Phylogeography and Ecology of New Zealand Freshwater Amphipoda (*Paracalliope*, *Paraleptamphopus* and *Phreatogammarus*)

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ABSTRACT

This thesis examines phylogenetic patterns in three New Zealand amphipod taxa in relation to current geographic distributions and historical climatic (e.g. glaciation, marine inundation) and geological (e.g. mountain building) events using DNA sequencing and distributional data. It also examines how recognition behaviour can be used to delineate potential species boundaries and to assess the role of sexual selection.

The endemic genus *Phreatogammarus* has been found in only a limited number of sites and is not very abundant. An analysis of the genetic variation of two species within the genus using allozyme electrophoresis revealed high levels of genetic differentiation among populations but low levels within populations. This suggested that limited dispersal occurred among habitats with one population possibly representing a cryptic species.

The endemic freshwater genus *Paraleptamphopus* is thought to contain a large number of undescribed species with a number of these existing in small waterbodies such as seepages. Examination of the phylogeographic patterns using both mtDNA (CO1) and nuclear DNA (28S) showed that a number of distinct genetic lineages exist, with CO1 revealing 21 haplotypes with genetic distance of over 20%. Using a molecular clock rate of 2.4%, most haplotypes diverged approximately 8-12 million years ago during the Miocene era, possibly as a result of greater land availability increasing habitat diversity or by allopatric speciation. Morphological and genetic differences were not congruent, with morphologically similar taxa appearing among highly genetically distinct lineages, and some morphologically distinct forms appearing within single lineages.

The distribution and habitat variables of 419 sites were analysed to determine what was affecting the presence or absence of *Paraleptamphopus*. The presence of native vegetation in catchments had a positive affect on *Paraleptamphopus* distribution suggesting that large anthropogenic changes in catchment vegetation could have a negative effect on their abundance. I found smaller waterbodies to be more important than larger ones highlighting the need to study such sites as rare taxa may be ignored. A better understanding is needed on the role of small waterbodies in promoting overall species diversity in catchments.

Examination of *Paracalliope fluviatilis* phylogenetic patterns using the mtDNA gene CO1 showed that a number of separate clades existed suggesting long term isolation and limited dispersal among catchments. Due to the large genetic divergences among some populations there was the possibility that cryptic species might exist. Species recognition experiments were conducted on seven populations to help determine whether cryptic species were present. For the three most genetically divergent crosses there was bias against inter-population pairings, suggesting that there were between two or three separate species.

Using a combined field and laboratory approach, size assortative mating was examined in *Paracalliope fluviatilis.* The field study showed positive size assortative mating and that larger females carried more eggs, suggesting they were more fecund. A series of laboratory experiments examining four existing theories explaining the phenomenon found that none adequately explained positive size assortative mating in *P. fluviatilis.* I therefore presented two new explanations to explain size assortative mating: a combination of female resistance and size-related variation in a male's capacity to amplex larger females or a form of indirect intra-sexual competition.

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INTRODUCTION

The development of evolution and genetics

By the nineteenth century most of the concepts needed for a theory of organic evolution were present and large advances in our understanding were made (Strickberger, 1995). Darwin (1859) in “The Origin of Species” was fundamental in the advancement of evolution, linking geographical isolation with speciation processes and describing how natural selection could operate to produce change in species: “As many more individuals of each species are born than can possibly survive; and as, consequently, there is a frequently recurring struggle for existence, it follows that any being, if it vary however slightly in any manner profitable to itself, under the complex and sometimes varying conditions of life, will have a better chance of surviving, and thus be naturally selected. From the strong principle of inheritance, any selected variety will tend to propagate its new and modified form”. In combination with natural selection, Darwin (1871) considered sexual selection, which he defined as “the advantage which certain individuals have over others of the same sex and species solely in respect of reproduction”, separate to natural selection which could also lead to change in species.

Darwin did not know the nature of the heritable units involved in natural and sexual selection, though Mendel’s work with peas showed that organisms inherited biological characteristics by means of discrete units (Mendel, 1866), later discovered to be genes. James Watson and Francis

Crick discovered the structure of the heritable unit, DNA (Watson and Crick, 1953). This enabled the field of genetics to progress rapidly through the later half of the twentieth century and the close relationship between evolution and genetics became apparent. For example, Dobzhansky (1937) commented that “...genetics has so profound a bearing on the problem of the mechanisms of evolution that any evolution theory which disregards the established genetic principles is faulty at its source”.

