Assessing the Diversity of New Zealand's Freshwater Copepods (Crustacea:Copepoda) using Mitochondrial DNA (COI) Sequences

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Abstract

The diversity of New Zealand's freshwater copepods has been largely understudied. In order to enhance our understanding of this taxon, I used the mitochondrial cytochrome *c* oxidase subunit one (COI) gene sequences to examine the diversity of two orders of New Zealand's freshwater copepods; Cyclopoida and Harpacticoida. Where possible, I also assessed the global affinities of taxa using available sequences from global databases (e.g. GenBank, BOLD). Specimens were collected from several sites across both the North Island and South Island of New Zealand. From these, DNA was extracted from 246 individuals, of which COI sequences were successfully obtained from 84 (a success rate of 34%). Sequences represented 17 species of freshwater copepod; nine species of cyclopoid and eight species of harpacticoid; all species were clearly delineated by the COI gene. Intraspecific sequence divergences were generally <1% whereas interspecific divergences usually exceeded 13%. For the cyclopoid copepods, three taxa (two distinct species of *Eucyclops* cf. '*serrulatus'* and *Acanthocyclops americanus*) showed close molecular affinities to Northern Hemisphere populations (<1% divergent in all cases); and are likely to represent recent human-mediated introductions to New Zealand. Additionally, *Mesocyclops* '*leuckarti*' was <1% divergent to an undescribed cyclopoid species from South Australia, likely *Mesocyclops australiensis*. For the harpacticoids, specimens of *Elaphoidella bidens* and two geographically distinct populations of *Bryocamptus pygmaeus* showed high levels of intraspecific diversity (>12% and >18% divergent respectively), suggesting the presence of cryptic taxa. These results suggest that the diversity of New Zealand's freshwater cyclopoid and harpacticoid copepods is underestimated and several non-indigenous taxa may be present among the New Zealand copepod fauna. I conclude that the COI gene will be a useful tool in assessing New Zealand's native copepod biodiversity and also in identifying invasive species.

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Chapter I

Introduction to Thesis

1.1 Introduction

Copepods are among the most diverse taxa on Earth. They are abundant in both fresh and marine waters (Dussart & Defaye, 1995) and are also found in some terrestrial habitats such as soil and leaf litter (Reid, 2001). The evolutionary history of copepods is somewhat limited by their poor representation in the fossil record (Schram, 1982). However, it is generally accepted that copepods originated in the marine environment sometime during the Lower Cretaceous era (Huys & Boxshall, 1991). They later colonized inland waters through a succession of invasion events beginning before the breakup of Pangea and continuing through to after the Pleistocene glaciations (Boxshall & Jaume, 2000). Today, three orders dominate the world's freshwater; the Calanoida, Cyclopoida and Harpacticoida (Dussart & Defaye, 1995). Freshwater copepods are integral components of freshwater ecosystems, providing key links in food webs between algae and higher trophic levels such as macroinvertebrates and fish (Alheit & Scheibel, 1982; Lancaster & Robertson, 1995). Copepods have also become increasingly popular in ecotoxicological studies (Kulkarni *et al.*, 2013), and can be useful as bioindicators of ecosystem health (Hanazato *et al.*, 1989; Hanazato & Yasuno, 1989; Ferdous & Muktadir, 2009).

From a biogeographic perspective, many freshwater copepods have circumscribed distributions, with over 90% of species endemic to a single zoogeographical region (Bayly, 1995; Boxshall & Defaye, 2008). For example, in the Southern Hemisphere, several calanoid taxa appear to have a Gondwanan affinity, with the calanoid family Centropagidae, and in particular the genus *Boeckella*, largely restricted to Australia, South America, New Caledonia and around the periphery of Antarctica (Bayly, 1992, 1995). Further, the Northern Hemisphere family Diaptomidae, dominant throughout the inland waters of Europe, Asia and North America, was prior to human intervention absent from New Zealand, Antarctica and most of Australia and South America (Bayly, 1995; Boxshall & Defaye, 2008). Similarly, Gondwanan affinities are noticeable among the harpacticoid fauna; the genera *Antarctobiotus* and *Loefflerella* and the two subgenera of *Attheyella*, *Delachauxiella* and *Chappuisiella*, are apparently restricted to Australasia, South America and the Antarctic (Lewis, 1984). On the other hand, the cosmopolitan family Parastenocaridae, known from all seven continents

(Boxshall & Defaye, 2008), is noticeably absent from New Zealand (Lewis, 1984). In contrast, biogeographic patterns for the Southern Hemisphere cyclopoid copepods are largely unclear. Many species have been considered cosmopolitan in distribution putatively being found in both the Northern and Southern Hemispheres. While many of these cosmopolitan taxa may prove to species complexes (e.g., (Kiefer, 1981; Alekseev *et al.*, 2011; Miracle *et al.*, 2013), some species may have been introduced by early European settlers to the Australasian region (Karanovic, 2005). Among the cyclopoids, New Zealand has a notable lack of the otherwise cosmopolitan genus *Cyclops* (Chapman *et al.*, 2011).

1.2 Diversity of New Zealand's freshwater copepod fauna

New Zealand has at least 67 known freshwater copepod species representing three orders; the Calanoida (11 species), Cyclopoida (21 species), and Harpacticoida (35 species) (Webber *et al.*, 2010). Several authors have noted that the diversity of New Zealand's freshwater copepods is unusually low, particularly when compared to locations of similar or smaller land size such as Tasmania or Great Britain (Maly & Bayly, 1991; Bayly, 1995; Chapman, *et al.*, 2011). This has been hypothesised to be at least partly the result of mass extinctions during the submergence of most (or all) of New Zealand during the Oligocene (*sensu* Stevens (1980)) (Maly & Bayly, 1991; Bayly, 1995). However, sampling of semi-terrestrial habitats may reveal additional species (Chapman, *et al.*, 2011).

The most well studied of New Zealand's freshwater copepods, the calanoid copepods, dominate the zooplankton of large lakes (Webber, *et al.*, 2010; Chapman, *et al.*, 2011). Most belong to the family Centropagidae, and of the 13 known species, two are introduced, three are considered endemic and several are shared with the Australian fauna (Chapman, *et al.*, 2011). Several species appear to show clearly defined distributions related to geography (Jamieson, 1998; Banks & Duggan, 2009). In contrast, New Zealand's freshwater cyclopoid taxa are poorly known. Although several endemic taxa have been discovered from groundwater and semi-terrestrial habitats (Harding, 1958; Karanovic, 2005), the majority of species are putatively cosmopolitan taxa originally described from the Northern Hemisphere (Chapman, *et al.*, 2011). New Zealand's freshwater Harpacticoida fauna has also received limited attention. Of the roughly 35 species

that are thought to occur in New Zealand only 19 have been formally described (Webber, *et al.*, 2010); and only one in the last 30 years (Wells, 2007). Most species belong to the predominately freshwater family Canthocamptidae, of which at least two genera are known only from New Zealand. All species are endemic, with the exception of three putatively cosmopolitan taxa and the Northern Hemisphere *Bryocamptus pygmaeus* (Lewis, 1984; Chapman, *et al.*, 2011). Unlike the Calanoida, the distribution of New Zealand's freshwater harpacticoid copepods appears to be more influenced by habitat preference than any clear biogeographic influence (Lewis, 1984). However, this may simply be due to inadequate sampling (Lewis, 1984).

1.3 Re-assessing the diversity of New Zealand's freshwater cyclopoid and harpacticoid copepods

Knowledge of the taxonomic diversity of New Zealand's freshwater cyclopoid and harpacticoid copepods is limited (Webber, *et al.*, 2010; Chapman, *et al.*, 2011). There are few trained taxonomists in New Zealand with expertise for these taxa (Webber, *et al.*, 2010), and research on the fauna has been, consequently, restricted (Lewis, 1984; Karanovic, 2005; Webber, *et al.*, 2010). A potential solution to bridge this global taxonomic impediment is to implore molecular techniques (*sensu* Hebert *et al.*, 2003) to assess the diversity of New Zealand's freshwater Cyclopoida and Harpacticoida. For crustaceans, the mitochondrial cytochrome *c* oxidase subunit one gene has been shown to be particularly useful at species level delineation (Costa *et al.*, 2007). Molecular techniques have also proven useful in the detection of morphologically conservative taxa, often revealing cryptic diversity (Hebert *et al.*, 2004; Gutiérrez-Aguirre *et al.*, 2014) and hidden biogeographic patterns (Knox *et al.*, 2012). Indeed, the application of such techniques to New Zealand's freshwater copepod fauna has been previously advocated (Webber, *et al.*, 2010; Chapman, *et al.*, 2011). Molecular techniques can also be useful in identifying non-indigenous taxa and elucidating potential invasion vectors (Makino *et al.*, 2010; Duggan *et al.*, 2012). The risk of non-indigenous freshwater copepod invasions in New Zealand has recently been highlighted by the discovery of two non-indigenous calanoid copepods, and two cladoceran in New Zealand lakes (Duggan *et al.*, 2006; Makino, *et al.*, 2010;

Duggan, *et al.*, 2012) as well as two non-indigenous harpacticoids found in freshwater aquaria (Duggan, 2010).

1.4 Thesis outline

The thesis consists of four chapters and two appendices. The first research chapter (Chapter II) is an examination of the diversity and genetic affinities of cyclopoid species collected from the North Island and South Island of New Zealand. COI sequences obtained from the New Zealand specimens were then compared with publically available freshwater cyclopoid COI sequences downloaded from GenBank and BOLD. This chapter investigated the cosmopolitan status for New Zealand's cyclopoid species and used molecular data to test the hypothesis of Karanovic (2005) that many of New Zealand's putatively cosmopolitan freshwater cyclopoid species were introduced by human-mediated translocation.

My second research chapter (Chapter III) uses the COI gene locus to examine the diversity of New Zealand's freshwater harpacticoid copepods. Harpacticoid copepods are perhaps the most diverse of New Zealand's freshwater copepod fauna, yet the most difficult to identify, and work on the fauna has been limited in the last three decades. Recently, similar research on Australia's subterranean harpacticoid copepod fauna has indicated that biodiversity there has been substantially underestimated (Karanovic & Cooper, 2012). Consequently, I tested the hypothesis that a re-evaluation of the New Zealand fauna using the COI gene locus would reveal cryptic diversity among the New Zealand harpacticoids. The final chapter (Chapter IV) provides a summary of the research findings from both research chapters and offers suggestions for future research on the fauna.

Appendix I contains a technical report that was produced for a research contract. Work undertaken during my thesis contributed to a larger project which aimed to develop a molecular-based identification tool for the routine monitoring of the New Zealand freshwater zooplankton. This senior-authored report was the product of this work.

Appendix II is a co-authored manuscript produced during my masterate tenure and contains the first record of the American calanoid copepod *Skistodiaptomas pallidus* in the South Island of New Zealand. This species was discovered in Lake Hood during sample collection for the thesis research.

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Chapter II

Evaluating the diversity and biogeography of New Zealand freshwater cyclopoids (Copepoda: Cyclopoida) using mitochondrial DNA (COI) sequences

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2.1 Abstract

We used the mitochondrial COI gene to examine the diversity of the New Zealand freshwater cyclopoid copepod fauna and to assess taxonomic affinities with the Northern Hemisphere taxa. COI sequences were obtained from 54 individuals representing eight species. A further 116 cyclopoid copepod sequences were obtained from GenBank or BOLD. The obtained COI gene sequences successfully delineated species and were congruent with known taxonomy. Intraspecific diversity amongst the New Zealand taxa was typically low (<1%), and the mean distance between species was usually above 13%. COI sequences from New Zealand *Mesocyclops* '*leuckarti*' specimens were >16% divergent to sequences of *M. leuckarti* collected from the Palearctic but <1% divergent to an unidentified cyclopoid copepod species collected from Southern Australia deposited on BOLD (likely *M. australiensis*). The New Zealand *Acanthocyclops* '*robustus*' specimens were >12% divergent from individuals of *A. robustus* collected from the type locality. We found two putative species of *Eucyclops serrulatus*, one genetically similar (<1% divergent) to Taiwanese specimens, and the other to specimens from the Ukraine and Russia. *Acanthocyclops americanus*, a species known from North America and Europe, was also recognised based on COI sequences. We suggest that both *A. americanus* and *E. serrulatus* are recent human-mediated introductions from the Northern Hemisphere. We conclude that the COI gene locus provides a useful tool for identifying New Zealand's freshwater cyclopoid taxa and will assist in the more rapid detection of non-indigenous species.

2.2 Introduction

Biogeographical studies of freshwater cyclopoid copepods have been limited by inadequate or incomplete taxonomic keys and consequently inaccurate distributional records (Kiefer, 1981; Reid, 1998; Karaytug, 1999; Miracle *et al.*, 2013). Species names for freshwater cyclopoid copepods, originally described from Northern Hemisphere specimens, have been routinely applied to species from elsewhere, resulting in many species showing an apparent cosmopolitan distribution (Reid, 1998; Dussart & Defaye, 2001). In recent years, however, the concept of species-level cosmopolitism amongst freshwater microcrustaceans has been challenged. Frey (1982; 1987) determined morphologically that the chydorid cladocerans, a group long thought to consist of only a few species with widespread distributions, was in fact much more diverse and consisted of species complexes with restricted distributions. Similar work has since followed with the freshwater cyclopoid copepods. Detailed analyses using micromorphology, cross-breeding experiments and genetic data have revealed that many taxa, once considered cosmopolitan, are also complexes of morphologically–similar, sibling species with restricted distributions (Alekseev *et al.*, 2006; Karanovic & Krajicek, 2012; Miracle, *et al.*, 2013). Consequently, putatively 'cosmopolitan' species have now been removed from the faunal lists of several countries. These include *Eucyclops serrulatus* - Australia (Morton, 1990), *Paracyclops fimbriatus* - Mexico (Gutiérrez-Aguirre *et al.*, 2003), and *Mesocyclops leuckarti* - USA (Reid, 1998). Indeed, Reid (1998) concluded that the concept of the cyclopoids as a relatively homogeneous group with widely distributed species had become outdated.

Further complicating the distribution of freshwater cyclopoid copepods has been the increase in dispersal of zooplankton via human activities (Reid, 2001; Bollens *et al.*, 2002; Havel & Shurin, 2004). Notable examples include the transfer of *Mesocyclops ogunnus* from Africa to South America (via African tilapiine fish; Reid & Pinto-Coelho, 1994) *Paracyclops bromeliacola* from South America into North America (via ornamental plants; Reid & Hribar, 2006), *Megacyclops viridis* into Great Lakes of North America (via ship ballast water; Reid & Hudson, 2008)

and the Eurasian *Thermocyclops crassus* into Mexico (unknown vector; Gutierrez-Aguirre & Suárez-Morales, 2000). Due to morphological similarities with native taxa, these species can remain undetected for years following their initial arrival (Matsumura-Tundisi & Silva, 2002). The growing number of translocated freshwater cyclopoid species led Gutiérrez-Aguirre, *et al.* (2003) to stress that future evaluations of biogeographical relationships must take into account the apparent ease with which planktonic cyclopoids can travel, as many 'cosmopolitan' taxa may have simply extended their ranges via human vectors.

Based on the global taxonomic revisions within the freshwater Cyclopoida, the status of New Zealand's fauna would also benefit from a careful re-evaluation. Chapman *et al.* (2011) listed 13 species of freshwater, surface-dwelling cyclopoid copepods in New Zealand; ten of these species are putatively cosmopolitan taxa originally described from the Palearctic. Most of these species are considered by others to be naturally distributed in the Northern Hemisphere only, such as *Mesocyclops leuckarti* (Kiefer, 1981), *Eucyclops serrulatus* (Alekseev, *et al.*, 2006), and *Paracyclops fimbriatus* (Karaytug & Boxshall, 1998) and their New Zealand status (introduced or endemic) remains questionable. Chapman, *et al.* (2011) regarded only two of the 'fully aquatic, surface dwelling' species to be endemic to New Zealand; *Metacyclops monocanthus*, which may also be shared with Australia (De Laurentiis *et al.*, 2001) and *Diacyclops crassicaudoides* which is known only from a single specimen and may indeed by a synonym for the cosmopolitan *Diacyclops bisetosus* (Morton, 1985; Karanovic, 2005; Chapman, *et al.*, 2011). Webber *et al.* (2010) adds *Paracyclops waiariki* to this list, however, as noted by Karaytug (1999) this species may be a synonym of the Australian *Paracyclops timmsi*.

Karanovic (2005) examined four New Zealand species, *Eucyclops serrulatus*, *Acanthocyclops robustus*, *Paracyclops fimbriatus* and *Diacyclops bisetosus*, and reported that he could find no morphological difference between them and European conspecific specimens. He consequently hypothesized that these species were introduced to New Zealand in casks of freshwater by early European settlers. However, this was later rejected by Chapman, *et al.* (2011), who advocated for a revision of the New Zealand taxa. More recently, (Karanovic & Krajicek, 2012)

used molecular techniques to investigate the global distribution of the putatively cosmopolitan cyclopoid *Macrocyclops albidus*. They showed that a shared 12S haplotype was found among populations from Australia, USA, and Germany, and suggested a human vector was necessary to explain such a widespread distribution (Karanovic & Krajicek, 2012).

In order to more thoroughly examine the diversity and global affinities of New Zealand's freshwater cyclopoids, we analysed sequence variation at the mitochondrial DNA cytochrome *c* oxidase subunit one (COI) gene locus and tested the hypothesis that the New Zealand taxa are genetically divergent from their global conspecifics.

2.3 Methods

2.3.1 Collection of specimens

Cyclopoid copepods were collected from a variety of freshwater habitats across the North and South Islands of New Zealand, between 2010 and 2014 (Figure 2.1). Habitats sampled included both constructed and natural lakes, small ponds (permanent and temporary), wetlands and bromeliads (semi-tropical plants that hold water). Copepods were collected with nets of varying mesh sizes (40 µm to 75 µm), pulled through the water from the shoreline, or by running a small sieve (75 µm) through the water in small ponds. A disposable plastic pipette was also used to collect water from smaller habitats, such as inside bromeliads, which was then passed through a fine mesh (40 µm) net. Samples were transferred to 250 ml wide-mouth, plastic containers and preserved with 95% ethanol. On return to the laboratory, samples were refrigerated at 4°C until needed for further processing.

Samples were initially identified under a dissecting or compound microscope at magnifications between 40 and 400 x, using Chapman, *et al.* (2011) or the more detailed keys of Miracle, *et al.* (2013) for *Acanthocyclops*, Alekseev *et al.* (2011) for *Eucyclops* and Karaytug (1999) for *Paracyclops*. Using Chapman, *et al.* (2011) identifications were based primarily on the number of antennal segments and the 5th leg (P5) of dissected females. However, the other keys utilised a variety of other morphological features. Specimens were photographed and then processed for genetic analysis.

2.3.2 Genetic analyses

Genetic analyses were carried out at both the University of Waikato and at the Canadian Centre for DNA Barcoding (CCDB) at the University of Guelph. At the University of Waikato a mixture of 10 µL of extraction solution and 2.5 µL of tissue preparation solution (Extract and Amp, Tissue PCR Kit, Signma-Aldrich, St. Louis, MO, USA) was added to 0.6 ml snap-top PCR tubes (Porex Bio Products Group, Fairburn, GA, USA) each containing an individual (typically whole body) representative of each morpho-species. The tubes were centrifuged for approximately five seconds to ensure the organism was drawn to the bottom of

the tube and covered by reagent. The tubes were then left at room temperature for three hours in the dark (to avoid exposure to UV light). After this time, tubes were incubated in an Eppendorf Thermocycler at 95˚C for three minutes to stop the reaction. Following this, 10 µL of neutralising solution was added to each tube and mixed by vortexing. DNA extracts were then kept at 4˚C until needed for PCR.

