



***Lindavia intermedia*, the causative organism of New Zealand lake snow: relationships between New Zealand, North American and European populations according to molecular and morphological data**



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Contents

Summary	v
1 Introduction and objectives	1
2 Methods	2
2.1 Collection of New Zealand material for genome sequencing	2
2.2 DNA extraction for genome sequencing	2
2.3 Shotgun genome sequencing and assembly	2
2.4 Marker design.....	3
2.5 Initial screening	3
2.6 Obtaining foreign material	4
2.7 Morphological characterisation of new samples	7
2.8 Data acquisition.....	7
3 Results	9
3.1 Morphological comparison of New Zealand and overseas specimens	9
3.2 Genetic comparison of New Zealand and overseas specimens	9
4 Discussion	13
5 Recommendations.....	16
6 Acknowledgements	17
7 References.....	17
Appendix 1 – Fragment sizes recorded in each sample for the three robust (repeatable) nuclear markers developed in this study.....	21
Appendix 2 – Concatenated sequences recorded in each sample for the three robust chloroplast markers developed in this study (marker order is LC6, LC17, LC30).....	22

Summary

Project and client

- Otago Regional Council contracted Landcare Research to undertake an investigation into the genetics of *Lindavia intermedia* populations in New Zealand and overseas, including the development and implementation of new molecular markers suitable for determining intraspecific variation, and testing hypotheses of the origin of this species in New Zealand.

Objective

- To test whether *Lindavia intermedia*, the causative agent of lake snow in New Zealand, is a recent invader in New Zealand lakes.

Methods

- DNA extracted from *L. intermedia* sourced from three New Zealand lakes was subjected to shotgun genome sequencing using Illumina Miseq.
- Assembled sequences were used to generate potential new molecular markers as tools to address the research question.
- These markers were tested and screened on New Zealand material.
- Selected markers were applied to samples from New Zealand, North America (two sites) and Europe (one site).
- Genetic diversity and phylogeographic relationships among populations of the species from these three areas were examined.

Results

- Three new nuclear markers and three new chloroplast markers were regarded as robust and combined to generate new data from the study populations.
- Specimens from Lake Youngs (USA) and all New Zealand lakes tested were identical, both genetically and morphologically. Specimens from Cultus Lake (Canada) were distinct genetically according to all markers, and were slightly different according to morphological data. The Lac Léman sample was the least similar to the New Zealand material.

Conclusions

- Although limited by the number of samples available from overseas, the results in context provide strong circumstantial evidence for the recent invasion of New Zealand lakes by *L. intermedia*.

Recommendations

- The status of *L. intermedia* as an invasive organism in New Zealand is now fairly certain. However, further study is needed to determine management options, its history in different New Zealand lakes and its effect on lake ecology, and to resolve the long-outstanding taxonomic issues surrounding bodanicoid *Lindavia* species (for which the newly developed markers offer useful tools). Such studies require funding at a national level.

1 Introduction and objectives

In 2008 Dr Marc Schallenberg and his students from Otago University began to study the production of an unusually abundant slime occurring in Lake Wānaka. Marc identified this as the globally rare phenomenon known as 'lake snow' (so called due to the drifting appearance of the flocs when viewed underwater; Brachvogel et al. 2001; Grossart et al. 1997). The abundance of this slime has been significant enough to require upgrades to municipal water infrastructure, and it has proven disruptive to recreational and professional fishing on the lake (Williams 2017).

The same phenomenon was identified in Lake Coleridge by Landcare Research in 2015 (although it was known to local bach owners about a year beforehand), and the mucilaginous strands were found to be produced by the same species in both cases. This species was identified as the diatom *Lindavia intermedia* (Manguin ex Kociolek & Reviere) Nakov, Guillory, Julius, Theriot & Alverson ex W.C.Daniels, Novis & Edlund (Novis et al. in press), a name that required taxonomic validation (Daniels et al. 2016). Lake snow was discovered in Lake Wakatipu and Lake Hawea shortly thereafter, and water filtration issues similar to those at Wānaka became evident in Queenstown (Scott 2017).

During this apparent range expansion in New Zealand, Schallenberg and Novis coined the vernacular term 'lake snot' as a result of its appearance on fishing lines and filters, and in reference to *Didymosphenia geminata* (Lyngbye) Schmidt, an invasive diatom in clean New Zealand rivers that is sometimes known as 'rock snot'. Although not used to date in the scientific literature, the term 'lake snot' has become popular in the news media. Both 'lake snow' and 'lake snot' refer to the same phenomenon.

Historical cases of slime outbreaks in New Zealand lakes were re-examined on the basis of this new knowledge. A 2008 event in Lake Waikaremoana (North Island) was definitively shown to have been due to the same organism, thanks to a preserved specimen held at NIWA by Dr Cathy Kilroy. Another occurrence in Lake Benmore during the early 2000s was caused by an organism identified as *Cyclotella stelligera* (Cleve & Grunow) van Heurck (now *Discostella stelligera* (Cleve & Grunow) Houk & Klee). This species could certainly be confused with *L. intermedia*, but this is currently impossible to test because reference specimens were not collected at the time. *Lindavia intermedia* was reported by Reid (2005) from Lakes Aviemore and Hayes, but whether lake snow was present at the time is uncertain. Of all the lakes from which the species is currently known in New Zealand, the latter is the only one that is significantly nutrient-enriched. Further information on *L. intermedia* is given in Novis et al. in press.

No historical record of this species in New Zealand has been found prior to the early 2000s. Furthermore, it is either absent or found only in surface layers of sediment cores from Lakes Wānaka, Wakatipu and Coleridge (É. Saulnier-Talbot, pers. comm., 2017; Novis unpublished observations), the only relevant lakes cored to date. This strongly suggests that the species is a recent invader of New Zealand. Testing this status is of obvious interest: if *L. intermedia* is not a recent arrival, the development of persistent lake snow is almost certainly due to a recent change in conditions in the Southern Lakes. If it is a recent adventive, different containment strategies should be considered and additional funding streams for research and remediation may be available.

Methods of DNA-based analysis can offer considerable additional information to test hypotheses of the origins of species, especially given the increasing affordability and speed offered by high-throughput DNA sequencing. Otago Regional Council (ORC) therefore contracted Landcare Research to address the origin of New Zealand *L. intermedia* using molecular methods.

2 Methods

2.1 Collection of New Zealand material for genome sequencing

Material was collected from Lakes Wānaka, Wakatipu, Hawea and Coleridge by dragging a weighted line from a boat trolling at low speed for 500–1,000 m. The Wānaka, Wakatipu and Hawea samples were provided by ORC. The material adhering to the line was scraped off and transported cold and fresh to the laboratory. Microscopic examination revealed all these samples to be well dominated (at least 95%) by *Lindavia intermedia*.

