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Genetic diversity and potential responses of New Zealand Rotifera and Antarctic Collembola to environmental changes

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Abstract

Environmental changes can impact the diversity and abundance of biotic communities as well as their dispersal among geographic regions. The availability of sensitive molecular techniques has enabled assessments of diversity at levels previously unattainable and allowed investigation of how biota are influenced by their environment. This thesis examines the use of mitochondrial DNA sequence variation in New Zealand freshwater rotifers (Rotifera) and Antarctic springtails (Collembola) to assess diversity and determine responses to environmental changes.

Using mitochondrial cytochrome *c* oxidase subunit I (COI) DNA sequences, I examined the diversity of New Zealand rotifers. The rotifer phylum is comprised of a variety of species and individuals are often abundant in freshwater systems globally. Species are associated with specific trophic states and in New Zealand are routinely monitored as biological indicators of ecosystem health. At present, species' identification relies on morphological characters which is time consuming and requires appropriate taxonomic expertise. Here, I assessed the use of COI sequences as an alternative for the routine identification of New Zealand rotifers. A total of 177 individuals were sequenced from 45 morphologically-recognised taxa. High intraspecific variation was found among 14 species (3.4 - 39.0% divergence) and based on comparisons with sequences available on GenBank, 18 species were genetically distinct from their global conspecifics (>6.6% divergence). I conclude that these distinct haplotypes are potentially endemic among putatively cosmopolitan species. A better understanding of rotifer diversity in New Zealand will allow the detection and potential control of future non-indigenous species' incursions.

In order to assess the effects of environmental temperature changes on the genetic structure of populations, I examined the diversity of COI sequences of Antarctic springtails relative to the environmental conditions during which they were active. The target species, *Gomphiocephalus hodgsoni*, is known to harbour high genetic (COI) diversity as well as having considerable variation in their ability to tolerate sub-zero temperature – specifically, there appear to be "warm" and "cold" adapted individuals. I sequenced 151 individuals collected in pitfall traps near Spaulding Pond, Taylor Valley and found 19 unique COI haplotypes that separated into two distinct groups (1.6% divergence), with one haplotype group comprising 80% of the sequenced population. During two-hourly sampling, air temperature was the strongest predictor of activity between the two haplotype groups ($R^2 = 0.56$) and when combined with subsurface soil temperature, relative humidity and photosynthetically active radiation, explanatory power increased to $R^2 = 0.71$. Air temperatures are predicted to continue increasing across most of Antarctica which is expected to impact springtails by a detectable amount. Monitoring programmes focussed on the temporal and spatial changes in COI haplotype diversity may provide a sensitive measure of population responses to climate change.

Collectively, these two studies contribute baseline data of the current genetic diversity and population structure among New Zealand rotifers and Antarctic springtails and provide a platform by which to monitor future environmental changes.

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Chapter I

Thesis Introduction

The development and widespread access to molecular techniques such as DNA barcoding has facilitated a more accurate assessment of biological diversity across a wide range of taxa. We now have the capacity to thoroughly assess relationships within and amongst species, and between species and their environments (e.g. Green 1972; Nandini et al. 2005). Improving our capacity to accurately assess diversity as well as understanding the role of environmental drivers is crucial for addressing ecological questions such as those related to biomonitoring and climate change.

One particular molecular technique which has become widely used is known as DNA barcoding (*sensu* Hebert et al. 2003), which uses a 658 bp fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene. These sequence fragments usually exhibit levels of variability that can distinguish between species while maintaining enough conserved characters to detect individuals of the same species. It is now becoming routine to develop COI barcode libraries to document genetic diversity within natural populations (Knebelsberger et al. 2014; Moriniere et al. 2014; Raupach et al. 2014).

When genetic variation is subtle, such as one or more single nucleotide polymorphisms, each unique sequence is deemed a haplotype. However, if COI diversity exceeds a given intraspecific threshold (often around 2%; Knebelsberger et al. 2014), then those divergent individuals may represent potentially cryptic species. Genetic studies assessing this haplotype variation and cryptic speciation are now emerging for a range of animal taxa (e.g. Hebert et al. 2004).

In this thesis I sequenced the COI gene regions for both New Zealand freshwater rotifers and the Antarctic springtail *Gomphiocephalus hodgsoni* (Collembola) to understand how these taxa may be affected by changes in their environment. My

focus differs between the two taxa in that rotifers are a phylum (Rotifera) containing a diverse array of species, whereas for *G. hodgsoni*, my interest was intraspecific variability related to environmental factors. For rotifers I was primarily interested in assessing existing levels of diversity and evaluating the use of COI sequences for the routine assessment of species diversity. For *G. hodgsoni* my focus was on assessing subtle changes in population genetic structure related to changes in environmental temperatures. The two research chapters within this thesis are centred on each of these two taxa.

The first research chapter (Chapter II) investigates genetic diversity of rotifers collected from freshwater lakes and ponds throughout the North Island of New Zealand and assesses potential endemism through international comparisons. Rotifers are bilaterally symmetrical pseudocoelomates, ranging from 50 to 2000 µm in length, featuring a mastax (a specialised pharynx used to crush food), trophi (jaws) and coronal cilia for locomotion and food gathering (Wallace 2002; Wallace et al. 2006; Segers 2007).

Traditional taxonomy based on morphological characters may lack the ability to detect subtle changes among rotifer species. As such, the COI gene region is widely-used for assessing species diversity and has proven accurate at identifying rotifers to the species-level and in agreement with morphological assessments (e.g. García-Morales and Elías-Gutiérrez 2013). To date, the development of a global rotifer COI database has been limited and largely restricted to recent intensive surveys of few species.

In order to improve our understanding of rotifer biodiversity, there is a need to increase the range of geographic coverage (Gomez 2005). This is particularly relevant to New Zealand rotifers which are considerably understudied compared

to those in temperate regions of the Northern Hemisphere (Segers 2008). Here, I address this gap by sequencing New Zealand rotifers with particular focus on identifying potentially endemic cryptic species. Closer genetic assessments of rotifer species often result in additional endemic or unique variants revealed among those currently considered cosmopolitan (Shiel and Green 1996, Shiel et al. 2009). This is particularly relevant for New Zealand as few species are currently classified as endemic (<5%; Shiel et al. 2009). This is somewhat surprising considering New Zealand's geographical isolation over the past 80 million years and high endemism among other biota (Cooper and Millener 1993). Geographical isolation of populations leads to allopatric speciation and the evolution of endemic variants (Lande 1980) and phylogeographic studies have revealed that increasing geographical distances often result in greater differences between rotifer communities (see Gomez 2005 for review).

Rotifers exhibit species-specific responses to water quality, whereby certain species are associated with specific trophic states in New Zealand's freshwater systems. Recently, a rotifer trophic level index (TLI) has been developed where the presence and abundance of certain taxa is assessed as the biological indicator of water quality and ecosystem health (Duggan et al. 2001), and this is now included in routine surveys by some regional councils (Auckland Regional Council 2005; Duggan 2008). Incorporating biological data in monitoring programs is an improvement from past reliance on physical and chemical measurements such as secchi depth, total nitrogen, total phosphorus, dissolved oxygen and chlorophyll *a* that are used to infer the biological health of ecosystems (Burns et al. 1999). However, these biological assessments are based on morphology alone which is time-consuming and diapausing stages cannot be identified. To streamline this process, developments are underway to establish a

method for detecting rotifer COI sequence fragments in water samples through the use of next generation sequencing platforms.

The genetic database developed in this research chapter will improve our understanding of New Zealand's endemic fauna while contributing to a global DNA barcoding database (www.boldsystems.org). This will assist in the detection of future non-indigenous species' incursions. Furthermore, it will provide a reference library to facilitate next generation sequencing of rotifers from water samples as a reflection of ecosystem health. I hypothesised that rotifer haplotypes and potential cryptic species unique to New Zealand will be revealed through the assessment of mitochondrial COI variability.

The second research chapter (Chapter III) is focussed on assessing subtle haplotype variability of an Antarctic springtail relative to environmental temperatures, as an indication of how the populations might respond to climate change. The Antarctic environment is harsh with more than 99% of the continent permanently ice-covered and therefore uninhabitable by invertebrates (Hopkins et al. 2006). Available ice-free habitat is restricted mostly to coastal regions and is incredibly fragmented, with many barriers to dispersal and gene flow such as glaciers and mountain ranges (Stevens and Hogg 2002; 2003; Hawes 2011). Such habitat fragmentation has been even more pronounced during past glacial maxima, resulting in survival of invertebrates in isolated refugia followed by recolonization of the landscape under more favourable conditions (Fraser et al. 2014). Collectively, patchy habitat availability and isolation events promote allopatric speciation and have resulted in the high level of intraspecific genetic diversity currently observed among Antarctic springtails (Collembola) – the largest year-round organisms within these ice-free areas.

Most of the ice-free landscape of Antarctica, approximately 4800 km^2, lies within the Dry Valleys of Southern Victoria Land, where average annual temperatures range from -14.8°C to -30°C, snow precipitation is low at <100 mm (Bromley 1985; Doran et al. 2002) and ablation exceeds annual precipitation at 150 to >1000 $mm.a^{-1}$ (Henderson et al. 1965; Clow et al. 1988). Together, these conditions create a hyper arid, cold-polar desert environment, unable to support complex trophic structures. In the Dry Valleys, *Gomphiocephalus hodgsoni* is the only springtail species present and several studies have focussed on its physiological and genetic variability.

Physiologically, considerable variation in the tolerance of *G. hodgsoni* to cold has been revealed through metabolic rate and supercooling point analyses (e.g. Sinclair and Sjursen 2001; McGaughran et al. 2008; 2011). The logistical and seasonal challenges involved with Antarctic research, coupled with the current inability to fully assess biological diversity (Savage 1995), have resulted in a limited genetic baseline dataset to date. There are four previous papers that have assessed COI sequences of *G. hodgsoni* specifically. Stevens and Hogg (2003) compared diversity between continental and island sites, then Nolan et al. (2006) assessed haplotype variation throughout Taylor Valley, followed by McGaughran et al. (2008) where rates of evolution were compared between *G. hodgsoni* and the mite *Stereotydeus mollis.* Subsequently, McGaughran et al. (2010a) compared variation between the continental *G. hodgsoni* and the Antarctic Peninsula springtail *Cryptopygus antarcticus antarcticus*. Collectively, these four genetic studies as well as unpublished MSc research (Demetras 2010; Bennett 2013), and my current study have contributed to the present database of around 70 *G. hodgsoni* COI haplotypes.

To bring the physiological and genetic aspects together, my second research chapter assesses the environmental conditions during which certain *G. hodgsoni* haplotypes are active. Specifically, I focused on the COI gene region as it is well-studied, has proven sensitive to environmental changes (e.g. Pichaud et al. 2012) and has been weakly linked to metabolic rate in other Antarctic springtails (e.g. McGaughran et al. 2010b). Accordingly, the hypothesis tested in this chapter was that the activity of *Gomphiocephalus hodgsoni* COI haplotypes would correspond to temperatures during the collection period. Ultimately, we aim to develop a sensitive genetic marker for detecting subtle changes in populations of *G. hodgsoni* in response to increasing temperature as a result of climate change.

The final chapter of this thesis (Chapter IV) summarises the findings and suggests avenues for future research related to my two projects.

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Chapter II

Diversity and global affinities of New Zealand rotifers assessed using mitochondrial DNA (COI) barcodes*

*To be published under the same title, as: Collins GE, Hogg ID, Duggan IC, Beet CR, Banks JC, Knox MA

ABSTRACT

In order to assess the diversity and endemicity of New Zealand freshwater rotifers, we sequenced the cytochrome *c* oxidase subunit I (COI) gene region of 177 individuals and obtained 73 unique sequences from 45 morphologically-recognised species. From these, 14 species had high intraspecific sequence variation (3.4 – 39.0% divergence). We found that 18 species had sequences that were divergent (>6.6%) from their conspecifics elsewhere (available on GenBank), suggesting cryptic endemism in New Zealand for putatively cosmopolitan species. Two species, *Lecane bulla* and *Brachionus calyciflorus*, were up to 19.7% and 21.4% divergent, respectively, from their conspecifics elsewhere. Of the 45 species we assessed, 27 had no international data available, highlighting the need for an enhanced international effort in order to facilitate comparisons to assess the global biodiversity of this phylum. Based on our data, we suggest that COI provides a valid method for the rapid identification of New Zealand rotifers. We also suggest that endemism among New Zealand rotifers may be higher than originally suggested on the basis of morphological assessments. From an applied perspective, these data will be essential for detecting non-indigenous species' incursions globally and can also be used to develop local water quality monitoring programs.

INTRODUCTION

With over 2000 species globally, the phylum Rotifera is highly diverse and representatives are ubiquitous in freshwater environments (Segers 2008). They are also integral components of freshwater ecosystems, particularly in the littoral zones of standing waters, serving as filter-feeders and contributors to aquatic food webs (Duggan 2001). Variation in the diversity and abundance of rotifer taxa occurs at both spatial and temporal scales, driven in part by environmental factors such as temperature and nutrient availability. Spatially, rotifers have high dispersal abilities due to their small size and stress-resistant diapausing stages, facilitating gene flow among populations (De Meester *et al*. 2002). While some long-distance dispersal events have been reported, communities tend to vary with increasing geographical distances (see Gomez 2005 for review). Temporally, diversity and abundance are highest during summer months, mostly due to higher water temperatures (Castro *et al*. 2005; Nandini *et al*. 2005), although this seasonality is less marked in New Zealand compared with temperate Northern Hemisphere habitats (Shiel *et al*. 2009).

Rotifers are also highly sensitive to nutrient loading and the presence of pollutants within freshwater systems. They have been found to elicit species-specific responses to water quality, whereby different species are found in water bodies of specific trophic states (Duggan *et al*. 2001; 2002; Castro *et al*. 2005). Accordingly, rotifers have been suggested as an ideal biological indicator to assess aquatic ecosystem health (Duggan *et al*. 2001), and can be used to supplement physical and chemical measurements (Gannon & Stemberger 1978; Lougheed & Chow-Fraser 2002; Bianchi *et al*. 2003). Unfortunately, species-level identifications based on morphological characters are particularly challenging for rotifers due to their small size and phenotypic plasticity (Briski *et al*. 2011). Furthermore, the

availability of taxonomic expertise to assist with identifications is declining (Segers 2008). Molecular approaches using mitochondrial cytochrome *c* oxidase subunit I (COI) sequences have been suggested as an alternative to facilitate the routine identification for a range of taxa (Hebert *et al*. 2003), and may be particularly useful for identifying rotifers (García-Morales & Elías-Gutiérrez 2013). Such data would also serve to assess genetic similarities among locations.