From each extraction, Polymerase Chain Reactions (PCR) were used to amplify a 710bp fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene. A master mix containing 5.5 µL of iNtRON® PCR Master Mix (iNtRON Biotechnology Inc., Korea), 0.5 µL of COI primers (LCO1490 and HCO2198 (Folmer *et al.*, 1994) or LepF1 and LepR1 (Hebert *et al.*, 2004) and 5.5 µL of deionised (Milli –Q) water per sample was then aliquoted into PCR tubes (0.2 mL) using a 200 µL pipette. One µL of sample extract was then added to each tube. Negative controls using deionised water as the template were run alongside the DNA extracts to test for any contamination. Reaction conditions varied slightly for different taxa; a typical reaction would include an initial denaturing step at 94˚C for five minutes, followed by 35 cycles of 94C for one minute, 52˚C for one minute and 30 seconds and 72˚C for one minute, with a final extension step of 72˚C for 5 minutes. For samples yielding no PCR product the annealing temperature was lowered (e.g. 49.1˚C) to facilitate primers binding to the template DNA. A 3 µL subsample from each PCR product was pipetted into comb set wells on a 2% agarose gel containing SYBR® Safe DNA Gel Stain (Life Technologies Corporation, NY, USA, 1 µL per 10 µL gel at 10000 x concentration). Gels were set in TBE buffer and run at 70 volts for 30 minutes and products visualised under UV light using a MultiImage™ light cabinet (Alpha Innotech/ProteinSimple, CA, USA).

PCR products were purified using Exo-SAP IT® (Affymetrix, USB, Cleveland, USA) to remove primers and any unincorporated dNTPs. A master mix containing 0.2 µL of ExonucleaseI (EXO), 0.1 µL of Shrimp Alkaline Phosphate (SAP) and 2.7 µL of deionised water per sample was created. Three µL of the master mix was aliquoted using a 10 µL pipette directly into the 0.2 mL PCR tubes. PCR tubes were then incubated at 37˚C for fifteen minutes to degrade any remaining

primers and nucleotides, followed by 80˚C for an additional fifteen minutes to inactivate the Exo-SAP IT® reagent. Purified PCR products were sent to the University of Waikato DNA Sequencing Facility for bidirectional sequencing on an ABI3130XL sequencer using the same primers as for amplification. All generated sequences and trace files were uploaded to the Barcode of Life Database (www.boldsystems.org), under the project NZCYC.

At the Canadian Centre for DNA Barcoding (CCDB), University of Guelph, extractions were performed using a Glassfiber Plate DNA Extractions (AcroPrep) method (Ivanova *et al.*, 2006). Polymerase Chain Reactions (PCR) were carried out in 12.5 µl volumes consisting of the following: 2 µl of DNA template, 6.25 µl 10% trehalose, 2 µl of ddH20, 1.25 µl 10× PCR buffer, 0.625 µl MgCl2 (50 mM), 0.125 µl of each forward and reverse primer (10 µM), 0.0652 µl of dNTPs (10mM) and 0.06 µl of Platinum® Taq Polymerase. Primers used were ZplankF1_t1 and ZplankR1_t1 ((Prosser *et al.*, 2013). Thermocycling conditions were: an initial denaturing step of 1 min at 94˚C, 40 cycles of 40 s at 94˚C, 40s at 52˚C and 1 min at 72˚C, and finally 5 min at 72˚C on an Eppendorf® Mastercycler® ep gradient thermocycler. PCR products were electrophoresed on Invitrogen™ pre-cast agarose gels for 6-12 min using a Mother E-BASE™ (Invitrogen™, Life Technologies Coperation, NY, USA). PCR products were cleaned up using Sephadex® G-50 (Sigma-Aldrich Milwaukee, WI, USA) and bi-directionally sequenced on an ABI3730xl DNA Analyzer (Applied Biosystems, Inc) using the sequence primer pair M13F (-21) M13R (-27) (Messing, 1983) .All sequences and trace files were uploaded to the Barcode of Life Datasystems (BOLD) database (www.boldsystems.org) under the project NZCYC.

Sequences were aligned using Geneious® version 6.1.2 or GeneiousPro® version 5.4.2 and checked for stop codons. Primer sequences were removed and sequences were verified as being derived from Cyclopoida using the GenBank BLAST algorithm or BOLD sequence identification tools. A further 116 available COI cyclopoid sequences were downloaded from either GenBank or BOLD to give a total of 170 sequences. Sequences were further trimmed to provide a 612bp (204 codons) alignment for all taxa. The homogeneity of base substitution patterns between sequences was tested using the Disparity Index test in MEGA (Kumar &

Gadagkar, 2001), which showed that many sequences likely evolved under different substitution patterns. jModel Test (v2.1.4) (Darriba *et al.*, 2012) was used to determine the appropriate model of evolution for Maximum Likelihood Tree construction using the following settings: 11 substitution schemes (88 models), including models with unequal base frequencies (+F), invariable sites (+I) and rate variation amongst sites (+G). The optimum model based on the lowest likelihood score (-1nL) value was the General Time Reversible Model (GTR) (Tavaré, 1986) with invariable sites (+I) and Gamma distributed heterogeneity +G (-1nL = 6,693.947, AIC = 14106.1469, BIC = 15698.5052). Neighbour Joining (NJ), Maximum Parsimony (MP) and Maximum likelihood (ML) trees were constructed using Molecular Evolutionary Genetic Analysis (MEGA) software for Windows version 6 (Tamura *et al.*, 2013), with 1000 Bootstrap replicates (Felsenstein, 1985). The Neighbour Joining tree was constructed using the Kimura 2-Parameter model (Kimura, 1980), while Maximum Parsimony and Maximum Likelihood trees were constructed using the GTR model (Tavaré, 1986). For each tree, 1000 bootstrap replicates were used. Average DNA sequence divergence within taxa from geographically separated locations and between different taxa were calculated using the Kimura 2-Parameter model (Kimura, 1980) in MEGA.

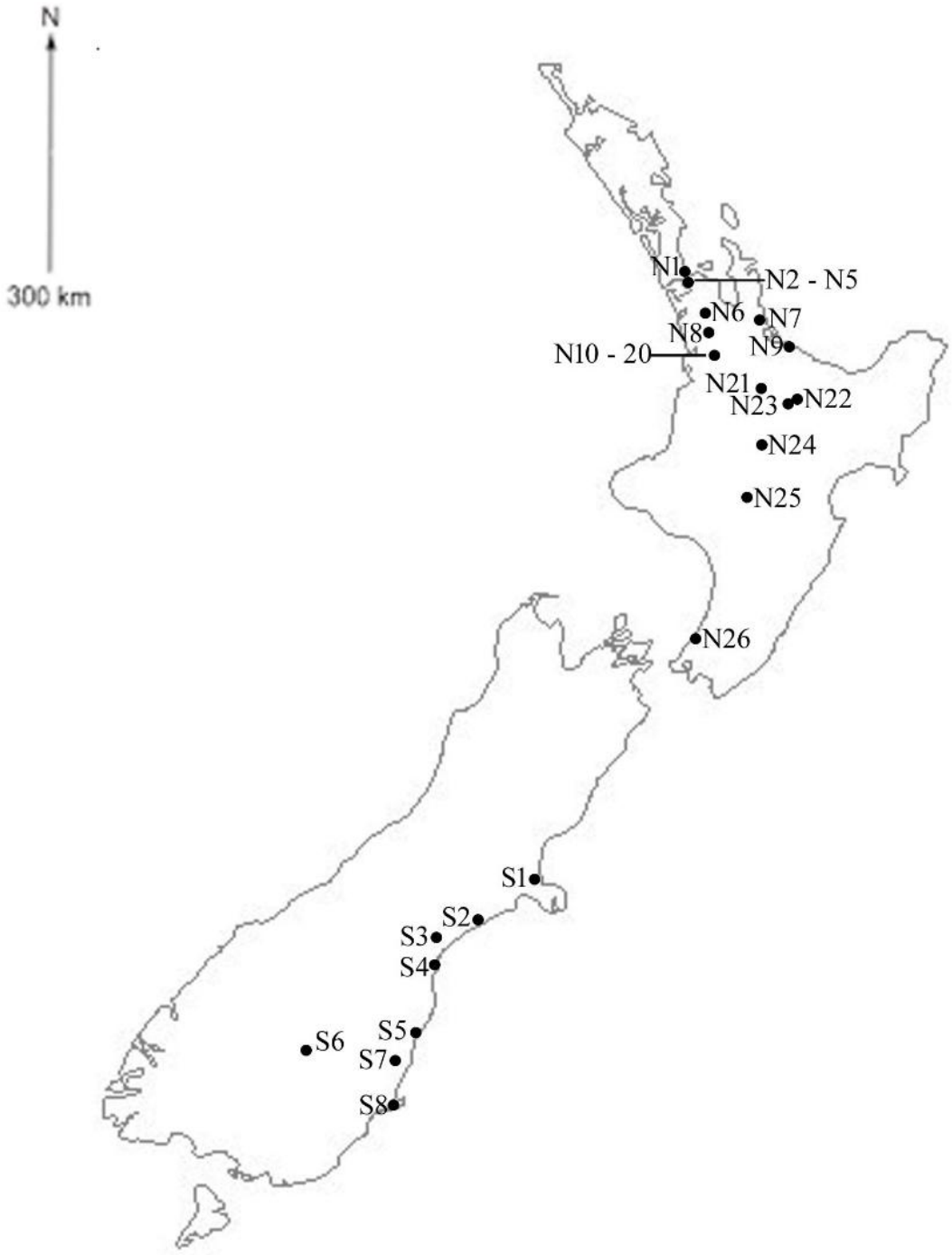

Figure 2.1 Map of New Zealand showing collection sites. Numbers refer to sample locations in Table 2.1

Table 2.1 Species found and Location Data. Key refers to sample locations as indicated on map in Figure 2.1

Key	Sample Location	Species Found	Date	Latitude	Longitude	Habitat
N1	Lake Pupuke	ML	31/05/2013	36°46'48.58"S	174°45'57.98"E	Lake
N2	Albany Pond	P1	09/01/2012	36°43'34.18"S	174°42'35.57"E	Pond
N3	Auckland Bromeliads	P1	06/08/2013	36°52'11.63"S	174°37'37.24"E	Phytotelmata
N4	Auckland Botanical Gardens	P1	18/02/2014	37° 0'42.60"S	174°54'21.59"E	Leaf litter in stream
N5	Auckland Duck Pond	E1	07/09/2013	36°51'20.16"S	174°46'26.80"E	Littoral sample
N6	Whangamarino Wetland	ML, P1, E2	12/12/2013 03/06/2010	37°22'47.75"S	175° 7'54.45"E	Wetlands
N7	Gilmour Lake	ML	17/11/2011	37°23'38.92"S	175°51'0.62"E	Lake
N8	Lake Puketirini	ML, A1	10/10/2013	37°34'12.08"S	175° 8'24.33"E	Littoral sample
N9	Tauranga	A1	08/01/2014	37°40'43.10"S	176°10'11.29"E	Constructed pond
N10	Ruakura	P1	13/08/2013	37°46'18.01"S	175°18'11.23"E	Water trough
N11	Wairere Drive Drain	A1, E1	17/08/2013	37°44'58.37"S	175°17'35.04"E	Drain
N12	Lake Rotoroa	ML	08/07/2010	37°47'46.32"S	175°16'30.00"E	Lake
N13	Jubilee Pond	ML	13/01/2011	37°46'31.88"S	175°17'28.10"E	Littoral sample
N14	Turtle Lake	A1, ML, E1	08/07/2010 01/09/2013 13/01/2011	37°48'18.00"S	175°18'14.40"E	Lake
N15	Lab Aquarium	E2	08/07/2013	37°47'22.20"S	175°19'4.80"E	Benthic gravel
N16	Kahikatea	P1	12/01/2012	37°47'18.83"S	175°19'18.69"E	Puddle
N17	Fern Garden Pond	A1	03/10/2013	37°47'18.83"S	175°19'18.69"E	Pond
N18	Lake Magellan	ML	22/07/2013	37°43'39.67"S	175°14'48.37"E	Lake
N19	Waikato University	A1, P1	11/01/2012 03/10/2013	37°47'18.52"S	175°18'54.29"E	Lakes

N20	Woodlands	ML	31/08/2013	37°40'14.90"S	175°18'22.70"E	
N21	Lake Moananui	A1, E1	16/01/2012	38°14'2.35"S	175°51'11.43"E	Lake
N22	Lake Ngahewa	ML, MA	12/02/2014	38°18'55.27"S	176°22'25.63"E	Lake
N23	Lake Rotowhero	P2	11/08/2013	38°19'20.17"S	176°22'26.77"E	Lake
N24	South Taupo Wetland	T	11/02/2011	38°58'11.86"S	175°50'29.77"E	Wetlands
N25	Lake Moawhango	P1	11/03/2014	39°23'27.84"S	175°45'13.85"E	Lake
N26	Queen Elizabeth II Park	ML	01/12/2013	40°56'56.95"S	175°40'9.99"E	Littoral sample
S1	Lake Victoria	A1, E1	28/01/2014	43°31'39.72"S	172°37'21.05"E	Lake
S2	Lake Hood	A2, E1	18/01/2014	43°58'7.43"S	171°46'13.46"E	Lake
S3	Geraldine Oakenro Pond	E1	27/01/2014	44° 4'52.33"S	171°10'28.52"E	Pond
S4	Timaru Botanic Gardens	A2	20/01/2014	44°24'36.28"S	171°15'9.73"E	Pond
S5	Oamaru Gardens	A1	28/01/2014	45° 5'54.33"S	170°57'37.86"E	Pond
S6	Alexandra Duck Pond	A1	23/01/2014	45°15'51.06"S	169°22'43.84"E	Pond
S7	Dunback Pond	E1	22/01/2014	45°22'27.50"S	170°38'10.42"E	Natural pond
S8	Dunedin Botanic Gardens	D, P1	24/01/2014	45°51'26.90"S	170°31'12.01"	Drain

Table Key	**Species**	**Table Key**	**Species**
E1	*Eucyclops* cf. *serrulatus* (Group A)	P1	*Paracyclops fimbriatus*
E2	*Eucyclops* cf. *serrulatus* (Group B)	P2	*Paracyclops waiariki*
A1	*Acanthocyclops* cf. *robustus*	T	*Tropocyclops prasinus*
A2	*Acanthocyclops americanus*	P3	*Paracyclops* sp.
ML	*Mesocyclops leuckarti*	MA	*Macrocyclops albidus*
D	*Diacyclops bicuspidatus*		

2.4 Results

Cyclopoid copepods were collected from 33 sites throughout New Zealand and eight species were identified using the key of (Chapman, *et al.*, 2011), including seven putatively cosmopolitan species and one endemic species. A full list of species and collection data are provided in Table 2.1. From these collections, we extracted DNA from 161 representative individuals and obtained COI sequences from 54; a success rate of 33.54%. Sequences covered six currently recognised species: *Eucyclops serrulatus*, *Acanthocyclops robustus*, *Mesocyclops leuckarti*, *Tropocyclops prasinus*, *Paracyclops fimbriatus*, and *Paracyclops waiariki.* Two species, *Diacyclops bicuspidatus* and *Macrocyclops albidus*, were not successfully amplified. Three additional species were also found with high COI divergences from other specimens with the same tentative identifications. These were subsequently confirmed to be morphologically different and included an undescribed species of *Paracyclops*, a variant of *Eucyclops serrulatus*, and *Acanthocyclops americanus*. For clarity, we have referred to the two putative species of *Eucyclops serrulatus* as *E.* cf. *serrulatus* (group A) and *E.* cf. *serrulatus* (group B). Using the key of Alekseev, *et al.* (2011) *Eucyclops* cf. *serrulatus* (group A) conforms closely to the description *E.* cf. *serrulatus sensu stricto* while *E*. cf. *serrulatus* (group B) did not match any described species.

Of the 612 bp used for sequence analysis, 318 codon positions were conserved and 294 were variable, of which 286 were parsimony informative. Nucleotide composition averaged across all sequences was A = 25.2%, C = 15.9%, T = 37.2% and G = 21.8%, revealing a slight AT bias. Intraspecific variation was generally low (<1%) whereas interspecific divergence was generally above 13%. Two New Zealand taxa showed high 'intraspecific' variation from their European conspecifics; *Acanthocyclops robustus* (>11% divergent) and *Mesocyclops leuckarti* (>15%). Sequence divergence between the New Zealand *Mesocyclops leuckarti*, and an unidentified cyclopoid copepod from Southern Australia was <1 %. Close relationships with international populations were found for three additional New Zealand taxa; *E*. cf. *serrulatus* (group A) with *E*. cf. *serrulatus* from the Ukraine and Russia (<1% divergent), *E*. cf. *serrulatus* (group B) with *E.* cf. *serrulatus* from Taiwan (<1% divergent), and *A. americanus* among *A. americanus* specimens from Europe and North America (<1% divergent). An

unpublished *T. prasinus* sequence from a Spanish population was more than 20% divergent from our *T.* '*prasinus*' specimen (based on a BOLD identification search). Further, the New Zealand *T.* '*prasinus*' sequence was also more than 18% divergent from published sequences of the subspecies *T. prasinus aztequei*, and *T.* cf. *aztequei* collected from Mexico (Figure 2.2).

These relationships were supported with high bootstrap values from all tree construction methods (NJ, ML and MP) which all showed the same topology. The ML tree is shown Figure 2.2 and the NJ tree in Figure 2.3 (MP tree not shown). All tree constructions separated species in accordance with their morphological identifications and showed separation of the two cyclopoid subfamilies Cyclopinae and Eucyclopinae, although these deeper relationships were not as well supported by bootstrap values.

Table 2.2. Sequence divergences of the COI gene locus within and among taxa. Divergences were calculated using Kimura 2-Parameter distances.