2.2 DNA extraction for genome sequencing

The samples were subjected to a protocol, established earlier, that successfully extracts DNA from fresh samples of lake snow (Novis et al. in press). Briefly, a large spoonful of material was resuspended in ADB buffer (Zymo Research, Irvine, CA, USA), which is usually intended to dissolve agarose gels, in 50 mL centrifuge tubes and incubated at 50–60°C for up to 2 h. The samples were then centrifuged briefly and resuspended in deionised water, before grinding and incubation in CTAB buffer for 30 min at 65°C. The material was centrifuged and the supernatant subjected to a phenol-chloroform extraction followed by clean-up in a Zymo Clean & Concentrate column (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The resulting extracts were assessed using Nanodrop measurements, with the column step ultimately undertaken twice to satisfy DNA quality requirements for Illumina library preparation.

2.3 Shotgun genome sequencing and assembly

Paired-end 2 × 250 Illumina Miseq was carried out by New Zealand Genomics Ltd (NZGL), with all three samples pooled on a single lane. Sequence contigs were generated using the 'de novo assembly' option on the processed sequences obtained from NZGL in Geneious 8.1 (<http://www.geneious.com>; Kears e et al. 2012), and consensus sequences generated on the basis of at least 100× coverage, with sequences of <100 bp removed (leaving approximately 6 million reads for each of the three lake samples). Additional assemblies were run to obtain chloroplast genomes using the 'assemble to reference' option, with the published chloroplast genomes of *Cyclotella* sp. WC03_2 (Genbank accession number KJ958481) and *Cyclotella* sp. L04_2 (Genbank accession number KJ958480) as reference sequences.

2.4 Marker design

The following approaches were taken to marker design.

a. Chloroplast markers.

The assembled chloroplast genomes from the three lakes were aligned using the Geneious alignment function. Primer pairs were designed in regions where sequence diversity was suspected by comparison to the *Cyclotella* genomes referenced above. At least one of each primer pair was overlapped from a coding region into an intergenic spacer (conferring high specificity that would exclusively amplify the target from a mixed sample).

b. Manually designed nuclear markers.

Contigs from all three lakes were compared by eye to select repeat regions that appeared polymorphic. Primer pairs were then designed using sequences of high complexity bordering these repeat regions.

c. Automated design of nuclear markers.

The software MSATCOMMANDER v0.8.2 (Faircloth 2008) was used to generate primer pairs from the processed Wakatipu Miseq reads. The library was searched for di- to pentanucleotide repeat regions with at least four repeat units, and flanked by appropriate regions for primer design. Primers were designed via the default settings of Primer3 (Rozen & Skaletsky 2000) as implemented in MSATCOMMANDER. Fifty primer pairs were chosen for screening from the MSATCOMMANDER output to supplement the additional 42 primer pairs (25 chloroplast and 17 nuclear, above) designed from existing sequence information, using an M13 tag (TGTAACAACGACGGCCAGT) on the 5' end of the forward primer for subsequent fluorescent labelling. The 50 automated pairs were arbitrarily chosen from a range of expected fragment sizes.

2.5 Initial screening

Sixty-seven of the primer pairs were tested on two samples (from Lake Coleridge and Lake Wanaka). DNA was extracted by the standard CTAB method (Doyle & Dickson 1987), with modifications as above. PCR was performed in 15 µL reactions consisting of 1 µL DNA at 5–50 ng and final concentrations of 1 × KAPA Plant PCR Buffer + dNTPs (KAPABIOSYSTEMS, Cape Town, South Africa) (0.2 mM each dNTP and 1.5 mM MgCl₂), 0.3 U KAPA3G Plant DNA Polymerase (KAPABIOSYSTEMS), 0.08 µM forward M13 tagged primer, 0.32 µM reverse primer, and 0.32 µM M13 labelled primer. PCR conditions were as follows: 95°C 5 min; 30 cycles of 95°C 20 s, 57°C 15 s, 72°C 30 s; 15 cycles of 95°C 20 s, 51°C 15 s, 72°C 30 s; 72°C 10 min.

PCR products were separated on 2% agarose gels, and the 15 primers that produced clear bands for both samples and that were potentially polymorphic were then used to amplify additional samples (see below).

2.6 Obtaining foreign material

The following methods were undertaken to attempt to obtain samples containing *L. intermedia*.

- An initial request for material corresponding to the *Lindavia bodanica* complex was sent on the global email server Diatom-L (subscribers to which are generally practising diatomists). I received one response to this email, from Dr Will Daniels (Brown University), with whom I was already in contact, offering material from Alaskan lakes. Unfortunately the species proved to be absent from these samples.
- Drs Andrew Alverson (University of Arkansas) and Edward Theriot (University of Texas) were approached for DNA from their former cultured strain of *Cyclotella bodanica* (now *Lindavia bodanica*, by far the closest relative of *L. intermedia* for which published sequence data are currently available). This they agreed to provide, but unfortunately it could not be found in their freezer storage. Dr Alverson was able to send scanning electron micrographs of the strain (some of which are reproduced with permission in Novis et al. in press).
- Dr Moya Joubert (Seattle Public Utilities), with whom we were previously in contact, provided a sample rich in *L. intermedia* from Lake Youngs (Washington, USA), the only other site worldwide where the species is known to produce lake snow.
- Joanna Gauthier (McGill University) provided samples from Cultus Lake (British Columbia, Canada). These comprised a sediment sample and several DNA samples from the lake water column. The sediment was rich in *L. intermedia*, and one of the DNA samples was collected when *L. intermedia* was present. Contact with Joanna was established by our colleague Dr Émilie Saulnier-Talbot (affiliated with Laval University) during the 2016 International Diatom Symposium in Québec City.
- Dr Sergei Genkal, who has published on *L. intermedia* from Russia (Genkal et al. 2013), was approached through email and ResearchGate messaging. No response was received.
- Dr Peter Kroth (Universität Konstanz) was approached via a previous contact in Germany and agreed to arrange for samples to be collected and sent from Bodensee (Lake Constance; Germany, Switzerland and Austria). However, after communicating with a local phycologist it transpired that the species had not been found in Lake Constance for 5 years, so these samples were not collected.
- Dr Joachim Hürlimann (AquaPlus, Switzerland) was contacted directly to obtain samples from Zugersee (Lake Zug, Switzerland). However, unlike many European lakes it transpired that this lake is still eutrophic following enrichment in the mid-20th century. Consequently *Lindavia bodanicoid* species have not been recorded there since the 1950s, and no samples were collected.

- Dr Arielle Cordonier (DETA, Switzerland) was contacted via Dr Hürlimann and agreed to arrange collection of samples from Lac Léman (Lake Geneva; Switzerland, Italy and France). Her colleague, Sophie Lavigne, sampled the lake monthly between January and May 2017 with plankton tows and filtration. The sample collected in February was found to contain small numbers of *Lindavia*; the genus appeared to be absent from the other samples.

Table 1 lists the final samples and associated details that were successfully used in this project, and Figure 1 shows their locations in map form.

