equally parsimonious trees for *Isoëtes* based on the ITS-1, ITS-2 and the intervening 5.8s gene. Numbers above the branches refer to branch lengths, and are proportional to the amount of character change along each branch. Sample names in **bold** refer to this study, others are from Hofstra & Gemmill (1999).

Figure 2. One of the equally parsimonious trees for *Isoëtes* based on truncated *rbc*L sequences for New Zealand and GenBank samples. Numbers above the branches refer to branch lengths, and are proportional to the amount of character change along each branch. Sample names in **bold** refer to this study. Samples not in bold font, are from GenBank, including *I. kirkii* from Lake Omapere (AF404499) (Rydin & Wikström 2002).



The *rbc*L primers amplified a c. 1150 bp region of DNA, which has proved difficult to sequence. Sequence data was available for at least one individual from each population of interest, but only covering approximately half of the region amplified (i.e. c. 600 bp from either end of the *rbc*L gene which did not always overlap). Parsimony analysis including these single sequences and GenBank accessions resulted in 252 equally parsimonious trees of 17 steps, CI of 0.88 and 37 parsimony informative characters were identified. All *Isoëtes* samples from New Zealand aligned closely with *I. kirkii* from GenBank (historical Lake Omapere) and were more distinctly different from other species of *Isoëtes* with the exception of *I. humilior* from Australia.

In addition, parsimony analyses were also carried out for truncated *rbc*L sequences (using only the first half of the *rbc*L gene, 616 bp) for both the New Zealand samples from this study and those from GenBank (Figs 2 and 3). The parsimonious tree (Fig. 2) shows at least one base difference between the *I. alpinus* and the *I. kirkii* samples, as well as IkOmp5 and IkTpo63 for this region. The 50% majority rule tree (see glossary, Appendix 1) for truncated *rbc*L sequence data (Fig. 3) also shows a separate clade for the *I. alpinus* and the *I. kirkii* (including *I. kirkii* from GenBank and the *I. humilior*) samples, as well as non-New Zealand taxa.

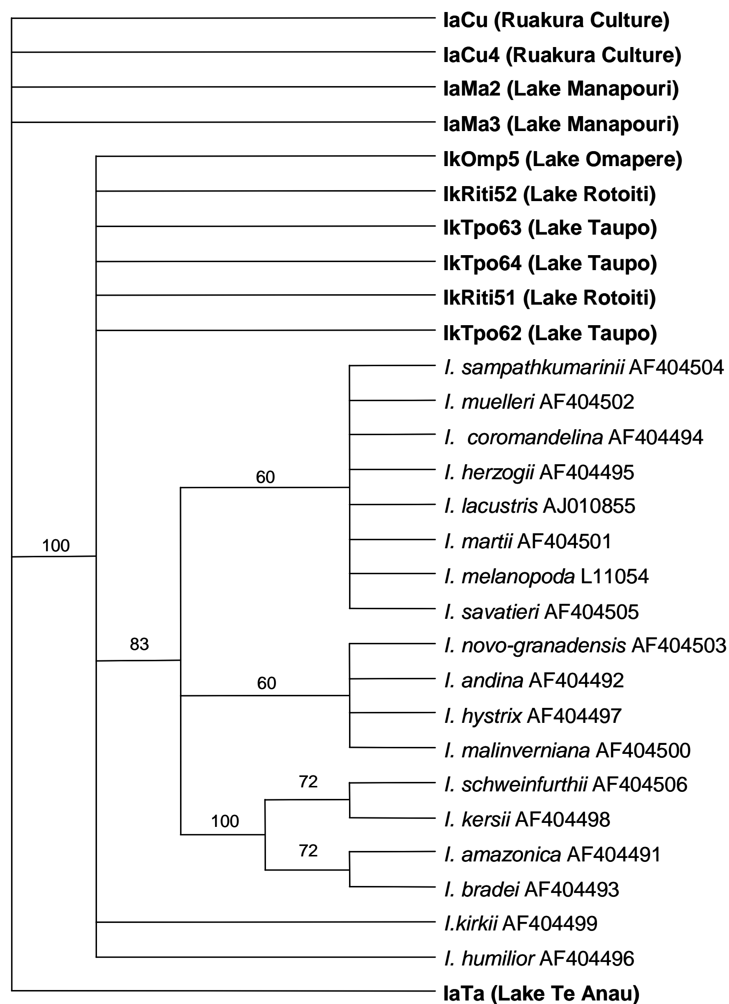


Figure 3. Fifty percent majority rule tree for *Isoëtes* based on truncated *rbc*L sequences for New Zealand and GenBank samples. Sample names in **bold** refer to this study, other samples are from GenBank including an *I. kirkii* plant from Lake Omapere (AF404499) (Rydin & Wikström 2002).