Within New Zealand, endemicity among rotifers is currently considered to be low (Shiel *et al*. 2009). This is surprising given New Zealand's geographical isolation and high level of endemicity for other biotia (Cooper & Millener, 1993). Accordingly, a more thorough molecular assessment may reveal endemic variants among putatively cosmopolitan species (Shiel & Green 1996, Shiel *et al*. 2009). This is supported by recent genetic studies demonstrating cryptic diversity among rotifer species elsewhere (e.g., Walsh *et al*. 2009; Garcia-Moralez & Elias-Guitierrez 2013; Velasco-Castrillion *et al*. 2014). Furthermore, molecular analyses often reveal considerable genetic variation among morphologically similar specimens, and such cryptic diversity may be particularly high in rotifers due to their niche partitioning and habitat heterogeneity (Segers 2008).

In order to evaluate the diversity and global affinities of New Zealand rotifers, and to develop a genetic database for rotifer identification and water quality management, here we assess New Zealand freshwater rotifers at the COI gene locus. We hypothesize that cryptic and potentially endemic taxa will be revealed among putatively cosmopolitan species.

MATERIALS AND METHODS

Rotifers were collected between January 2011 and May 2014 from freshwater lakes and ponds throughout the North Island, New Zealand (Fig. 1), using a 40 µm plankton net hauled manually from the shoreline. Samples were immediately preserved in 70% ethanol and later identified to species level using available taxonomic keys (e.g., Nogrady *et al*. 1993; Shiel 1995) prior to genetic analyses.

Total genomic DNA was extracted from between one and eight whole individuals of the same species per reaction tube using the Red Extract n Amp (Sigma-Aldrich) kit following the manufacturer's protocol with slight modification: per sample, 20 µL extract solution (Ex) and 5 µL tissue preparation solution (TP) was used, followed by incubation in darkness at room temperature for 3 hours, heating to 95˚C for 3 minutes then addition of 20 µL neutralising solution (N).

A 658 bp region of the mitochondrial cyctochrome *c* oxidase subunit I (COI) gene was PCR amplified using either the primer pair LepF1 (5'-ATTCAACCAATCATAAAGATATTGG-3') and LepR1 (5'-TAAACTTCTGGATGTCCAAAAAATCA-3') (Ratnasingham & Hebert 2007) or LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al*. 1994). The 15 µL PCR reactions contained 7.5 µL PCR master mix solution (i-Taq) (Intron Biotechnology), 0.3 µM each of the forward and reverse primers and 1 µL DNA extract (unquantified). Thermal cycling conditions consisted of an initial denaturation of 5 min at 94ºC; five cycles of 1 min at 94ºC, 1.5 min at 48ºC, and 1 min at 72ºC; 36 cycles of 1 min at 94ºC, 1.5 min at 52ºC, and 1 min at 72ºC; and a final extension of 5 min at 72ºC. PCR products were visualised under UV light in a 1.5% agarose gel containing RedSafe Nucleic Acid Staining Solution (20,000x)

according to manufacturer's instructions (5 µL/100mL). Successfully amplified PCR products were cleaned using Exonuclease I (EXO) and Shrimp Alkaline Phosphate (SAP) before sequencing in both directions on a capillary electrophoresis ABI 3130XL genetic analyser at the University of Waikato Sequencing Facility.

Sequences >500 bp generated in this study were verified using the GenBank BLASTn algorithm then aligned using MUSCLE as implemented in Geneious v6.1.2 (Drummond *et al*. 2010) and trimmed to exclude primer regions. No stop codons were detected, indicating that only mitochondrial DNA was amplified. To remove ambiguities, the alignment was further trimmed to 526 bp to include positions 1583-2110 when aligned with the *Drosophila yakuba* complete mitochondrial genome reference sequences (GenBank Accession numbers X03240 and NC_001322), and sequences short of this alignment were removed. The resulting alignment of 149 specimens, including the bdelloid rotifer *Rotaria neptunia* as an outgroup, was used as the basis for phylogenetic analyses. All unique sequences were verified by visually inspecting original traces. Full-length sequences for all 149 specimens, ranging from 526 to 658 bp, were deposited in the Barcode of Life Datasystems (BOLD) database under the project Freshwater Rotifers of New Zealand (NZPLR) along with supporting information, and cross referenced to GenBank®. Chi-square tests (χ^2) as implemented in PAUP* 4.0b10 (Swofford 2001) were used to determine whether base frequencies were equal among all sites, parsimonious-informative sites, and first, second or third codon positions for all 148 monogonont sequences.

Duplicate sequences were then removed from the alignment and the remaining 74 sequences formed the basis of phylogenetic tree construction. Neighbour-Joining

(NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) trees were generated in MEGA v5.2.2 (Tamura *et al*. 2011) each with 1000 bootstrap replicates. The final tree was based on ML topology, with the incorporation of 75% majority rule bootstrap consensus of NJ and MP trees. The model of sequence evolution for ML tree construction was General Time Reversible with gamma distribution and invariant sites (GTR + I + G) as predicted by jModelTest v2.1.1 (Darriba *et al*. 2012). For construction of MP and NJ trees the Jukes-Cantor model was used, with gamma distribution. A pairwise genetic distance matrix was also generated in MEGA v5.2.2 as the basis for assessment of intraspecific divergences. The off-stated 2% value of divergence was used as the cut-off for determining potential cryptic speciation.

Barcode index numbers (BINs) were determined by Refined Single Linkage (RESL) analyses performed in BOLD (Ratnasingham & Hebert 2013) and used as a surrogate for operational taxonomic units (OTUs). The assignment of BINs has shown to be highly congruent with traditional morphological taxonomy (Ratnasingham & Hebert 2013). We therefore considered BINs as an indicator of potentially cryptic species.

Sequences of species assessed in the current study available on GenBank (Benson *et al*. 2012), some of which were cross-referenced to BOLD, were downloaded to Geneious v6.1.2 and included in the sequence alignment. As many GenBank sequences were shorter than those obtained in this study, sequences were further trimmed to give a final 482 bp alignment of 1591 specimens that was used for subsequent analyses. A NJ tree was constructed in MEGA v5.2.2 with the Jukes-Cantor model of evolution and gamma distribution. Bootstrap values (1000 replicates) providing at least 75% branch support were included in the final tree.

As several taxa, notably *Brachionus calyciflorus* and *Polyarthra dolichoptera*, have been extensively studied overseas, large GenBank sequence clusters were simplified in FigTree (Rambaut 2012), resulting in triangles proportional to the variation collapsed. Unfortunately, not all sequences on GenBank have been cross-referenced to BOLD and collection information was absent for some specimens downloaded from GenBank. Visual inspection of the NJ tree indicated New Zealand sequences that differed from international comparisons, and distance matrices for these species were calculated in Geneious to determine intra-specific divergence values.

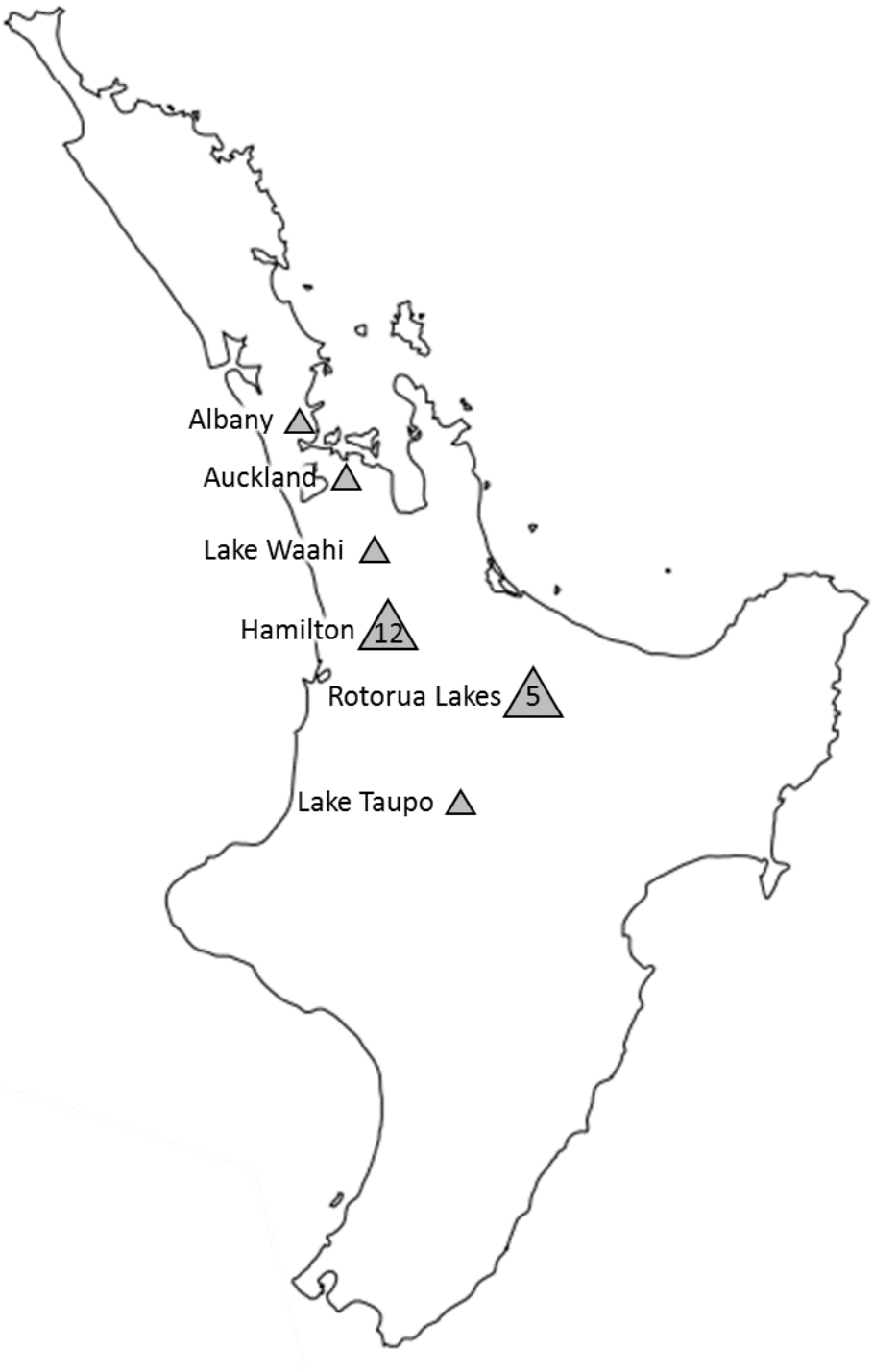


Fig. 1 Map of the North Island, New Zealand, showing approximate location of sampling sites for rotifers included in the final alignment. Rotifers were collected from twelve different lakes and ponds in the Hamilton area, from five lakes in the Rotorua area and from one site per additional labelled location on the map.

RESULTS

We sequenced 177 individuals representing 45 morphologically-identified species. Nucleotide composition across all positions for the 148 sequences in the final alignment, excluding the outgroup *Rotaria neptunia*, showed an A-T bias (A = 21.6%, T = 42.9%, G = 17.6%, C = 17.9%). Base frequencies were not homogeneous across sequences for all sites (χ^2_{441} = 863.15, $p < 0.001$), for parsimony informative sites (A-T = 68.2%; χ^2_{441} = 1456.84, $p < 0.001$) nor third codon positions (A-T = 80.2%; χ^2_{441} = 2096.52, $p < 0.001$). However, base frequencies were homogeneous across sequences for first (A-T = 54.5%; χ^2_{441} = 287.02, p = 1.0) and second (A-T = 58.9%; χ^2_{441} = 41.62, p = 1.0) codon positions. Of the 526 bp analysed, 316 sites were parsimony informative, 9 of the variable sites were parsimony uninformative and the remaining 201 sites were constant.