Table 1 Details of *Lindavia intermedia* samples from New Zealand and overseas used in this study

Site	Country	Approx. locality	Collector	Date	Allan Herbarium voucher
Lake Coleridge	New Zealand	43.333924S, 171.541807E	P. Novis, Landcare Research	22/7/16	CHR589916
Lake Hawea	New Zealand	44.590408S, 169.273291E	N. Manning, Otago Regional Council	26/6/17	-
Lake Wānaka	New Zealand	44.659884S, 169.100325E	A. Uytendaal, Otago Regional Council	23/9/16	CHR589915
Lake Wakatipu	New Zealand	45.056293S, 168.644162E	A. Uytendaal, Otago Regional Council	23/9/16	CHR589911
Lake Youngs	USA	47.419110N, 122.122227W	E. Johnson, Seattle Public Utilities	4/5/2017	CHR644492
Cultus Lake	Canada	49.050800N, 121.988273W	J. Gauthier, McGill University	?/6/15	CHR644493
Lac Léman (Lake Geneva)	Switzerland/ France/Italy	46.443638N, 6.481979E	S. Lavigne, DETA	21/2/17	CHR644494

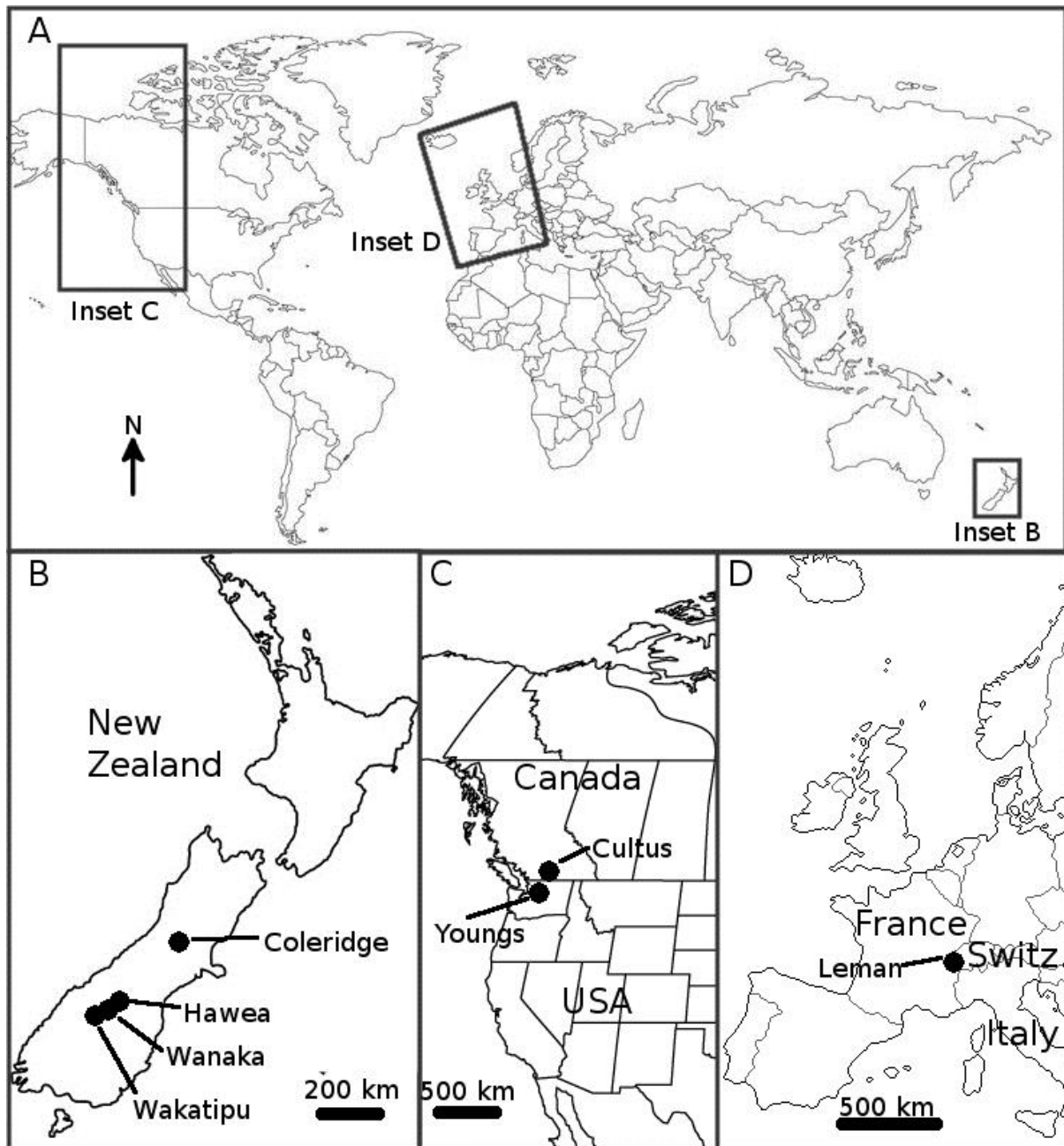


Figure 1 Maps showing the locations from which samples that were successfully sequenced using newly developed molecular markers were collected. A. World map showing relative locations of collecting areas. B. Collecting sites in New Zealand. C. Collecting sites in the Pacific Northwest of North America. D. Collecting site in Central Europe.

2.7 Morphological characterisation of new samples

Samples were examined on a Leica DMLB compound microscope with Nomarski Differential Interference Contrast optics, and images were taken using a Canon DS126271 digital camera. In order to observe valve ornamentation, diatoms were cleaned directly on coverslips using the muffle furnace method described by Biggs and Kilroy (2000), and permanent slides created using Naphrax (Brunel Microscopes Ltd, Wiltshire, UK) as a mountant. The diameters of valves and central areas and the number of striae per 10 μm (using the arc method) were measured on 40 valves from each sample for the Youngs and Cultus samples, and nine valves from the Léman sample (which contained much less material).

In order to observe ultrastructural surface features of diatoms in the samples, a cleaned coverslip from each sample was gold coated in a Desk Sputter Coater DSR1 (Nanostructured Coatings Co., Tehran, Iran) and examined on a TM3030Plus benchtop SEM (Hitachi, Tokyo, Japan). Typical operating conditions for the latter were 15 kV accelerating voltage and 5 mm working distance, using a mix of backscattered and secondary electron detectors, with the settings appropriate for conductive material.

2.8 Data acquisition

DNA from foreign samples was extracted either with the method described above or with a Maxwell 16 Tissue DNA purification kit (Promega Corporation, Madison, USA), or the DNA was received already extracted from a collaborator (in the case of Cultus Lake water column samples).

a. Nuclear markers.

The amplification method was as described above. One microlitre from each PCR product was added to 10 μL Hi-Di formamide (Applied Biosystems, Waltham, MA, USA) and 0.2 μL GeneScan 600 LIZ size standard (Applied Biosystems), before being separated on a 3500xl genetic analyser (Applied Biosystems) using a DS-33 dye set at the Landcare Research sequencing laboratory (Auckland, New Zealand). Fragments were visualised and scored using Geneious v8.0.5 (Biomatters, Auckland, New Zealand), and polymorphism at each locus was assessed. Seven of the loci tested produced polymorphic fragments and no more than two alleles per individual (Table 2). A further six amplified reliably but were monomorphic, and two produced results that were not scoreable. A genetic distance matrix and principal coordinates analysis (PCoA) was performed using GenAlEx v6.501, and a UPGMA tree was constructed in MVSP v3.22 (Kovach 2007) from the same genetic distance matrix. The markers were tested for repeatability by repeating a duplicate set, and three were retained as robust.

b. Chloroplast markers.