The *Isoëtes trn*L sequences that were amplified were approximately 600 bp long. After sequencing and editing the truncated alignment was 450 bp with little variation between samples (i.e. one mutation at position 356 where *I. kirkii* have an A and *I. alpinus* have a G).

LFY was amplified for all *Isoëtes* isolates (Table 1). Initial PCRs resulted in more than one product (region amplified) that required separation from the desirable region. This was achieved through optimising the PCR protocol, and subsequent gel electrophoresis to separate a 1050 bp and 1100 bp product, with further PCR and gel band purification.

For the sample IaCu, both products (1050 and 1100 bp) were sequenced; for all other isolates only the 1100 bp product was purified for sequencing because this was considered the more informative (S. Hoot, University of Wisconsin, pers. comm. 1999). A sequence from the IaCu sample has been provided by the University of Waikato DNA sequencing facility (with modifications to the standard cycle sequencing protocol), however the remaining 1100 bp PCR products (other isolates) did not yield sequences of adequate quality.

We were able to align four sequences of the 1100 bp *LFY* product generated from the same IaCu DNA under different protocols as part of the optimisation process. We then used these four sequences to generate a consensus sequence

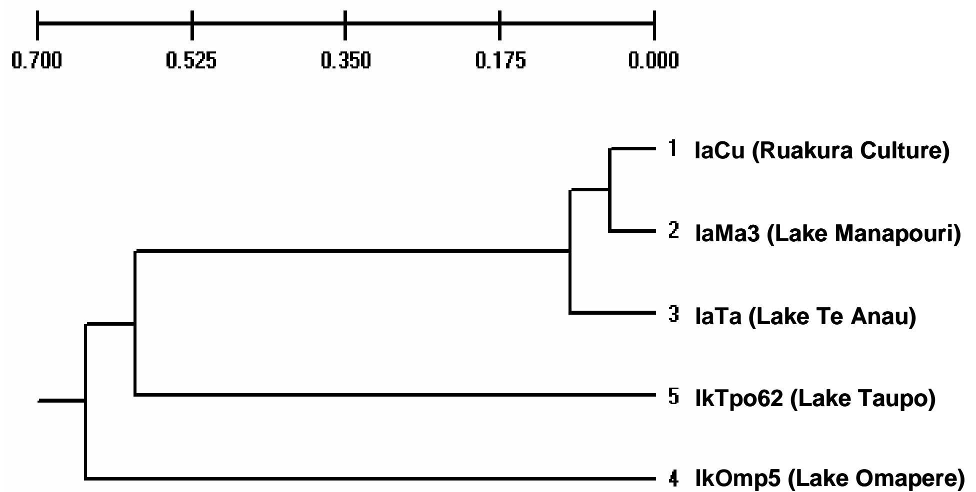


Figure 4. UPGMA cluster diagram generated from *Isoëtes* RAPD data using Nei's (1972) genetic distance.

based both strands of the DNA of c. 1023 bp. We compared these sequences to those in GenBank via the BLASTn search; no matches were found for more than 21 bp of sequence indicating that no similar taxa for this region are contained within the GenBank database.

From the IaCu 1050 bp *LFY* product a consensus sequence of 1008 bp was obtained. This sequence had a 98% match for c. 60 bp region of *I. asiatica* AB050087 from Japan (Himi et al. 2001).

RAPDs were generated for five *Isoëtes* samples, including one from each population, except Lake Rotoiti. As samples from Lake Rotoiti did not produce single band products for PCR with universal ITS primers it was suspected that the DNA from these samples was possibly contaminated with epiphytic algae.

Of the 30 primers originally screened for RAPDs using IaCu DNA, 8 produced multiple bands, and were subsequently used to generate RAPDs for the remaining *Isoëtes* (IaMa3, IaTa, IkOmp5, IkTpo62). The 60 RAPD loci (represented by band presence or absence) reveal distinct differences between each of the *I. kirkii* samples and between the *I. kirkii* and *I. alpinus* at c. half of the loci, with differences amongst *I. alpinus* isolates at c. 5–6 loci (Fig. 4).

3.2 *Nitella*

DNA was obtained from all *Nitella* samples in Table 2. Initial PCR with ITS primers resulted in more than one band for some isolates and failure to amplify for others. Subsequent use of smaller amounts of young shoot material reduced the chances of extracting DNA from unseen epiphytic algae, without the need (and time delay that would be required) for developing axenic cultures.