Species Concepts

One of the oldest problems in biology that is still very much applicable today is the definition of a biological species. Early species concepts were not based on evolutionary principles but focused on groups of organisms that shared the same essence (the essentialist species concept). This concept was accepted by Christians, who believed that God created all species and that each one was separate and different from the other. Later John Ray (1627-1705) introduced reproduction in the species concept "no surer criterion for determining species has occurred to me than the distinguishing features that perpetuate themselves in propagation from seed". Therefore, individuals within a species shared the same essence and had common descent.

Carl Linnaeus (1707-1778) helped instigate the modern binominal classification system whereby each species is defined by a genus and species tag e.g. *Homo sapiens*. However, his species concept was essentially the same as Jon Rays that each species was created, constant and

that no new species could arise. Georges Buffon (1707-1788) introduced the idea that species distinctions should be made on the basis of whether there were reproductive barriers to crossbreeding between groups.

The permanent and constant nature of species meant that evolution had no role in species concepts. Therefore, Baptiste de Lamarck (1744-1829) discarded species distinctions as man-made in an attempt to establish the possibility of evolution. Though his evolutionary mechanisms were wrong it did introduce the idea that species could change and provided a platform for Darwin's (1872) The Origin of Species (Strickberger, 1995).

After Darwin's (1859) Origin of Species many taxonomists still continued to use only morphological features to describe species where the degree of morphological difference used to determine which species an individual was assigned to (Morphological species concept). This concept was flawed in that it had difficulty in dealing with sibling or cryptic species and conspicuous morphs. Numerous species concepts based on interbreeding and reproductive isolation were formed in the early part of the 20th century. The concept that eventually became adopted was Mayr’s (1942) biological species concept (BSC) "species are groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups". This concept emphasised isolating mechanisms, defined by Mayr (1963) as " biological properties of individuals which prevent the interbreeding of populations that are actually or potentially sympatric". The obvious problem with this is that the majority of species have evolved via allopatric speciation. Proponents of BSC (e.g. McKitrick and Zink, 1988) suggest that

reproductive isolation has no consistent genotypic or phenotypic correlates and cannot be used to predict reproductive isolation. Therefore, the species status of two distinctive populations in allopatry can only be inferred unless artificially brought together which is not usually practical. Some advocates of the MSC were against the BSC because morphologically similar populations that were reproductively isolated (cryptic species) were given species status. It has been suggested that various species concepts have to be employed to cater for the diverse range of situations species may arise in (Scudder, 1974).

A number of current species concepts attempt to improve upon the BSC. The phylogenetic species concept (PSC) (Cracraft, 1983) considers "taxa are monophyletic clusters of individuals and species are the smallest diagnosable cluster". However, there are problems with this. For instance, what characters are diagnostic and how do you delineate what is the smallest cluster? There have been suggestions about a compromise between BSC and PSC "genealogical concordance" (Avise and Ball 1990) where elements of both BSC and PSC are employed. This concept emphasises the use of concordant genetic partitions across multiple, independent, genetically (or morphological) traits while still retaining the use of reproductive barriers in delineating species boundaries.

At the moment there is not a species concept that adequately addressees all the issues raised by various biologists which is why a number of species concepts currently exist. In practise species are identified using a variety of characters (morphological, genetical, behavioural, ecological etc) and are usually described using physical descriptions.

Phylogeography

The relatively new discipline of phylogeography (Avise et al. 1987) emphasises the importance of geographic barriers to gene flow as it relates to the geographical distributions of genealogical lineages and has gained rapid acceptance in the field of evolutionary genetics (Avise, 1998). In particular, it was seen as a bridge linking micro and macro-evolutionary processes. The pre-cursor for the development of phylogeography has been the creation of relatively fast and cheap methods for mtDNA sequencing and the majority of phylogeographic studies are based on animal mtDNA sequence data. The use of animal mtDNA-based phylogeography studies is thought to have lead to improved descriptions of geographical distributions, phylogenetic relationships and genetic distances among lineages (Bermingham and Moritz, 1998).