Species	*Acanthocyclops americanus* - Spain	*Eucyclops.* cf. *serrulatus* - Europe 1	*Eucyclops* . cf. *serrulatus* - Europe 2	*Eucyclops* . cf. *serrulatus* - Taiwan	*Tropocyclops* cf. *aztequei* - Mexico	*Mesocyclops leuckarti* - Russia	*Acanthocyclops americanus* - USA	*Acanthocyclops americanus* - France	*Acanthocyclops robustus* - Norway	*Acanthocyclops vernalis* - Russia	*Acanthocyclops americanus* - Mexico	Cyclopoida sp. - Australia	*Eucyclops* cf. *serrulatus* (Group B) - NZ	*Mesocyclops 'leuckarti'* - NZ	*Paracyclops waiariki* - NZ	*Paracyclops fimbriatus* - NZ	*Eucyclops* cf. *serrulatus* (Group A) - NZ	*Paracyclops* sp. - NZ	*Acanthocyclops robustus* - NZ	*Tropocyclops prasinus* - NZ	*Acanthocyclops americanus* - NZ	*Tropocyclops prasinus aztequei* - Mexico
Acanthocyclops americanus - Spain																						
Eucyclops. cf. *serrulatus* - Europe 1	0.297																					
Eucyclops . cf. *serrulatus* - Europe 2	0.277	0.234																				
Eucyclops . cf. *serrulatus* - Taiwan	0.229	0.220	0.186																			
Tropocyclops cf. *aztequei* - Mexico	0.277	0.296	0.246	0.224																		
Mesocyclops leuckarti - Russia	0.164	0.231	0.217	0.179	0.231																	
Acanthocyclops americanus - USA	0.001	0.300	0.278	0.231	0.278	0.167																
Acanthocyclops americanus - France	0.001	0.302	0.279	0.233	0.278	0.168	0.000															
Acanthocyclops robustus - Norway	0.161	0.276	0.302	0.216	0.248	0.159	0.163	0.164														
Acanthocyclops vernalis - Russia	0.136	0.264	0.266	0.208	0.224	0.151	0.137	0.137	0.155													
Acanthocyclops americanus - Mexico	0.008	0.299	0.277	0.234	0.273	0.169	0.008	0.008	0.163	0.138												
Cyclopoida sp. - Australia	0.202	0.290	0.300	0.223	0.270	0.108	0.205	0.206	0.197	0.192	0.202											
Eucyclops cf. *serrulatus* (Group B) - NZ	0.298	0.268	0.232	0.006	0.301	0.240	0.301	0.303	0.281	0.284	0.303	0.283										
Mesocyclops 'leuckarti' - NZ	0.208	0.284	0.302	0.226	0.271	0.109	0.211	0.212	0.197	0.194	0.208	0.006	0.286									
Paracyclops waiariki - NZ	0.255	0.270	0.285	0.229	0.267	0.252	0.256	0.256	0.285	0.251	0.264	0.276	0.293	0.274								
Paracyclops fimbriatus - NZ	0.225	0.255	0.236	0.186	0.238	0.170	0.227	0.228	0.212	0.181	0.228	0.202	0.255	0.210	0.238							
Eucyclops cf. *serrulatus* (Group A) - NZ	0.275	0.233	0.005	0.188	0.255	0.223	0.276	0.277	0.304	0.274	0.274	0.303	0.235	0.306	0.290	0.237						
Paracyclops sp. - NZ	0.223	0.258	0.262	0.200	0.269	0.207	0.225	0.224	0.252	0.198	0.223	0.239	0.265	0.244	0.241	0.151	0.266					
Acanthocyclops robustus - NZ	0.171	0.300	0.271	0.234	0.238	0.149	0.172	0.173	0.125	0.146	0.178	0.205	0.301	0.206	0.290	0.217	0.275	0.257				
Tropocyclops prasinus - NZ	0.291	0.334	0.287	0.187	0.215	0.251	0.292	0.292	0.267	0.263	0.294	0.289	0.254	0.293	0.296	0.245	0.286	0.256	0.281			
Acanthocyclops americanus - NZ	0.005	0.305	0.279	0.233	0.278	0.167	0.003	0.004	0.167	0.142	0.012	0.205	0.303	0.210	0.257	0.228	0.277	0.228	0.175	0.294		
Tropocyclops prasinus aztequei - Mexico	0.255	0.342	0.323	0.222	0.204	0.246	0.256	0.257	0.238	0.239	0.252	0.269	0.301	0.273	0.265	0.258	0.329	0.265	0.248	0.213	0.257	

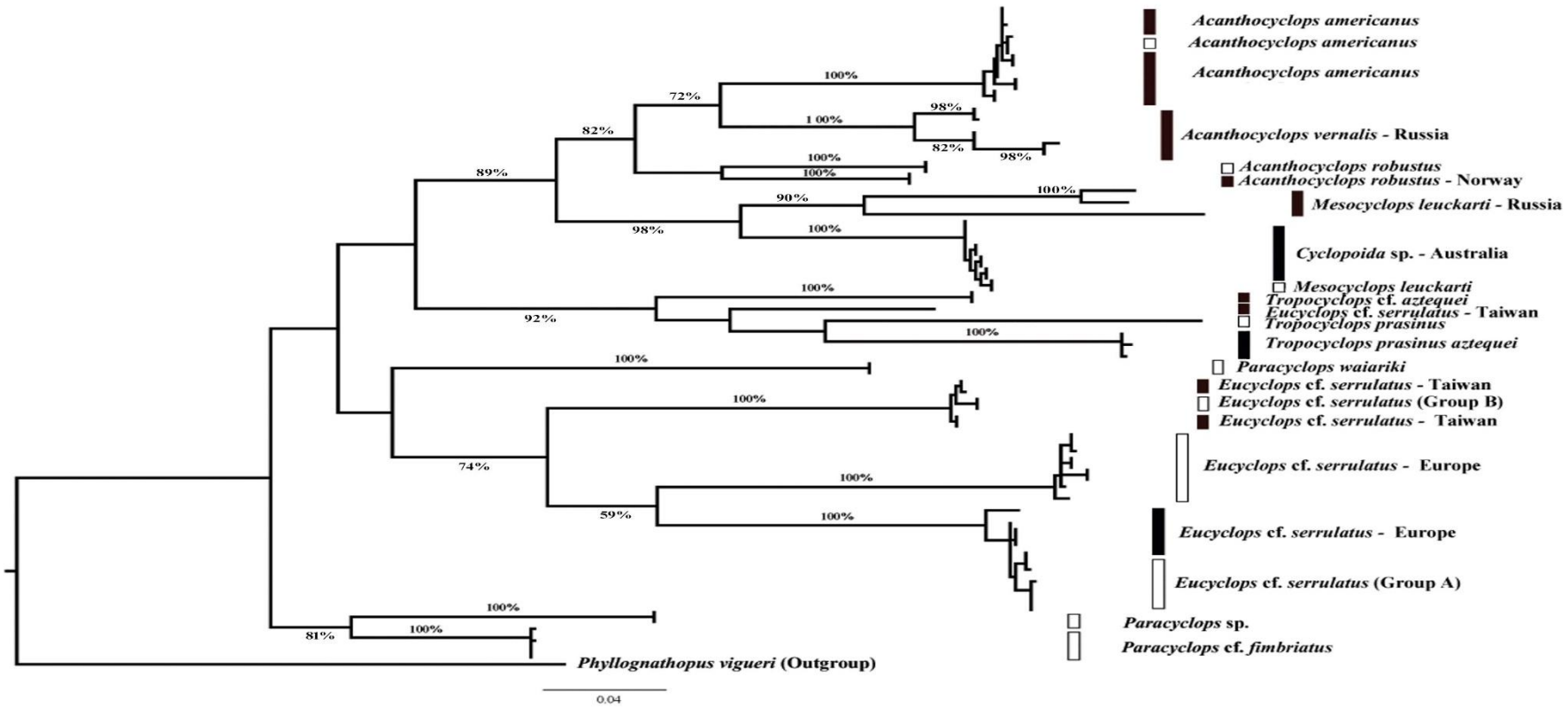

Figure 2.2. Phylogram based on a 612bp fragment of the mtCOI gene from 170 cyclopoid copepod individuals constructed using Maximum Likelihood analysis based on the GTR model. The numbers on branches indicate support derived from 1000 bootstrap replicates. Species and geographical location are indicated on the right hand side by a solid bar corresponding to their position on the tree. New Zealand species are identified by white bars, black bars represent species downloaded from BOLD or GenBank. The harpacticoid copepod *Phyllognathopus viguieri* has been used as an out group.

Figure 2.3. Phylogram based on a 612bp fragment of the mtCOI gene from 170 cyclopoid copepod individuals constructed using Neighbour joining analysis based on the Kimura 2-Parameter model. The numbers on branches indicate support derived from 1000 bootstrap replicates. Species and geographical location are indicated on the right hand side by a solid bar corresponding to their position on the tree. New Zealand species are identified by white bars, black bars represent species downloaded from BOLD or GenBank. The harpacticoid copepod *Phyllognathopus viguieri* has been used as an out group.

2.5 Discussion

Based on our analysis of COI gene sequences we found that at least one of the cosmopolitan taxa (*Eucyclops serrulatus*) has a close affinity to a European population whereas two species (*Mesocyclops leuckarti* and *Acanthocyclops robustus*) were highly genetically divergent from their European conspecifics. Specifically, *Eucyclops* cf. *serrulatus* (group A) showed a close affinity to *E*. cf. *serrulatus* populations from Ukraine and Russia whereas *Eucyclops* cf. *serrulatus* (group B) specimens showed a close affinity a population from Taiwan. Using the key of Alekseev, *et al.* (2011) our *Eucyclops* cf. *serrulatus* (group A) specimens appeared morphologically most like *Eucyclops serrulatus sensu stricto*, which Alekseev, *et al.* (2006) suggests is naturally restricted to the Palearctic and parts of Asia. Indeed, Karanovic (2005) deemed that this species was morphologically indistinguishable from European conspecifics, and due to the eastern European affinity of our specimens, an early European introduction seems plausible. A second species, *Mesocyclops* '*leuckarti*' was over 16% divergent from *M. leuckarti* specimens collected from Russia. Indeed *Mesocyclops leuckarti sensu stricto* is now thought to be restricted to the Palearctic (Kiefer, 1981) and this species is likely to have been misidentified for the New Zealand fauna. Instead, we suggest that the affinity of this species to sequences from an unidentified cyclopoid species from Southern Australia (<1% divergent) supports the hypothesis of Bayly (1995) who considered that the New Zealand species of *Mesocyclops* was in fact as *Mesocyclops australiensis*. *Mesocyclops australiensis* has previously been recorded from the Waikato River in New Zealand by Burger *et al.* (2002), who similarly considered that the New Zealand *Mesocyclops* species morphologically belonged to *M. australiensis*. It is unclear why Chapman, *et al.* (2011) did not similarly accept this designation. The Australian *Mesocyclops* are thought to have originated in South-East Asia (Wyngaard *et al.*, 2010), and to have invaded the South Pacific Islands at least twice from Australia (Hołyńska & Stoch, 2012). It therefore seems likely that *M. australiensis* has dispersed from Australia to New Zealand. Further, the apparent restriction of this species to the North Island of New Zealand, relative to its widespread distribution throughout south-eastern Australia and Tasmania (Hołyńska & Brown, 2002) supports this Australian origin. We recorded this species from two lakes; one in the Rotorua region (Lake Ngahewa), and one in the Auckland region (Lake Pupuke) where

this species has not been recorded in ecological work during the 1960s to 1970s, indicating that the geographic spread within the country may be very recent (Green, 1967; Forsyth & McColl, 1975). The dispersal of *M.* '*leuckarti*' may be related to human activities such as the transport of fishing or boating equipment (Duggan *et al.*, 2012). However, passive dispersal is also known for freshwater zooplankton (Havel & Shurin, 2004) and two species of *Mesocyclops* are thought to have been introduced to Yukon Territory Canada from the neotropics via migrating shorebirds (Reid & Reed, 1994).

Acanthocyclops robustus was more than 12% divergent from specimens of the same species from the type locality (Oslo, Norway). This species (*sensu stricto*) may not exist outside the Northern Hemisphere (Mirabdullayev & Defaye, 2002) and is likely to be misidentified among the New Zealand fauna. Indeed, Miracle, *et al.* (2013) suggested that cryptic species will be discovered among the *Acanthocyclops robustus* morphotype as more populations are screened for genetic variation. Distributional records for both *A. robustus* and *A. vernalis* in New Zealand should be re-examined, as *Acanthocyclops americanus* has previously been synonymised with both species (Kiefer, 1976; Einsle, 1996). Owing to the geographic locations of both Lake Hood and Lake Victoria, *Acanthocyclops americanus* may have been mistaken for *Acanthocyclops vernalis sensu stricto*, which has been recorded from a few localities in Otago and South Canterbury (Chapman, *et al.*, 2011). Additionally, morphological traits previously used to distinguish species of the *Acanthocyclops* genus such as the presence of a seta or a spine on the outer margin of the terminal segment of leg 4, (mentioned in Chapman, *et al.* 2011) have been considered too variable for reliable identification (Miracle, *et al.*, 2013).

Analysis of specimens from which we obtained unexpected high divergences revealed three new species unknown from New Zealand, *Paracyclops* sp. *Eucyclops* cf. *serrulatus* (group B) and *Acanthocyclops americanus*, all of which were confirmed as morphologically different from their originally presumed identifications. Owing to the habitats where these species were found, and the genetic affinity of *E.* cf. s*errulatus* (group B) and *A. americanus* with Northern Hemisphere specimens, we suggest that these species are likely to be recent

invaders. *Paracyclops* sp. was found only from one location; within bromeliads from a private garden in the Auckland region of New Zealand. As bromeliads are a subtropical plant, native to the Neotropics and West Africa (Givnish *et al.*, 2007; 2011), it is possible this species was introduced incidentally with the exotic plants. Bromeliads have been suggested as an invasion vector for the Neotropical cyclopoid species *Paracyclops bromeliacola* into North America. Indeed, Duggan *et al.* (2006) cites the botanical plant trade as a possible invasion vector for the Japanese calanoid copepod *Sinodiaptomus valkanovi*, which has been recorded in ponds in the Auckland Domain. Likewise, *Eucyclops* cf. *serrulatus* (group B) was found on only two occasions, once from a laboratory aquarium and once from a wetland. The strong affinity of this species to specimens from Taiwan suggests an Asian origin. The freshwater aquarium trade has also been identified as being a likely invasion vectors for copepods by Duggan (2010), and may have been important in the introduction of this species to New Zealand. The discovery of *Acanthocyclops americanus* in Lake Hood, a constructed lake popular with recreational boaters and fishers, is of particular interest as this species has been hypothesised to have spread from North America into Europe, Asia and Africa by human-mediated translocations (Miracle, *et al.*, 2013). Lake Hood is also home to an introduced population of the North American calanoid copepod species *Skistodiaptomas pallidus* (Duggan *et al.*, 2014). These species may have been introduced into the lake via incidental transport on fishing or boating gear.The North American cladoceran *Daphnia* '*pulex*' and the nuisance diatom Didymo (*Didymosphenia geminata*) are also thought to have been introduced to South Island, New Zealand, lakes in this manner (Kelly, 2009; Duggan, *et al.*, 2012). Similarly, the spread of the invasive cladoceran *Bythotrephes longimanus* throughout North America has been attributed primarily to human fishing activities (MacIsaac *et al.*, 2004; Panov & Caceres, 2007; Yan *et al.*, 2011). Alternatively, the stocking of domestically cultured grass carp (*Ctenopharyngodon idella*), which were introduced into Lake Hood in 2005, may have also provided a dispersal vector (Duggan, *et al.*, 2014).

We were unable to determine the genetic affinities of the other New Zealand species we found in this study; *Paracyclops fimbriatus*, *Paracyclops waiariki*, *Tropocyclops prasinus*, *Macrocyclops albidus* and *Diacyclops bicuspidatus*. For

the first three species, no comparable sequences were found on Genbank or BOLD (with the exception of one unverified *T. prasinus* record from a private collection on BOLD). For the latter two species, amplification failed and fresh samples were unobtainable. However, further investigation of these and other New Zealand species is likely to be fruitful. For example, Karanovic (2005) considered *Paracyclops fimbriatus* to be synonymous with *Paracyclops chiltoni*, a species originally described from New Zealand (Thomson, 1883). However, both are recognised as separate species by Karaytug (1999) who, after completing a global revision of the genus, suggested that Southern Hemisphere records of *Paracyclops fimbriatus* probably refer to *Paracyclops chiltoni*. Indeed, using the key of (Karaytug, 1999), our species clearly keyed out as *Paracyclops chiltoni* and the question remains to whether *P. fimbriatus* and *P. chiltoni* deserve independent species status. Further, the 'cosmopolitan' statuses of New Zealand *Macrocyclops albidus*, *Tropocyclops prasinus*, *Diacyclops bicuspidatus* and *Acanthocyclops vernalis* need revaluating as other authors have indicated that these species may be species complexes (Stoch, 2001; Lee & Chang, 2007; Bláha *et al.*, 2010; Karanovic & Krajicek, 2012).

2.5.1 Conclusion

We suggest that a substantial proportion of New Zealand's freshwater cyclopoid fauna may be non-indigenous. Indeed, in this study we found three species with strong COI affinities to Northern Hemisphere species, and one to an unidentified Australian species. Considering this and the recent discoveries of non-indigenous fauna amongst New Zealand's freshwater calanoid copepods, harpacticoid copepods, and cladocereans (Duggan, *et al.*, 2006; Duggan, 2010; Makino *et al.*, 2010; Duggan, *et al.*, 2012; Duggan, *et al.*, 2014), it appears that human-mediated translocations are increasingly homogenizing the world's freshwater microcrustacean fauna. New Zealand appears to have relatively few native freshwater copepod species (Karanovic, 2005; Chapman, *et al.*, 2011), and this may be the result of mass extinctions during the Oligocene when up to two thirds of New Zealand was submerged under sea water (Stevens, 1980; Bayly, 1995; Webber, *et al.*, 2010). The lack of native fauna may have provided an ideal

environment for any non-indigenous taxa, transported to New Zealand, to establish. However, there may be higher levels of endemism for the subterranean and semi terrestrial fauna, with some taxa showing relationships to other Gondwanan countries (Lewis, 1984; Karanovic, 2005). It is possible that the fauna capable of existing in semi-terrestrial and subterranean refugia were capable of surviving the Oligocene submergence which would have salinized much of the open waters.

In summary, we suggest that the COI gene locus is useful in elucidating global affinities for both New Zealand's putatively cosmopolitan and endemic taxa and can more easily reveal species which may be overlooked due to morphological conservatism. The presence of non-indigenous taxa in New Zealand is of concern, as invasive species can result in significant economic and ecological disruption (Pimentel *et al.*, 2005). By creating a reference DNA database of sequences from both New Zealand and elsewhere such invasive taxa may be more easily identified at an early stage, and potential invasion vectors identified and minimised.

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2.8 Appendix

Appendix 1. Primer sequences used in this study

Primer	Sequence (5' – 3')	Reference
LepF1	ATTCAACCAATCATAAAGATATTGG	Herbert *et al*. (2004)
LepR1	TAAACTTCTGGATGTCCAAAAAATCA	
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer *et al*. (1994)
LCO1490	GGTCAACAAATCATAAAGATATTGG	
ZplankF1_t1	TCTASWAATCATAARGATATTGG	Prosser *et al.* (2013)
ZplankR1_t1	TTCAGGRTGRCCRAARAATCA	
M13F(-21)	TGTAAAACGACGGCCAGT	Messing (1983)
M13R (-27)	CAGGAAACAGCTATGAC	

Appendix 2. GenBank and BOLD specimen data

GenBank Ascension Number	BOLD Number	Species	Country	Reference
KC016141	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016142	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016143	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016144	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016145	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016146	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016147	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016148	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016149	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016150	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016151	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016152	N/A	*Acanthocyclops americanus*	Washington DC, USA	Miracle *et al*. (2013)
KC016153	N/A	*Acanthocyclops americanus*	Arizona, USA	Miracle *et al*. (2013)
KC016154	N/A	*Acanthocyclops americanus*	Arizona, USA	Miracle *et al*. (2013)
KC016155	N/A	*Acanthocyclops americanus*	Arizona, USA	Miracle *et al*. (2013)
KC016156	N/A	*Acanthocyclops americanus*	Arizona, USA	Miracle *et al*. (2013)
KC016157	N/A	*Acanthocyclops americanus*	Wisconsin, USA	Miracle *et al*. (2013)
KC016158	N/A	*Acanthocyclops americanus*	Wisconsin, USA	Miracle *et al*. (2013)
KC016159	N/A	*Acanthocyclops americanus*	France	Miracle *et al*. (2013)
KC016160	N/A	*Acanthocyclops americanus*	France	Miracle *et al*. (2013)
KC016161	N/A	*Acanthocyclops americanus*	France	Miracle *et al*. (2013)

KC016162	N/A	*Acanthocyclops americanus*	France	Miracle *et al*. (2013)
KC016163	N/A	*Acanthocyclops americanus*	France	Miracle *et al*. (2013)
KC016164	N/A	*Acanthocyclops americanus*	France	Miracle *et al*. (2013)
KC016165	N/A	*Acanthocyclops americanus*	France	Miracle *et al*. (2013)
KC016166	N/A	*Acanthocyclops americanus*	France	Miracle *et al*. (2013)
KC016167	N/A	*Acanthocyclops americanus*	France	Miracle *et al*. (2013)
KC016168	N/A	*Acanthocyclops americanus*	France	Miracle *et al*. (2013)
KC016169	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016170	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016171	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016172	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016173	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016174	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016175	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016176	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016177	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016178	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016180	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC016181	N/A	*Acanthocyclops americanus*	Spain	Miracle *et al*. (2013)
KC616763	ZPII1339-11	*Acanthocyclops americanus*	Mexico	Prosser *et al*. (2013)
KC617189	ZPIII983-12	*Acanthocyclops americanus*	Mexico	Prosser *et al*. (2013)
KC617426	ZPLIV609-11	*Acanthocyclops americanus*	Mexico	Prosser *et al*. (2013)
KC617430	ZPLIV709-11	*Acanthocyclops americanus*	Mexico	Prosser *et al*. (2013)
KC617431	ZPLIV605-11	*Acanthocyclops americanus*	Mexico	Prosser *et al*. (2013)
KC617432	ZPLIV606-11	*Acanthocyclops americanus*	Mexico	Prosser *et al*. (2013)