Nitella DNA was amplified with primers for seven different regions of the DNA, three of which were then optimised (Table 3). Sequence data is available for ITS (nrDNA, including ITS-1 through ITS-2), the *rbc*L gene and a region of the cpDNA encoding the rRNA genes and including the *trn*I intron, although not for all individuals due to lack of observed sequence variation.

PCR amplification of the cpDNA using the Tur1 and Tur9 primers (Table 3) produced a c.1000 bp product for six samples (NhHaw18, NhGunn3, NhOha16, NhWak17, NtLyn14, NtWan15 and NtPea13). Despite apparently high quality PCR products, they proved difficult to sequence, with only partial sequence data obtained for four of the samples. NhHaw18, Ntlyn14, NtWan15 and NhOha16 were identical for the 585 bp sequenced.

The *rbc*L gene was amplified by either set of primers (Table 3) resulting in a PCR product of c. 1000 bp. Sequence data has been aligned and edited for eight samples: NtPea13, NhTeA6, NcMoe10, NcRaira1, NcTau4, NcTau5, NcTau8 and NcBru12. Parsimony analyses produced eight equally parsimonious trees of 126 steps and a CI of 0.73, in which samples NhTeA6 and NtPea13 are unresolved, but form a separate clade to the *N.* aff. *cristata* samples and other species of *Nitella* (Figs 5 and 6).

The ITS region of the *Nitella* samples was amplified using ITS5Hp and ITS4 primers (Table 3). A c. 650 bp region was amplified for samples of *N. hookeri* and *N.* aff. *cristata* (Table 2), however complete sequence data was only obtained for NtLyn14, NtPea13 and NcBru12. For these three samples a 526 bp region was aligned for which NtLyn14 and NtPea13 were identical, but differed

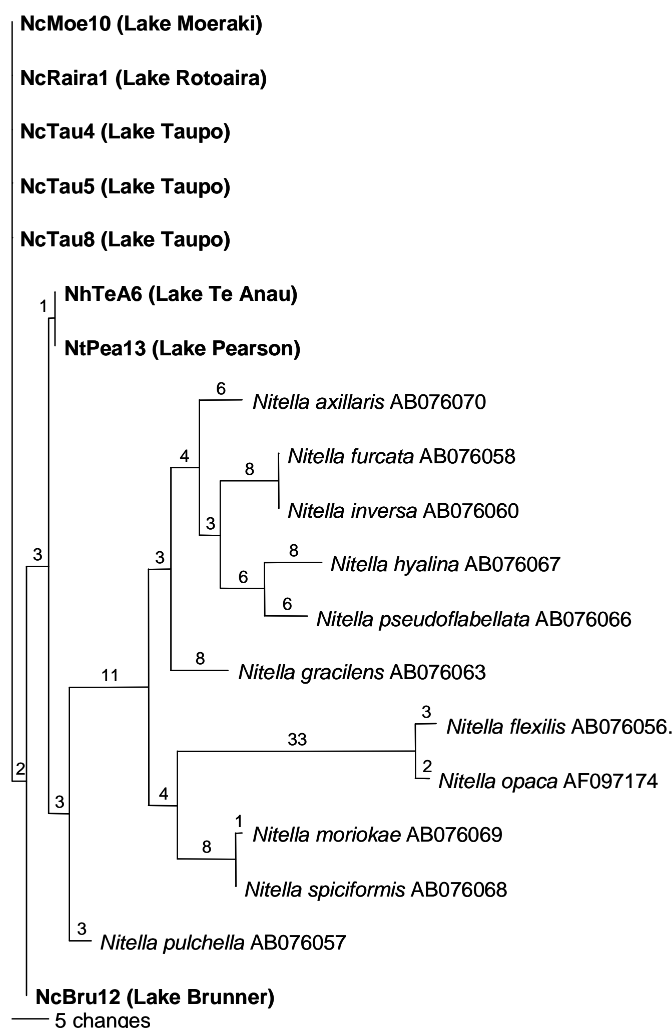
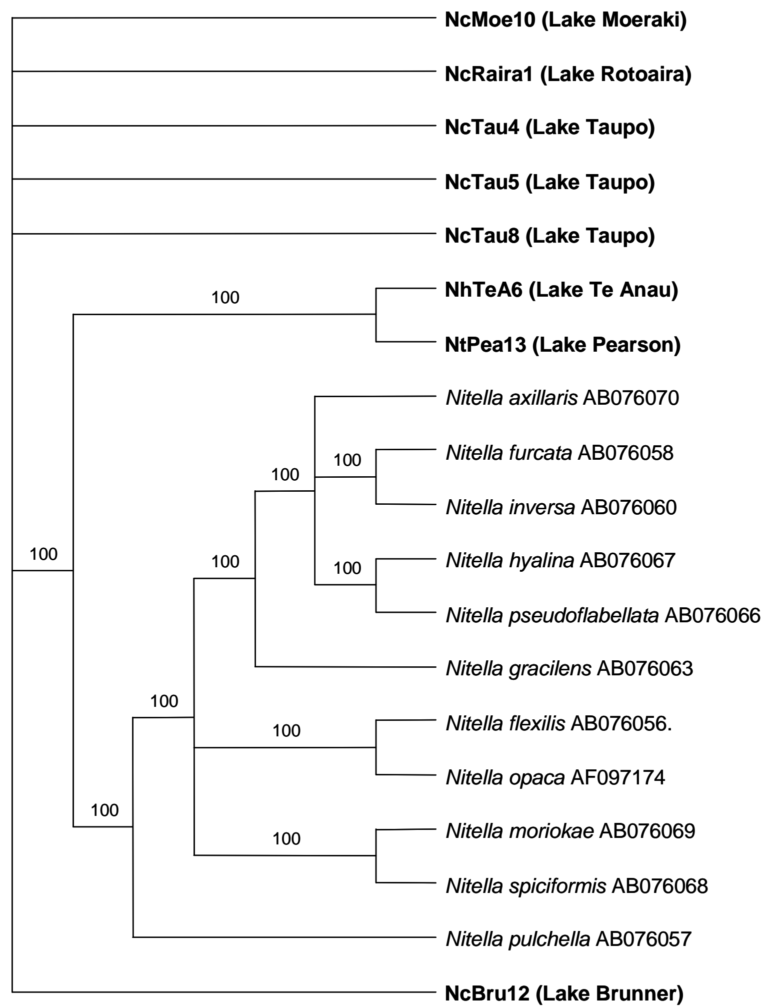