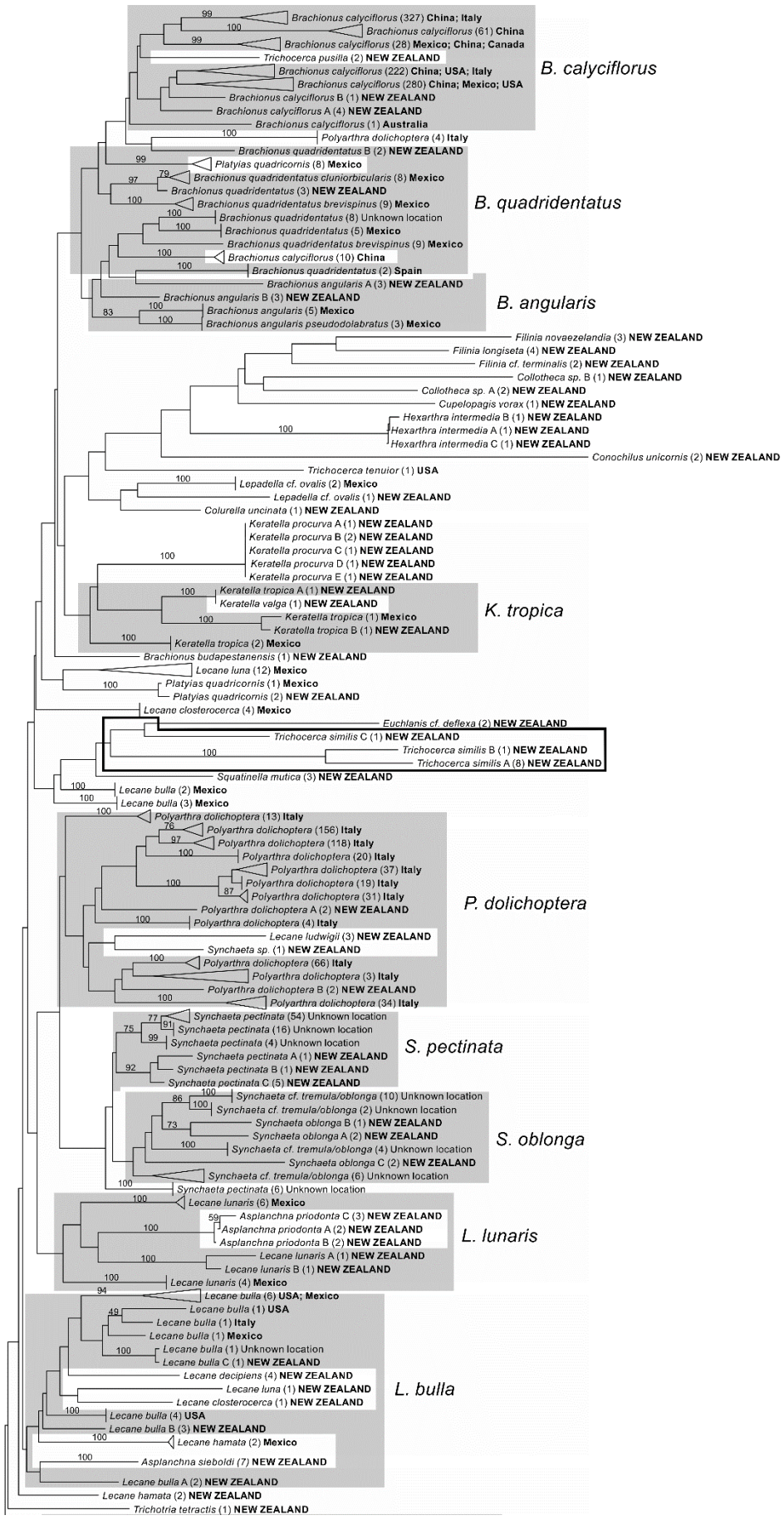
The ML tree derived from the New Zealand representative dataset is provided in Fig. 2. Fourteen of the 45 species showed high intraspecific divergences (>3.4%) as well as having been assigned unique BIN designations using the BOLD algorithm (*sensu* Ratnasingham and Hebert 2013; see appendix 1 for a complete list of BINs). While some genera formed distinct clusters (e.g., *Brachionus*, *Synchaeta*, *Asplanchna* and *Filinia*), species from other genera were dispersed throughout the tree (e.g., *Lecane, Keratella, Trichocerca*) and the highly diverse *Lecane bulla* was found at two distinct locations within the tree. Two taxonomic uncertainties were also highlighted; *Keratella tecta* was genetically similar to one of the two *Keratella cochlearis* sequences (1% divergence), and *Keratella valga* was identical to *Keratella tropica*. Overall, maximum intraspecific divergence values range from 0-39% (Table 1).

Sequences from 14 species (*Ascomorpha ovalis*, *Brachionus calyciflorus*, *B. angularis*, *Lecane closterocerca*, *L. hamata*, *L. luna*, *L. lunaris*, *Lepadella* cf. *ovalis*, *Polyarthra dolichoptera*, *Synchaeta pectinata*, *S. oblonga*, *Trichocerca stylata*, *T. tenuior* and *Trichotria tetractis*) were all distinct from their international conspecifics (>6.6% divergence). For four additional species (*Brachionus quadridentatus*, *Keratella cochlearis*, *K. tropica* and *Lecane bulla*) we found sequences that were reasonably similar to those on GenBank (1.9 – 5.0% divergence), as well as sequences that were unique to New Zealand (12.0 – 22.4% divergence). Of the New Zealand sequences that were similar to GenBank sequences, *B. quadridentatus* and *K. tropica* were most similar to individuals from Mexico. Individuals of *L. bulla* and *K. cochlearis* were also similar to those on GenBank (1.9 – 2.3% divergence). However, no location data were provided for these individuals on GenBank. For a further 27 species, no records were available on GenBank (Table 1).

Fig. 2 Maximum Likelihood tree comprising 74 unique sequences among 46 rotifer species. Unique sequences for each species are labelled from A to E and those with unique BINs are indicated by grey boxes. The number of duplicate sequences are shown in parentheses. Bootstrap support (1000 replicates) where >75% are shown on branches for ML/NJ/MP analyses.

Table 1 The number of individuals sequenced (*n*), number of unique sequences and maximum intraspecific COI divergences (calculated in MEGA) for the 45 rotifer species assessed and the outgroup *Rotaria neptunia*. The number of GenBank sequences included for each species and the number of New Zealand sequences unique from GenBank (>6.6% divergence as calculated in Geneious and indicated with bold typeface) are also shown.

Species	*n*	Unique seqs	Unique BINs	Max. Intraspecific divergence (%)	GenBank sequences	Unique from GenBank
Ascomorpha ovalis	**2**	**1**	**1**	**0.0**	**23**	**1**
Ascomorpha sp.	3	1	1	0.0	-	-
Asplanchna priodonta	8	3	1	1.2	-	-
Asplanchna sieboldi	7	2	1	0.2	-	-
Brachionus angularis	**6**	**2**	**2**	**21.0**	**8**	**2**
Brachionus budapestanensis	1	1	1	-	-	-
Brachionus calyciflorus	**5**	**2**	**2**	**12.0**	**714**	**2**
Brachionus quadridentatus	**5**	**2**	**2**	**22.0**	**33**	**1**
Collotheca sp.	3	2	2	30.1	-	-
Colurella uncinata	1	1	1	-	-	-
Conochilus unicornis	2	1	1	0.0	-	-
Cupelopagis vorax	1	1	1	-	-	-
Euchlanis cf. *deflexa*	2	1	1	0.0	-	-
Euchlanis meneta	3	2	2	13.5	-	-
Euchlanis pyriformis	3	1	1	0.0	-	-
Filinia cf. *terminalis*	2	1	1	0.0	-	-
Filinia longiseta	4	1	1	0.0	-	-
Filinia novaezelandia	3	1	1	0.0	-	-
Hexarthra intermedia	3	3	1	0.4	-	-
Keratella cochlearis	**5**	**2**	**2**	**17.6**	**65**	**1**
Keratella procurva	6	5	2	0.6	-	-
Keratella tecta	4	1	1	0.0	-	-
Keratella tropica	**7**	**2**	**2**	**14.5**	**3**	**1**
Keratella valga	1	1	1	-	-	-
Lecane bulla	**6**	**3**	**3**	**22.5**	**19**	**2**
Lecane closterocerca	**1**	**1**	**1**	**-**	**4**	**1**
Lecane decipiens	4	1	1	0.0	-	-
Lecane hamata	**2**	**1**	**1**	**0.0**	**2**	**1**
Lecane ludwigii	3	1	1	0.0	-	-
Lecane luna	**1**	**1**	**1**	**-**	**12**	**1**
Lecane lunaris	**2**	**2**	**2**	**8.7**	**10**	**2**
Lepadella* cf. *ovalis	**1**	**1**	**1**	**-**	**2**	**1**
Notommata pseudocerberus	1	1	1	-	-	-
Platyias quadricornis	2	1	1	0.0	9	-
Polyarthra dolichoptera	**4**	**2**	**2**	**29.7**	**497**	**2**
Rotaria neptunia (outgroup)	1	1	1	-	-	-
Squatinella mutica	3	1	1	0.0	-	-
Synchaeta oblonga	**5**	**3**	**3**	**21.5**	**22**	**3**
Synchaeta pectinata	**7**	**3**	**3**	**3.4**	**80**	**3**
Synchaeta sp.	1	1	1	-	-	-
Trichocerca pusilla	2	1	1	0.0	-	-
Trichocerca similis	10	3	3	39.0	-	-
Trichocerca sp.	2	2	2	20.9	-	-
Trichocerca stylata	**2**	**2**	**1**	**1.0**	**7**	**1**
Trichocerca tenuior	**1**	**1**	**1**	**-**	**1**	**1**
Trichotria tetractis	**1**	**1**	**1**	**-**	**6**	**1**
46 species in total	149	74	63		1517	27


Brachionus calyciflorus (327) China; Italy
Brachionus calyciflorus (61) China
Brachionus calyciflorus (28) Mexico; China; Canada
Trichocerca pusilla (2) NEW ZEALAND
Brachionus calyciflorus (222) China; USA; Italy
Brachionus calyciflorus (280) China; Mexico; USA
Brachionus calyciflorus B (1) NEW ZEALAND
Brachionus calyciflorus A (4) NEW ZEALAND
Brachionus calyciflorus (1) Australia
B. calyciflorus
Polyarthra dolichoptera (4) Italy
Brachionus quadridentatus B (2) NEW ZEALAND
Platyias quadricornis (8) Mexico
Brachionus quadridentatus cluniorbicularis (8) Mexico
Brachionus quadridentatus (3) NEW ZEALAND
Brachionus quadridentatus brevispinus (9) Mexico
Brachionus quadridentatus (8) Unknown location
Brachionus quadridentatus (5) Mexico
Brachionus quadridentatus brevispinus (9) Mexico
Brachionus calyciflorus (10) China
Brachionus quadridentatus (2) Spain
B. quadridentatus
Brachionus angularis A (3) NEW ZEALAND
Brachionus angularis B (3) NEW ZEALAND
Brachionus angularis (5) Mexico
Brachionus angularis pseudodolabratus (3) Mexico
B. angularis
Filinia novaezelandia (3) NEW ZEALAND
Filinia longiseta (4) NEW ZEALAND
Filinia cf. terminalis (2) NEW ZEALAND
Collotheca sp. B (1) NEW ZEALAND
Collotheca sp. A (2) NEW ZEALAND
Cupelopagis vorax (1) NEW ZEALAND
Hexarthra intermedia B (1) NEW ZEALAND
Hexarthra intermedia A (1) NEW ZEALAND
Hexarthra intermedia C (1) NEW ZEALAND
Conochilus unicornis (2) NEW ZEALAND
Trichocerca tenuior (1) USA
Lepadella cf. ovalis (2) Mexico
Lepadella cf. ovalis (1) NEW ZEALAND
Colurella uncinata (1) NEW ZEALAND
Keratella procurva A (1) NEW ZEALAND
Keratella procurva B (2) NEW ZEALAND
Keratella procurva C (1) NEW ZEALAND
Keratella procurva D (1) NEW ZEALAND
Keratella procurva E (1) NEW ZEALAND
Keratella tropica A (1) NEW ZEALAND
Keratella valga (1) NEW ZEALAND
Keratella tropica (1) Mexico
Keratella tropica B (1) NEW ZEALAND
Keratella tropica (2) Mexico
K. tropica
Brachionus budapestanensis (1) NEW ZEALAND
Lecane luna (12) Mexico
Platyias quadricornis (1) Mexico
Platyias quadricornis (2) NEW ZEALAND
Lecane closterocerca (4) Mexico
Euchlanis cf. deflexa (2) NEW ZEALAND
Trichocerca similis C (1) NEW ZEALAND
Trichocerca similis B (1) NEW ZEALAND
Trichocerca similis A (8) NEW ZEALAND
Squatinella mutica (3) NEW ZEALAND
Lecane bulla (2) Mexico
Lecane bulla (3) Mexico
Polyarthra dolichoptera (13) Italy
Polyarthra dolichoptera (156) Italy
Polyarthra dolichoptera (118) Italy
Polyarthra dolichoptera (20) Italy
Polyarthra dolichoptera (37) Italy
Polyarthra dolichoptera (19) Italy
Polyarthra dolichoptera (31) Italy
Polyarthra dolichoptera A (2) NEW ZEALAND
Polyarthra dolichoptera (4) Italy
Lecane ludwigii (3) NEW ZEALAND
Synchaeta sp. (1) NEW ZEALAND
Polyarthra dolichoptera (66) Italy
Polyarthra dolichoptera (3) Italy
Polyarthra dolichoptera B (2) NEW ZEALAND
Polyarthra dolichoptera (34) Italy
P. dolichoptera
Synchaeta pectinata (54) Unknown location
Synchaeta pectinata (16) Unknown location
Synchaeta pectinata (4) Unknown location
Synchaeta pectinata A (1) NEW ZEALAND
Synchaeta pectinata B (1) NEW ZEALAND
Synchaeta pectinata C (5) NEW ZEALAND
S. pectinata
Synchaeta cf. tremula/oblonga (10) Unknown location
Synchaeta cf. tremula/oblonga (2) Unknown location
Synchaeta oblonga B (1) NEW ZEALAND
Synchaeta oblonga A (2) NEW ZEALAND
Synchaeta cf. tremula/oblonga (4) Unknown location
Synchaeta oblonga C (2) NEW ZEALAND
Synchaeta cf. tremula/oblonga (6) Unknown location
Synchaeta pectinata (6) Unknown location
S. oblonga
Lecane lunaris (6) Mexico
Asplanchna priodonta C (3) NEW ZEALAND
Asplanchna priodonta A (2) NEW ZEALAND
Asplanchna priodonta B (2) NEW ZEALAND
Lecane lunaris A (1) NEW ZEALAND
Lecane lunaris B (1) NEW ZEALAND
Lecane lunaris (4) Mexico
L. lunaris
Lecane bulla (6) USA; Mexico
Lecane bulla (1) USA
Lecane bulla (1) Italy
Lecane bulla (1) Mexico
Lecane bulla (1) Unknown location
Lecane bulla C (1) NEW ZEALAND
Lecane decipiens (4) NEW ZEALAND
Lecane luna (1) NEW ZEALAND
Lecane closterocerca (1) NEW ZEALAND
Lecane bulla (4) USA
Lecane bulla B (3) NEW ZEALAND
Lecane hamata (2) Mexico
Asplanchna sieboldi (7) NEW ZEALAND
Lecane bulla A (2) NEW ZEALAND
L. bulla
Lecane hamata (2) NEW ZEALAND
Trichotria tetractis (1) NEW ZEALAND

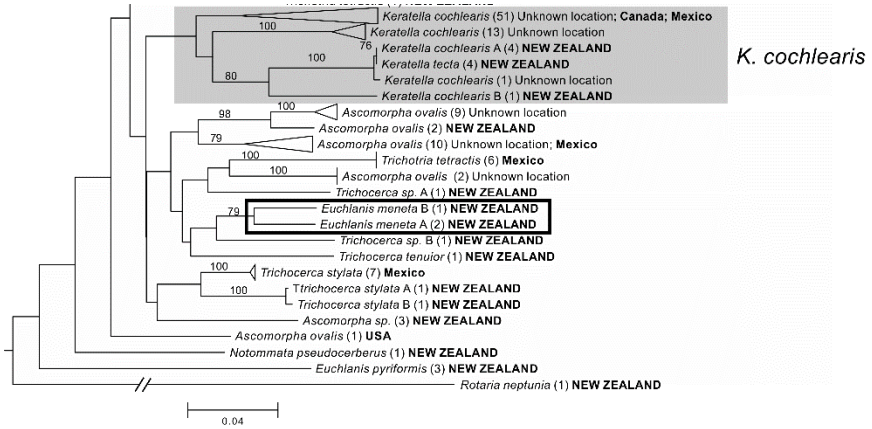


Fig. 3 NJ tree based on a 482 bp alignment of 1591 sequences comprised of the 45 New Zealand rotifer species, sequences available on GenBank for those species and the outgroup *Rotaria neptunia* (Bdelloidea). Large GenBank clusters are collapsed with triangles representative of collapsed diversity. Numbers of individuals are shown in parentheses. Grey boxes highlight cryptic taxa with GenBank comparisons and black borders highlight cryptic taxa for which no sequences were found on GenBank. Country of collection is bolded. Bootstrap support (1000 replicates) where >75% are indicated on branches.