KC016182	N/A	*Acanthocyclops robustus*	Oslo, Norway	Miracle *et al*. (2013)
KC016183	N/A	*Acanthocyclops robustus*	Oslo, Norway	Miracle *et al*. (2013)
KC016184	N/A	*Acanthocyclops robustus*	Oslo, Norway	Miracle *et al*. (2013)
KC016185	N/A	*Acanthocyclops robustus*	Oslo, Norway	Miracle *et al*. (2013)
KC016186	N/A	*Acanthocyclops robustus*	Oslo, Norway	Miracle *et al*. (2013)
KC016187	N/A	*Acanthocyclops robustus*	Oslo, Norway	Miracle *et al*. (2013)
KC016188	N/A	*Acanthocyclops vernalis*	Russia	Miracle *et al*. (2013)
KC016189	N/A	*Acanthocyclops vernalis*	Russia	Miracle *et al*. (2013)
KC016190	N/A	*Acanthocyclops vernalis*	Russia	Miracle *et al*. (2013)
KC016191	N/A	*Acanthocyclops vernalis*	Russia	Miracle *et al*. (2013)
KC016192	N/A	*Acanthocyclops vernalis*	Russia	Miracle *et al*. (2013)
N/A	MSCP178-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP179-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP181-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP182-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP188-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP192-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP193-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP194-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP195-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP196-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP199-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP200-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP202-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP203-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished

N/A	MSCP204-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP205-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP206-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP207-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP208-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP210-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP223-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP224-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP226-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
N/A	MSCP227-09	Cyclopoida sp.	South Australia	Adamowicz Unpublished
KC627279	ACSD146-11	*Eucyclops* cf. *serrulatus*	St. Petersburg, Russia	Sukhikh unpublished
KC627298	ACSD182-11	*Eucyclops* cf. *serrulatus*	Ukraine	Sukhikh unpublished
KC627299	ACSD181-11	*Eucyclops* cf. *serrulatus*	Ukraine	Sukhikh unpublished
KC627300	ACSD180-11	*Eucyclops* cf. *serrulatus*	Ukraine	Sukhikh unpublished
KC627301	ACSD179-11	*Eucyclops* cf. *serrulatus*	Ukraine	Sukhikh unpublished
KC627302	ACSD101-11	*Eucyclops* cf. *serrulatus*	St. Petersburg, Russia	Sukhikh unpublished
KC627303	ACSD102-11	*Eucyclops* cf. *serrulatus*	St. Petersburg, Russia	Sukhikh unpublished
KC627305	ACSD104-11	*Eucyclops* cf. *serrulatus*	St. Petersburg, Russia	Sukhikh unpublished
KC627306	ACSD105-11	*Eucyclops* cf. *serrulatus*	St. Petersburg, Russia	Sukhikh unpublished
KC627312	ACSD136-11	*Eucyclops* cf. *serrulatus*	Udmuritja, Russia	Sukhikh unpublished
KC627313	ACSD178-11	*Eucyclops* cf. *serrulatus*	Ukraine	Sukhikh unpublished
KC627314	ACSD177-11	*Eucyclops* cf. *serrulatus*	Ukraine	Sukhikh unpublished
KC627315	ACSD176-11	*Eucyclops* cf. *serrulatus*	Ukraine	Sukhikh unpublished
KC627316	ACSD175-11	*Eucyclops* cf. *serrulatus*	Ukraine	Sukhikh unpublished
KC627317	ACSD142-11	*Eucyclops* cf. *serrulatus*	Norway	Sukhikh unpublished

KC627318	ACSD143-11	*Eucyclops* cf. *serrulatus*	Norway	Sukhikh unpublished
KC627319	ACSD144-11	*Eucyclops* cf. *serrulatus*	Norway	Sukhikh unpublished
KC627320	ACSD145-11	*Eucyclops* cf. *serrulatus*	St. Petersburg, Russia	Sukhikh unpublished
KC627321	ACSD183-11	*Eucyclops* cf. *serrulatus*	Ukraine	Sukhikh unpublished
KC627322	ACSD174-11	*Eucyclops* cf. *serrulatus*	Ukraine	Sukhikh unpublished
KC627324	ACSD116-11	*Eucyclops* cf. *serrulatus*	Taiwan	Sukhikh unpublished
KC627325	ACSD117-11	*Eucyclops* cf. *serrulatus*	Taiwan	Sukhikh unpublished
KC627326	ACSD118-11	*Eucyclops* cf. *serrulatus*	Taiwan	Sukhikh unpublished
KC627328	ACSD124-11	*Eucyclops* cf. *serrulatus*	Taiwan	Sukhikh unpublished
KC627329	ACSD126-11	*Eucyclops* cf. *serrulatus*	Taiwan	Sukhikh unpublished
GU055748	N/A	*Mesocyclops leuckarti*	Lake Baikal, Russia	Mayor et al. (2010)
HQ336795	N/A	*Mesocyclops leuckarti*	Kazan City, Russia	Frolova et al. (2010)
KF357729	N/A	*Mesocyclops leuckarti*	Russia	Zagoskin et al. (2013)
KC617424	ZMIII668-12	*Tropocyclops* cf. *aztequei*	Mexico	Prosser *et al*. (2013)
KC617425	ZMIII763-12	*Tropocyclops* cf. *aztequei*	Mexico	Prosser *et al*. (2013)
KC617732	ZPLIV628-11	*Tropocyclops* cf. *aztequei*	Mexico	Prosser *et al*. (2013)
KC617184	ZPII1356-11	*Tropocyclops prasinus aztequei*	Mexico	Prosser *et al*. (2013)
KC617185	ZPII1361-11	*Tropocyclops prasinus aztequei*	Mexico	Prosser *et al*. (2013)
KC617733	ZPLIV457-11	*Tropocyclops prasinus aztequei*	Mexico	Prosser *et al*. (2013)

Chapter III

Assessing the diversity of New Zealand freshwater harpacticoid copepods (Crustacea) using mitochondrial DNA (COI) barcodes

To be submitted for publication under the same title as: Watson, N.T.N, Duggan, I.C., and Hogg, I.D.

3.1 Abstract

Taxonomic and ecological studies of New Zealand's freshwater harpacticoid copepods have been limited, with little progress over the past three decades. Consequently, taxonomic diversity within the group remains largely unknown. One factor limiting the study of this group is the ability to easily and accurately identify specimens. Here, we test the use of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene sequences as a tool for assessing the diversity of New Zealand's freshwater Harpacticoida. We extracted DNA from 85 specimens from the North and South Islands of New Zealand from which we obtained 30 useable sequences. Successful sequences represented two families, five genera and nine species, including the non-indigenous *Elaphoidella sewelli*. All species were delineated by the COI gene, although high intraspecific diversity was evident between individuals of *Elaphoidella bidens* (>12%), and North and South Island populations of *Bryocamptus pygmaeus* (>18%), potentially indicating the presence of a morphologically cryptic taxa. We suggest that mtDNA (COI) sequences can provide a useful tool for the routine identification of New Zealand's freshwater harpacticoid copepods. Applications of these data include assessing species diversity and biogeography as well as assisting with the detection of non-indigenous species and invasion pathways.

3.2 Introduction

Harpacticoid copepods are an integral component of standing and running water ecosystems, providing a food source for higher trophic levels such as macroinvertebrates and fish (Alheit & Scheibel, 1982; Coull, 1990; Lancaster & Robertson, 1995) as well as playing an important role in the recycling of organic nutrients (O'Doherty, 1985; Perlmutter & Meyer, 1991). Further, they are useful in aquatic toxicology studies (Bengtsson, 1978; Diz *et al.*, 2009; Ward *et al.*, 2011) and can be biogeographically informative due to a high degree of species-level endemism (Boxshall & Defaye, 2008). However, the diversity of freshwater harpacticoid copepods is probably greatly underestimated ((Reid, 2001; Webber *et al.*, 2010) and previously unknown species are regularly being discovered (Karanovic, 2010; Gaviria & Defaye, 2012; Tran & Chang, 2012; Fiers & Jocque, 2013).

Unfortunately, in New Zealand, little progress has been made in assessing the diversity of the freshwater harpacticoid fauna since the work of Harding (1958) and Lewis, (1972a; 1972b, 1984)), with the exception of one new species described by Wells (2007). Currently 19 species have been formally described (Webber, *et al.*, 2010), although Lewis (1984) suggests that at least 45 species are present. One factor limiting progress on fully assessing the species diversity of New Zealand's harpacticoid copepod fauna is the difficulty in determining species-level identifications. Due to the morphological conservatism and small size of individuals, identification typically involves the dissection and examination of all appendages (including the mouth parts) under a microscope (Chapman *et al.*, 2011). Furthermore, both male and female specimens are generally required for examination as they can differ quite markedly in size and structure (Chapman, *et al.*, 2011).

In order to more accurately assess the diversity of morphologically conservative taxa, molecular markers such as the cytochrome *c* oxidase subunit I gene (COI) have been suggested as particularly useful (Hebert *et al.*, 2003; Costa *et al.*, 2007; Knox *et al.*, 2012). Sequences from the COI gene locus have been applied to the

Australian subterranean harpacticoid copepod fauna where biodiversity was shown to be highly underestimated (Karanovic & Cooper, 2011; Karanovic & Cooper, 2012). Unfortunately, no similar molecular work has been carried out for New Zealand's freshwater harpacticoid copepods. Accordingly, we examined the diversity of New Zealand freshwater harpacticoid copepods using the COI gene locus and tested congruence of sequences with known taxonomy

3.3 Methods

3.3.1 Collection of specimens and identification

Harpacticoid copepods were collected from 15 locations from the North Island and South Island of New Zealand (Figure 1). Habitats sampled included damp leaf litter, river bank moss, aquarium sediment, bromeliads (semi-tropical plants which often hold water) and permanent and temporary water bodies (Table 1). Samples were persevered in 95% ethanol and taken back to the laboratory for analysis. Leaf litter and moss samples were rinsed in beakers of water to displace microfauna, which were then collected by pouring the water through a fine mesh (40 µm) sieve. Samples with only small amounts of organic material were poured directly through the sieve. Harpacticoid copepods were identified by dissection and examination of appendages under a compound microscope at magnifications of between 40 and 400 x, and using the key of (Chapman, *et al.*, 2011) for New Zealand freshwater harpacticoid copepods. Selected individuals from each identified species were photographed and subsequently used for genetic analysis. Any individuals that showed high intraspecific COI divergences were re-examined using international harpacticoid copepod keys, including (Lang, 1948).

3.3.2 Genetic analyses

Genetic analyses were carried out at both the University of Waikato and at the Canadian Centre for DNA Barcoding (CCDB) at the University of Guelph. At the University of Waikato a mixture of 10 µL of extraction solution and 2.5 µL of tissue preparation solution (Extract and Amp, Tissue PCR Kit, Signma-Aldrich, St. Louis, MO, USA) was added to 0.6 ml snap-top PCR tubes (Porex Bio Products Group, Fairburn, GA, USA) each containing an individual (whole body) representative of each morpho-species. The tubes were centrifuged for approximately five seconds to ensure the organism was drawn to the bottom of the tube and consequently the reagents covered the organism. The tubes were then left at room temperature for three hours in the dark (to avoid exposure to UV light). After this time, tubes were incubated in an Eppendorf Thermocycler at 95˚C for three minutes to stop the reaction. Following this, 10 µL of neutralising solution

was added to each tube and mixed by vortexing. DNA extracts were then kept at 4˚C until needed for PCR.

From each extraction, Polymerase Chain Reactions (PCR) were used to amplify a 710bp fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene. A master mix containing 5.5 µL of iNtRON® PCR Master Mix (iNtRON Biotechnology Inc., Korea), 0.5 µL of COI primers (LCO1490 GGTCAACAAATCATAAAGATATTGG and HCO2198 TAAACTTCAGGGTGACCAAAAAATCA (Folmer *et al.*, 1994) or Lep F1 ATTCAACCAATCATAAAGATATTGG and Lep R1 TAAACTTCTGGATGTCCAAAAAATCA (Hebert *et al.*, 2004)) and 5.5 µL of deionised (Milli –Q) water per sample was created and then aliquoted into PCR tubes (0.2 mL) using a 200 µL pipette. One µL of extraction solution from each sample was then added into each one of the tubes. To test for any contamination, negative controls using deionised water as the template were run alongside the DNA extracts. Reaction conditions varied slightly for different taxa. However, a typical reaction included initial denaturing at 94˚C for five minutes, followed by 35 cycles of 94˚C for one minute, 52˚C for one minute and 30 seconds and 72˚C for one minute, with a final extension step of 72˚C for 5 minutes. For problematic samples (samples where a DNA band could not be visualised after electrophoresis on an agarose gel), the annealing temperature was lowered (e.g. 49.1˚C) to facilitate primers binding to the template DNA.

A 3 µL subsample from each PCR product was pipetted into comb set wells on a 2% agarose gel containing SYBR® Safe DNA Gel Stain (Life Technologies Corporation, USA, 1 µL per 10 µL gel at 10000 x concentration). Gels were set in TBE buffer and run at 70 volts for 30 minutes. Products were visualised under UV light using a MultiImage™ light cabinet (Alpha Innotech/ProteinSimple, CA, USA)

PCR products were purified using Exo-SAP IT® (Affymetrix, USB, Cleveland, USA) to remove primers and any unincorporated dNTPs. A master mix consisted of 0.2 µL of ExonucleaseI (EXO), 0.1 µL of Shrimp Alkaline Phosphate (SAP) and 2.7 µL of deionised water. A 3 µL aliquot of the master mix was added

directly into the 0.2 mL PCR tubes. PCR tubes were then incubated at 37˚C for fifteen minutes to degrade any remaining primers and nucleotides, followed by 80˚C for an additional fifteen minutes to inactivate the Exo-SAP IT® reagent. Purified PCR products were sent to the University of Waikato DNA Sequencing Facility for bidirectional sequencing on an ABI3130XL sequencer using the same primers that were used for PCR amplification. All generated sequences and trace files were uploaded to the Barcode of Life Datasystems (BOLD) database (www.boldsystems.org).

At the Canadian Centre for DNA Barcoding (CCDB), University of Guelph, extractions were performed using a Glassfiber Plate DNA Extractions (AcroPrep) method (Ivanova *et al.*, 2006). Polymerase Chain Reactions (PCR) were carried out in 12.5 µl volumes consisting of the following: 2 µl of DNA template, 6.25 µl 10% trehalose, 2 µl of ddH20, 1.25 µl 10× PCR buffer, 0.625 µl MgCl2 (50 mM), 0.125 µl of each forward and reverse primer (10 µM), 0.0652 µl of dNTPs (10mM) and 0.06 µl of Platinum® Taq Polymerase. Primers used were ZplankF1_t1 (5'-TGTAAAACGACGGCCAGTTCTASWAATCATAARGATATTGG-3') and ZplankR1_t1 (5'-CAGGAAACAGCTATGACTTCAGGRTGRCCRAARAATCA3-') (Prosser *et al.*, 2013). Thermocycling conditions were: an initial denaturing step of 1 min at 94ºC, 40 cycles of 40 s at 94ºC, 40s at 52ºC and 1 min at 72ºC, and finally 5 min at 72ºC on an Eppendorf® Mastercycler® ep gradient thermocycler. PCR products were electrophoresed on Invitrogen™ pre-cast agarose gels for 6-12 min using a Mother E-BASE™ (Invitrogen™, Life Technologies Coperation, NY, USA). PCR products were cleaned using Sephadex® G-50 (Sigma-Aldrich Milwaukee, WI, USA) and bi-directionally sequenced on an ABI3730xl DNA Analyzer (Applied Biosystems, Inc) using the sequence primer pair M13F (-21) (GTAAAACGACGGCCAGT) and M13R (-27) (CAGGAAACAGCTATGAC) (Messing, 1983). All sequences and trace files were uploaded to the Barcode of Life Datasystems (BOLD) database (www.boldsystems.org). Sequences and trace files from all specimens used in this study can be viewed in the dataset NZHARP.

Sequences were aligned and checked for stop codons, and primer sequences removed using Geneious® version 6.1.2 or GeneiousPro® version 5.4.2

(Drummond *et al.*, 2011). Sequences were verified as being derived from Harpacticoida using the GenBank BLAST algorithm or BOLD sequence identification tools. Sequences were further trimmed to provide 623bp (207 codons) of alignment for all taxa. jModel Test (v2.1.4) (Darriba *et al.*, 2012) was used to determine the appropriate model of evolution (substitution model). The optimum model based on the lowest likelihood score (-1nL) value was the General Time Reversible Model (GTR) (Tavaré, 1986) with invariable sites (+I) and Gamma distributed heterogeneity +G (-1nL = 6,213.79, AIC = 12575.5797, BIC = 12900.8165). Using this model, Maximum Parsimony (MP) and Maximum likelihood (ML) trees were constructed using Molecular Evolutionary Genetic Analysis (MEGA) software for Windows version 6 (Tamura *et al.*, 2013) with 1000 Bootstrap replicates (Felsenstein, 1985). A Neighbour Joining tree was similarly constructed in MEGA using the Kimura 2-Parameter model (Kimura, 1980). Average DNA sequence divergence within and among taxa were calculated using uncorrected P distances in MEGA.

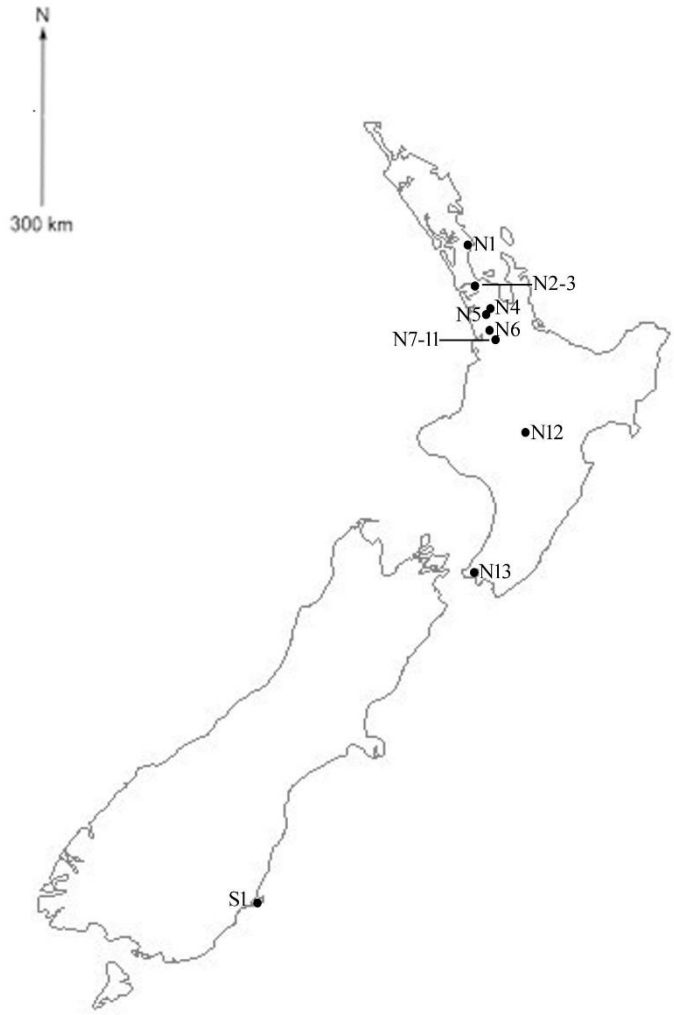

Figure 3.1. Map showing sample locations throughout New Zealand. Numbers refer to sample locations provided in Table 3.1

Table 3.1. Species collected and collection data

Species	Sample Location	Map Key	Habitat Type	Collection Date	Co-ordinates		COI sequence
					Latitude	Longitude	
***Attheyella* (*C*) *lewisae* (Wells, 2007)**	Waipakihi, Tongariro River	N12	Dripping mossy banks	12/03/2014	39° 2'12.24"S	175°48'59.65"E	Yes
***Attheyella* (*C*) *rotoruensis* (Lewis, 1972)**	Rangiriri, Waikato River	N5	River plankton Sample	05/05/2014	37°25'55.91"S	175° 8'15.15"E	Yes
***Antarctobiotus triplex* (Lewis, 1972)**	Waipakihi, Tongariro River	N12	Dripping mossy banks	12/03/2014	39° 2'12.24"S	175°48'59.65"E	Yes
Elaphoidella bidens	Auckland Botanical Gardens	N3	Leaf litter in stream	18/02/2014	37° 0'42.60"S	174°54'21.59"E	Yes
	Lake Rotoroa	N8	Littoral sample	08/07/2010	37°47'46.32"S	175°16'30.00"E	No

***Elaphoidella sewelli* (Chappuis, 1928)**	Laboratory Aquarium	N9	Bottom Gravel	08/07/2013	37°47'22.20"S	175°19'4.80"E	Yes
***Bryocamptus pygmaeus* (Sars, 1863)**	Hamilton	N10	Dripping mossy banks	31/10/2013	37°47'50.97"S	175°17'22.66"E	Yes
***Bryocamptus pygmaeus* (Sars, 1863)**	Dunedin Botanical Gardens	S1	Leaf litter in creek	24/01/2014	45°51'26.90"S	170°31'12.01"	Yes
***Bryocamptus* sp.**	Ngaruawahia	N6	Bromeliads	10/10/2013	37°40'4.25"S	175° 8'50.72"E	Yes
	Warkworth	N1	Bromeliads	04/08/2013	36°23'53.14"S	174°39'39.66"E	No
Phyllognathopus viguieri	Hamilton	N11	Leaf litter in paint tray,	02/10/2013	37°46'20.29"S	175°18'0.31"E	Yes
	Auckland	N2	Bromeliads	06/08/2013	36°52'11.63"S	174°37'37.24"E	Yes
***Phyllognathopus volcanicus* (Barclay, 1969)**	Whangamirino Wetlands	N4	Submerged macrophytes	12/12/2013 03/06/2010	37°22'47.75"S	175° 7'54.45"E	Yes
***Antipodiella chappuisi* (Brehm, 1928)**	Waipakihi, Tongariro River	N12	Dripping mossy banks	12/03/2014	39° 2'12.24"S	175°48'59.65"E	No

3.4 Results

Ten species of New Zealand harpacticoid copepod were collected and identified. Of these, *Phyllognathopus viguieri* was the most widely collected and was found in a diverse range of habitats including a paint tray with damp leaf litter, within bromeliads and from net hauls taken from a eutrophic lake. *Attheyella lewisae*, *Antarctobiotus triplex* and *Antipodella chappusi* were all recovered from a single sample of damp moss collected from the Tongariro River, while *Bryocamptus pygmaeus*, the only species found in both the North Island and South Island, was collected from dripping moss alongside the banks of the Waikato River in the North Island and from a shallow drain in the Dunedin Botanical Gardens in the South Island. An undescribed species of *Bryocamptus* was found in association with bromeliads from private gardens separated by over 100km. Collection locations are shown in Figure 3.1 and complete collection details are presented in Table 3.1.