The 25 chloroplast primer pairs were used to sequence all samples, using the same PCR mix (omitting the M13 tagged primer and adjusting primer concentrations to equimolar) and the cycling conditions were as follows: 95°C 5 min; 35 cycles of 95°C 20 s, 57°C 15 s, 72°C 30 s; 72°C 10 min. Sequencing was performed on the same genetic analyser described above. Electropherograms were checked by eye and assembled using Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, MI, USA). A multiple sequence alignment was created using MEGA6 (Tamura et al. 2013) from the four primer pairs that amplified products from all samples (except Lac Léman, which did not amplify reliably), data gaps and sites containing ambiguous nucleotides were omitted, and a UPGMA tree was created using MEGA6, with ‘number of differences’ as the distance metric (a more complex model was unnecessary, since all sequences were identical except one; see Results). Where possible, sequences from the Cultus Lake DNA sample extracted from water by Joanna Gauthier were compared to sequences from the Cultus Lake sample extracted from sediment (which were extracted by us and from which we could obtain morphological data); these were always identical.

The final set of six primer pairs is shown in Table 2.

Table 2 Characteristics of six polymorphic loci developed in *Lindavia intermedia*. The LN and SSR prefixes refer to nuclear markers, LC to chloroplast markers

Locus	Primer sequences (5'-3') ¹	Repeat motif ²	Allele size range (bp)	T _a ³ (°C)
LN11	F: GCATATTTTATGTGCAGG R: TATGTTGTTTCGATATACC	GA	436–454	57
LN26	F: TATGACAAAGGCCAGGGC R: CACTGCATCGGAAGGTGC	TA	194–196	57
SSR34	F: ATCACCCTAGCTACCGCTTG R: AACCGGACCAGCGATTTGG	CAG	141–151	57
LC6	F: AACTGAGTTCTAACAGCG R: GAAGAAAGCAAATTGGC	-	195	57
LC17	F: CCCAGTAGGAATCGAACC R: AGCGGTAGAGTGTCTGCC	-	141	57
LC30	F: TAGCCTACGGAAGTAGCG R: GACCATTCGACATAATAG	-	120	57

¹Note that an M13 tail (TGAAAACGACGGCCAGT) was added to the 5' end of each forward primer.

²Not applicable for chloroplast markers, which were sequenced rather than scored by size, and distinguished taxa by base substitutions.

³Annealing temperature used in PCR.

3 Results

3.1 Morphological comparison of New Zealand and overseas specimens

A selection of scanning electron micrographs of *Lindavia intermedia* from New Zealand and overseas localities, recorded from the samples used in this study, is shown in Figure 2. Lake Youngs specimens were indistinguishable from New Zealand specimens according to stria density, cell size, and ratio of central area width to valve width (Figure 3). These characters are among those used to distinguish species within *Lindavia* (e.g. Novis et al. in press); Figure 2A indicates their positions on the diatom valve face.

However, Cultus Lake and Lac Léman specimens were slightly different, with a slightly increased range and median density of striae and central area:valve width ratio (Figure 3). Figure 4 places this in the context of other species of *Lindavia*: while the criteria placed the New Zealand and Lake Youngs material in agreement with currently accepted boundaries of the species *L. intermedia* (Daniels 2012), the Cultus Lake and Lac Léman specimens were found to range between these and the criteria currently accepted for *L. bodanica* (Burge & Edlund 2017).

3.2 Genetic comparison of New Zealand and overseas specimens

Each of the three nuclear markers and three chloroplast markers produced the same key result: all New Zealand material was identical to that from Lake Youngs. The sample from Cultus Lake differed from this slightly (in the case of the chloroplast markers, this difference amounted to seven base substitutions over 434 base pairs of chloroplast genome spacer region). The sample from Lac Léman amplified from only one of the three chloroplast markers; therefore, a conservative approach was taken and this sequences was excluded. If it was included, and considering that marker alone, the Lac Léman specimens were the most distant from the New Zealand specimens. The three robust nuclear markers amplified successfully from the Lac Léman sample, similarly placing it slightly more distant from the New Zealand material than the material from Cultus Lake. These results are shown in the form of a UPGMA phylogeny in Figure 5.