DISCUSSION

We assessed the genetic diversity for 45 recognised species of New Zealand freshwater rotifer. COI sequences for 18 of the species were genetically divergent (6.6 – 22.4%) when compared with international conspecifics available from GenBank and BOLD. Furthermore, 14 of the New Zealand species were found to have high levels of intraspecific COI diversity (3.4 – 39.0%), suggestive of cryptic taxa among putatively cosmopolitan species. A total of 27 species had no available sequences on global databases (e.g., GenBank or BOLD) to enable a more thorough comparison. Of the 1517 GenBank and BOLD sequences included in this study, approximately 700 were from *Brachionus calyciflorus*, roughly 500 for *Polyarthra dolichoptera* and the remaining 300 represented a further 17 species. Overall, this meant that only 19 of the 45 species we sequenced could be compared with their international conspecifics. This is unfortunate, as several of these New Zealand species harboured high intraspecific diversity which enhances their potential to be distinct from those collected elsewhere. In the absence of more complete international data, we suggest that the potential for these taxa to be unique to New Zealand is high.

The occurrence of cryptic species among some of the highly divergent taxa we identified in New Zealand has also been reported from elsewhere (e.g., Gilbert & Walsh 2005). For example, we found two different sequences of *Brachionus calyciflorus* that are potentially endemic to New Zealand. Previous work has suggested that *B. calyciflorus* is a species complex and has reproductive isolation even at relatively small geographical scales (Gilbert & Walsh 2005; Li *et al*. 2010; Xiang *et al*. 2010; 2011). We found unique sequences for *Ascomorpha ovalis*, *Brachionus calyciflorus*, *Keratella cochlearis*, *Lecane bulla* and *L. lunaris*, all of

which have had cryptic species reported from Mexico (Walsh *et al*. 2009; Garcia-Morales & Elias-Gutierrez 2013).

Our COI sequences highlighted potential mis-identifications for *Keratella tecta* and *K. valga* as they both shared BINs and had low sequence divergences (<1%) with *K. cochlearis* and *K. tropica*, respectively. Indeed, there are inconsistencies within the taxonomic literature and records on GenBank for some rotifer taxa. For example, *K. tecta* is considered a subspecies of *K. cochlearis* in some cases (e.g., Derry *et al*. 2003), whereas others (e.g., Shiel *et al*. 2009) consider it as a separate species. The use of both genetic and morphological identifications may help resolve some of these taxonomic uncertainties.

The baseline data and COI reference library that we have begun to assemble here will also facilitate the use of next generation sequencing platforms for community-level analyses by detecting rotifer species present in environmental samples. The current methods for the routine assessment of rotifer species' diversity are time-consuming and rely on morphological identification. By assessing rotifer communities at a molecular level, it may be possible to enhance taxonomic resolution and provide rapid information about the health of ecosystems as well as gauge the progress and success of restoration efforts. It is also likely to provide data to enable the detection of non-native species. Two major zooplankton groups (Copepoda and Cladocera) are now well represented in New Zealand by non-indigenous species, and it is likely that rotifers are similar. For example, the North American copepod *Skistodiaptomus pallidus*, the Japanese *Sinodiaptomus valkanovi* as well as the Australian *Boeckella minuta* and *B. symmetrica*, are all present within New Zealand waters (Banks & Duggan 2009; Makino *et al*. 2010; Duggan *et al*. 2014). Non-indigenous cladocerans present in

New Zealand waters include *Daphnia pulex* and *D. galeata* (Duggan *et al*. 2006; 2012).

The geographical and reproductive isolation of New Zealand taxa has likely promoted the genetic divergence of rotifers found in our study. For example, isolation-by-distance has been reported within the *Brachionus plicatilis* species complex, with cryptic diversity at local (Gomez *et al*. 2002; 2007) and global (Mills *et al*. 2007) scales. Similarly, bdelloid species on different continents were found to be genetically distinct (Fontaneto *et al*. 2008). Indeed, allopatric speciation as a result of geographical isolation is a well-known concept (Lande 1980). It is also likely that the currently reported high cosmopolitanism and low endemicity in rotifers is due to the lack of thorough genetic diversity assessments. When assessed in greater detail, it is likely that further instances of endemicity will be found (Shiel & Green 1996; Segers 2008; Shiel *et al*. 2009). The identification of genetic diversity within and among natural populations will ultimately enhance our global understanding of phylogeographical patterns. We suggest that the COI gene locus will be particularly helpful in this regard.

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Chapter III

Temperature-related activity of *Gomphiocephalus hodgsoni* (Collembola) mitochondrial DNA (COI) haplotypes in Taylor Valley, Antarctica*

*Submitted under the same title, as: Collins GE and Hogg ID

ABSTRACT

Gomphiocephalus hodgsoni (Collembola) is the most common and widely distributed arthropod in the Dry Valleys of Southern Victoria Land, and is genetically diverse with over 70 mitochondrial cytochrome *c* oxidase subunit I (COI) haplotypes. There is also considerable physiological variation among *G. hodgsoni* individuals in their cold tolerance and metabolic activity. Here, we assessed genetic haplotypes of *G. hodgsoni* relative to the environmental conditions during which individuals were active. We sequenced 151 individuals collected in pitfall traps from three sites within Taylor Valley and found 19 unique COI haplotypes that separated into two distinct groups (1.6% divergence), with one haplotype group comprising 80% of the sequenced population. During two-hourly sampling air temperature was the strongest predictor of activity between the two haplotype groups (R^2 = 0.56) and when combined with subsurface soil temperature, relative humidity and photosynthetically active radiation, explanatory power increased to R^2 = 0.71. With steadily increasing air temperatures predicted for much of Antarctica, it is likely that some haplotypes will have a selective advantage and potentially result in decreased genetic variability within populations. We suggest that temporal monitoring of the relative proportions of COI haplotypes or other appropriate genetic markers may provide a subtle measure of biological responses to environmental changes within Antarctic terrestrial ecosystems.

INTRODUCTION

Current environmental changes in Antarctica include cooling in Eastern Antarctica and rapid warming in parts of Western Antarctica, driving an overall warming air temperature trend for the continent (Steig et al. 2009). Air temperatures in the Antarctic Peninsula are warming at one of the fastest rates in the world with a steady 0.56°C increase per decade and are now approximately 2.5°C warmer than 50 years ago (Vaughn et al. 2003; Turner et al. 2005). More recent data have also shown that warming has been occurring over much of Western Antarctica including the Ross Sea Region (Steig et al. 2009).

Steadily increasing temperatures along with other similar "press" perturbations (*sensu* Bender et al. 1984) are likely to cause considerable disruption to Antarctic terrestrial ecosystems (Convey 2011; Hogg and Wall 2011; Smith et al. 2012). Ecosystem responses may include shifts in population densities and distributions as well as the establishment of new or invasive species potentially filling new niches, enhancing trophic complexity and increasing diversity (see Convey 2011; Nielsen and Wall 2013 for reviews). There are also likely to be more subtle responses at the individual level including behavioural, physiological and genetical changes. Behavioural responses may include shifting of preferred microhabitats to more sheltered areas such as under rocks or deeper in the soil profile. Physiologically, more energy may be available for foraging and reproduction activities and less energy required for cold tolerance strategies (Convey 2011). From a genetic perspective, elevated temperatures may provide a selective advantage for some individuals while others may decline or become extinct resulting in an overall decrease in genetic diversity. This, in turn, could decrease the evolutionary potential of populations to respond to concomitant or future environmental changes (Reed and Frankham 2002).

Collembola (springtails) have among the narrowest environmental tolerances of Antarctic terrestrial animals making them sensitive to changes in environmental conditions such as temperature, humidity and pollutants (Hopkin 1997; Kohler et al. 1999). In many cases, they are effectively living at their physiological limits and any environmental changes are expected to have a detectable effect on their dispersal and reproductive success (Sinclair and Stevens 2006). The distribution of Antarctic springtails is restricted to ice-free areas which encompass roughly 0.3% of the continent, most of which lies within the Dry Valleys of Southern Victoria Land (Hopkins et al. 2006). The lack of available habitat, combined with landscape features such as glaciers and mountain ranges, limits the distribution and gene flow between springtail populations (Stevens and Hogg 2002; 2003; Hawes 2011). The availability of liquid water further restricts springtail distribution to habitats such as the edge of ponds and streams (Tilbrook 1967; Kennedy 1993).

Here, we focus on *Gomphiocephalus hodgsoni* as it is the most widely distributed springtail taxon within Southern Victoria Land (Wise 1967) and is also among the most intensively studied (McGaughran et al. 2011). While *G. hodgsoni* can remain active at temperatures near freezing, there is considerable variation in their survival of colder temperatures. Supercooling points (SCP) can provide an accurate indication of cold tolerance (e.g. Cannon and Block 1988), and this has formed the basis of several studies focussing on *G. hodgsoni* (Janetschek 1963; Fitzsimons 1971; Block 1985; Sinclair and Sjursen 2001). The distribution of SCPs within *G. hodgsoni* populations is bimodal (Sinclair and Sjursen 2001) and this could be important in a warming environment as individuals with high SCPs (less cold tolerant) may be more active in an extended summer season and therefore have a selective advantage over those with low SCPs.

In order to test whether genetic variation is related to the activity of individuals under different environmental conditions, we examined variability in mitochondrial cytochrome *c* oxidase subunit I (COI) sequences of individuals relative to the environmental parameters during which they were captured. We targeted the COI gene as it is part of critical energy pathways within all eukaryotes and is widely used for species identification (Hebert et al. 2003). Previous studies have also shown that COI is sensitive to environmental changes (e.g. Pichaud et al. 2012), and has been linked, albeit weakly, to metabolic rates in *G. hodgsoni* (McGaughran et al. 2010). Accordingly, our aim was to determine whether temporal patterns of activity were influenced by genetic variation in mitochondrial COI haplotypes. Using these data, we tested the hypothesis that the temporal activity of *G. hodgsoni* COI haplotypes was influenced by environmental factors including subsurface soil temperature, air temperature, relative humidity and photosynthetically active radiation.

MATERIALS AND METHODS

Individuals of *G. hodgsoni* were collected from three sites around the shoreline of Spaulding Pond (S 77° 39.517' E 163° 06.485'), Taylor Valley, in Southern Victoria Land from 12-26 January 2014 (Fig. 1). This location is known to harbour high levels of allozyme and COI haplotype diversity, including two sympatric COI haplotype groups (Stevens and Hogg 2003; Nolan et al. 2006). Sampling sites were chosen to maximise collection of this genetic diversity with Site 1 positioned at the Glacier meltwater inflow, Site 2 on the northern shoreline and Site 3 at the pond outflow.

Miniaturised pitfall traps (4.5cm diameter, 6 cm depth; McGaughran et al. 2011) were used to capture active springtails. As inactive individuals are normally found under rocks or in the soil, traps will collect only those individuals active on the soil surface. Traps were flush with the ground and consisted of an outer PVC sleeve and an inner container partially filled with glycerol (1 cm depth) to prevent subsequent escape of animals. When traps were cleared, inner containers were immediately replaced with another container. Contents were examined under the microscope in the field and individuals were isolated and immediately preserved in 70% ethanol. Springtails were then transferred to 95% ethanol upon arrival in New Zealand.

To assess temporal activity, Sites 1 and 2 each initially consisted of six pitfall traps, six iButtons (DS1921; Dallas Semiconductors, Dallas, USA) and one temperature/light data logger (Hobo; OneTemp Pty. Ltd., Adelaide, Australia). The Hobo loggers measured photosynthetically active radiation (PAR) and air temperature (T_{air}) at each site, while iButtons located by each pitfall trap measured soil temperature 1 cm below the surface (T_{soil}), and one iButton per site also

measured relative humidity (RH). Traps were cleared every two hours from 0700 to 2300 during 13-17 January 2014, encompassing three 24h periods as an intensive sampling regime. To ensure a more thorough assessment of COI variability at Spaulding Pond, two traps each from Sites 1 and 2 were then removed and used for Site 3, resulting in four traps, four iButtons and one PAR detector at each of the three sites for the remaining sampling duration, on a 12-hourly schedule with traps cleared at 0900 and 2100. All samples taken from Site 1 were on the hour, 15 minutes past the hour for Site 2, 30 minutes past the hour for Site 3 to allow for travel between sites.