In total, DNA was extracted from 85 individuals collected from locations throughout New Zealand, and representing 11 species from six genera and two families (Table 3.1). From these, a 623 bp fragment of the COI gene was obtained from 30 individuals, representing a success rate of 35% and covering eight of the 11 species found. Nucleotide composition averaged across all sequences was T = 37.4% C = 15.6% A = 24.5% G = 22.5%, showing an A-T bias. Of the 623 positions, 323 sites were conserved and 306 sites were variable, of which 293 were parsimony informative. All putative species were clearly delineated with the COI gene using all tree construction methods and tree topologies were similar. The Neighbouring Tree and Maximum Likelihood tree are provided in Figure 3.2, and Figure 3.3, respectively. Inter-specific COI sequence divergences ranged from 17 to 30% (Table 3.2). In contrast, intraspecific divergences were generally low (<3%) except for *Elaphoidella bidens* (>11%), which showed two distinct groups from the same sample and two geographically separated populations of *Bryocamptus pygmaeus* (>16%) (Table 3.2). *Elaphoidella bidens* specimens, and both populations of *Bryocamptus pygmaeus*, were re-examined using the international harpacticoid key of Lang (1948), which confirmed their initial

species identifications. No obvious morphological variation among individuals of either species was detected. No COI sequences from conspecific specimens were available for comparison on either GenBank or BOLD.

Five unique COI lineages were derived from specimens taken from a single moss sample in the splash zone of the Tongariro River. Only two of these lineages could be confidently attributed to morphologically-recognised species. Further morphological assessment of specimens in the sample revealed the presence of *Antipodiella chappuisi*. However, we were unable to determine whether this species was represented in the COI lineages, as examination of specimen photos were inconclusive.

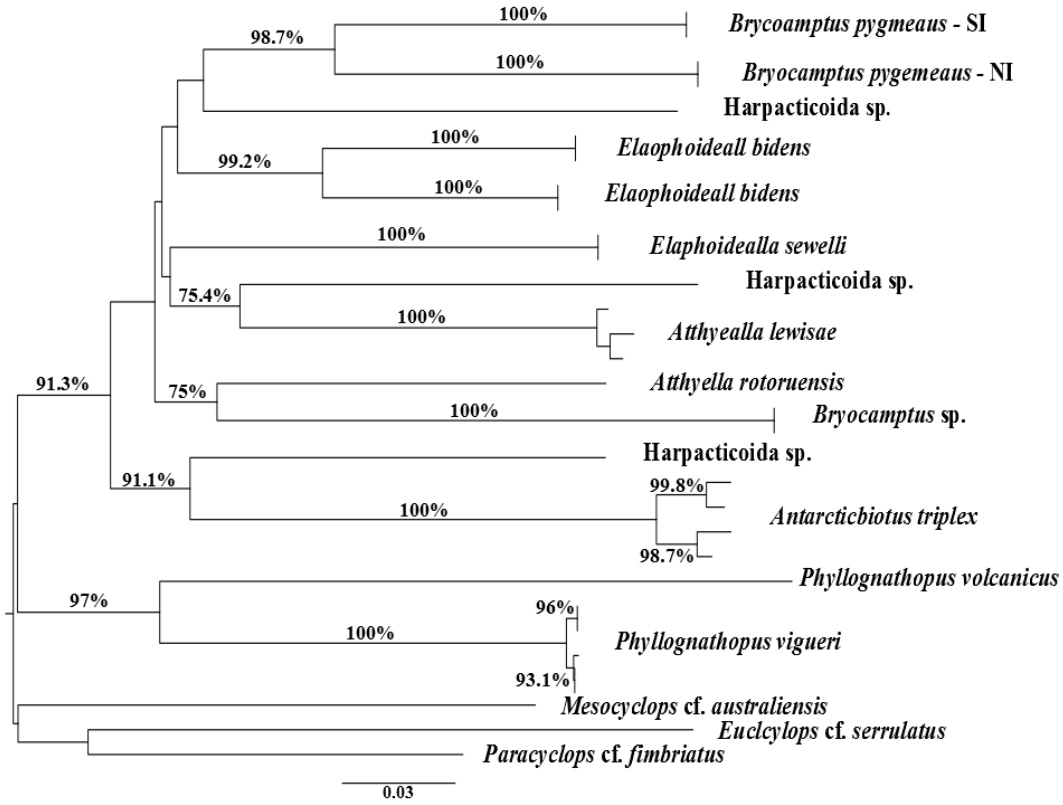

Figure 3.2. Neighbour joining tree of 30 sequences obtained from New Zealand freshwater Harpacticoida. The tree was constructed using the Kimura-2-Parameter model of evolution with 1000 bootstrap replicates. SI and NI after *Bryocamptus pygmaeus* indicates South Island and North Island populations respectively. Three species of cyclopoid were used as an outgroup.

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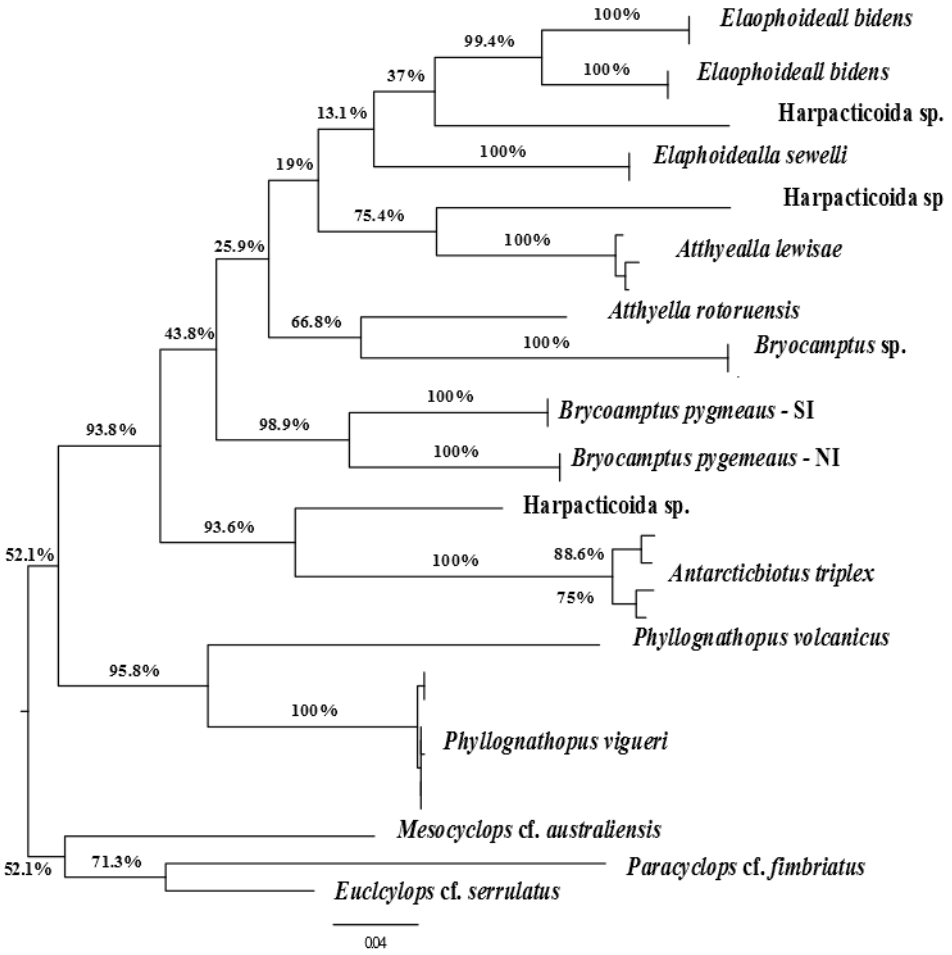

Figure 3.3. Maximum Likelihood tree of 30 sequences obtained from New Zealand freshwater Harpacticoida. The tree was constructed using the Kimura-2-Parameter model of evolution with 1000 bootstrap replicates. SI and NI after *Bryocamptus pygmaeus* indicates South Island and North Island populations respectively. Three species of cyclopoid were used as an outgroup.

Table 3.2 The divergence between a 623 bp section of the COI gene (number of base substitutions per site) between and amongst taxa calculated using uncorrected P distances. The letters in brackets (A) and (B) after Elaphoidella are used two denote two different haplotypes found for the species.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*Phyllognanthopus volcanicus*														
2	*Bryocamptus* sp.	0.302													
3	*Antarctobiotus triplex*	0.296	0.269												
4	*Harpacticoida* sp.	0.280	0.256	0.211											
5	*Bryocamptus pygmaeus* - South Island	0.298	0.240	0.243	0.211										
6	*Bryocamptus pygmaeus* - North Island	0.299	0.262	0.234	0.219	0.165									
7	*Attheyella rotoruensis*	0.293	0.213	0.250	0.218	0.200	0.213								
8	*Harpacticoida* sp.	0.278	0.254	0.268	0.234	0.222	0.226	0.216							
9	*Harpacticoida* sp.	0.298	0.245	0.250	0.234	0.210	0.218	0.222	0.227						
10	*Attheyella lewisae*	0.281	0.241	0.252	0.232	0.228	0.228	0.201	0.189	0.211					
11	*Elaphoidella sewelli*	0.274	0.229	0.233	0.218	0.222	0.224	0.205	0.206	0.219	0.206				
12	*Phyllognathopus viguieri*	0.232	0.284	0.276	0.253	0.261	0.249	0.248	0.274	0.271	0.246	0.253			
13	*Elaphoidella bidens* (A)	0.301	0.237	0.244	0.229	0.216	0.202	0.203	0.224	0.194	0.172	0.198	0.233		
14	*Elaphoidella bidens* (B)	0.282	0.219	0.240	0.224	0.211	0.214	0.195	0.214	0.192	0.195	0.176	0.242	0.117	

3.5 Discussion

Based on our analysis of COI sequences for New Zealand freshwater harpacticoid copepods, we support the diversity of New Zealand's freshwater harpacticoid copepods being underestimated (e.g. Lewis, 1984; Webber, *et al.*, 2010; Chapman, *et al.*, 2011). We recorded an undescribed species of the *Bryocamptus* genus amongst bromeliads, and found genetically divergent taxa, that could not be identified using available taxonomic keys. Such taxa can be ecologically unique from their sibling species (Hebert, *et al.*, 2004) and recognition can be important for both conservation efforts and for understanding biogeographical patterns (Bickford *et al.*, 2007; Sattler *et al.*, 2007). In New Zealand, it has generally been thought that only one species of freshwater harpacticoid is likely to be present in any one sample, and seldom more than three (Lewis, 1984). However, we discovered five genetically divergent COI lineages among specimens from a single moss sample, from which only three species corresponded to known taxonomy. We also demonstrated that the two putative species of Phyllognathopodidae known from New Zealand, *Phyllognathopus volcanicus* and *Phyllognathopus viguieri*, are likely to represent different species (*sensu* Chapman, *et al.*, 2011), and not conspecific morphotypes (*sensu* Karanovic & Ranga Reddy, 2004).

Biogeographic patterns within New Zealand's freshwater harpacticoid copepods may also be underestimated. Indeed, (Lewis, 1984) observed few obvious distribution trends within New Zealand, but acknowledged a lack of adequate sampling. We identified two populations of *Bryocamptus pygmaeus* with highly divergent COI sequences (>18%), which may indicate separate species between the North and South Islands. Such north – south differences have previously been suggested for the New Zealand freshwater calanoid copepods *Boeckella dilatata* and *Boeckella hamata* (Jamieson, 1998). Further, distinct North-South divergences have been identified using molecular methods amongst New Zealand's freshwater insects (Hogg *et al.*, 2002) and amphipods (Hogg *et al.*, 2006). However, as *B. pygmaeus* was the only species we found in both the North and South Islands of New Zealand, no definitive inferences can be made for other New Zealand harpacticoid taxa.

The majority of the New Zealand freshwater harpacticoid fauna show strong Southern Hemisphere affinities with the genera *Antarctobiotus* and *Loefflerella* and the two subgenera of *Attheyella*, *Delachauxiella* and *Chappuisiella*, apparently restricted to Australasia, South America and the Antarctic (Lewis, 1984). Furthermore, New Zealand has two endemic genera, *Antipodiella* and an undescribed genus (Lewis, 1984), and there is a notable absence of the cosmopolitan family Parastenocaridiae, known from all continents except Antarctica (Huys & Boxshall, 1991; Boxshall & Defaye, 2008). However, three putatively cosmopolitan species; *Elaphoidella bidens*, *Epactophanes richardi* and *Phyllognathopus viguieri*, and one Holarctic species *Bryocamptus pygmaeus*, are known from the New Zealand fauna (Lewis, 1984; Chapman, *et al.*, 2011). The presence of these species in New Zealand, is biogeographically interesting as the family Phyllognathopidae and the genus *Bryocamptus*, are supposedly absent from Australia (Lewis, 1984). Indeed (Lewis, 1984) notes that understanding of the global biogeographical relationships of New Zealand's freshwater harpacticoid fauna is limited by a lack of knowledge for the Australian species; of all the species known to occur in New Zealand only the putatively cosmopolitan *Elaphoidella bidens* has been found in Australia (Tang & Knott, 2009). In contrast, several species of freshwater calanoid copepod and cyclopoid copepod are shared between the two countries (Jamieson, 1998; De Laurentiis *et al.*, 2001; Chapter 2). Comparisons of COI barcodes from both the Australian and New Zealand fauna, as well as other Gondwanan countries (e.g. South America) may ultimately reveal a much closer relationship.

COI sequences may also be useful in the detection of non-indigenous harpacticoid species. Freshwater harpacticoid copepods live in a variety of semi-terrestrial habitats (Reid, 2001) and incidental transportation has led to intercontinental species incursions (Horvath *et al.*, 2001; Reid & Hudson, 2008). In New Zealand, the risk of species' introductions was recently highlighted by Duggan (2010), who found two non-indigenous harpacticoid species in New Zealand freshwater aquaria; *Nitokra pietschmanni* and *Elaphoidella sewelli*. Further, species already established in New Zealand may have been incidentally introduced by early European settlers (Karanovic, 2005). Indeed, Lewis (1984) suggests that the putatively cosmopolitan species *Elaphoidella bidens* could be a relatively recent

arrival to the country. Unfortunately, in the absence of a comprehensive taxonomic inventory, an assessment of the native or non-native status of species is challenging. COI barcodes have been used to investigate the origin of non-indigenous species and consequently can be used to highlight potential invasion vectors by identifying possible source populations (Makino *et al.*, 2010; Duggan *et al.*, 2012). Further, identification of non-indigenous species can be achieved by comparison of unknown sequences with sequences in a reference database (e.g. BOLD, GenBank) (Armstrong *et al.*, 2003; Armstrong & Ball, 2005). Unfortunately, there are currently few such reference sequences (DNA barcodes) for freshwater harpacticoids.

In conclusion, our analyses of the mitochondrial COI gene locus have proven useful for the routine identification of harpacticoid taxa and have identified of two potentially cryptic species. COI data may also be useful in revealing biogeographic patterns amongst New Zealand's fauna and can provide a powerful tool in the early detection of non-indigenous species and determining possible dispersal vectors. Currently, there are few available sequences for freshwater harpacticoids in reference databases (e.g. BOLD, GenBank). The sequences we provide here thus contribute crucial baseline data on harpacticoid copepods for a global reference library, providing a foundation for future ecological and evolutionary studies.

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Chapter IV

Thesis Conclusion

4.1 Summary of Thesis

In this thesis, I used the mitochondrial COI gene locus to examine the diversity of two orders of New Zealand's freshwater copepods; the Cyclopoida and Harpacticoida. Both of these orders have been relatively understudied in New Zealand in comparison to their sister order the Calanoida. Based on my thesis results, I suggest that the mitochondrial COI gene provides a useful tool for assessing the diversity of freshwater copepod taxa. All morphologically identified species were successfully delineated using the COI gene sequences. Furthermore, three cyclopoid species and two potentially cryptic species of harpacticoid not previously known from New Zealand were also recognised. I suggest that these, and other, taxa are likely to have been overlooked in routine sampling due to their morphological similarity with other New Zealand taxa. This was highlighted by the discovery of *Acanthocyclops americanus*, a species morphologically similar to both *Acanthocyclops robustus* and *Acanthocyclops vernalis* in Lake Hood (Chapter II), and the discovery of five unique harpacticoid COI lineages (putative species) from a single moss sample, from which only three species could be morphologically identified (Chapter III). It is important that such species are recognised, as an accurate understanding of biodiversity is vital for conservation efforts. For example, Tang and Knott (2008) discovered a new harpacticoid species in the Yanchep National Park of Western Australia. This species was found primarily in caves amongst submerged tuart root mats – a habitat in threat of destruction due to a declining water table in the region (Tang & Knott, 2008). As there are still likely many undescribed species of freshwater harpacticoid in New Zealand (Lewis, 1984; Webber *et al.*, 2010), some of these may be threatened or at risk of extinction.

The homogenization of the world's fauna via human-mediated dispersal vectors has been well documented (Bollens *et al.*, 2002; Havel & Shurin, 2004) and Chapter II provides further evidence of this for the New Zealand cyclopoid fauna. Using the COI gene locus and online reference databases (GenBank and BOLD), I examined the global affinities of several putatively cosmopolitan taxa among New Zealand's freshwater cyclopoid fauna. Four New Zealand species showed close

affinities to international populations; three to their Northern Hemisphere conspecifics, and one to an unidentified Australian species. Due to the geographical distance from the Northern Hemisphere to New Zealand, it seems likely that the presence of these species among the New Zealand fauna is the result of human-mediated translocation. Indeed, in New Zealand there have been several recent discoveries of Northern Hemisphere taxa amongst the calanoid copepod and cladoceran fauna (Duggan *et al.*, 2006; Duggan *et al.*, 2012; Duggan *et al.*, 2014) and two non-indigenous harpacticoid copepods have been found in freshwater aquaria in New Zealand (Duggan, 2010). It is also possible, as discussed in Chapter II, that some copepod fauna invaded New Zealand during the arrival of early European settlers (Karanovic, 2005), and have remained undetected since then.