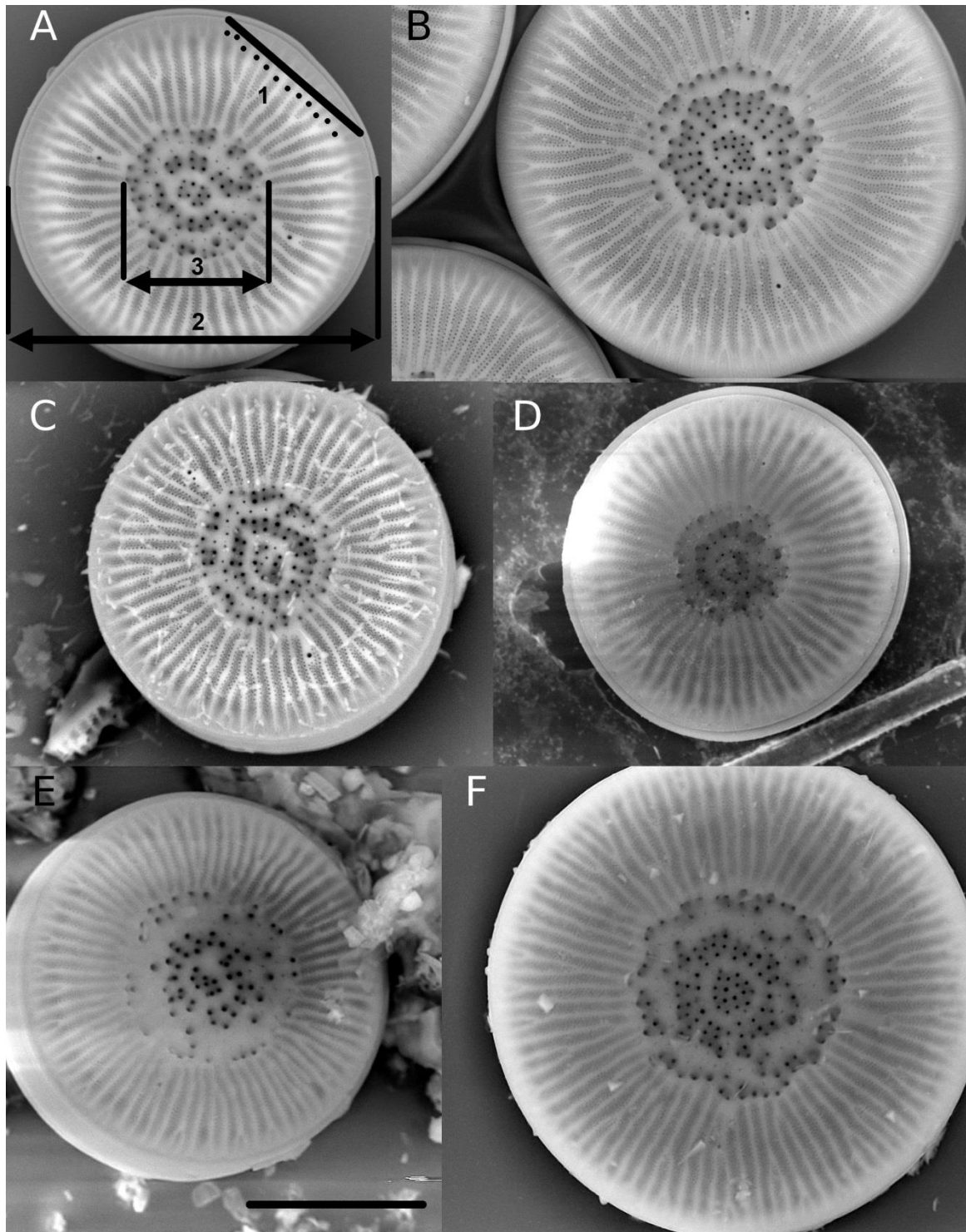


Figure 2 Scanning electron micrographs showing typical valves of *Lindavia intermedia* from New Zealand and elsewhere, and characters used in morphological analysis. A. Lake Coleridge, showing morphological characters enumerated in later figures: 1, number of striae (12 in this case) in a 10 µm arc; 2, cell width; 3, central area width, typically combined with cell width to give a ratio. B. Lake Wānaka. C. Lake Wakatipu. D. Lake Youngs (WA, USA). E. Cultus Lake (BC, Canada). F. Lac Léman (Switzerland). All shown at the same scale (the bar in E. is 10 µm).

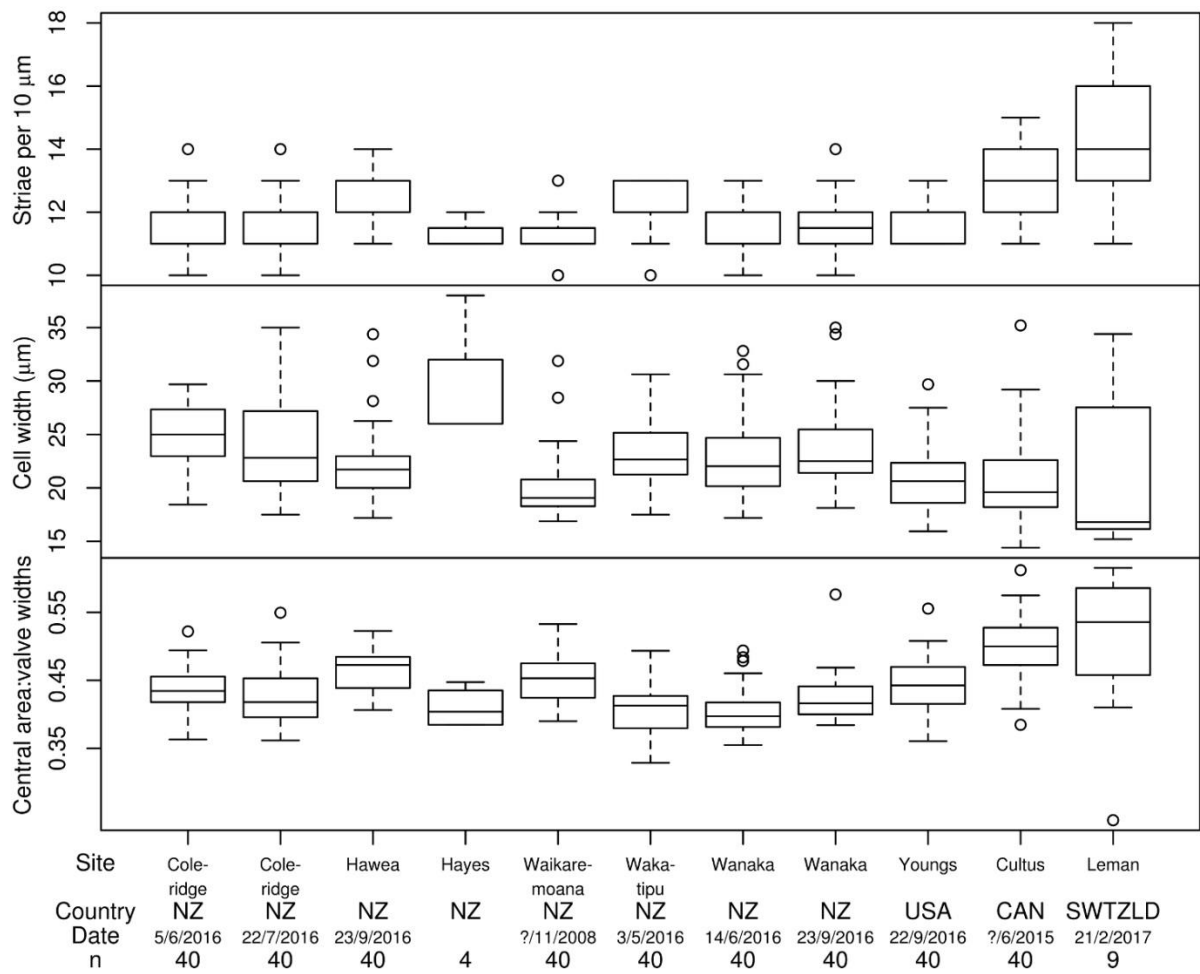


Figure 3 Values for three characters commonly used for identification of bodanoid diatoms (see Figure 2A), scored for *Lindavia intermedia* populations in New Zealand and from populations from Lake Youngs (USA), Cultus Lake (Canada) and Lac Léman (Switzerland). Boxes define (from top to bottom) the upper quartile, median and lower quartiles; whiskers the limits of the nominal ranges inferred from the quartiles; and open circles the outliers. 'Date' refers to the date of collection, and 'n' to the number of individuals measured from the population.