Cryptic species and Molecular Markers

Though cryptic species have long known to exist, Mayr (1942) described the term, they were generally referred to as biologically races (Thorpe 1930; 1940). However, numerous studies using molecular markers such as allozymes and DNA sequencing have revealed large genetic differences in morphologically similar populations, suggesting that cryptic species are quite common (Witt and Hebert, 2000; Stevens and Hogg,

2004). The use of molecular markers has been suggested as a method to supplement traditional morphology-based taxonomy (Tautz, et al. 2003). Indeed, Hebert et al. (2003a) have suggested that species could be identified on the basis of one or two gene sequence fragments alone (e.g. barcoding using CO1; Hebert et al. 2003a; Hebert et al. 2003b). However, the ability to use molecular markers to identify and separate species has been questioned (e.g. Mallet and Willmot, 2003), and where cryptic species exist other forms of differentiating between potential species have been recommended (Will and Rubinoff, 2004).

Behaviour and species

"Ever since Lamarck and Darwin, it has been clear that recognition behaviour plays an important evolutionary role both in species isolation and as a source of adaptation in the formation of new species" (Colgan 1983). The ability to recognise, and hence mate with, members of the same species is an important component of hybrid avoidance and ultimately speciation (Colgan, 1983). Accordingly, deviation from random mating between different populations may indicate the existence of different species or the beginnings of speciation. Furthermore, recognition

behaviour could potentially be used to help validate cryptic species. Recognition behaviour occurs at a number of different levels (e.g. mate, kin, species) and plays a critical part in sexual selection (e.g. larger males may be stronger competitors in aggressive encounters and hence pass on the genes for large size to their progeny). Recognition behaviour at various levels is therefore important in understanding micro and macro-evolutionary processes.

New Zealand geology and geography

The isolated archipelago of New Zealand has undergone a number of geological (volcanism, mountain building) and climatic (glaciation, marine inundation) events (Fleming, 1979; Stevens, 1995) since it separated from the super-continent Gondwana approximately 80 million years ago.

During the Paeocene (65-55 mya) New Zealand was approximately the same size as it is currently with a sub-tropical climate. As Antarctica become separated from surrounding landmasses (80-40 mya) the circum-Antarctic current system developed which had a significant cooling affect on it and nearby landmasses. During the Eocene (53-37 mya) and Oligocene (37-24 mya) times the sub-tropical environment was gradually replaced by more temperature climates. This cooling period also coincided with a gradual but significant reduction in landmass due to

erosion. By the Oligocene New Zealand consisted of a few small islands that were relatively flat and cold that probably contained a relatively low level of biodiversity.

The Miocene (24-5 mya) saw an increase s volcanic activity and mountain building processes caused by New Zealand's position straddling the boundaries of the India-Australian and Pacific plates. This caused an increase in land area and biodiversity in New Zealand. Furthermore, due to re-arrangements of largely South-East Asian islands tropical water was able to reach the coast of New Zealand during the Early Miocene which significantly increased temperatures to that 5-7 degrees ˚C higher than today. However, during the middle and late Miocene (15-5 mya) a period of gradual cooling ensured due to a build up of ice in Antarctica.

The Pliocene period (5-2 mya) saw increased mountain building activity with the the uplift of the Southern Alps occurring, the large mountain chain extending most of the length of South Island of New Zealand. Uplifting of blocks of land in created new ranges and depressions in southern New Zealand. The North Island experienced the creation and eruptions of several large volcanoes. The end of the Pliocene and during the Pleistocene (2- 0.01 mya) a massive cooling phase caused New Zealand to experience a period of ice ages interspersed with warmer periods. This created glacial climates in southern New Zealand where ice covered most of southern New Zealand and only the northern part of New Zealand maintained a temperate forest remnant with tussock grassland the dominant vegetation type (Stevens, 1995).

Chapter Introduction

There is a paucity of information on invertebrates from many southern hemisphere regions and here I address this by examining three freshwater amphipod taxa in New Zealand that have different distributions, habitat preferences and general ecologies. Amphipods lack a specific dispersal stage and freshwater taxa have little between catchment dispersal creating isolated, fragmented populations. This combined with New Zealand's turbulent climatic and geological history suggests that they would serve as ideal models to examine phylogeographic processes and to examine the usefulness of molecular markers, (e.g. allozyme and DNA sequences) to delineate species boundaries by examining morphology and species recognition patterns.

This thesis is presented in five chapters. Chapter I examines the population genetics (allozymes) of the New Zealand endemic amphipod *Phreatogammarus helmsii* (Fig. 1) and *P. waipoua*, specifically looking at total genetic variation within and among populations and their distribution.