Although non-native species can result in economic and ecological costs to their receiving environments (Pimentel *et al.*, 2005), the effect of these species on the New Zealand fauna is largely unknown. However, Duggan, *et al.* (2014); (Appendix II) showed that the North American calanoid copepod *Skistodiaptomas pallidus* may compete with the native calanoid *Calamoecia lucasi*. As a preventative measure, the application of molecular identification tools to biosecurity protocols may allow for such non-indigenous taxa to be identified 'at the border', thus limiting possible introductions. Further, by examining the global affinities of introduced species, a source population for the non-indigenous species can often be determined, and potential invasion vectors identified and minimised (Armstrong *et al.*, 2003; Armstrong & Ball, 2005; Makino *et al.*, 2010).

The COI gene locus can be used for assessing the biogeographic relationships of New Zealand's freshwater copepod fauna. Several genera of New Zealand's freshwater calanoid and harpacticoid copepods appear to have a strong Gondwanan affinity (Chapter I). However, such relationships are less apparent among New Zealand's freshwater cyclopoid fauna, for which most species have been considered cosmopolitan (Webber, *et al.*, 2010; Chapman *et al.*, 2011). In Chapter II, two such putatively cosmopolitan species were found to have high sequence divergences from their Palearctic 'conspecifics'. One of these species, *Mesocyclops 'leuckarti'*, showed a remarkably close relationship to an

unidentified species from Australia. Further genetic comparisons between copepod fauna from New Zealand, particularly of putatively cosmopolitan taxa, with fauna from other Southern Hemisphere countries may reveal a closer 'Gondwanan' relationship among the cyclopoid fauna, similar to that found among the Calanoida and Harpacticoida.

COI barcodes may also be useful in identifying biogeographic patterns of copepods within New Zealand. Although few biogeographic trends have been found for New Zealand's freshwater harpacticoid copepods (Lewis, 1984), I found high levels of sequence divergence between two populations of *Bryocamptus pygmaeus* between populations in the North and South Island (Chapter III). Other freshwater taxa have also shown distinct North Island - South Island divergences (Hogg *et al.*, 2002; Hogg *et al.*, 2006) and the speciation of the freshwater calanoids *Boeckella dilatata* and *Boeckella hamata* has been hypothesised to be the result of vicariance during glacial periods (Jamieson, 1998). Consequently, as other copepod taxa are more rigorously sampled, genetic variability may indicate north – south vicariance among the New Zealand copepod fauna.

4.2 The Future

Sequencing technology is advancing rapidly with operational costs dropping at a remarkable rate (Shendure & Ji, 2008). Consequently, molecular techniques such as DNA barcoding are becoming more accessible and sequence reference databases are becoming more inclusive. In this thesis, I provided the first available sequence records on the Barcode of Life Datasystems (BOLD) database for New Zealand's freshwater cyclopoid and harpacticoid copepods. These data will be useful in the future for assessing diversity, revealing non-indigenous taxa and potentially elucidating biogeographic patterns within the Copepoda. To this extent, it is imperative that reference COI sequences be obtained from more copepod species, from both New Zealand and elsewhere. Further, as Next Generation Sequencing techniques continue to be become more readily accessible it is conceivable that the identification of zooplankton using molecular techniques will become almost entirely automated. Appendix I provides an applied example of

how such a COI reference library could be used as a tool for the routine identification of zooplankton. It is hoped that this thesis will serve as a useful foundation for future molecular studies into New Zealand's freshwater copepods.

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Appendix I

A Molecular-Based Assessment Tool for Characterising New Zealand Freshwater Zooplankton Communities

2014

ERI report number 39

Client report prepared for Lake Ecosystem Restoration New Zealand

by

Watson, N.T.N., Duggan, I.C., Collins, G.E., Beet, C.R., Woods, S.M., Banks, J.C., &
Hogg, I.D.

EXECUTIVE SUMMARY

The ability to adequately assess ecosystem health is essential for informed resource management. Freshwater zooplankton respond rapidly to environmental changes in pest fish populations and nutrient loads and can therefore be used to monitor ecosystem health and provide a surrogate for lake biodiversity. The Zooplankton Molecular-Based Assessment (ZooMBA) described here is a technique for assessing zooplankton communities using short fragments of DNA sequences and a recently developed, online database of reference sequences ("DNA barcodes"). Users can collect their own zooplankton samples using standard collection techniques and either pre-process samples or send samples directly to appropriate laboratory facilities for molecular analyses. Resulting data can then be used to provide accurate species inventories, or cumulatively, can be used to compute indices of lake trophic status (e.g. rotifer Trophic Level Index).

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1.0 INTRODUCTION

Zooplankton are key components of freshwater food webs and respond quickly to environmental changes (Ferdous & Muktadir, 2009; Hanazato & Yasuno, 1989; Kirk, 1991). As such, changes in the composition of zooplankton communities can be used as an indication of ecosystem health and function, and as a surrogate for overall lake biodiversity. For example, zooplankton communities can be affected by the introduction of pest fish such as carp, perch and *Gambusia*. Such species can rapidly deplete populations of large grazing zooplankton (i.e. copepod and cladoceran crustaceans) through both predation and resource competition (Attayde & Hansson, 2001; Hurlbert et al., 1972; Jeppesen et al., 1997). Furthermore, the resuspension of sediments in the water column caused by benthic-feeding fish can interfere with the ability of filter feeders such as cladocerans to obtain phytoplankton (Kirk, 1991; Kirk & Gilbert, 1990). This can lead to a proliferation of algae in the water column.

By integrating the effect of multiple variables over time, zooplankton can provide a holistic view of the overall health of the ecosystem (Bianchi et al., 2003; Gannon & Stemberger, 1978; Lougheed & Chow-Fraser, 2002). In particular, smaller zooplankton, such as the rotifers, can have species-specific tolerances to various trophic states and therefore be used as indicators of water quality. In New Zealand, the rotifer-inferred Trophic Level Index (rotifer TLI; Duggan et al. 2001) has been used by both the Waikato and Auckland regional councils as a means of assessing water quality in North Island lakes (Auckland Regional Council, 2005; Duggan, 2007, 2008). The rotifer TLI incorporates the varying sensitivities of different rotifer species to environmental parameters as a surrogate for the water quality measurements needed to assess the New Zealand Trophic Level Index (TLI) (Burns et al., 1999).

However, the accurate identification of zooplankton to a species level using morphology alone is both difficult and time consuming. To allow for a more simplified and rapid approach for zooplankton identification, we have employed a molecular approach; the Zooplankton Molecular-Based Assessment (ZooMBA).

The ZooMBA utilizes ‘DNA barcodes’; short, standardised segments of DNA, to differentiate between animals to a species level (Hebert et al., 2003). Comparing DNA barcodes from unknown zooplankton against a reference database allows for the rapid and accurate identification of taxa.

In this report we provide details on using the molecular approach as a tool for the identification of New Zealand freshwater zooplankton species. We discuss applications of the technique for assessing species diversity, detecting invasive species and generating community-level data from environmental samples including a molecular version of the rotifer TLI.

2.0 METHODS

2.1 Building the DNA Barcode Reference Database

2.1.1 Collection of specimens

Zooplankton were collected from a variety of freshwater habitats, primarily in the North Island of New Zealand, between 2006 and 2013 (Figure 1) and from south-eastern Australia between 2006 and 2011 (Figure 2). These latter samples were added to the database to enable identification of any species that may have been introduced from Australia. Habitats sampled included both constructed and natural lakes, small ponds, wetlands, aquatic plants (bromeliads) and small temporary waters. Zooplankton were collected with nets of varying mesh sizes (40 µm to 75 µm), generally pulled through the water from the shore, or by running a small sieve (75 µm) through the water in small ponds. A turkey baster was used to collect water from difficult to reach places, such as inside bromeliads, which was also passed through a fine mesh. Samples were transferred from the sampling device to plastic honey pots or similar containers and 95% ethanol was added to preserve samples. On return to the laboratory, samples were refrigerated at 4°C until needed for further processing.

Samples were identified under a dissecting or compound microscope at magnifications between 40 and 400 x, using the keys of Shiel (1995) and Voigt and Koste (1978) for rotifers and Chapman et al. (2011) for crustaceans. The identification of calanoid copepods involved dissection of the male 5^{th} leg, which was placed on a glass slide and viewed under a compound microscope at 100 x magnification or greater, as needed. Cyclopoid copepod identification was based primarily on the 5^{th} leg of dissected females. The identification of rotifers was based on body morphology, or of trophi (tiny calcified jaw like structures) morphology following erosion of the soft tissues with sodium hypochlorite. Cladocerans were identified based on body morphology. Selected specimens were then photographed and processed for genetic analysis.

Figure 1: Sampling locations of zooplankton from New Zealand

Figure 2: Sampling locations of zooplankton from eastern Australia and Tasmania

2.1.2 Genetic analyses

A mixture of 10 µL of extraction solution and 2.5 µL of tissue preparation solution (Extract and Amp, Tissue PCR Kit, Signma-Aldrich, St. Louis, MO, USA) was added to 0.6 ml snap-top PCR tubes (Porex Bio Products Group, Fairburn, GA, USA) each containing an individual (whole body) representative of each morpho-species. The tubes were centrifuged for approximately 5 seconds to ensure the organism was drawn to the bottom of the tube and consequently the reagents covered the organism. The tubes were then left at room temperature for 3 hours in the dark (to avoid exposure to UV light). After this time, tubes were incubated in an Eppendorf Thermocycler at 95˚C for 3 minutes to stop the reaction. Following this, 10 µL of neutralising solution was added to each tube and mixed by vortexing. DNA-extracted samples were refrigerated at 4˚C.

Polymerase Chain Reactions (PCR) were used to amplify the mitochondrial cytochrome *c* oxidase subunit I (COI) gene from each extraction. A master mix containing 5.5 µL of iNtRON® PCR Master Mix (iNtRON Biotechnology Inc., Korea), 0.5 µL of COI primers (LCO1490 GGTCAACAAATCATAAAGATATTGG and HCO2198 TAAACTTCAGGGTGACCAAAAAATCA or Lep F1 ATTCAACCAATCATAAAGATATTGG and Lep R1 TAAACTTCTGGATGTCCAAAAAATCA) and 5.5 µL of deionised (Milli –Q) water per sample was created and then aliquoted into PCR tubes (0.2 mL) using a 200 µL pipette. 1 µL of extraction solution from each sample was then added into each one of the tubes. To check for contamination, negative controls using deionised water as the template were run alongside the DNA extracts. Reaction conditions varied slightly for different taxa, however, a typical reaction would include an initial denaturing step at 94˚C for five minutes, followed by 35 cycles of 94˚C for one minute, 52˚C for one minute and 30 seconds and 72˚C for one minute, with a final extension step of 72˚C for 5 minutes. For problematic samples, (i.e., samples where no visible DNA band could be seen after electrophoresis) the annealing temperature was lowered as low as 49.1°C to encourage the primers to bind to template DNA.

A 3 µL subsample from each PCR product was pipetted into comb set wells on a 2% agarose gel containing SYBR® Safe DNA Gel Stain (Life Technologies Corporation, USA, 1 µL per 10 µL gel at 10000 x concentration). Gels were set in TBE buffer and run at 70 volts for 30 minutes. Products were visualised under UV light using a MultiImage™ light cabinet (Alpha Innotech/ProteinSimple, CA, USA).

PCR products were purified using Exo-SAP IT® (Affymetrix, USB, Cleveland, USA) to remove primers and any unincorporated dNTPs. A master mix containing 0.2 µL of ExonucleaseI (EXO), 0.1 µL of Shrimp Alkaline Phosphate (SAP) and 2.7 µL of deionised water per sample was created. 3 µL of the master mix was aliquoted using a 10 µL pipette directly into the 0.2 mL PCR tubes. PCR tubes were then incubated at 37˚C for fifteen minutes to degrade any remaining primers and nucleotides, followed by 80˚C for an additional fifteen minutes to inactivate the Exo-SAP IT® reagent. Purified PCR products were sent to the University of Waikato DNA Sequencing Facility for bidirectional sequencing on an ABI3130XL sequencer using the same primers that were used for amplification. Primer sequences were identified and trimmed and each sequence was checked for stop codons using Geneious® version 6.1.2 or GeneiousPro® version 5.4.2. All generated sequences and trace files were uploaded to the Barcode of Life Database *(www.boldsystems.org)*, under the campaign WG1.7 Freshwater Biosurveillance. Barcode gap analysis was performed using the Barcode Gap Analysis algorithm on the BOLD website, using the BOLD Aligner (Amino Acid Based HMM) algorithm to align sequences.

3.0 RESULTS

3.1 The Reference Database

A total of over 480 DNA barcodes, representing 99 freshwater zooplankton species, has been added to the BOLD database. These include 50 species of rotifer, 21 species of calanoid copepod, 14 species of cladoceran, 8 species of harpacticoid copepod and 6 species of cyclopoid copepod. A complete list of the barcoded species is provided in Table 1. Analysis of all COI sequences showed that some species have high levels (>10%) of intraspecific divergence (Table 1). In contrast, the minimum interspecific divergence was 0.95%, and the mean interspecific distance between neighbouring species was 18.72% (Figure 3). However, despite the range of intra- and interspecific divergences, all taxa could be unambiguously assigned to their nominate species.

The interspecific distance between the two rotifer species *Keratella tecta* and *K. cochlearis*, represented the smallest interspecific divergence (0.95%) and the relationship between these two species is currently being examined (Collins et al., unpublished). Aside from this instance, there was >6% divergence between all other species included in the reference dataset. Consequently, there should be no ambiguity in the identification of unknown zooplankton using this database, providing the collected species are similar to those in the dataset.

Table 1: Species of New Zealand Freshwater zooplankton for which mitochondrial DNA, cytochrome c oxidase subunit I (COI), barcodes have been obtained.

Cladocera	
	Bosmina meridionalis
	Penilia avirostris
	Daphnia carinata
	Ceriodaphnia dubia
	Simocephalus vetulus
	Daphnia galeata
	Ilyocryptus sordidus
	Chydorus sp.
	Chydorus sphaericus
	Alona sp.
	Graptoleberis testudinaria
	Daphnia pulex
	Eodiaptomus lumholtzi
	Undescribed species (Duggan et al., unpublished.)
Calanoid Copepods	
	Sinodiaptomus valkanovi
	Gladioferens pectinatus
	Bockella symmetrical
	Bockella fluvialis

	Bockella triarticulalra
	Bockella hamata
	Bockella pseudochelae
	Bockella delicata
	Bockella montana
	Bockella propinqua
	Bockella tanea
	Bockella minuta
	Calamoecia lucasi
	Calaniecia ampulla
	Calamoecia tasmanica
	Skistodiaptomus pallidius
	Hemiboeckella
	Sulcanus conflictis
	Eodiaptomus lumholtzi
	Centropagidae sp.
	Calmoecia lucasi
Cyclopoid Copepods	
	Eucyclops cf. serrulatus
	Acanthacyclops robustus
	Mesocyclops cf. leukarti
	Paracyclops fimbriatus
	Paracyclops waiariki

	Tropocyclops prainsus
Hapacticoid Copepods	
	Phyllognathopus viguieri
	Phyllognathopus volcanicus
	Bryocamptus pgmeaus
	Elaphoidella bidens
	Elaphoidella sewelli
	Attheyella leisae
	Attheyella maorica
	Antarctobiotus triplex
Rotifers	
	Ascomorpha ovalis
	Ascomorpha sp.
	Asplanchna priodonta
	Asplanchna sieboldi
	Brachionus angularis
	Brachionus budapestanensis
	Brachionus calyciflorus
	Brachionus quadridentatus
	Collotheca sp.
	Collotheca cf. pelagica
	Conochilus unicornis
	Cupelopagis vorax

	Euchlanis cf. deflexa
	Euchlanis meneta
	Euchlanis pyriformis
	Filinia cf. terminalis
	Filinia longiseta
	Filinia novaezelandia
	Hexarthra intermedia
	Keratella cochlearis
	Keratella procurva
	Keratella tecta
	Keratella tropica
	Keratella valga
	Lecane bulla
	Lecane closterocerca
	Lecane decipiens
	Lecane hamata
	Lecane ludwigii
	Lecane luna
	Lecane lunaris
	Lepadella cf. ovalis
	Lepadella patella
	Lophocharis salpina
	Notommata pseudocerberus

	Platyais quadricornis
	Polyarthra dolichoptera
	Pompholyx sp.
	Rotaria neptunia
	Squatinella mutica
	Synchaeta grimpii
	Synchaeta oblonga
	Synchaeta pectinata
	Synchaeta sp.
	Trichocerca marina
	Trichocerca pusilla
	Trichocerca similis
	Trichocerca tenuior
	Trichotria tetractis
	Trichocerca sp.

Table 2: Mean intraspecific diversity and distance to Nearest Neighbour of barcoded rotifer TLI species. Where only one individual has been sequenced from a particular species, intraspecific variation is marked Not-Applicable (NA). Number of individuals sequenced is provided in parentheses following species name.

Species	Maximum Intraspecific COI Divergence (%)	Interspecific COI Divergence to Nearest Neighbour (%)
Polyarthra dolichoptera (5)	25.65	21.84
Conochilus unicornis (2)	0	40.08
Ascomorpha ovalis (2)	0	21.61
Lecane closterocerca (1)	NA	17.58
Lecane bulla (species complex) (7)	19.66	16.69
Synchaeta oblonga (7)	19.27	15.95
Asplanchna priodonta (11)	3.61	19.15
Synchaeta pectinata (14)	12.46	9.51
Collotheca sp. (3)	25.54	25.43
Trichotria tetractis (1)	NA	19.97
Trichocerca tenuior (2)	1.6	17.72
Trichocerca similis (species complex) (12)	32.12	26.92
Keratella cochlearis (species complex) (5)	16.71	0.95
Filinia novaezelandia (3)	0	24.6
Trichocerca pusilla (2)	0	19.14
Hexarthra intermedia (2)	0.16	30.82
Keratella procurva (6)	3.85	19.93
Asplanchna sieboldi (6)	0.31	17.49
Keratella tropica (6)	0.31	13.68
Brachionus quadridentatus (species complex**)** (5)	19.59	18.16
Keratella tecta (8)	0.87	0.95
Brachionus calyciflorus (species complex) (5)	10.91	15.83
Filinia longiseta (4)	0.87	42.6
Brachionus budapestanensis (1)	NA	19.2

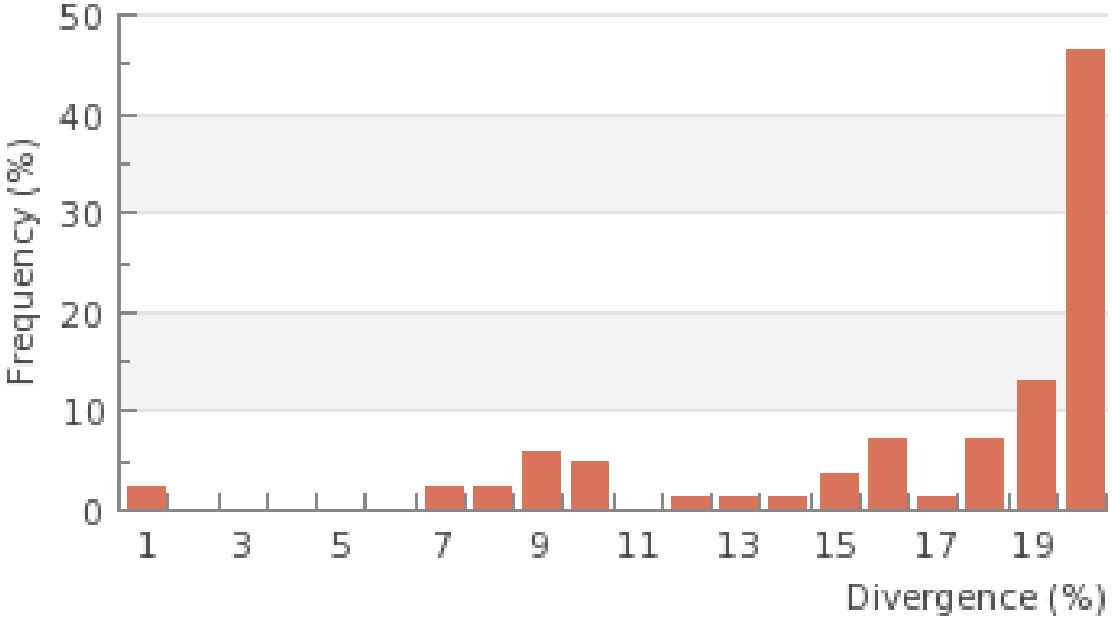

Figure 3: Genetic divergence values between "nearest neighbours" for zooplankton species used in our study

4.0 DISCUSSION

4.1 Assessing Zooplankton Communities Using DNA Barcodes (ZooMBA)

Using the DNA barcodes included in the reference library, a Zooplankton Molecular Based-Assessment (ZooMBA) can be used for the routine identification of unknown zooplankton from environmental samples. Here, individuals from habitats can be identified by comparison with the reference database and then compiled to assess community composition. The key steps involved in this process are outlined below:

4.1.1 Sample acquisition, documentation and submission

Zooplankton can be collected using existing institutional sampling methods or using standard methods such as those outlined in Chapman et al. (2011). Typically, collection involves casting a fine mesh conical net from the shore and dragging it through the water using a rope. Contents can then be transferred directly from the collection net into a plastic honey pot or similar container. Excess water should be carefully drained off, and replaced with 95% ethanol and refrigerated at 4˚C for best preservation. The use of formaldehyde or other preserving fluids (e.g. Kahles) must be avoided as this will degrade the DNA. Further it is important to keep samples out of direct sunlight as UV light degrades DNA. For shipping purposes, samples should be placed in a suitable insulated container (e.g. chilly bin) and kept cool with standard ice-packs (or similar).