Figure 5. One of the equally parsimonious trees for *Nitella* based on *rbc*L sequences for New Zealand and GenBank samples. Numbers above the branches refer to branch lengths, and are proportional to the amount of character change along each branch. Sample names in **bold** refer to this study. Samples not in bold font are from GenBank.

Figure 6. Fifty percent majority rule tree for *Nitella* based *rbc*L sequences for New Zealand and GenBank samples. Sample names in **bold** refer to this study, those samples not in bold are from GenBank.



from NcBru12 by 71 point mutations (Fig. 7). Initial problems in obtaining clean DNA samples of *Nitella* meant that RAPDs were not considered a suitable method for determining genetic variation amongst these samples. However, following optimised protocols for DNA extraction, and the resultant single band products that were obtained for ITS PCR (indicative of contaminant-free *Nitella* DNA), an initial screening of 30 RAPD primers was undertaken. Of the 30 primers screened using *N. pseudoflabellata* DNA or *Chara australis* DNA (extracted from a NIWA culture sample), OPP11, 13 and 14 generated multiple band patterns, indicative of primers that may be useful for future RAPD analyses.

Figure 7. ITS sequence data for *Nitella* samples. Differences between samples are in **bold** font.

'NtPea13' T**:A** GTA CCC CTC A**A**G T**G**G **A**CC **TTA** **A**GG **AAA** GCT TG**G** GCG GCT G**T**C T**C**T

'NtLyn14' T**:A** GTA CCC CTC A**A**G T**G**G **A**CC **TTA** **A**GG **AAA** GCT TG**G** GCG GCT G**T**C T**C**T

'NcBru12' T**CT** GTA CCC CTC A**T**G T**C**G **T**CC **CTC** **T**GG **GAT** GCT TG**A** GCG GCT G**G**C T**T**T

'NtPea13' CGG G**TC** AG**C** CGG GCT CCC **C::** **:::** **:::** **:::** **:**GC **AAG** GG**T** G**AG** T**C**G **GC**A

'NtLyn14' CGG G**TC** AG**C** CGG GCT CCC **C::** **:::** **:::** **:::** **:**GC **AAG** GG**T** G**AG** T**C**G **GC**A

'NcBru12' CGG G**CT** AG**T** CGG GCT CCC **GCT** **AGC** **TAT** **TGC** **A**GC **TTC** GG**G** G**GT** T**G**G **CA**A