DNA extractions, PCR amplifications and sequencing were jointly carried out at the University of Waikato and at the Canadian Centre for DNA Barcoding (CCDB) at the University of Guelph. At the CCDB, total DNA was extracted from specimens following a glass fibre plate method (AcroPrep) (Ivanova et al. 2006) or using a Red Extract n Amp kit (Sigma-Aldrich) at the University of Waikato. A 658 bp region of the COI gene was amplified using the universal primers HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') (Folmer et al. 1994) at the University of Waikato, and the primers LepF1 (5'-ATTCAACCAATCATAAAGATATTGG-3') and LepR1 (5'-TAAACTTCTGGATGTCCAAAAAATCA-3') (Ratnasingham and Hebert 2007) at CCDB. At the University of Waikato, PCR amplifications for each specimen were carried out in 15 µL volumes containing 7.5 µL PCR master mix solution (i-Taq) (Intron Biotechnology), 0.2 µM (0.3 µL) of each primer and 1 µL of DNA extract (unquantified). Thermal cycling conditions were: 94ºC for 5 min followed by 5 cycles (94ºC for 1 min, 48ºC for 1.5 min and 72ºC for 1 min) then 35 cycles (94ºC for 1 min, 52ºC for 1.5 min and 72ºC for 1 min) of denaturation and

polymerase amplification, with a final elongation at 72ºC for 5 min. At CCDB, thermal cycling conditions were: 94ºC for 1 min followed by 5 cycles (94ºC for 1 min, 45ºC for 1.5 min and 72ºC for 1.5 min) then 35 cycles (94ºC for 1 min, 50ºC for 1.5 min and 72ºC for 1 min) of denaturation and polymerase amplification, with a final elongation at 72ºC for 5 min. Successful amplification products were cleaned with 0.1 μL ExonucleaseI (EXO) (10 U/μL) and 0.2 μL Shrimp Alkaline Phosphate (SAP) (1 U/μL) (Illustra from GE Healthcare) at 37°C for 30 min then 80°C for 15 min at the University of Waikato, or Sephadex at CCDB. Sequencing was carried out in forward and reverse directions using an ABI 3130 at the University of Waikato, or an ABI 3730x1 sequencer at CCDB.

Sequences were verified as *G. hodgsoni* using the GenBank BLASTn algorithm then were aligned using MUSCLE as implemented in Geneious v6.1.2 (Drummond et al. 2010) and trimmed to exclude primer regions. Sequences were further trimmed to remove ambiguous or missing characters and the resulting 632 bp alignment was used for all further analyses. No insertion, deletion or stop codon was detected, indicating that only mitochondrial DNA was amplified. All haplotypes were verified by visually inspecting original traces. Sequences were aligned with the two complete mitochondrial genomes of *G. hodgsoni* available on GenBank (Accession numbers: AY191995 and NC_005438) to ascertain the reading frame for indicating any amino acid changes. Chi-square tests (χ^2) as implemented in PAUP* 4.0 (Swofford 2002) were used to determine whether base frequencies were equal among all sites, parsimonious-informative sites, and third codon positions for all sequences. Duplicate sequences were removed and representative sequences for each of the 19 unique haplotypes were used for phylogenetic analyses. A Neighbour Joining tree was generated in MEGA v5.2.2

(Tamura et al. 2011) with 1000 bootstrap replicates. A pairwise genetic distance matrix was also generated in MEGA v5.2.2.

Statistical analyses were performed using Primer v6 (Clarke and Gorley 2006). Springtails were pooled for each sampling site owing to the low number of individuals collected per pitfall trap. COI haplotype abundances were arranged in three formats to include: 1) all haplotypes Gh1 to Gh19; 2) the two main haplotypes Gh1 and Gh13 only; and 3) all haplotypes separated into two main phylogenetic groups (Gh1-12 and Gh13-19). Environmental data consisted of subsurface soil temperature (T_{soil}), relative humidity (RH), photosynthetically active radiation (PAR) and air temperature (T_{air}) measurements logged at 10-minute intervals. Values for each sampling time were averaged at each of the three sites and then used for statistical analyses such that haplotypes could be associated with the environmental conditions during collection.

RH and PAR values were log(x) transformed to correct for an asymmetrical distribution. All environmental data were then normalised so that the four variables were directly comparable, and a resemblance matrix was generated using Euclidean distance. The three formats of biological data were all square root transformed to minimise the influence of rare haplotypes. Resemblance matrices were then generated using Bray-Curtis similarity, with a dummy value of 1.0 due to high proportion of zeros in the haplotype abundance dataset.

Multidimensional scaling (MDS) and principle coordinate ordination (PCO) analyses were performed on four resemblance matrices (3 biological; 1 environmental). The three biological matrices were compared with the environmental matrix using the RELATE (Spearman) function in Primer, where Rho values closest to 1 indicate greater similarities. The biological matrix with the

Rho value closest to 1 was run through a distance-based linear model (DistLM) against environmental data using BEST (AICc) function in Primer. The DistLM examines the variation in communities to identify which environmental parameters are the strongest drivers of haplotype activity based on R^2 values.

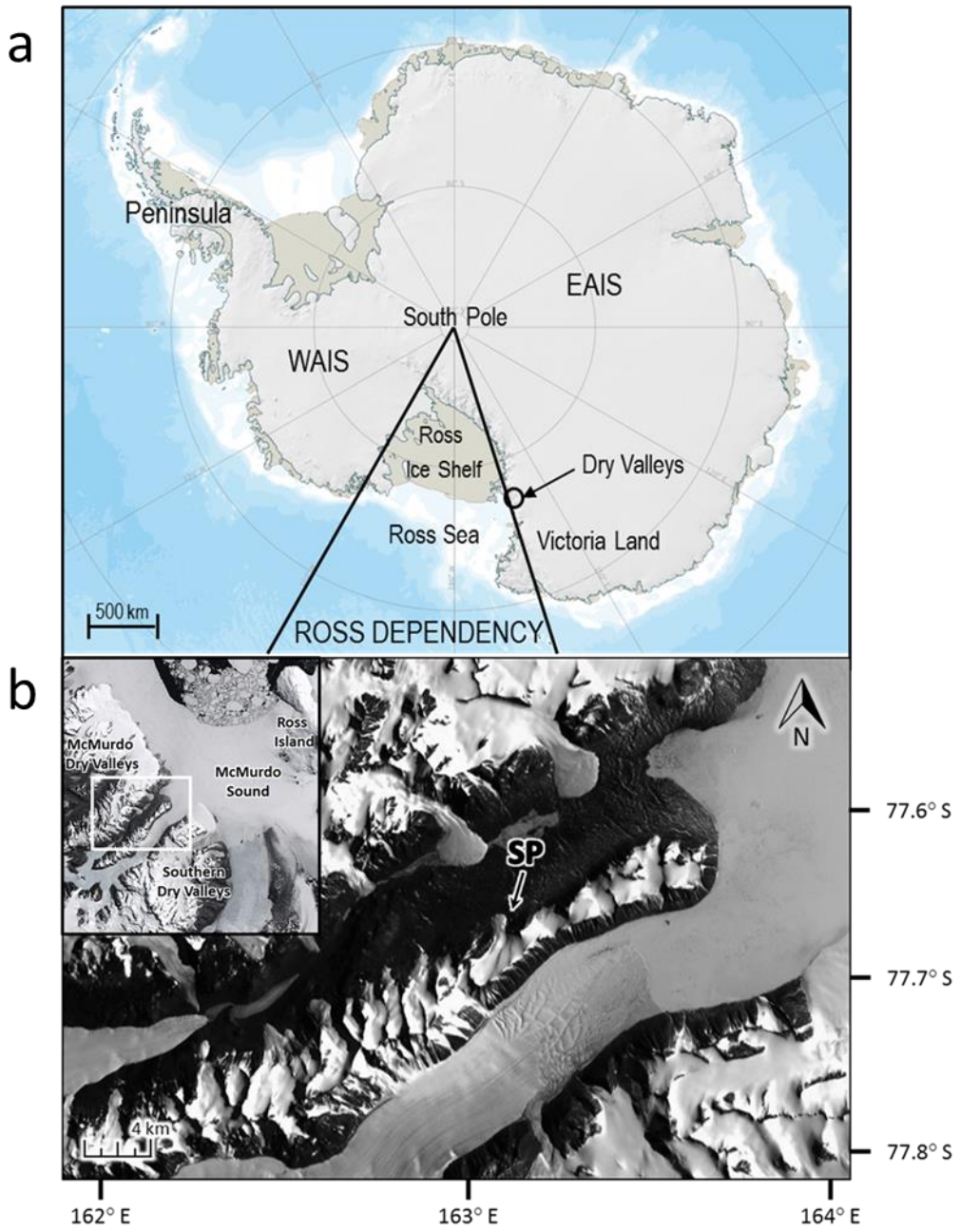

Fig. 1 a) Antarctica, showing the location of the study area within the Ross Sea Region of the Ross Dependency. The Western and Eastern Antarctic Ice Sheets (WAIS and EAIS, respectively) are also shown. Modified from the Antarctic Digital Database v6.0, British Antarctic Survey (http://www.add.scar.org/home/add6); b) Location of Taylor Valley (inset) and Spaulding Pond (SP) in Southern Victoria Land.

RESULTS

A total of 178 live springtails were captured in 12 pitfall traps across three sites at Spaulding Pond, Taylor Valley during a two-week period in January 2014. No springtails were captured between 2300 and 0700 throughout the 2-hourly sampling at Sites 1 and 2 during which time air temperatures were below 2.2°C and 6.9°C, respectively (Fig. 2). More springtails were captured at Site 1 than Site 2 (Fig. 2), although Site 2 was generally warmer than Site 1, especially at the subsurface level (Fig. 3). Light, soil and air temperatures were co-variables, whereas humidity showed an inverse relationship (Fig. 4).

Using a 632 bp alignment of 151 *G. hodgsoni* COI sequences, nucleotide composition across all positions was biased for A-T at 64.2% (A = 27.7%, T = 36.5%, G = 16.9%, C = 18.9%). Base frequencies were homogeneous across sequences for all sites (χ^2_{456} = 6.08, p = 1.00), for parsimony informative sites (A-T = 53.1%; χ^2_{456} = 306.21, p = 1.00) and for third codon positions (A-T = 84.3%; χ^2_{456} = 49.48, p = 1.00). Of the 632 bp analysed, 11 sites were parsimony informative, 13 of the variable sites were parsimony uninformative and the remaining 608 sites were constant. We found 19 haplotypes among the 151 individuals analysed. Fourteen of these haplotypes were found at Sites 1 and 2 with the additional five found only at Site 3. Six amino acid changes occurred as a result of parsimony uninformative variation only.

Neighbour-joining (NJ) analyses of representative haplotype sequences separated the 19 haplotypes into two main X (Gh13-19) and Y (Gh1-12) groups (1.6% divergence), with strong bootstrap support (Fig. 5). The main group of 120 individuals with the haplotypes Gh1 to Gh12 (0.3% divergence) made up 80% of the sequenced population and the remaining 31 individuals with the haplotypes

Gh13 to Gh19 (0.5% divergence) made up the remainder. There were 9 mutational steps between the main Gh1 and Gh13 haplotypes, with 6 of those haplotype mutations not found in our samples (haplotype network not shown).

COI haplotypes versus environmental parameters

A significant correlation was found at Site 1 between the environmental and biological matrices when haplotypes were merged into their respective X or Y groups (RELATE (dummy = 1.0): Rho = 0.402; $p < 0.001$). Multi-dimensional scaling (MDS) analyses with overlaying vectors also show the close relationship between co-variables T_{soil}, T_{air} and PAR, whereas RH had an inverse relationship (Fig. 6). During conditions to the right of the MDS ordination (between 2300 and 0700 daily; Fig. 6A), no springtails were collected (Fig. 6B). The biological resemblance matrix for haplotypes merged as X and Y had the greatest Rho value (Rho = 0.402; $p < 0.001$) and was used for the DistLM analysis. T_{air} alone explained 56% of the variation in haplotype abundance ($R^2 = 0.563$; $p < 0.001$). Explanatory power gradually increased with each additional variable, and with all variables together explained 71% of the haplotype activity ($R^2 = 0.716$; $p < 0.001$).

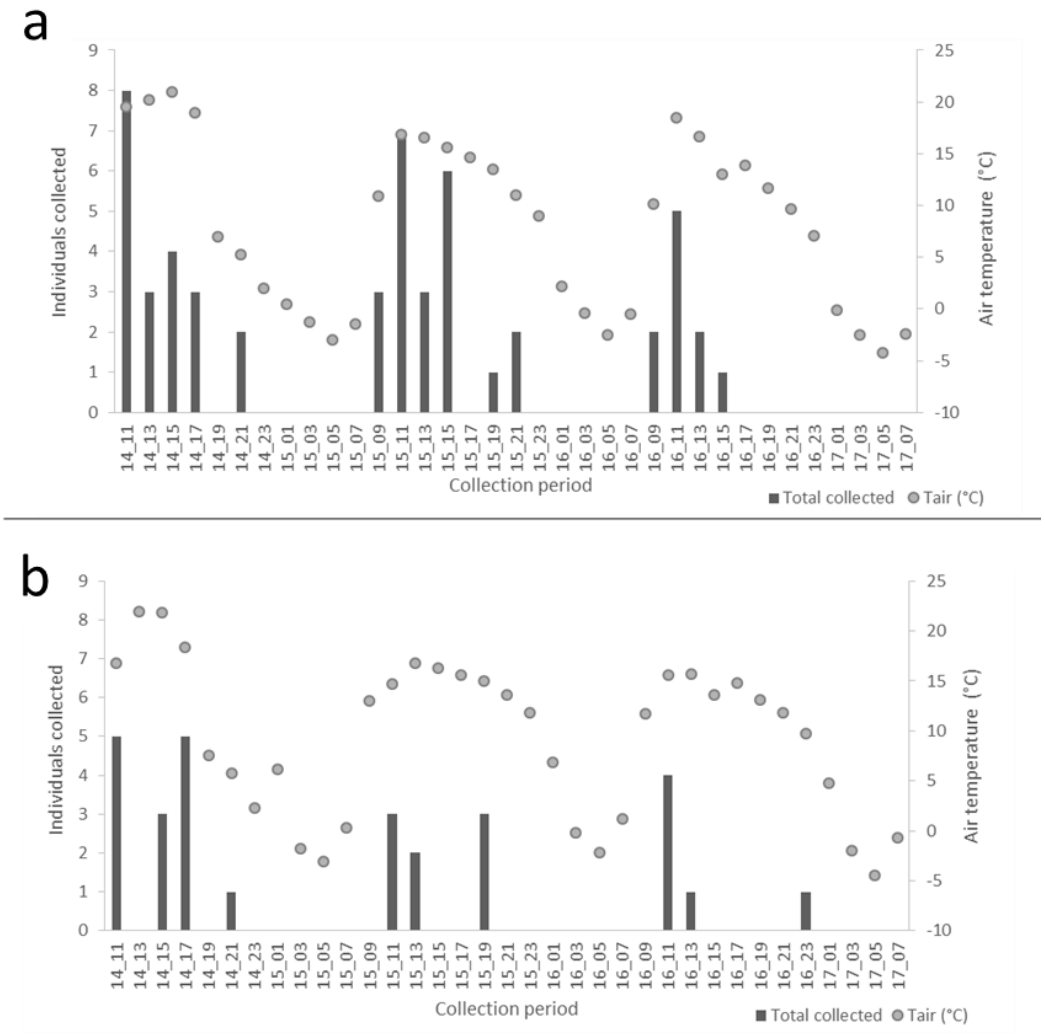

Fig. 2 Number of *Gomphiocephalus hodgsoni* individuals collected and mean air temperatures at: a) Site 1; and b) Site 2. Collections were made every two hours during January 2014 near Spaulding Pond, Taylor Valley, Antarctica.