Documentation required for each sample includes sampling date and location (including latitidue and longitude). Samples and documentation should be couriered to a suitable DNA Sequencing facility, such as the Pacific Barcoding Research Laboratory (University of Waikato), within 48 hours of collection.

4.1.2 Laboratory analyses

Upon arrival at the processing laboratory, samples are filtered through a sieve (40 µm mesh) to remove zooplankton. Specimens are then transferred to a petri dish filled with 95% ethanol for examination under a stereomicroscope at 4x (or higher) magnification.

Rotifers and microcrustaceans are separated and the latter are sorted into their four main orders; Cladocera, Calanoida, Cyclopoida and Harpacticoida, using simplified identification keys (e.g. Shiel 1995; Chapman et al. 2011). Rotifers are sorted together within their phylum. Additional taxa outside these five groups should be noted, although will not usually be included in the molecular analysis.

Based on previous sampling, the selection of five representatives from the crustacean groups and 20 representatives for the rotifers are likely to provide an initial assessment of diversity using genetic analyses. However, within each of the taxonomic groups it is essential to target morphologically-distinct individuals (i.e. morpho-species) to ensure that an adequate coverage of species is obtained.

4.1.3 Genetic analyses

Extraction of DNA, COI amplification, and sequencing of representative individuals is completed as per the methods used in creating the reference database and presented under the Methods section of this report. In most cases, PCR products are sequenced in a single direction only as this will usually provide sufficient information for a species designation and reduce costs. The resulting COI sequences are then searched against the reference database on BOLD using the available search engine to provide information on the identity of each specimen.

All users can obtain a personal account on BOLD by visiting the website www.boldsystems.org and following the on-screen instructions. Alternatively, there is also a public search function available which allows for the querying of sequences or taxonomic data against the reference database.

4.2 The Molecular Rotifer TLI (MoRTLI)

Of the 44 species used in the rotifer TLI (Duggan et al., 2001) 24 have been barcoded and are now included in the BOLD reference database. Additional species will be collected and can be added to the database to fill in gaps for key taxa as required. The existing species in the database represent the most common North Island, New Zealand species and cover the entire tolerance range presented by Duggan et al. (2001). A list of the currently available species and their susceptibility index scores is provided in Table 3. Using the molecular data generated using the ZooMBA, the rotifer TLI can then be calculated by matching identified rotifer species to their TLI optimum and TLI tolerance scores as per Duggan et al. (2001).

Table 3: Weighted average (WA) optima and tolerance data for TLI for abundant North Island rotifer species for which COI barcodes have been obtained. Species are ordered by TLI optima.

Species	TLI optimum	TLI tolerance
Polyarthra dolichoptera	3.44	1.36
Conochilus unicornis	3.80	1.12
Ascomorpha ovalis	3.96	0.87
Lecane closterocerca	4.14	0.60
Lecane bulla	4.17	0.74
Synchaeta oblonga	4.39	1.29
Asplanchna priodonta	4.40	1.39
Synchaeta pectinata	4.50	0.98
Collotheca sp.	4.52	1.66
Trichotria tetractis	4.69	0.16
Trichocerca tenuior	4.70	0.12
Trichocerca similis	4.77	0.90
Keratella cochlearis	4.83	1.19
Filinia novaezelandia	4.84	1.48
Trichocerca pusilla	4.86	0.79
Hexarthra intermedia	5.09	1.48
Keratella procurva	5.23	1.11

Asplanchna sieboldi	5.62	1.31
Keratella tropica	5.85	1.09
Brachionus quadridentatus	5.92	0.97
Keratella tecta	6.02	1.11
Brachionus calyciflorus	6.16	0.42
Filinia longiseta	6.40	0.72
Brachionus budapestanensis	6.53	0.45

4.3 Applications

The molecular-based identification approach for zooplankton (ZooMBA) that we describe here provides a capacity for the fast and accurate identification of specimens without the routine need for a highly-skilled taxonomic expert. For the sequences currently on the Barcode of Life Datasystems (BOLD) database, we were able to successfully differentiate among the currently recognised species on the basis of their COI sequences. The high intraspecific divergences we observed in some instances were likely due to the presence of species complexes, or morphologically 'cryptic species'. However, we caution that this could also be the result of out-dated taxonomy and/or cross-contamination of samples resulting from the amplification of non-target DNA (e.g. stomach contents). Regardless, we were able to unambiguously assign all individuals to their appropriate species designations. By applying these data to unknown communities the molecular-based assessment (ZooMBA) can provide accurate assessments of species' composition. We anticipate the reduced cost of zooplankton community characterisation coupled with a streamlined and easy-to-use, standardised method will make the molecular-based approach a useful tool for routine water quality monitoring required by regulatory bodies. Further uses for a molecular-based assessment include the accurate assessment of population and species-level diversity as well as biosecurity applications such as the detection of non-indigenous or invasive species.

4.3.1 Assessing variability within and among species

Molecular approaches can assist in the rapid identification of cryptic or "new" species that may be missed by traditional, morphological approaches due to morphological conservatism. Such species can be revealed by the subtle differences in DNA sequences at the COI gene locus (Hebert et al., 2004; Gutiérrez-Aguirre et al., 2014). Three potential cryptic species of freshwater zooplankton have already been identified in the assembly of our DNA barcode reference library. One of these species is currently undergoing formal description as a new species (I.C. Duggan et al., unpublished), while the remaining two await a more detailed examination. The recognition of cryptic species can be important

from both a conservation perspective as well as the accurate interpretation of community-based changes, as cryptic species are likely to respond differently to similar environmental stressors (Hogg et al., 1998; Rocha-Olivares et al., 2004; Feckler et al., 2014).

The gap between intraspecific and interspecific variation of the COI gene (Hebert et al., 2003) – referred to as the 'barcoding gap' – can be used as a proxy for species diversity when taxonomic data are unavailable or limited. Such closely related sequences, or Molecular Operational Taxonomic Units (MOTUs), can be identified on BOLD by Barcode Index Numbers (BINS) which are assigned to clusters of closely related sequences (Ratnasingham & Hebert, 2013). Knox et al. (2012) used MOTUs derived from COI sequences to act as a surrogate for species diversity in the deep sea amphipods of New Zealand – a taxonomically understudied group. By combining these data with biogeographic information, inferences could be made about the relationship between amphipod diversity and habitat heterogeneity. As a barcoding gap appears to be present between species of New Zealand freshwater zooplankton, a similar approach could be used for analysis of COI gene sequences from freshwater zooplankton communities when species present are undescribed or have not yet been added to the BOLD database.

Molecular data can also be useful in assessing intraspecific diversity, as individuals from geographically distinct populations will often have subtle differences in COI sequences (haplotypes), typically the result of divergent evolution. Analysis of such haplotypes can reveal information about gene flow – or lack thereof – between populations. Understanding patterns of gene flow and intraspecific diversity can provide vital information for conservation biologists (Arif & Khan, 2009; Hardy et al., 2011; Ludwig et al., 2003).

4.3.2 Biosecurity

Molecular-based identification will provide a valuable tool for assessing biosecurity threats in New Zealand. The advantages of using DNA barcoding within the New Zealand context have already been highlighted by Armstrong & Ball (2005) who conducted two case studies; one on exotic species of tussock

moth, the other on a fruit fly intercepted at a New Zealand border security checkpoint. In these cases, DNA barcoding allowed previously unknown specimens to be identified to likely genus and species level; important information as invasion risk can vary markedly between closely related species (Armstrong & Ball, 2005). Additionally, larvae of fruit flies could be identified using molecular data, something very difficult to do morphologically (Armstrong & Ball, 2005). In this manner, comparison of DNA barcodes from the BOLD database could potentially aid in the identification of unknown zooplankton specimens stopped at the border (e.g. aquarium fish trade).

Analysis of DNA barcodes from introduced species can also reveal vital information about the country of origin of the species and potential invasion vectors. Recently Makino et al. (2010) traced the origin of the recent invader, *Sinodiaptomus valkonovi*, a calanoid copepod back to the north-eastern region of Japan using haplotype networking of COI gene sequences. Similarly, Duggan et al. (2012) traced the exotic cladoceran *Daphnia pulex* back to North America. Such information is invaluable in assessing the risk of specific invasion vectors, and consequently focusing preventative efforts on those pathways which pose the most risk.

4.4 The Future

There are several species of New Zealand zooplankton yet to be barcoded, particularly for freshwater rotifers. However, the reference database can be continually updated as new specimens are obtained. When species are analysed that are not currently in the BOLD database an exact species-level identification will not be possible, although comparison against international records will likely give a match to the higher taxonomic level possible, such as order. For any currently undescribed or cryptic species, a Barcode Index Number (BIN) will be assigned by BOLD to allow for similar, unidentified sequences to be grouped together as a Molecular Operational Taxonomic Unit (MOTU).

The molecular rotifer TLI (MoRTLI) presented in this report contains 24 of the 44 species included in the rotifer TLI. However, these species cover the entire susceptibility range presented by Duggan et al. (2001) and can, therefore, be used

in assessing the trophic state of North Island Lakes. We anticipate that ongoing sampling will further enhance the reference database.

We expect the capabilities of the ZooMBA to grow over time with technological advancements. Sequencing technology is advancing rapidly, with sequencing costs dropping at an unprecedented rate (Shendure & Ji, 2008). Consequently, the cost of using a molecular-based approach such as ZooMBA is likely to decrease over time. The ZooMBA is currently focused primarily on describing the species diversity of zooplankton communities. However, future developments are also likely to allow for the quantification of species within such communities. Techniques such as quantitative PCR (qPCR) have proved useful in the estimation of koi carp (*Cyprinus carpio)* biomass (Takahara et al., 2012) and amphibian population abundance (Lodge et al., 2012) in aquatic ecosystems. Accordingly, qPCR-based biomass quantification could be applied to the COI sequences of freshwater zooplankton and subsequently allow for the molecular quantification of abundant species.

Finally, the application of Next Generation Sequencing (NGS) platforms to environmental samples has the potential to revolutionise the efficiency of molecular-based approaches. NGS platforms, such as the Illumina MiSec 2000 and the Ion Torrent (Life Technologies), allow for the metabarcoding of DNA directly from environmental samples (Baird & Hajibabaei, 2012; Metzker, 2010; Quail et al., 2012). It is therefore possible that an entire freshwater zooplankton community could be characterised directly from an environmental sample. NGS techniques have already been applied to marine zooplankton community samples with some success (Lindeque et al., 2013; Machida et al., 2009). By integrating NGS techniques into our molecular approach, the process of characterising freshwater zooplankton communities could become more automated. In this case, zooplankton samples could simply be collected, stored in ethanol as a bulk sample, and then sent to a sequencing lab for NGS sequencing. The resulting sequences could then be compared against the BOLD reference database to gain species level identification. Consequently, once a complete reference database is created there would be much less need for morphological identification of samples. The potential of applying NGS approaches for the New Zealand zooplankton is

currently being investigated at the University of Waikato as part of a large-scale pest fish study at the Hamilton Zoo (Woods et al. unpublished. data).

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Appendix II

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Research Article

Skistodiaptomus pallidus (Copepoda: Diaptomidae) establishment in New Zealand natural lakes, and its effects on zooplankton community composition

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Abstract

The North American calanoid copepod *Skistodiaptomus pallidus* is an emerging invader globally, with non-indigenous populations recorded from constructed waters in New Zealand, Germany and Mexico since 2000. We examined the effects of *S. pallidus* establishment on the zooplankton community of a natural lake, Lake Kereta, where it was first recorded in late-2008, coincident with releases of domestically cultured grass carp (*Ctenopharyngodon idella*). Although not present in any of our samples prior to August 2008, *S. pallidus* was found in all samples collected in the subsequent five years. ANOSIM indicated zooplankton community composition significantly differed between samples collected before and after *S. pallidus* invasion, whether the invader was included in the analysis or not. Zooplankton species affected most greatly were the copepods *Calamoecia lucasi* and *Mesocyclops* sp., which decreased in their relative importance, and the cladocerans *Bosmina meridionalis* and *Daphnia galeata*, which increased. Rotifer species were relatively unaffected. As the length of grass carp released were >6.5 cm, direct predatory effects by this species on the zooplankton community are unlikely. Associated reductions in macrophyte biomass could explain increases in the relative abundances of planktonic cladocerans (*B. meridionalis* and *D. galeata*). However, the effect of macrophyte reduction by grass carp on zooplankton communities is considered to be limited elsewhere, while the reduced macrophyte biomass cannot explain the decrease in relative abundance of the native planktonic calanoid copepod *C. lucasi*. Competition between *C. lucasi* and *S. pallidus* is the most compelling explanation for the reduction in importance of the native calanoid copepod species. *Skistodiaptomus pallidus* appears to have undergone a "boom-and-bust" cycle in Lake Kereta, increasing in relative abundance in the first three years following establishment, before declining in importance.

Key words: boom-and-bust, *Ctenopharyngodon idella*, exotic species, calanoid copepods, constructed waters

Introduction

A common pattern observed in aquatic invasions is the relative ease of establishment in constructed waters (e.g., retired quarry and mine pits, dams constructed for water supply or electricity generation, and ornamental ponds) compared with natural waters. New water bodies have increased the number, area and spatial distribution of lakes and ponds in many areas globally, and have seemingly aided in the establishment and spread of non-indigenous species (Havel et al. 2005; Johnson et al. 2008; Banks and Duggan 2009; Parkes and Duggan 2012). The African cladoceran *Daphnia lumholtzi* G.O. Sars, 1885, for example, spread through the United States after first establishing in a Texan reservoir, primarily with dams at the invasion front (Havel et al. 2005). Johnson et al. (2008), similarly, found five other widespread, high profile, invaders in Wisconsin and Michigan, USA (Eurasian watermilfoil, zebra mussel, rusty crayfish, spiny waterflea and rainbow smelt) to have higher rates of occurrence in dams than natural waters. New Zealand has similar examples, with four calanoid copepod species recorded exclusively in constructed water bodies (Banks and Duggan 2009). Parkes and Duggan (2012) found zooplankton community composition to vary between natural and constructed waters in New Zealand; natural waters housed species

better adapted to pelagic conditions, with composition largely governed by trophic state, while constructed waters had more varied assemblages and composition related to opportunity for colonisation (i.e., closeness to other lakes and number of water bodies nearby were an important determinant). Reduced biotic resistance from poorly adapted species in newer water bodies may allow for easier establishment of non-indigenous species. Once established in constructed waters, however, these water bodies can provide sites of high propagule supply to other water bodies, allowing greater opportunities for invasion of natural waters through large release sizes and frequencies. As such, species can commonly spread to natural water bodies following invasion of constructed water bodies. For example, *D. lumholtzi* spread from dams to natural waters in North America, while the calanoid copepod *Boeckella triarticulata* (Thomson, 1883) first invaded constructed ponds in northern Italy before spreading to other Italian constructed and natural waters (Ferrari 1991; Ferrari and Rossetti 2006; Alfonso and Belmonte 2008).

Here we report on the invasion of the North American diaptomid copepod *Skistodiaptomus pallidus* (Herrick, 1879) into two natural North Island, New Zealand, lakes, a species previously only recorded there from constructed waters. We also provide the first record for this species in the South Island, from a constructed lake. All of the new distribution records are in lakes where domestically produced grass carp (*Ctenopharyngodon idella* (Valenciennes, 1844)) have been introduced for aquatic macrophyte control. *Skistodiaptomus pallidus* is an invader of emerging importance globally, having expanded its geographical distribution in North America (Byron and Saunders 1981), and recently establishing populations in constructed waters in Germany and Mexico (Brandorff 2011; Suárez-Morales and Arroyo-Bustos 2012). In New Zealand, *S. pallidus* was first recorded in 2000 in ponds at the Auckland Regional Botanic Gardens, but may have been present for some time prior to this (Duggan et al. 2006). Banks and Duggan (2009) expanded these records, finding the species in other New Zealand localities (again, all constructed). As there are few studies documenting the effects of non-indigenous freshwater zooplankton globally, including *S. pallidus,* we examined the consequences of establishment of this species over a six year period following invasion on the zooplankton community in one natural lake, Lake Kereta.

Methods

Skistodiaptomus pallidus was recorded from a series of samples from Lake Kereta, North Island (36°35'34.66"S, 174°16'50.26"E) from late 2008, from a one-off sample from Lake Omapere, North Island (35°20'40.58"S, 173°47'21.76"E) from mid-June 2012, and a one-off sample from Lake Hood, South Island (43°58'00.3"S, 171°46'16.4"E) on 18 January 2014. Lake Kereta is a eutrophic lake, with a maximum depth of 1.5 m. Zooplankton samples were collected from Lake Kereta briefly in 1997, and then quarterly from September 2001, using vertical hauls from a central lake station with a 40 µm plankton net. Samples were preserved in ethanol (>50% final concentration). While samples have been lost in the intervening period, 13 samples from Lake Kereta were available prior to the first record of *S. pallidus* on 13 August 2008, with 20 samples available to 20 May 2013. Where possible, samples were enumerated in aliquots until at least 300 zooplankton individuals were counted. Because net hauls provide only semi-quantitative results, we calculated the relative abundances of zooplankton in each sample for all analyses. Non-metric multi-dimensional scaling (MDS) and analysis of similarities (ANOSIM) were used to infer whether changes have occurred in the Lake Kereta zooplankton community composition before and after the invasion of *S. pallidus* (Primer v.6.1.13; Primer-E Ltd 2009). MDS is a multivariate ordination technique that builds a 2-D 'map' of samples based on their similarity to each other as defined by a distance metric; a stress value measures the goodness of fit of the map, showing how well correlated the distances on the map are to those of the underlying similarity matrix. We performed MDS on the Bray-Curtis similarity matrix. Zooplankton data were log(x+1) transformed to down weigh the influence of dominant species in the analyses. Only zooplankton species found in two or more samples were included in our analyses, to reduce the influence of rare species potentially sampled by chance. ANOSIM was applied to the similarity matrix to test whether differences in zooplankton community composition between samples collected before and after the first detection of *S. pallidus* were statistically significant. For all tests, 999 permutations were executed. SIMPER analysis was used to explore the contribution of individual species to the average dissimilarity of samples collected before and after

Table 1. Zooplankton species observed in Lake Kereta during the study period.