'NtPea13' ACC **ATT** **TT**A CAC ACC CAA C**T**A A**T**A TGT CTG TCT GAA GAT GAT TCG **GC**T

'NtLyn14' ACC **ATT** **TT**A CAC ACC CAA C**T**A A**T**A TGT CTG TCT GAA GAT GAT TCG **GC**T

'NcBru12' ACC **CAA** **CC**A CAC ACC CAA C**C**A A**A**T TGT CTG TCT GAA GAT GAT TCG **AT**T

'NtPea13' CAG CAC TGC TG**G** TCG ATT AAC TAA GAC AAC TCT CAA CAA CGG ATA TCT

'NtLyn14' CAG CAC TGC TG**G** TCG ATT AAC TAA GAC AAC TCT CAA CAA CGG ATA TCT

'NcBru12' CAG CAC TGC TG**A** TCG ATT AAC TAA GAC AAC TCT CAA CAA CGG ATA TCT

'NtPea13' **A**GG CTC TCG CAA CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AGT GTG

'NtLyn14' **A**GG CTC TCG CAA CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AGT GTG

'NcBru12' **T**GG CTC TCG CAA CGA TGA AGA ACG CAG CGA AAT GCG ATA CGT AGT GTG

'NtPea13' AAT TGC AGA ATT CCG TGA ATC ATC GAA TCT TTG AAC GCA TAT TGC GGT

'NtLyn14' AAT TGC AGA ATT CCG TGA ATC ATC GAA TCT TTG AAC GCA TAT TGC GGT

'NcBru12' AAT TGC AGA ATT CCG TGA ATC ATC GAA TCT TTG AAC GCA TAT TGC GGT

'NtPea13' CGA GGC TTC GGC **T**GA GAC CAT GTC TGC CTC AGC GTC GGT G**TG** A**A**C CCT

'NtLyn14' CGA GGC TTC GGC **T**GA GAC CAT GTC TGC CTC AGC GTC GGT G**TG** A**A**C CCT

'NcBru12' CGA GGC TTC GGC **C**GA GAC CAT GTC TGC CTC AGC GTC GGT G**AA** **AC**C CCT

'NtPea13' CA**C** T**:**C C**T**C CCG C**A**C G**G**G TGG A**G**T GGA ACT GG**C** CTC CCC ATT GCT TGC

'NtLyn14' CA**C** T**:**C C**T**C CCG C**A**C G**G**G TGG A**G**T GGA ACT GG**C** CTC CCC ATT GCT TGC

'NcBru12' CA**T** **TC**C C**A**C CCC C**GG** G**T**G TGG A**A**T GGA ACT GG**T** CTC CCC ATT GCT TGC

'NtPea13' **G**AT GGG **C**TG GCT GAA ATT CAG AGG **A**TT **G**AG CTT GGA CCT GTA TGG CAA

'NtLyn14' **G**AT GGG **C**TG GCT GAA ATT CAG AGG **A**TT **G**AG CTT GGA CCT GTA TGG CAA

'NcBru12' **A**AT GGG **T**TG GCT GAA ATT CAG AGG **T**TT **A**AG CTT GGA CCT GTA TGG CAA

'NtPea13' CAG CAA GGT AGG TAG **A**TT **T**AC TAC TCC AGC TGA TGC TTT GGG CAC T**C**G

'NtLyn14' CAG CAA GGT AGG TAG **A**TT **T**AC TAC TCC AGC TGA TGC TTT GGG CAC T**C**G

'NcBru12' CAG CAA GGT AGG TAG **C**TT **G**AC TAC TCC AGC TGA TGC TTT GGG CAC T**T**G

'NtPea13' CAC GA**C** CCC GCA GGA ATT C**:**T CAC C**C**T TTC GAC CTG AGA TCA GG**C** A

'NtLyn14' CAC GA**C** CCC GCA GGA ATT C**:**T CAC C**C**T TTC GAC CTG AGA TCA GG**T** A

'NcBru12' CAC GAA CCC GCA GGA AAT CAT CAC CTT TTC GAC CTG AGA TCA GGC A

4. Discussion

4.1 *Isoëtes*

Sequence data, in conjunction with the RAPDs and previous assessments of morphology and cytology (Marsden 1979; D. Brunton Consulting Services, pers. comm. 2003), support the separation of *Isoëtes* into *I. alpinus* and *I. kirkii*. Previously, ITS was not able to adequately resolve differences between *I. kirkii* from Lake Omapere and other localities within New Zealand (Hofstra & Gemmill 1999). It was revisited in the present study, because it was possible that plants from additional localities (e.g. *I. alpinus* from Lake Te Anau and Lake Manapouri) may be more divergent or genetically distinct from the *I. kirkii* examined previously. However, our results show only a single base change between the *I. alpinus* and *I. kirkii* samples and do not resolve the relationships amongst New Zealand *Isoëtes* (and even with *I. gunnii* from Australia) any more clearly than previous studies.