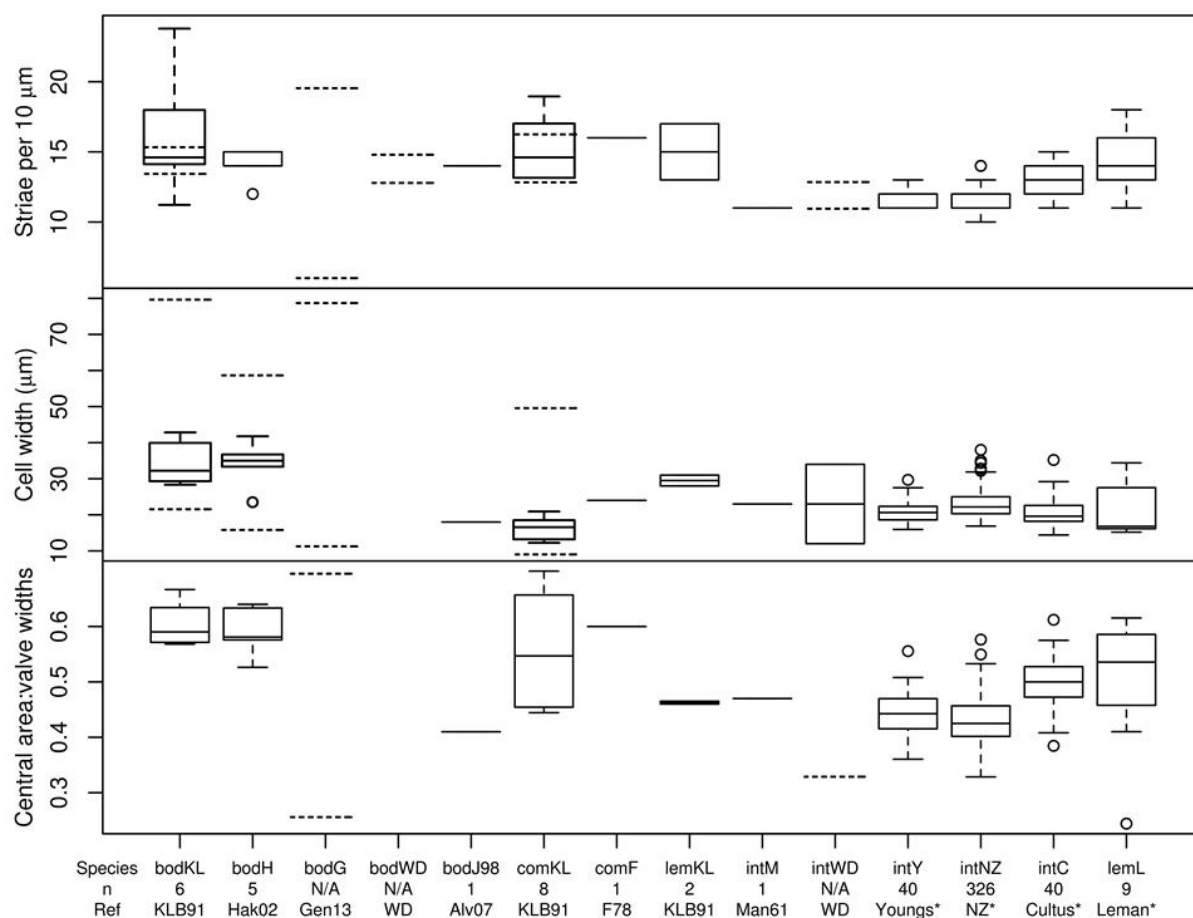


Figure 4 Values for the three characters scored in the previous figure, applied to representatives of the *Lindavia bodanica* complex from a variety of sources. Boxplots refer to measurements made on illustrative material from references or (in the case of Youngs, Cultus, Léman and New Zealand) on field populations. Dashed lines represent ranges specified in descriptions from the references given below. Under ‘Species’, ‘bod’ refers to *Lindavia bodanica*, ‘com’ to *L. comta*, ‘int’ to *L. intermedia*, and ‘lem’ to *L. lemanensis*. The initials following the species name refer to the associated reference or site. The identification of *L. lemanensis* for material from Lac Léman is assumed here but uncertain. References are: KLB91, Krammer & Lange-Bertalot 1991; Hak02, Häkansson 2002; Gen13, Genkal et al. 2013; WD, Western Diatoms; Alv07, Alverson et al. 2007; SEM images kindly sent by the author); F78, Foged 1978; Man61, Manguin 1961. Western Diatoms includes two references: Daniels 2012 for *L. intermedia* and the striae count for *L. bodanica*, and Burge & Edlund 2017 for the remaining data for *L. bodanica*. Asterisks refer to the populations measured by us from field material.

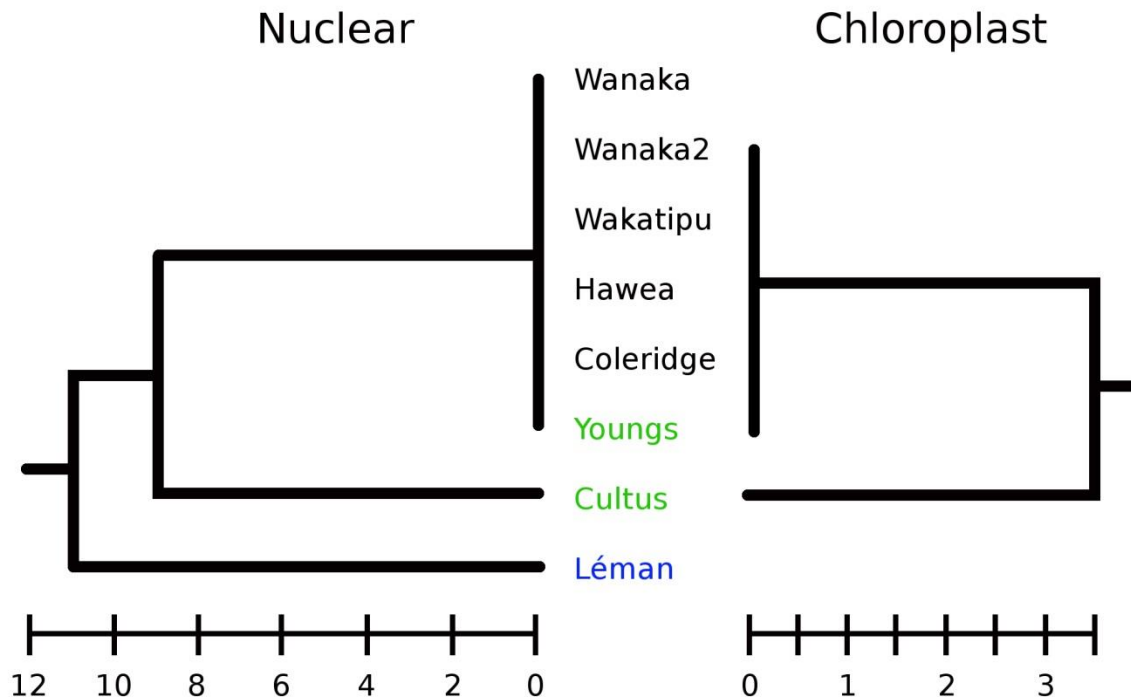


Figure 5 Trees constructed using the UPGMA method for genetic distance data generated from three newly developed nuclear (left) and chloroplast (right) molecular markers for *Lindavia intermedia*. New Zealand sites are in black, North American in green and European in blue. Scales refer to the codominant genetic distance for microsatellites calculated by GenAlEx for nuclear markers, and number of base substitutions for the chloroplast markers.

4 Discussion

Although tempered by the modest samples from overseas, this study produced a clear result. According to analysis of newly developed markers suitable for examining intraspecific variation, *Lindavia intermedia* from Lake Youngs (USA) is identical to the species currently producing lake snow in large, clean lakes in New Zealand, and different from other forms identified as *L. intermedia* and *L. lemanensis* (two species that Krammer and Lange-Bertalot (1991) placed in synonymy). Furthermore, morphological measurements tend to support the genetic data and indicate a range of values spanning the intervals currently circumscribing separate species (Daniels 2012; Burge & Edlund 2017). This is similar to findings published by Genkal et al. (2013), prompting them to propose synonymy of *L. intermedia* with *L. bodanica*. As described in Daniels et al. (2016) and Novis et al. (in press), the taxonomy of this group has a complicated history and species boundaries remain uncertain.