Rotifera	
Ascomorpha ovalis (Bergendal, 1892)	*Keratella tropica* (Apstein, 1907)
Ascomorphella volvocicola (Plate, 1886)	*Lecane bulla* (Gosse, 1851)
Asplanchna brightwelli Gosse, 1850	*Lecane closterocerca* (Schmarda, 1859)
Asplanchna priodonta Gosse, 1850	*Lecane flexilis* (Gosse, 1886)
Bdelloids	*Lecane luna* (Müller, 1776)
Brachionus caudatus Barrois & Daday, 1894	*Lecane lunaris* (Ehrenberg, 1832)
Brachionus quadridentatus Hermann, 1783	*Lepadella acuminata* (Ehrenberg, 1834)
Cephalodella gibba (Ehrenberg, 1832)	*Macrochaetus collinsi* (Gosse, 1867)
Collotheca sp.	*Mytilina ventralis* (Ehrenberg, 1832)
Cupelopagis vorax (Leidy, 1857)	*Polyarthra dolichoptera* Idelson, 1925
Dicranophorus epicharis Harring & Myers, 1928	*Pompholyx complanata* Gosse, 1851
Dicranophorus grandis (Ehrenberg, 1832)	*Proales* cf. *alba* (Wulfert, 1938)
Euchlanis calpidia (Myers, 1930)	*Synchaeta oblonga* Ehrenberg, 1832
Euchlanis dilatata Ehrenberg, 1832	*Synchaeta pectinata* Ehrenberg, 1832
Euchlanis meneta Myers, 1930	*Testudinella mucronata* (Gosse, 1886)
Filinia longiseta (Ehrenberg, 1834)	*Testudinella patina* (Hermann, 1783)
Filinia terminalis (Plate, 1886)	*Trichocerca brachyura* (Gosse, 1851)
Hexarthra intermedia (Wiszniewski, 1929)	*Trichocerca pusilla* (Jennings, 1903)
Keratella cochlearis (Gosse, 1851)	*Trichocerca similis* (Wierzejski, 1893)
Keratella procurva Thorpe, 1891	*Trichocerca tenuior* (Gosse, 1886)
Keratella tecta (Gosse, 1851)	
Cladocera	
Alona sp.	*Daphnia galeata* G.O. Sars, 1864
Bosmina meridionalis G.O. Sars, 1904	*Ilyocryptus sordidus* (Liévin, 1894)
Ceriodaphnia dubia Richard, 1894	*Simocephalus vetulus* (Müller, 1776)
Chydorus sp.	*Graptoleberis testudinaria* (Fischer, 1851)
Copepoda	
Calamoecia lucasi Brady, 1906	*Mesocyclops* sp.
Eucyclops serrulatus (Fischer, 1851)	*Skistodiaptomus pallidus* (Herrick, 1879)
Macrocyclops albidus (Jurine, 1820)	
Ostracods	

invasion of *S. pallidus*. Analyses were undertaken using *S. pallidus* included (a combined total of copepodites and adults), to determine whether the presence of this species significantly influenced community composition. The analyses were repeated with *S. pallidus* excluded, to infer whether the invasion of this species has altered the community composition of other zooplankton present. Copepod nauplii were excluded from all analyses as these could not be confidently ascribed to species.

Results

The last samples collected without *S. pallidus* in Lake Kereta were on 2 April 2008, with its first detection on 13 August 2008. *Skistodiaptomus pallidus* was present in all samples collected following this date. Fifty-five taxa were observed in the lake during the study period, comprising 41 rotifers, 8 cladocerans and 5 copepod species (Table 1). Prior to the invasion of *S. pallidus*, the native calanoid copepod *Calamoecia lucasi* Brady, 1906 was typically present, and occasionally dominated the community (e.g., March 2003, January 2005; Figure 1). At various times, rotifers, cyclopoid copepods or cladocerans dominated the community. Following invasion, *S. pallidus* generally increased in relative abundance through time, commonly dominating the community (>25% of numbers), and particularly so in August 2010 (94.1%) and June 2011 (91.8% of individuals counted). Following this time, the relative abundance of *S. pallidus* decreased (always <25% of total numbers over the last 13 months). Excluding *S. pallidus*, cladocerans and rotifers (as a group) became relatively more important post-invasion, and other copepods (including *C. lucasi*) decreased in their relative importance, compared to before the invasion.

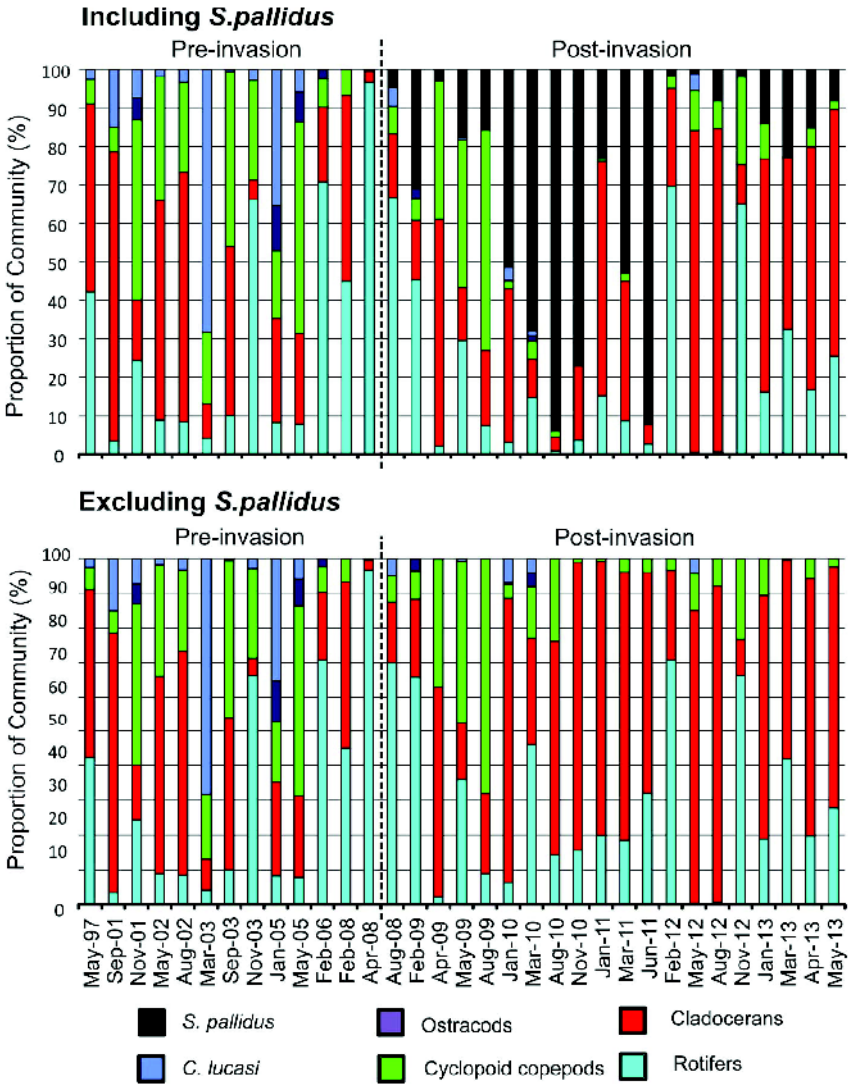

Figure 1. Relative abundances of the major zooplankton groups in Lake Kereta between 1997 and 2013, with *S. pallidus* included (top) and excluded (bottom).

The MDS undertaken with *S. pallidus* included revealed a clear separation between samples before and after *S. pallidus* invasion (Figure 2). ANOSIM indicated community composition significantly differed before and after the invasion (Global R=0.501; P=0.001). SIMPER analysis indicated that species contributing most greatly (>5%) to the average dissimilarity calculated between sample groups before and after invasion were crustaceans: *S. pallidus* (responsible for 13.5% of the dissimilarity between sample groups before and after invasion), *Mesocyclops* sp. (7.3%; decreased post-invasion), *Bosmina meridionalis* G.O. Sars, 1904 (7.2%; increase), *Daphnia galeata* G.O. Sars, 1864 (6.9%; increase) and *C. lucasi* (6.8%; decrease). This indicates the dominance of *S. pallidus* in many samples following its invasion was the most important species differentiating the sample groups before and after the invasion.

The MDS excluding *S. pallidus* showed a separation in samples before and after the *S. pallidus* invasion, with 'before' samples distributed primarily on the left of the ordination, and 'after' samples on the right. However, the separation of 'before' and 'after' samples was not as clearly defined as in the ordination where *S. pallidus* was included (Figure 2). Nevertheless, ANOSIM

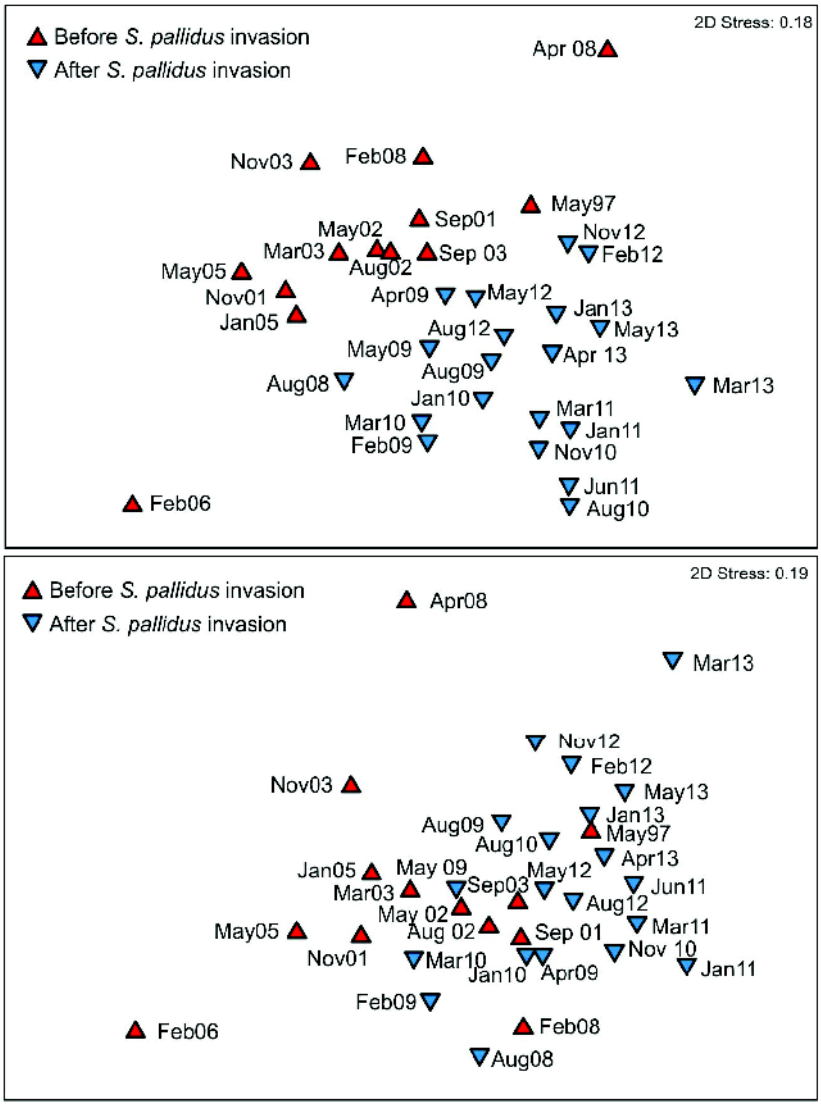

Figure 2. Multidimensional scaling plots showing change in species composition before and after invasion of *Skistodiaptomus pallidus* into Lake Kereta. Analyses were undertaken with *S. pallidus* included (above) and excluded (below) from the dataset.

inferred community composition to significantly differ before and after the invasion (Global R=0.246; P=0.002). SIMPER analysis indicated that species contributing most greatly (>5%) to the dissimilarity calculated between samples before and after invasion were *D. galeata* (9.5%; increase), *B. meridionalis* (7.8%; increase), *C. lucasi* (7.3%; decrease), *Mesocyclops* sp. (6.8%; decrease), and *Chydorus* sp. (5.2%; decrease). Due to the clear statistical differences between community composition in samples collected before and after *S. pallidus* invasion, missing samples in the dataset were unlikely to have affected the statistical results.

Discussion

Skistodiaptomus pallidus was first recorded in Lake Kereta on 13 August 2008. Although not present in 13 samples prior to this date, it was present in all 20 samples collected in the following five years. First detection of *S. pallidus* in Lake Kereta coincided with releases of grass carp (*Ctenopharyngodon idella*) into the lake to aid in the control of hornwort (*Ceratophyllum demersum* L., 1753); hornwort had invaded the lake prior to 1999. Between March 2008 and April 2009, four grass carp releases were made into Lake Kereta, comprising almost 15000 fish (de Winton 2012).

Translocations in association with fish appears to be a common transportation vector for zooplankton invasions globally with, for example, *Daphnia lumholtzi* potentially having been released into North America with introductions of Nile Perch (Havel and Hebert 1993), while the first introduction of *Boeckella triarticulata* to northern Italy likely also occurred with introductions of fish (Ferrari 1991). Lake Omapere, where we also recorded *S. pallidus*, had over 40000 grass carp released as a method of controlling Brazilian waterweed *Egeria densa* Planch., 1849 in 2000 (Champion 2004; Ray et al. 2006), while Lake Hood had over 2000 grass carp released in 2005. Grass carp utilized in New Zealand are domestically produced, from stock originally derived from Hong Kong (McDowall 1990), and it may be that ponds for the stocking of carp are contaminated with *S. pallidus*. However, *S. pallidus* has also been recorded among live fish food (*Daphnia carinata* King, 1852) in aquarium stores (Duggan et al. 2006), and is present in several constructed Auckland lakes, leaving the possibility that it may have been spread by vectors other than grass carp. *Skistodiaptomus pallidus* has not yet been recorded from some lakes where grass carp have been released, for which we have zooplankton samples (Western Springs and Lake Wainamu; de Winton and Edwards 2012). However, the consistent timing of the first records of *S. pallidus* in Lake Kereta with grass carp releases appears compelling.

The first records of *S. pallidus* in the North Island of New Zealand were from constructed waters (Duggan et al. 2006; Banks et al. 2009). Here we present the first record from the South Island, in a lake constructed for water sports activities such as fishing, rowing and water skiing. Taylor and Duggan (2012) demonstrated that *S. pallidus* could readily establish populations in experimental tanks where native New Zealand calanoid copepods (primarily *C. lucasi*) were absent, while invasions were repelled where calanoid copepods were present. However, these authors noted that any community could be invaded if presented with adequate propagule supplies, ultimately overwhelming biotic resistance (see also von Holle and Simberloff 2005). Abundant propagule supplies of *S. pallidus* could have been provided to the natural lakes in this study in association with large releases of grass carp, if ponds used in the culture of this fish species contained *S. pallidus*. While recent invasions of *S. pallidus* in Germany (Brandorff 2011) and tropical Mexico (Suárez-Morales and Arroyo-Bustos 2012) are currently restricted to constructed water bodies, it is likely a matter of time before they spread to natural waters also (as has also been the case for *D. lumholtzi* in North America, and *B. triarticulata* in Italy). In New Zealand, the Japanese calanoid copepod *Sinodiaptomus valkanovi* Kiefer, 1938, and Australian species' *Boeckella minuta* G.O. Sars, 1896 and *B. symmetrica* G.O. Sars, 1908, are seemingly still confined to constructed waters (Banks and Duggan 2009; Makino et al. 2010), but may also establish in natural waters given time.

A significant change in zooplankton community composition coincided with the establishment of *S. pallidus* in Lake Kereta. When considering *S. pallidus* as part of the community, the assemblages before and after the invasion differed significantly, as *S. pallidus* commonly dominated. However, even when *S. pallidus* was removed from the analyses, the zooplankton assemblages were still found to be significantly different, primarily due to changes in the relative abundances of other crustacean species. Most notably, the native calanoid copepod *C. lucasi* decreased in relative abundance. Calanoid copepods of similar size, or with significant dietary overlap, cannot co-exist, such that in many lakes coexistence is rare unless they are of significantly different size (e.g., Hutchinson 1967; Maly and Maly 1997). Similar to the current study, the non-indigenous population of *S. pallidus* in the Tahoe Keys marina, southern Lake Tahoe, United States, was found to displace the native calanoid copepods *Leptodiaptomus tyrelli* (Poppe, 1888) and *Epischura nevadensis* Lilljeborg, 1889 (Byron and Saunders 1981). In the current study, the planktonic cladocerans *Bosmina meridionalis* and *Daphnia galeata* increased in relative abundance, and the cyclopoid copepod *Mesocyclops* sp. and the cladoceran *Chydorus* sp. decreased, while no rotifer species contributed greater than 5% to the average dissimilarity between zooplankton communities before and after *S. pallidus* invasion.

Complicating the identification of potential effects of *S. pallidus* on the zooplankton community was the concomitant release of grass carp and associated reduction in macrophyte biomass. However, studies of grass carp introductions have typically found limited effects on zooplankton communities. Grass carp introduced to Lake Kereta were all greater than 6 cm in length (de Winton 2012). Although grass carp fry utilize zooplankton as a primary food source, these were not released to the lake, and adults rarely eat anything except plant material (Richard et

al. 1985). Grass carp transition from a diet of zooplankton or benthos to aquatic macrophytes at approximately 5.5 cm (He et al. 2013). For example, fish >6.3 cm in a Florida pond were found to be strict herbivores (Colle et al. 1978). Indeed, Prowse (1971) noted that adult grass carp may starve in ponds lacking vegetation even if they have high abundances of zooplankton. As such, direct effects of grass carp are unlikely. Macrophyte biomass decreased markedly following grass carp introduction. Observations in 2008 showed hornwort to have >75% coverage in the lake, while >99.9% of biomass was removed when the lake was resurveyed in February 2012 (de Winton 2012). Conditions may have thus become more favourable for some species by providing more open water for planktonic species, while the reduced extent of weedbeds may have reduced the abundances of typically littoral species. This reduction in macrophytes could explain increases in the planktonic cladoceran species, *B. meridionalis* and *D. galeata*, and the associated decrease in the littoral *Chydorus* sp. However, the reduction in macrophytes cannot provide an explanation for the decreased importance of the planktonic *C. lucasi*. Most studies on the indirect effects of grass carp on zooplankton, through macrophyte removal, have found limited effects also. Fry and Osbourne (1980) found grass carp additions to have little direct or indirect effect upon the zooplankton in three experimental Florida ponds, while Pípalová et al. (2009) found no changes in zooplankton composition or abundance associated with increasing grass carp densities from 29 kg/ha to 92 kg/ha in a Czech Republic pond, despite a reduction in macrophyte density from 109 g/m^2 to 33 g/m^2. Kirkagac and Demir (2004) found increases in zooplankton abundance in ponds stocked with increasing densities of grass carp (>19.5 cm) in Turkey, largely as a result of a greater availability of nutrients for phytoplankton, providing food for zooplankton. Where effects on composition have been noted, it is usually related to subsequent increases in the abundances of planktivorous fishes following macrophyte reduction (e.g., Maceina et al. 1992; Richard et al. 2005). However, New Zealand zooplankton communities are likely to be under reduced pressure from zooplanktivorous fish relative to elsewhere (Chapman and Green 1987). Overall, competitive interactions with *S. pallidus* provide the most convincing explanation for the reduction in *C. lucasi*, rather than any direct or indirect effects of grass carp.

Skistodiaptomus pallidus appears to have undergone a "boom-and-bust" cycle in Lake Kereta. The species gradually increased in its numerical importance following establishment, peaked in relative abundance in late-2010 and mid-2011 when it comprised >90% of individuals counted, and thereafter declined dramatically in its importance, comprising <25% of the relative abundance over the last 13 months of study. Such boom-and-bust cycles are typically expected when food of the invader is previously under-utilised by the existing species, leading to a boom period for the invader, followed by a decline towards an equilibrium with available resources (bust) (Williamson 1996). Alternatively, but less likely, pathogens or predators may also have played a role in reducing the importance of this species. Further monitoring is required to determine whether *S. pallidus* remains integrated in the lake community at low densities, is extirpated, or if the species may continue to dominate in a cyclic or irregular manner (*sensu* Strayer and Malcom 2006). Such monitoring is also important to determine the long-term effects on the native calanoid copepod, *C. lucasi*, whose population had not attained their pre-invasion importance following the decline in *S. pallidus*.

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