Similarly the *rbc*L gene had only single sequence changes between the *I. alpinus* and *I. kirkii* samples, and *I. kirkii* samples were placed in the same clade as the Australian *I. humilior*. Although, only half of the amplified region (c. 600 of 1000 bp) was sequenced and analysed, these results were in agreement with other published data for *Isoëtes rbc*L. For example a GenBank *I. kirkii* sample (Rydin & Wikström 2002) from Lake Omapere (Chinnock 447 specimen) was identical in *rbc*L sequence to the Lake Omapere sample used in the present study (IkOmp5). Although the *rbc*L appears conservative in the *Isoëtes* (Rydin et al. 1999), a greater level of variation could have been anticipated in the *trn*L intron. The *trn*L intron has been reported with sequence divergence three to five times higher than *rbc*L in some taxa (Soltis & Soltis 1998). However, the *trn*L intron also showed little variation with only one base change between *I. alpinus* and *I. kirkii*. Although, it is worth noting that levels of variation such as these (i.e. single base changes) or no sequence variation at all, have been observed in other morphologically distinct taxa, that are recognised as separate species (Gemmill et al. 2002).

The fourth region of *Isoëtes* DNA for which sequence data was obtained was *LFY*—a region that is variable at the species and population level (Hoot & Taylor 2001), and has recently been employed to confirm species in *Isoëtes* (S. Hoot, University of Wisconsin, pers. comm. 1999) which have been described as 'notorious for the difficulties they present in identification' (Taylor & Hickey 1992). *LFY* sequence has only been obtained from one *I. alpinus* sample to date and when compared with GenBank data, it matched over a short portion of sequences with another *Isoëtes* species (*I. asiatica*), indicating that this was the *LFY* intron of interest. Unfortunately no other *Isoëtes* data have been deposited in GenBank so we are not able to conduct further phylogenetic analyses.

The close relationship of New Zealand and Australian *Isoëtes* is a recurring theme in both the sequence data from this study and published literature, which requires additional sequence data to resolve. For example, a combination of *LFY* and ITS data was used to delimit species within the North American

Isoëtes complex (Hoot & Taylor 2001). In addition, the ETS region, which is more variable in some taxa than ITS and *trn*L, and increasingly being employed (Markos & Baldwin 2001, 2002; Starr et al. 2003), may also better resolve differences amongst New Zealand *Isoëtes*, if developed for this taxa in the future.

RAPDs have provided the best evidence for genetically distinct entities amongst New Zealand *Isoëtes*, with differences between *I. kirkii* and *I. alpinus*, and the two *I. kirkii* samples (from Lake Omapere and Lake Taupo) at c. 50% of the loci analysed. These results support previous morphological and cytological descriptions of the *Isoëtes*. For example, the *I. alpinus* plants, which are diploids but differed in plant size, only show differences at c. 10% of the RAPD loci, whereas the *I. kirkii* plants (IkOmp5 and IkTpo), which differ in morphological character states (e.g. *I. kirkii* var. *flabellata* (Marsden 1979) from Lake Omapere) and are diploid and tetraploid respectively, are genetically more variable (at 50% of the loci). Further investigation at the population-level (within lakes) and between lakes is essential before final conclusions can be drawn.

4.2 *Nitella*

DNA sequence data for *Nitella* samples confirm the separation of *N*. aff. *cristata* from *N. hookeri*, while the *N. hookeri* complex (including *N. hookeri* var. *tricellularis*) remains unresolved. *Nitella* aff. *cristata* and *N. hookeri* complex samples were differentiated by both the *rbc*L and the ITS sequence data with 6 and 71 base pair changes, respectively. Samples from within the *N. hookeri* complex were identical for *rbc*L and *trn*I sequence data (and not widely analysed for ITS). The results for the *rbc*L sequence data support earlier work on *Nitella* from New Zealand and Australia (Karol, University of Maryland, pers. comm. 1993), in which *N*. aff. *cristata* and *N. hookeri* were clearly differentiated but differences amongst the *N. hookeri* complex were unresolved. By comparison, one of the few other studies investigating relationships amongst *Nitella* at the species level has shown that even recognised species (*N. furcata* and *N. inversa*) may not be distinguished by *rbc*L sequences (Sakayama et al. 2002). These results indicate that a more variable region than *rbc*L (or potentially cpDNA) is required to better resolve lower level taxonomic relationships in *Nitella*, such as the ITS. For example, there were a comparatively larger number of base changes between the recognised species *N*. aff. *cristata* and *N. hookeri* in ITS than for *rbc*L for the same taxa. ITS has the potential to elucidate lower level taxonomic relationships amongst *Nitella*.