The original goal of the study was to determine whether *L. intermedia* is a recent invader to New Zealand and where it may have come from. The paucity of material we were able to obtain from overseas was undoubtedly a hindrance in addressing these questions. Nonetheless, some very pertinent information has been obtained.

- New Zealand *Lindavia intermedia* is genetically and morphologically identical to specimens from Lake Youngs (USA), which is the only other site worldwide in which this species conclusively produces significant quantities of lake snow. In Cultus Lake (Canada) it is not certain if lake snow occurs. There are apparently no reports of this phenomenon from fishers, but some researchers experience problems with net clogging at some times of the year, and this may not be during fishing season (J. Gauthier, McGill University, pers. comm., 2017).
- Specimens from Cultus Lake, which closely resemble the material from New Zealand and Lake Youngs and have been identified as *L. intermedia* by researchers working on the lake, belong to a different genotype. They also appear to differ slightly in morphology. There is thus a greater genotypic diversity within this species, as well as within the genus, in the Northern Hemisphere than occurs in New Zealand, where only one genotype (and species of *Lindavia* for that matter) appears to be present.
- Material corresponding to this species (or the possibly conspecific *L. lemanensis* or even *L. bodanica*) appears to have become scarce in the lakes of Europe. This is at least partly due to the trophic perturbations experienced by these lakes over the previous 60 years. In some cases, such as Zugersee, eutrophication persists and excludes *Lindavia*; the genus has recolonised other lakes following phosphorous reduction programmes and oligotrophication. However, the populations persisting in European lakes prior to eutrophication in the mid-20th century were doubtless exterminated by the enrichment (as shown in the sedimentary record of Zugersee; Elber et al. 2001). Whether the restored populations reflect the earlier genotypes is unknown, and probably impossible to test. This is unfortunate, since some of these lakes were type localities for species of *Lindavia* (*L. bodanica* from Bodensee, *L. lemanensis* from Lac Léman). The increased rarity of *Lindavia* in these lakes also reduces the chance of Europe being a source of new material in New Zealand, consistent with the result in Figure 5.
- Sediment core samples taken from Lakes Wanaka, Wakatipu and Coleridge all indicate that the species has appeared in these lakes in the recent past (M. Schallenberg, University of Otago, and É. Saulnier-Talbot, pers. comms., 2017).

Taken together, these observations represent a strong circumstantial case for transfer of *Lindavia intermedia* to New Zealand from the Northern Hemisphere, particularly North America.

Given the presence of the same genotype in Lake Youngs as in New Zealand, it may be worth reviewing knowledge of the environment of Lake Youngs. It is a water reservoir for local communities and is therefore relatively clean. Published data on the lake are scarce; the best source found is Canale et al. 1997, which predates the occurrence of lake snow in Lake Youngs, but provides some indication of its water quality (phosphorous loadings are not thought to have risen prior to the advent of lake snow, unless possibly from the deep sediments; Zisette 2009). These data are compared with other lakes in the study in Table 3. The two North American lakes are notably smaller than the New Zealand lakes and Lac Léman, but are deep enough to stratify, and all three foreign lakes contain more phosphorous than the New Zealand lakes. However, none would be categorised as more than mesotrophic.

Table 3 Selected properties of the lakes in this study, based on the variables available for Lake Youngs in Canale et al. 1997

Lake	Maximum depth (m)	Surface area (km ²)	Total P (µg L ⁻¹)	Source ¹
Coleridge	200	36.9	2–5	LAWA
Hawea	384	137.6	2–7	LAWA
Wānaka	311	180	2–7	LAWA
Wakatipu	380	289	2	LAWA
Youngs	30.5	2.8	4–15	Canale et al. 1997 ²
Cultus	41.8	6.3	4.3–15	Selbie 2013
Léman	309	580	12–20	Jacquet et al. 2014

¹ LAWA = Land, Air, Water Aotearoa. website: www.lawa.org.nz

² Reporting data from 1992.

It is tempting to hypothesise Lake Youngs as a possible source of *Lindavia* in New Zealand. Although Lake Youngs is a reservoir for local drinking water, and is nominally fenced off from the public, this probably does not isolate it from being a potential source of material for transfer elsewhere. Local internet forums can easily be found in which subscribers enthusiastically describe the size of the fish in Lake Youngs and recommend that others ‘hop the fence ... and start casting’ (e.g. www.washingtonflyfishing.com/forum/threads/why-no-trophy-lake-in-south-king-county.46625/; www.northwestfishingreports.com/forum/viewtopic.php?t=3812).

However, counter to the hypothesis of Lake Youngs as a possible source of New Zealand *Lindavia* is the timing of lake snow in that lake, first reported in earnest in 2008 (Zisette 2009). The phenomenon is thought to have begun prior to this in Lake Wānaka, with the species thought to have become detectable in lake sediments in approximately 2004 (É. Saulnier-Talbot, pers. comm., 2017). The earliest suspected lake snow event in New Zealand is in Lake Benmore in the early 2000s. However, it does not appear to be known how long *L. intermedia* has been present in Lake Youngs prior to the onset of lake snow production.

Another strong possibility is that other North American lakes harbour this species without lake snow being produced or perhaps detected; the latter could be quite likely in more isolated alpine lakes. Regardless, it now seems very likely that the genotype responsible for lake snow diversified somewhere in the Northern Hemisphere prior to a recent invasion of New Zealand, either natural or human-mediated.

5 Recommendations

A large number of pressing questions remain to be addressed. One of these, highlighted by the present study is: Why is lake snow produced by *L. intermedia* so rare in the rest of the world? A related question is, Why has it proven persistent in some lakes (Wānaka since at least 2008, Coleridge since 2015) but apparently ephemerally in others (probably in Benmore; confirmed in Waikaremoana)? The New Zealand lakes that have been cored to date are those in which lake snow has been persistent, and demonstrate a gradual increase in *L. intermedia* in the core surface layers; sediment cores taken from Benmore and Waikaremoana may reveal much more periodicity, which could help to link abundances to water chemistry and thus population drivers, where such data are available. Coring a wider variety of lakes is therefore an important line to pursue.

Determining possible management interventions requires more than cell quantification, however. Methods for accurately quantifying lake snow production must be developed to determine the extent of its decoupling from population growth and the conditions under which it occurs. If lake snow consists largely of β -chitin, as seems likely (Gügi et al. 2015), large-scale production of this nitrogen-containing polysaccharide may indicate a delicate imbalance between available nitrogen and phosphorous. Ultimately, laboratory experiments will be needed to study the physiology of *L. intermedia*, requiring the ability to culture the organism. A close relative from Wyoming was cultured with difficulty by Alverson et al. (2007); our newly established contact with these researchers would probably prove very useful in efforts to grow the New Zealand material artificially.

The effect of *Lindavia* on lake ecology also demands attention. Lake snow transforms the water column of microtrophic lakes from essentially a carbon 'desert' to a series of carbon- (and potentially nitrogen-) rich oases much more favourable for bacterial growth. The further impact of this on higher trophic levels in the food chain is unknown, but suggests a tipping-point scenario towards further change in species and resource composition of lake ecosystems.