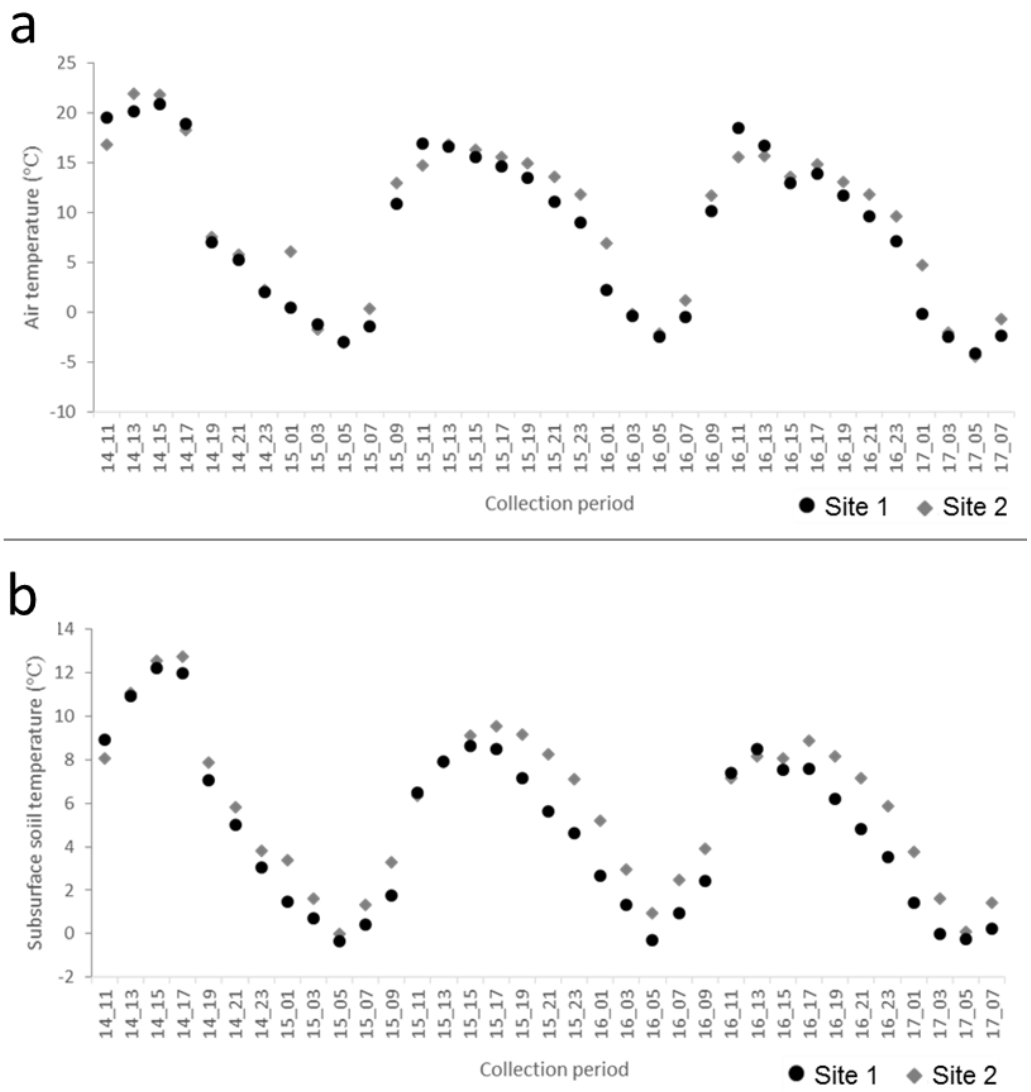

Fig. 3 a) Air temperature (Tair); and b) subsurface soil temperature (Tsoil) measurements at Sites 1 and 2 near Spaulding Pond, Taylor Valley. Measurements were taken every two hours in January 2014.

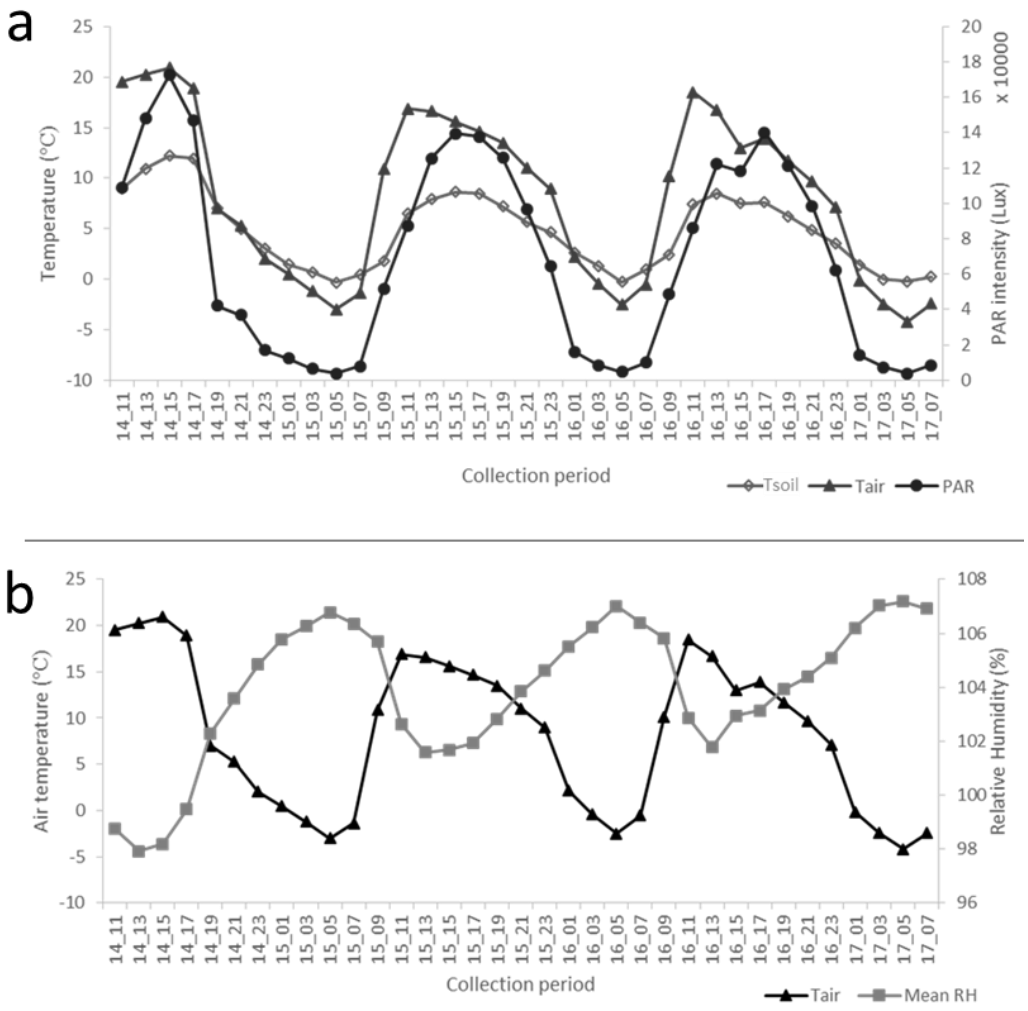

Fig. 4 Subsurface soil temperature (T_{soil}), air temperature (T_{air}), relative humidity (RH) and photosynthetically active radiation (PAR) measurements taken every two hours at Site 1, Spaulding Pond, Taylor Valley (January 2014).

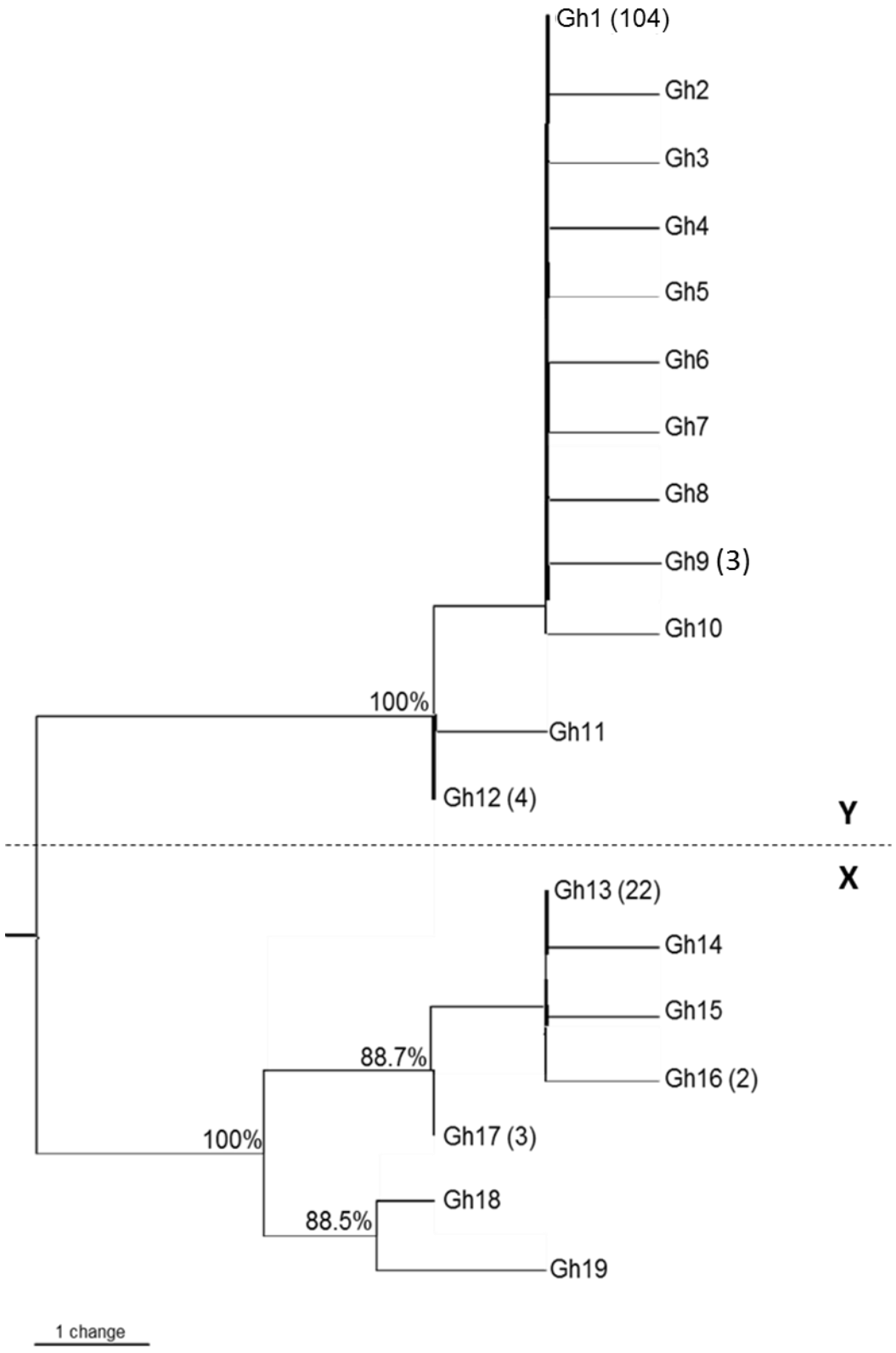

Fig. 5 Neighbour-joining tree for 19 COI haplotypes from the 151 *G. hodgsoni* individuals, showing the two main groups (X and Y). The number of individuals for each haplotype when >1 are shown in parentheses. Bootstrap confidence limits (1000 replicates) greater than 75% are shown at nodes.