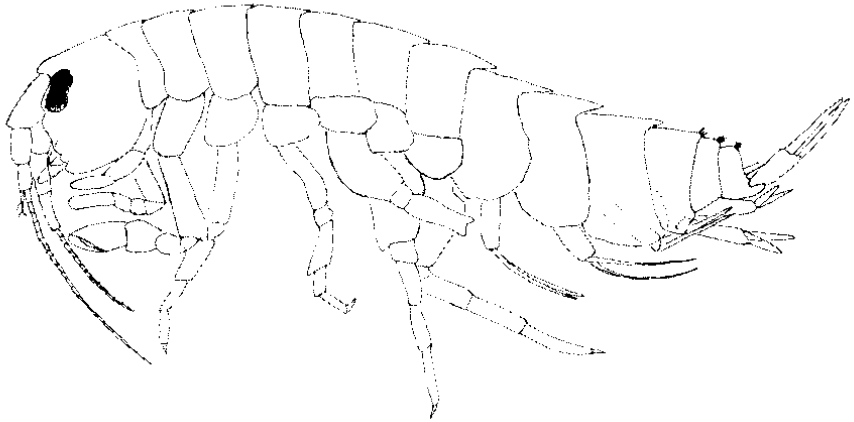

Figure 2. Drawing of the amphipod *Phreatogammarus helmsii* modified from Chapman (2003). The species is found in small rivers usually close to the coast.

Chapter II examines the phylogeography of the endemic New Zealand amphipod genus *Paraleptamphopus* (Fig. 2) using both mitochondrial and nuclear DNA sequences to assess patterns of diversity and how they relate to geography and past natural events.

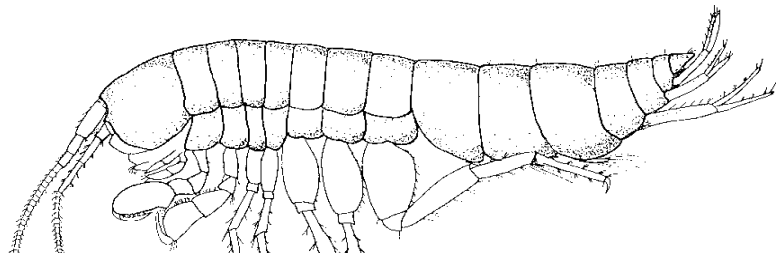

Figure 2. Drawing of the amphipod *Paraleptamphopus subterraneus* modified from Chapman and Lewis (1976). The species is found in groundwater in the Canterbury region of New Zealand

Little is known about the phylogenetics of spring and seepage fauna in New Zealand and none on the *Paraleptamphopus*.In Chapter III the species richness, distribution and habitat preferences of the genus *Paraleptamphopus* was examined. In particular, the hypotheses that the genus would prefer 1) small waterbodies versus large waterbodies and 2) more natural areas (assessed by native vegetation cover) were tested.

Chapter IV examines phylogeography and species recognition in the *Paracalliope fluviatilis* (Fig. 3) species complex. The mitochondrial gene cytochrome c oxidase 1 was sequenced for populations covering the entire species range and the evolutionary relationships among populations

analysed with respect to divergence times between lineages and how New Zealand biogeography. In the second part of the chapter, laboratory experiments were used to test the hypothesis that males preferred same population females to females from genetically divergent populations. Preference for same population females over highly genetically distant foreign females may indicate that cryptic species exist.

Chapter V examining the role of size assortative mating in the pre-copulatory mate guarding amphipod *P. fluviatilis*. Studies on amphipod species displaying pre-copulatory mate guarding, such as *Gammarus pulex*, have demonstrated positive size-assortative mating where small males are more likely to pair with small females while large males are more likely to pair with large females (Elwood & Dick 1990). The first part of chapter V examines whether *P. fluviatilis* demonstrates size assortative mating and whether larger females have more eggs. The second part of Chapter V uses a series of laboratory experiments to test several hypotheses that describe mechanisms that explain size assortative mating in amphipoda. The thesis concludes with a summary of main findings and suggestions for future research

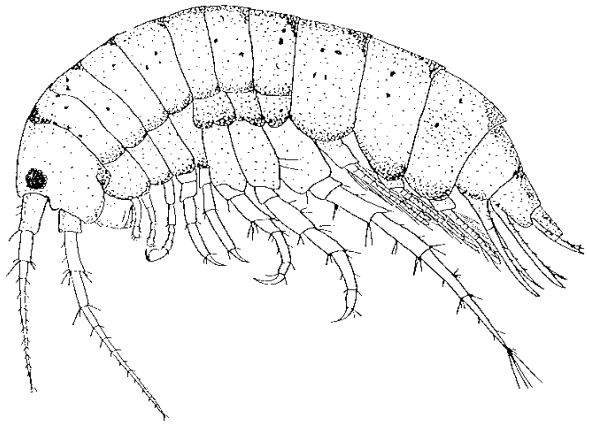

Figure 3. Drawing of the amphipod *Paracalliope fluviatilis* modified from Chapman and Lewis (1976). Adults are between 2-4 mm long and live in freshwater habitats throughout New Zealand.