A limitation to resolving lower level genetic differences amongst *Nitella* within this study (i.e. further ITS, or RAPD analyses) was the purity of the DNA template. Epiphytic and endophytic micro-organisms associated with the *Nitella* were difficult to detect and although due care was taken to ensure samples were clean prior to DNA extraction, some contaminated PCR products were initially obtained. DNA that is even potentially contaminated cannot be used for generating RAPDs, and consequently RAPDs were not undertaken for

Nitella in this study, even though their utility for resolving species and population level genetic differences has been documented. Consequently a significant investment was made to develop a protocol that would minimise the likelihood of extracting DNA from associated algae along with the *Nitella*, without having to develop recommended axenic *Nitella* cultures (K. Karol, University of Maryland, pers. comm. 2003), which was precluded by the timeframe, and without having to strip cell contents for DNA extraction to avoid endophytic algae (Ragan et al. 1994). This was achieved during the study and sequences have been subsequently amplified for some samples and an initial screening for RAPD primers successfully undertaken.

4.3 IMPLICATIONS FOR BIODIVERSITY

This study has provided genetic evidence in support of recent morphological and cytological investigations (D. Brunton Consulting Services, pers. comm. 2003; M. Casanova, Royal Botanic Gardens, pers. comm. 2005) that detected diverse and distinct entities of *Isoëtes* and *Nitella* in New Zealand. Specifically, we found evidence that *I. alpinus, I. kirkii* var. *flabellata* and *I. kirkii* (tetraploid) are genetically distinct from one another, as are the *N.* aff. *cristata* from the *N. hookeri* complex.

These findings have significant implications for New Zealand biodiversity because some populations of these endemic plants are under threat. *Isoëtes kirkii* is extinct in the lower Waikato lakes, with the subsequent loss of the type material for this species. *Isoëtes kirkii* var. *flabellata* is likely to be extinct at the only site it has been recorded, Lake Omapere, following a large deterioration in water quality of that lake (P. Champion, NIWA, pers. comm. 2005), with the culture maintained by NIWA based on plants recovered in 1998. Deterioration in water quality, such as documented for the Rotorua lakes, impacts on both these endemic plants. For example, *I. 'kirkii'* is now very rare within eutrophic Lake Rotorua. Eutrophication is also associated with a reduction in the depth of colonisation of charophytes, which are also being replaced by alien weed species. For example, the alien weed *Ceratophyllum demersum* L. has recently invaded Lake Taupo and has demonstrated the potential to replace charophytes over most of their depth range (J. Clayton, NIWA, pers. comm. 2004).

The close relationship between New Zealand and Australian *Isoëtes* is a recurring theme in this study and the published literature. A number of native New Zealand submerged plants are in common with Australia and the potential for gene flow between Australasia/Pacific and this country may exist, particularly via migratory water birds (Champion & Clayton 2000). For example, the distribution of *Isoëtes* species has previously been linked to transport of spores by waterfowl (Hoot & Taylor 2001), with a similar mechanism determined for charophyte oospores (Proctor 1962). This study and others suggest some common lineage of New Zealand *Isoëtes* with Australian taxa, and it would be valuable to include Australian taxa in any future analyses.

The presence of apparently apomictic (asexual) *Isoëtes* in the Central North Island lakes has some implications for their genetic uniqueness. Usually an

apomictic population is genetically relatively uniform, and may behave like a discrete taxa, with consistent characters that separate it from other populations, although the development of any discrete morphological entities depends on whether there is still the potential for outcrossing with other populations.

Population level studies of genetic variation within, and between, lake populations would significantly contribute to our understanding of the biodiversity and uniqueness of *Isoëtes* and *N. hookeri* entities. Such studies could obtain detailed information (e.g. validate our findings of three distinct genetic *Isoëtes* entities that have been sampled from different geographical regions) for the development of selection criteria for the protection of lakes with threatened plant populations.

5. Recommendations

Based on the results presented in this study, the authors make the following recommendations:

- A population level investigation (within- and between-lakes) should be carried out on *Isoëtes* using RAPDs (in possible conjunction with *LFY* (1100) and/or ETS) to clarify the following issues: whether genetic distinctiveness of the three entities identified remain when more individuals and localities (where they exist) are tested; the scale of genetic variation within the New Zealand group with reference to known Australian species; whether *I. kirkii* (s.s., diploid entity) is present within lower risk, South Island lakes.
- ITS and RAPDs should be conducted to resolve the status of the *N. hookeri* complex since problems with DNA contamination have now been resolved. This work should focus on between-lake variation.
- Strategies to preserve cultures of *I. kirkii* var. *flabellata* (ex Lake Omapere) are required, at least until the unique genetic status of this variety is confirmed.
- Representative habitat for *Isoëtes* amongst lakes needs to be preserved, particularly where genetic entities appear geographically restricted (e.g. Central North Island). A survey of *Isoëtes* habitat would identify the best lake/site candidates for protection, while identifying potential risks (e.g. weed invasion, local development, stream influences).