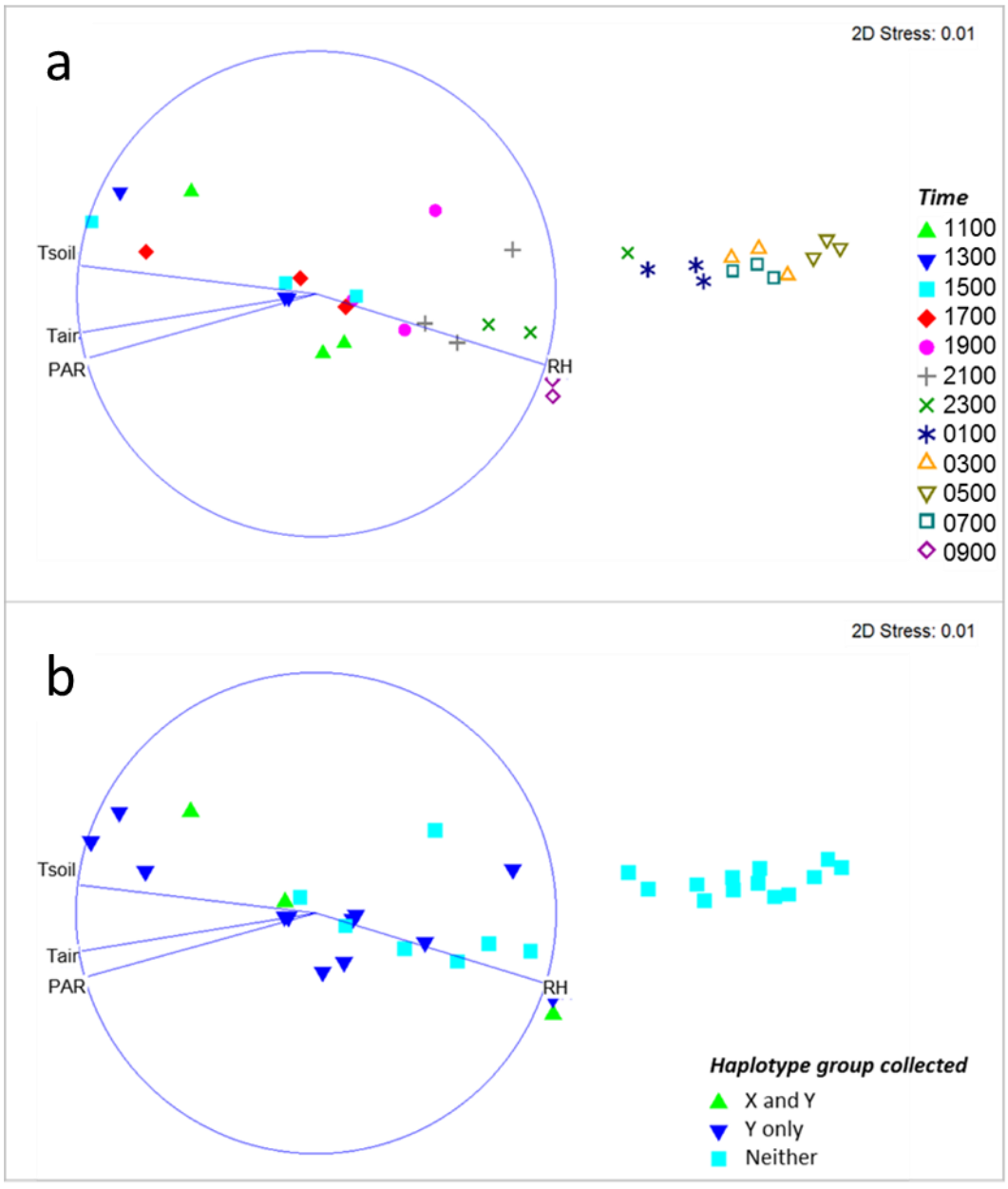

Fig. 6 Multidimensional scaling ordination of environmental data with overlaying vectors from 2-hourly sampling at Sites 1 and 2 near Spaulding Pond, Taylor Valley. Data are labelled by a) time; and b) haplotype grouping.

DISCUSSION

During our 2-hourly sampling, no springtails were captured during 'night' conditions (between 2300 and 0700), suggesting that environmental conditions were influencing activity. This was expected as temperature and the availability of liquid water are known drivers of activity in Antarctic terrestrial systems (Hogg et al. 2006). We also found that air temperature was strongly correlated with activity of different haplotypes at Site 1.

The 19 COI haplotypes we obtained from Spaulding Pond, Taylor Valley, were clustered into two distinct groups (1.6% divergence) and aligned with the X and Y groups described by Nolan et al. (2006), where haplotypes Gh1 (n=104) and Gh13 (n=22) were identical to the Nolan et al. (2006) haplotypes I and A, respectively. Nolan et al. (2006) suggested that the Y group haplotypes were likely to have originated from the upper Taylor Valley vicinity whereas X group haplotypes were sourced from along the Ross Sea coastline. These two sympatric groups were suggested by Nolan et al. (2006) to have accumulated their mutations while geographically isolated by Glacial Lake Washburn approximately 40,000 years ago (Denton and Hughes 2000; Denton and Marchant 2000). Indeed, the survival of isolated refugial populations during glacial periods followed by recolonization from surrounding areas is well known for a range of taxa (e.g. Stevens and Hogg 2003; Green et al. 2011; Fraser et al. 2014).

We identified six missing mutational steps between individuals in the distinct X and Y haplotype groups. As we sequenced 151 individuals, the probability of finding additional haplotypes was high. We therefore support previous suggestions (Stevens and Hogg 2003; Nolan et al. 2006) of two sympatric and possibly reproductively isolated populations in the vicinity of Spaulding Pond.

We also found much higher COI diversity at Spaulding Pond than previously recorded (Stevens and Hogg 2003; Nolan et al. 2006) most likely due to our more intensive and localised sampling effort. However, this does highlight the need for more comprehensive sampling in the future to accurately reflect genetic diversity at each site and to detect any changes in population genetic structure. Indeed, the addition of a third sampling site (Site 3) provided an additional five haplotypes not recorded at our primary sampling locations.

We found a dominance of the upper Taylor Valley Y group haplotypes (Gh1-12) comprising 80% of the sequenced individuals. Conditions in the upper Valley where Y group haplotypes originated are likely to have been colder than the lower Taylor Valley, due to the difference in elevation. If true, then the Spaulding Pond area is presently dominated by intrinsically cold-adapted individuals. If these individuals currently have a selective advantage at Spaulding Pond, then in a progressively warming environment we would predict a shift in dominance from Upper Valley Y group haplotypes to Lower Valley X group haplotypes. In particular, if variation in mitochondrial (COI) gene sequences is associated with activity under certain environmental conditions as we suggest, then the dominance of one haplotype group within *G. hodgsoni* populations at Spaulding Pond would be expected. The ability to detect such genetic shifts within natural populations will be essential for determining subtle biological responses to environmental changes prior to large-scale responses such as range expansion or an increase in abundance.

We conclude that temperature is a strong driver of springtail activity for the two haplotype groups found in Taylor Valley and is likely to influence the genetic structure of populations with predicted temperature increases. We suggest that

COI sequences for *G. hodgsoni* may provide an appropriately sensitive genetic indicator for monitoring the response of biological systems to temperature changes in Antarctica. Specifically, the annual monitoring of haplotype proportions within *G. hodgsoni* populations could be used to detect subtle ecosystem changes in response to a warming environment. This approach could also contribute to developing monitoring programs such as the McMurdo Terrestrial Observation Network (Levy et al. 2013). In order to more thoroughly examine the effects of environmental changes on the genetic structure of populations, future studies could focus on examining COI haplotype activity at a seasonal scale with the potential for screening additional nuclear or mitochondrial genetic markers to improve our understanding of population dynamics and biological responses to climate changes.

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Chapter IV

Thesis Summary and Conclusions

In this thesis, I analysed variation in the COI sequences of New Zealand freshwater rotifers and the Antarctic terrestrial springtail *Gomphiocephalus hodgsoni*. High intraspecific variation among several rotifer species was suggestive of cryptic variants unique to New Zealand (Chapter II), while *G. hodgsoni* haplotypes exhibited temperature-related activity (Chapter III). Together, these results emphasise the high level of hidden diversity among species, while demonstrating the potential for this diversity to align with environmental factors. Ultimately, this new information will provide the tools for monitoring responses of both aquatic and terrestrial biotic communities to a changing environment, through the use of rotifers as a measure of water quality and springtails to detect the effects of climate change.

In Chapter II, I identified high intraspecific COI diversity (3.4 – 39.0%) in 14 morphologically-recognised rotifer species from New Zealand. Based on comparisons with available sequences on GenBank, 27 sequences from 18 species were found to be distinct (>6.6% divergence), indicating potential cryptic speciation among putatively cosmopolitan species. This potential endemic diversity has most likely resulted from the accumulation of allopatric mutations, as New Zealand is geographically isolated and contains a relatively high proportion of other endemic taxa (Cooper and Millener 1993). Furthermore, isolation by distance is known to occur within rotifers (Gomez et al. 2002; 2007; Mills et al. 2007).

Of the potentially cryptic species I identified, *Ascomorpha ovalis*, *Brachionus calyciflorus*, *Keratella cochlearis*, *Lecane bulla* and *L. lunaris* have been previously recognised as genetically variant (Gilbert and Walsh 2005; Walsh et al. 2009; Li et al. 2010; Xiang et al. 2010; 2011; Garcia-Morales and Elias-Gutierrez

2013). However, the bulk of publically-available rotifer COI sequences is limited to a few species and species complexes, notably *Brachionus plicatilis* and *Brachionus calyciflorus*. Furthermore, sampling intensity is a major factor affecting our understanding of phylogeography (e.g. Fontaneto et al. 2012). This adds difficulty in identifying which genetic variants may be unique to New Zealand as many were lacking international comparison. As such, we encourage international efforts to assess the COI diversity of this phylum in order to improve our understanding of its biodiversity.

I conclude that COI is an appropriate genetic marker for the rapid identification of rotifer species and provides additional information that is useful to understand more about New Zealand's endemic variants. The rotifer database developed in this research project will also be useful in applications such as detecting non-indigenous species' incursions, and as a reference library for future next generation sequencing of rotifers as a rapid reflection of the health of New Zealand's freshwater ecosystems.

In Chapter III, I associated the activity of *G. hodgsoni* COI haplotypes (as collected in pitfall traps) at Spaulding Pond in Taylor Valley, Antarctica with the environmental conditions during which individuals were active. Haplotype diversity at Spaulding Pond was known to be high and two main phylogenetic groups were found at this site. The two haplogroups are likely to have evolved allopatrically and accumulated mutations due to geographical isolation. Their present sympatric occurrence would have been the result of recolonization of the Valley post-glacial maximum (Stevens and Hogg 2003; Nolan et al. 2006). This species is also known to harbour considerable variation at an individual level in their tolerance to cold (Sinclair and Sjursen 2001; McGaughran et al. 2011). By

combining these two genetical and physiological aspects, we were able to show that the two haplogroups have differing responses to temperature. Air temperature was able to explain 56% of the variation in haplotype activity, while explanatory power improved when air temperature, soil temperature, light, and relative humidity were combined ($R^2 = 0.71$).

I also showed that one haplogroup is currently dominating, at 80% of the sequenced population. This dominant haplogroup is thought to have evolved in the Upper Taylor Valley area (Nolan et al. 2006), where conditions were probably colder than in the Lower Valley area where the other haplotype group evolved. If these hypotheses are true, then we may expect to see a future shift in dominance from the intrinsically cold-adapted group to the warm-adapted group in response to increasing temperatures as predicted for most of Antarctica.

I conclude that COI is a sensitive genetic marker suitable for detecting the response of *G. hodgsoni* populations to changing environmental conditions. By monitoring the COI haplotype diversity and abundance within Antarctic springtail populations over time, we may have the ability to detect a biotic response to climate change as an early warning system as to how other biota may be affected.

FUTURE RESEARCH

The assessment of genetic diversity has been undertaken over the past few decades for a range of species globally (e.g. Vrijenhoek 1997). However, with the increased access to molecular techniques and initiatives such as DNA barcoding (e.g. Hebert et al. 2004; Lefébure et al. 2006; Bucklin et al. 2011), the acquisition of such data has increased at an unprecedented rate. New Zealand rotifers remain an understudied group with most work focused at the morphological level (e.g. Duggan 2008; Shiel et al. 2009). The data that I have begun to assemble (Chapter II) have revealed hidden diversity and local endemism. This highlights the need for continual investigation of mtDNA variation among rotifer populations in New Zealand, as well as overseas, to continue developing an international database for this phylum and to more thoroughly examine global phylogeographic patterns. The BOLD database is particularly useful as it includes a greater range of supplementary information and will aid in international comparisons. At a local scale, the COI sequencing for important bioindicator species in New Zealand (*sensu* Duggan et al. 2001) will enable the application of next generation sequencing techniques. A further refinement of the current taxonomic list based on the additional genetic information obtained is also now possible. Continual development of the global database for biodiversity assessment will be particularly relevant and as sequencing costs continue to decrease in the foreseeable future.

Physiological variation among Antarctic springtails has previously been investigated (e.g. Block 1985; Cannon and Block 1988; Sinclair and Sjursen 2001; McGaughran et al. 2010a; 2011) and studies have also assessed COI sequence variation in *Gomphiocephalus hodgsoni* specifically (Stevens and Hogg 2003; Nolan et al. 2006; McGaughran et al. 2008; 2010b). While this has provided a

baseline understanding of population diversity, findings in Chapter III show that there is also a link between genetic variability and physiological response to environmental conditions for *G. hodgsoni*. However, we are still at the embryonic stage of understanding the response of natural springtail populations in Antarctica to climate changes and there are certainly profitable avenues for future research. Of most relevance to the study undertaken in this thesis, a seasonal assessment of haplotype activity with associated environmental parameters would be informative to understand the broader–scale implications of how these populations are influenced by a suite of environmental variables. Vertical migration is known to occur in some springtail species (e.g. *Isotoma klovstadi*; Sinclair et al. 2003) and this could be assessed for *G. hodgsoni* as it would be of particular interest to see if there is depth stratification of the different haplotypes. Laboratory experiments could be carried out with live springtails, for example monitoring vertical migration of tagged individuals in core samples, or monitoring activity of individuals in a simulation situation where temperature can be controlled. Additional mitochondrial or nuclear genes could be assessed through either Sanger or NGS platforms for existing DNA extracts to utilise existing specimens. Once we have a basic understanding of how environmental factors influence the distribution and genetic structure of Antarctic populations, we can then better detect subtle changes in response to the climate and provide predictions for the future.

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APPENDICES

Appendix 1 Supporting information for 149 rotifer specimens sequenced.