The molecular markers developed here have the potential to unravel both the systematics and biogeography of bodanoid *Lindavia*. This would resolve long-standing taxonomic problems in the group and greatly assist with the biosecurity implications arising from our results; i.e. that this species should be regarded as an adventive that may threaten more lakes worldwide, and thus needs to be accurately detectable. Given better samples, more markers would likely be available for use. Such an enterprise would best be undertaken in a more coordinated fashion, with more time and funding available (i.e. at a national level) than has been available for this study.

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Appendix 1 – Fragment sizes recorded in each sample for the three robust (repeatable) nuclear markers developed in this study.

Lake	Country	Nuclear marker fragment sizes		
		LN11	LN26	SSR43
Coleridge	New Zealand	441, 454	196	151
Hawea	New Zealand	441, 454	196	151
Wānaka	New Zealand	441, 454	196	151
Wānaka 2	New Zealand	441, 454	196	151
Wakatipu	New Zealand	441, 454	196	151
Youngs	USA	441, 454	196	151
Cultus	Canada	436, 454	194	145
Léman	Switzerland/France/Italy	392, 392	175	143

Appendix 2 – Concatenated sequences recorded in each sample for the three robust chloroplast markers developed in this study (marker order is LC6, LC17, LC30).

>Coleridge

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ACTGAGTTCTAACAGCGAGCTTATTAATATGGAAAGGTTGATTTTATCAATTTTACAAATTAATAAAAAAT
TTTTAAATAAAATAACCTAAGTTAAACTTAGATTATTTAACGTTTGAATAATTGTTTTTTAGCTGACTTT
AATTTTTTTTAAATTACACAGTTTTTTGATAGAGCAGCCAATTTTCCCAGTAGGAATCGAACCTACATTAGA
AACTTAGAAGGTTCCCTGTCCTGATCCGTTAGACGATAGGCCCAAACAAAAAGCAATGGGTAATACAGGATT
TGAACCTGTGACCTTCTGCTTGTAAGGCATTAGCCTACGGAAGTAGCGTTCTTTAAGAGAAAAACTAATAT
TTGGACATTAATAAAAGATTCTTTACAAACCTCAACCATATGTAAAAACTATTAAGGAACTCTATTATGTC
GAATGGTC
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>Youngs

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ACTGAGTTCTAACAGCGAGCTTATTAATATGGAAAGGTTGATTTTATCAATTTTACAAATTAATAAAAAAT
TTTTAAATAAAATAACCTAAGTTAAACTTAGATTATTTAACGTTTGAATAATTGTTTTTTAGCTGACTTT
AATTTTTTTTAAATTACACAGTTTTTTGATAGAGCAGCCAATTTTCCCAGTAGGAATCGARCTACATTAGA
AACTTAGAAGGTTCCCTGTCCTGATCCGTTAGACGATAGGCCCAAACAAAAAGCAATGGGTAATACAGGATT
TGAACCTGTGACCTTCTGCTTGTAAGGCATTAGCCTACGGAAGTAGCGTTCTTTAAGAGAAAAACTAATAT
TTGGACATTAATAAAAGATTCTTTACAAACCTCAACCATATGTAAAAACTATTAAGGAACTCTATTATGTC
GAATGGTC
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>Wanaka

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ACTGAGTTCTAACAGCGAGCTTATTAATATGGAAAGGTTGATTTTATCAATTTTACAAATTAATAAAAAAT
TTTTAAATAAAATAACCTAAGTTAAACTTAGATTATTTAACGTTTGAATAATTGTTTTTTAGCTGACTTT
AATTTTTTTTAAATTACACAGTTTTTTGATAGAGCAGCCAATTTTCCCAGTAGGAATCGAACCTACATTAGA
AACTTAGAAGGTTCCCTGTCCTGATCCGTTAGACGATAGGCCCAAACAAAAAGCAATGGGTAATACAGGATT
TGAACCTGTGACCTTCTGCTTGTAAGGCATTAGCCTACGGAAGTAGCGTTCTTTAAGAGAAAAACTAATAT
TTGGACATTAATAAAAGATTCTTTACAAACCTCAACCATATGTAAAAACTATTAAGGAACTCTATTATGTC
GAATGGTC
```

>Hawea

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ACTGAGTTCTAACAGCGAGCTTATTAATATGGAAAGGTTGATTTTATCAATTTTACAAATTAATAAAAAAT
TTTTAAATAAAATAACCTAAGTTAAACTTAGATTATTTAACGTTTGAATAATTGTTTTTTAGCTGACTTT
AATTTTTTTTAAATTACACAGTTTTTTGATAGAGCAGCCAATTTTCCCAGTAGGAATCGAACCTACATTAGA
AACTTAGAAGGTTCCCTGTCCTGATCCGTTAGACGATAGGCCCAAACAAAAAGCAATGGGTAATACAGGATT
TGAACCTGTGACCTTCTGCTTGTAAGGCATTAGCCTACGGAAGTAGCGTTCTTTAAGAGAAAAACTAATAT
TTGGACATTAATAAAAGATTCTTTACAAACCTCAACCATATGTAAAAACTATTAAGGAACTCTATTATGTC
GAATGGTC
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>Wakatipu

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ACTGAGTTCTAACAGCGAGCTTATTAATATGGAAAGGTTGATTTTATCAATTTTACAAATTAATAAAAAAT
TTTTAAATAAAATAACCTAAGTTAAACTTAGATTATTTAACGTTTGAATAATTGTTTTTTAGCTGACTTT
AATTTTTTTTAAATTACACAGTTTTTTGATAGAGCAGCCAATTTTCCCAGTAGGAATCGAACCTACATTAGA
AACTTAGAAGGTTCCCTGTCCTGATCCGTTAGACGATAGGCCCAAACAAAAAGCAATGGGTAATACAGGATT
```


TGAACCTGTGACCTTCTGCTTGTAAGGCATTAGCCTACGGAAGTAGCGTTCTTTAAGAGAAAACTAATAT
TTGGACATTAATAAAGATTCTTTACAAACCTCAACCATATGTAAAACTATTAAGGAACTCTATTATGTC
GAATGGTC

>Cultus

ACTGAGTTCTAACAGCGAGCTTATTAATATGGAAAGGTTGATTTTATTAATTTTACAAATTAATAAAAAAT
TTTTAAATAAAATAACCTAAGTTAAACTTAGATTATTTAACGTTTGAATAACTGTTTTTTAGCTGACTTT
AATTTTTTTGAATCACACAGTTTTTGATAGAGCAGCCAATTTTTCCCAGTAGGAATCGAACCTACATTAGA
AACTTAGAAGGTTCTGTCCTGATCCGTTAGACGATAGGCCCAAATAAAAAGCAATGGGTAATACAGGATT
TGAACCTGTGACCTTCTGCTTGTAAGGCATTAGCCTACGGAAGTAGCGTTCTTCAAGAGAAAACTAATAT
TTSGACATTAATAAAGATTCTTTACGAACCTCAACCATATGTAAAACTATTAAGGAACTCTATTATGTC
GAATGGTC