6. Acknowledgements

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7. References

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Appendix 1

GLOSSARY AND ABBREVIATIONS

Allopolyploid Crossing of two or more closely related species or genera and having a chromosome set composed of two or more chromosome sets derived more or less completely from the parents. Hybrids are usually sterile, because they do not have sets of homologous chromosomes and, therefore, pairing cannot take place. However, if doubling of the chromosome number occurs in a hybrid derived from two diploid (2n) species, the resulting tetraploid (4n) is a fertile plant as it contains two sets of homologous chromosomes making pairing and crossing over possible.

aff. (= affinity) A very close resemblance to the stated species in structure or in the essential structural parts, but possessing differences that warrant a different identity.

Apomictic Defined broadly as asexual reproduction and narrowly, and more commonly, as seed production without fertilisation (i.e. generation of a sporophyte from a gametophyte without fertilisation). Such sporophytes have the same chromosome number as the gametophyte from which they have been derived (synonym = apogamous).

Autopolyploid A polyploid organism in which the multiple sets of chromosomes are all derived from the same species. For example, doubling of the chromosome number during mitotic cell division as a result of nondisjunction.

bp Base pairs The nucleotides of the DNA sequence.

cpDNA Chloroplast DNA. The plasmid DNA found in plants that encodes for many genes relating to photosynthesis.

Diploid A condition in which the chromosomes in the nucleus of a cell exist as homologous pairs (i.e. carrying the same set of genes) and are twice the number of the haploid set (i.e. those found in the gametes). The diploid number of chromosomes in a cell is expressed as 2n.

ETS The external transcribed spacer of nuclear ribosomal DNA. This spacer region follows the 26S gene of ribosomal DNA. It is transcribed and subsequently excised from the mRNA. The ETS, a relatively newly developed marker, evolves at a faster rate than ITS and is showing much promise for resolving phylogenetic relationships among closely related taxa.

Hybrid A cross-bred offspring where parents are of different species. Intraspecific hybrids are the result of crossing between distinct populations.

ITS The internal transcribed spacers of nuclear ribosomal DNA. There are two intron regions, ITS-1 and ITS-2, which flank the 5.8S ribosomal gene. This is a multiple gene family that is found in hundreds to thousands of copies in plants. The ITS is generally useful at the generic or species level.

LFY The second intron of the LEAFY gene. LEAFY is a meristem identity gene involved in shoot development. This recently developed marker seems to

evolve faster than ITS and as such may be useful resolving relationships at the species or subspecific level.

Majority rule tree A phylogenetic tree which represents a summary of all the equally parsimonious trees (i.e. the set of trees of the same, minimum length). Relationships shown in majority rule trees are those found in 50% or more of the individual trees.

nrDNA Nuclear ribosomal DNA. Nuclear ribosomal genes (18S, 5.8S, and 26S) are so highly conserved that 'universal' primers have been developed to allow amplification of these genes with primers across taxa as diverse as fungi to mammals.

Parsimony analysis Applied to phylogenetics, parsimony, or maximum parsimony, analyses use minimum tree length as the optimality criterion for choosing between alternative phylogenetic trees. The tree or set of trees with the least number of steps (evolutionary events) is/are preferred over those that are longer. Parsimony analyses can generate a single tree or thousands of equally parsimonious (all of the same number of steps) trees.

PCR Polymerase Chain Reaction DNA technology where millions of copies of DNA can be generated in vitro. This technique has revolutionised molecular biology to the point where DNA sequences can be generated from a single drop of blood or from the hairs of an extinct animal. PCR has also allowed for entire genomes to be sequenced.

Polyploid Describing an organism containing nuclei that contain more than two sets of chromosomes (see diploid). For example, triploid plants have three sets of chromosomes and tetraploid plants have four.

RAPDs Random Amplified Polymorphic DNA A PCR-based method that randomly surveys the genome for genetic variation without prior knowledge of the genome. RAPD products produce 'fingerprints' (multiple bands per individual) that are then scored for presence or absence as compared to other samples.

s.s. (= *sensu stricto*) In the strict sense of the species description (i.e. identical to a type description).

***trn*L Chloroplast trn L (UAA) intron sequence** This non-coding sequence region is frequently used in phylogenetic analyses of closely related species because of its relatively fast rate of evolution.

UPGMA Unweighted Pair Group Method of Arithmetic Averages This clustering method of analysis uses the genetic distance matrix to produce a dendrogram. The dendrogram is a visual representation of the genetic similarity among samples.