Species	Haplotype	Individuals	BINs	NZPL numbers
Ascomorpha ovalis	-	2	BOLD:ABV4107	857, 858
Ascomorpha sp.	-	3	BOLD:ACG5495	992, 993, 994
Asplanchna priodonta	A	2	BOLD:AAZ3168	880, 916
Asplanchna priodonta	B	2		846, 847
Asplanchna priodonta	C	3		859, 860, 861, 879
Asplanchna sieboldi	A	6	BOLD:AAV0423	563, 564, 945, 952, 953, 1103
Asplanchna sieboldi	B	1		545
Brachionus angularis	A	3	BOLD:ABU8353	662, 906, 907
Brachionus angularis	B	3	BOLD:AAZ3874	800, 801, 883
Brachionus budapestanensis	-	1	BOLD:ACG5388	987
Brachionus calyciflorus	A	4	BOLD:AAT8862	557, 844, 954, 955
Brachionus calyciflorus	B	1	BOLD:AAT8863	562
Brachionus quadridentatus	A	3	BOLD:ABA7390	795, 802, 884
Brachionus quadridentatus	B	2	BOLD:AAT9963	565, 845
Collotheca sp.	A	2	BOLD:ABU9596	856, 874
Collotheca sp.	B	1	BOLD:ACC8099	940
Colurella uncinata	-	1	BOLD:ACR4005	1104
Conochilus unicornis	-	2	BOLD:AAZ6072	676, 678
Cupelopagis vorax	-	1	BOLD:ABU8196	843
Euchlanis cf. deflexa	-	2	BOLD:ACA2873	912, 913
Euchlanis meneta	A	2	BOLD:ACC8729	936, 937
Euchlanis meneta	B	1	BOLD:ACC8200	934
Euchlanis pyriformis	-	3	BOLD:ABU8292	848, 946, 957
Filinia cf. terminalis	-	2	BOLD:ACA2813	908, 909
Filinia longiseta	-	4	BOLD:ABU8253	817, 818, 824, 825
Filinia novaezelandia	-	3	BOLD:ACG5383	990, 995, 996
Hexarthra intermedia	A	1	BOLD:AAU5719	560
Hexarthra intermedia	B	1		966
Hexarthra intermedia	C	1		1072
Keratella cochlearis	A	4	BOLD:AAZ5138	692, 863, 864, 865
Keratella cochlearis	B	1	BOLD:AAM3256	491
Keratella procurva	A	1	BOLD:AAM5102	805
Keratella procurva	B	2		821, 822
Keratella procurva	C	1		494
Keratella procurva	D	1		804
Keratella procurva	E	1	BOLD:ABU9212	885
Keratella tecta	-	4	BOLD:AAZ5138	650, 722, 723, 725
Keratella tropica	A	1	BOLD:AAW0344	1105
Keratella tropica	B	6	BOLD:AAU3067	558, 559, 566, 689, 690, 827
Keratella valga	-	1	BOLD:AAW0344	961
Lecane bulla	A	2	BOLD:ACC8387	932, 933
Lecane bulla	B	3	BOLD:ACA3088	900, 901, 902
Lecane bulla	C	1	BOLD:ABV4669	841
Lecane closterocerca	-	1	BOLD:ACM1597	973
Lecane decipiens	-	4	BOLD:ABU8977	829, 830, 886, 887
Lecane hamata	-	2	BOLD:ACC8864	944, 951
Lecane ludwigii	-	3	BOLD:ACC7351	947, 948, 949
Lecane luna	-	1	BOLD:ABU8861	826
Lecane lunaris	A	1	BOLD:ABU8293	862
Lecane lunaris	B	1	BOLD:ACA2793	899
Lepadella cf. ovalis	-	1	BOLD:ACA3261	897
Notommata pseudocerberus	-	1	BOLD:ACC8585	938

Platyias quadricornis	-	2	BOLD:AAN1828	488, 888
Polyarthra dolichoptera	A	2	BOLD:AAZ5884	682, 684
Polyarthra dolichoptera	B	2	BOLD:ACA3159	904, 905
Rotaria neptunia	-	1	BOLD:AAZ8510	975
Squatinella mutica	-	3	BOLD:ABV0111	840, 894, 895
Synchaeta oblonga	A	2	BOLD:AAZ4951	639, 641
Synchaeta oblonga	B	1	BOLD:AAM9605	492
Synchaeta oblonga	C	2	BOLD:ACA2798	892, 893
Synchaeta pectinata	A	1	BOLD:ACB9845	915
Synchaeta pectinata	B	1	BOLD:ACB9930	917
Synchaeta pectinata	C	5	BOLD:AAW0345	631, 632, 685, 686, 688
Synchaeta sp.	-	1	BOLD:AAZ9010	721
Trichocerca pusilla	-	2	BOLD:ACC6641	927, 928
Trichocerca similis	A	8	BOLD:AAV7895	556, 668, 669, 670, 672, 674, 720, 823
Trichocerca similis	B	1	BOLD:AAN0349	490
Trichocerca similis	C	1	BOLD:ACA2867	903
Trichocerca sp.	A	1	BOLD:ACG1767	976
Trichocerca sp.	B	1	BOLD:AAM9636	489
Trichocerca stylata	A	1	BOLD:ACR3832	1060
Trichocerca stylata	B	1		1069
Trichocerca tenuior	-	1	BOLD:AAZ8314	728
Trichotria tetractis	-	1	BOLD:ACG5331	991

Appendix 2 Genetic distance matrix generated in MEGA v5.2.2 for all 19 unique haplotypes (Gh1 – Gh19) from 151 *Gomphiocephalus hodgsoni* individuals for a 632 bp mtDNA (COI) fragment.

	Gh1	Gh2	Gh3	Gh4	Gh5	Gh6	Gh7	Gh8	Gh9	Gh10	Gh11	Gh12	Gh13	Gh14	Gh15	Gh16	Gh17	Gh18
Gh1																		
Gh2	0.002																	
Gh3	0.002	0.003																
Gh4	0.002	0.003	0.003															
Gh5	0.002	0.003	0.003	0.003														
Gh6	0.002	0.003	0.003	0.003	0.003													
Gh7	0.002	0.003	0.003	0.003	0.003	0.003												
Gh8	0.002	0.003	0.003	0.003	0.003	0.003	0.003											
Gh9	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.003										
Gh10	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003									
Gh11	0.003	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005								
Gh12	0.002	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.002							
Gh13	0.014	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.014	0.013						
Gh14	0.016	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.016	0.014	0.002					
Gh15	0.016	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.016	0.014	0.002	0.003				
Gh16	0.016	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.018	0.016	0.014	0.002	0.003	0.003			
Gh17	0.013	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.014	0.013	0.011	0.002	0.003	0.003	0.003		
Gh18	0.013	0.015	0.014	0.014	0.015	0.015	0.014	0.015	0.014	0.014	0.013	0.011	0.006	0.008	0.008	0.008	0.005	
Gh19	0.014	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.014	0.013	0.008	0.010	0.010	0.010	0.006	0.003

Appendix 3 The 24 variable nucleotide sites and 6 variable amino acids among 19 unique 632 bp mtDNA (COI) sequence fragments from 151 *Gomphiocephalus hodgsoni* individuals. The most common haplotype Gh1 was used as the reference sequence. Nucleotide sites that translate to an amino acid change are shown in bold.

	Nucleotide position																								Amino acid					
	2	**5**	6	**8**	1	1	1	1	1	2	**2**	2	2	2	2	3	3	3	**3**	**3**	3	4	4	6					1	1
	9	**2**	7	**9**	2	5	6	8	9	1	**1**	4	7	8	9	0	1	4	**6**	**7**	8	3	3	1	1	1	3	7	2	2
Haplotype	**9**	**2**	7	**9**	1	7	6	4	3	4	**5**	4	1	3	8	1	6	7	**9**	**1**	8	3	9	3	0	7	0	2	3	4
Gh1	G	C	G	G	C	T	A	G	T	T	G	A	C	C	A	G	A	T	C	T	G	A	A	C	Ala	Phe	Ala	Gly	Ser	Gly
Gh2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
Gh3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gh4	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Gh5	**A**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Thr	.	.	.	.	.
Gh6	.	.	.	.	.	.	.	.	.	.	**A**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Ser	.	.
Gh7	.	**A**	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Leu	.	.	.	.
Gh8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**A**	.	.	.	.	.	.	.	.	.	Ser
Gh9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
Gh10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	**T**	.	.	.	.	.	.	.	.	.	Phe	.
Gh11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	G	.	.	.	.	.	.	.	.
Gh12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.
Gh13	.	.	C	.	T	.	G	.	C	C	.	G	.	.	.	.	.	C	.	.	.	G	G	.	.	.	.	.	.	.
Gh14	.	.	C	**A**	T	.	G	.	C	C	.	G	.	.	.	.	.	C	.	.	.	G	G	.	.	.	Thr	.	.	.
Gh15	.	.	C	.	T	.	G	.	C	C	.	G	.	T	.	.	.	C	.	.	.	G	G	.	.	.	.	.	.	.
Gh16	.	.	C	.	T	.	G	.	C	C	.	G	.	.	.	.	.	C	.	.	A	G	G	.	.	.	.	.	.	.
Gh17	.	.	C	.	T	.	G	.	C	C	.	G	.	.	.	.	.	C	.	.	.	G	.	.	.	.	.	.	.	.
Gh18	**.**	**.**	**A**	**.**	**T**	**.**	**G**	**A**	**C**	**.**	**.**	**G**	**.**	**.**	**.**	**.**	**.**	**C**	**.**	**.**	**.**	**G**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
Gh19	**.**	**.**	**A**	**.**	**T**	**.**	**G**	**C**	**C**	**.**	**.**	**G**	**T**	**.**	**.**	**.**	**.**	**C**	**.**	**.**	**.**	**G**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**

Appendix 4 Summary of collection data for all 151 *G. hodgsoni* specimens comprising 19 COI haplotypes with their corresponding BOLD specimen IDs.

Haplotype	Site	Date	Time	Specimen ID
Gh1	1	13/01/2014	7:45	ANTSP393
			7:45-21:00	ANTSP394
		14/01/2014	09:00-11:00	ANTSP376 - ANTSP378, ANTSP488, ANTSP489
			11:00-13:00	ANTSP399, ANTSP477
			13:00-15:00	ANTSP400 - ANTSP402
			15:00-17:00	ANTSP404, ANTSP479
		15/01/2014	07:00-09:00	ANTSP440
			11:00-13:00	ANTSP441, ANTSP442, ANTSP485
			09:00-11:00	ANTSP381, ANTSP382, ANTSP481 - ANTSP484
			13:00-15:00	ANTSP445, ANTSP446, ANTSP486
			17:00-19:00	ANTSP383
			19:00-21:00	ANTSP384
		16/01/2014	07:00-09:00	ANTSP447, ANTSP448
			11:00-13:00	ANTSP449
			13:00-15:00	ANTSP451
			9:00-11:00	ANTSP386, ANTSP387, ANTSP498, ANTSP500, ANTSP501
		20/01/2014	17:00-21:00	ANTSP388
		21/01/2014	09:00-11:00	ANTSP390
		22/01/2014	09:00-11:00	ANTSP391
		24/01/2014	21:00-09:00	ANTSP433
	2	14/01/2014	09:15-11:15	ANTSP418, ANTSP490, ANTSP491
			13:15-15:15	ANTSP420, ANTSP421, ANTSP492
			15:15-17:15	ANTSP422, ANTSP423, ANTSP493, ANTSP494
			17:15-21:15	ANTSP425
		15/01/2014	09:15-11:15	ANTSP410, ANTSP411
			11:15-13:15	ANTSP412
		16/01/2014	09:15-11:15	ANTSP405, ANTSP407, ANTSP502
			21:15-23:15	ANTSP409
		20/01/2014	17:15-21:15	ANTSP430, ANTSP431
		21/01/2014	17:15-21:15	ANTSP429
		23/01/2014	17:15-21:15	ANTSP428
	3	21/01/2014	09:30-21:30	ANTSP370, ANTSP372, ANTSP373, ANTSP435, ANTSP437, ANTSP438, ANTSP504, ANTSP506, ANTSP507
		22/01/2014	09:30-21:30	ANTSP367 -ANTSP369, ANTSP471, ANTSP476, ANTSP510
			21:30-09:30	ANTSP366
		23/01/2014	09:30-21:30	ANTSP363, ANTSP364, ANTSP455, ANTSP456, ANTSP458 - ANTSP463, ANTSP466, ANTSP467, ANTSP513, ANTSP515 - ANTSP517
			21:30-09:30	ANTSP470, ANTSP474, ANTSP475

Haplotype	Site	Date	Time	Specimen ID
Gh1 (cont.)	3	24/01/2014	21:30-09:30	ANTSP468
		26/01/2014	09:30-21:30	ANTSP360 - ANTSP362, ANTSP472
Gh2	3	23/01/2014	09:30-21:30	ANTSP365
Gh3	1	14/01/2014	19:00-21:00	ANTSP379
Gh4	3	26/01/2014	09:30-21:30	ANTSP465
Gh5	3	23/01/2014	09:30-21:30	ANTSP457
Gh6	2	14/01/2014	15:15-17:15	ANTSP424
Gh7	3	23/01/2014	21:30-09:30	ANTSP473
Gh8	1	14/01/2014	11:00-13:00	ANTSP398
Gh9	1	15/01/2014	07:00-09:00	ANTSP439
			09:00-11:00	ANTSP380
			19:00-21:00	ANTSP385
Gh10	1	16/01/2014	11:00-13:00	ANTSP450
Gh11	1	22/01/2014	17:00-21:00	ANTSP392
Gh12	1	14/01/2014	09:00-11:00	ANTSP396
			15:00-17:00	ANTSP403
		25/01/2014	21:00-09:00	ANTSP434
		20/01/2014	17:00-21:00	ANTSP503
Gh13	1	14/01/2014	09:00-11:00	ANTSP377, ANTSP487
		15/01/2014	13:00-15:00	ANTSP443, ANTSP444
		15/01/2014	07:00-09:00	ANTSP480
	2	16/01/2014	09:15-11:15	ANTSP406
		16/01/2014	11:15-13:15	ANTSP408
		15/01/2014	11:15-13:15	ANTSP413
		15/01/2014	17:15-19:15	ANTSP414
		14/01/2014	21:15-09:15	ANTSP415
		14/01/2014	09:15-11:15	ANTSP417, ANTSP419
		26/01/2014	09:15-21:15	ANTSP426
		15/01/2014	9:15-11:15	ANTSP495
	3	21/01/2014	09:30-21:30	ANTSP371
		23/01/2014	09:30-21:30	ANTSP464
		23/01/2014	21:30-09:30	ANTSP469
		21/01/2014	09:30-21:30	ANTSP505
		22/01/2014	09:30-21:30	ANTSP509, ANTSP511, ANTSP512
		23/01/2014	09:30-21:30	ANTSP514
Gh14	1	13/01/2014	7:45-21:00	ANTSP395
Gh15	1	14/01/2014	21:00-09:00	ANTSP375
Gh16	2	15/01/2014	17:15-19:15	ANTSP416
	3	21/01/2014	09:30-21:30	ANTSP508
Gh17	1	20/01/2014	17:00-21:00	ANTSP389
	3	21/01/2014	09:30-21:30	ANTSP436
	2	26/01/2014	09:15-21:15	ANTSP427
Gh18	3	21/01/2014	21:30-09:30	ANTSP374
Gh19	1	24/01/2014	21:00-09:00	ANTSP432