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CHAPTER I

ALLOZYME VARIABILITY AMONG POPULATIONS OF *PHREATOGAMMARUS* (CRUSTACEA: AMPHIPODA): REFLECTS RESTRICTED DISTRIBUTION AND LIMITED DISPERSAL

Keywords: Population genetics**,** *Phreatogammarus helmsii*, Distribution, New Zealand, genetic variation

ABSTRACT

Phreatogammarus helmsii and *P. waipoua* are freshwater amphipod species endemic to New Zealand. *Phreatogammarus helmsii* has a widespread distribution but both species are restricted to relatively few sites, often in low abundances, and hence, may be considered rare. Given their restricted occurrence, we tested the hypothesis that *P. helmsii* and *P. waipoua* would show low levels of genetic variation within sites, and that most variation would be found among populations. From 419 sites sampled throughout New Zealand, we found *P. helmsii* at four sites and *P. waipoua* at two sites confirming their rare status. Cellulose acetate electrophoresis was used to assess genetic (allozyme) variation among populations. We found very high levels of genetic differentiation between *P. waipoua* and *P. helmsii* (D = 0.81) and relatively high levels of genetic differentiation among *P. helmsii* populations (F_{ST} = 0.616) indicating limited gene flow. We suggest that *P. helmsii* populations are effectively isolated with infrequent dispersal among current populations. Limited dispersal may be a consequence of restricted habitat availability and this is likely to have been exacerbated by human activities.

Introduction

Rare species are thought to have low levels of total genetic variation (Cole 2003). This is due to two main reasons. Firstly, rare species usually have small populations and therefore genetic drift will be stronger, reducing variation. Secondly, rare species often have populations that are isolated from one another, causing low rates of migration that will also act to limit variation (Frankham 1995). Gitzendanner and Soltis (2000) showed that this was not necessarily correct in their study of congeneric pairings of rare and common plant species. They found that common species did have higher levels of genetic variation but for approximately one quarter of rare species levels of genetic diversity were similar to that of common species. Hogg et al. (1998) found a similar trend for amphipod species. Contrary to expectations, Hogg et al. (1998) found amphipod species in fragmented, discrete habitats often had higher levels of total genetic diversity than more widespread, and usually more common, species in continuous habitats.

The New Zealand endemic amphipod genus *Phreatogammaurus* currently contains four described species with *P. helmsii* being the only widespread epigean species in the genus (Hurley 1975). *P. waipoua* has been reported from two localities in the far north of New Zealand (Chapman 2003), and *P. propinquus* from three localities from Stewart Island in the far south of New Zealand (Chapman 2004). Until recently *P. helmsii* was known from only five localities (Hurley 1954), even though the species was described over 86 years ago (Chilton 1918). Chapman (2003) expanded this distribution to include a further 12 sites including sites in two new areas, Waikato and Taranaki. Even with these new sites

P. helmsii and *P. waipoua* are still relatively rare amphipod species compared with other New Zealand species such as *Paracalliope fluviatilis* and *Paracorophium excavatum* (Hurley 1975). Nijoer and Verdonschot (2004) suggested that macroinvertebrates inhabiting 0.15-0.5% of sites should be considered rare and >0.5-1.5% of sites classed as uncommon. Based on this classification *P. helmsii* was uncommon and *P. waipoua* rare. Accordingly, *Phreatogammarus* spp. may have restricted habitat requirements and/or limited dispersal capacity. This, in turn, may lead to high levels of genetic differentiation among populations.

In order to test if rare/ uncommon species did have relatively high inter-population genetic differentiation and low intra-population differentiation , we examined the levels of genetic variation among populations of the two relatively rare species, *P. helmsii* and *P. waipoua*.

Materials and Methods

Field sites and sampling

We sampled 419 sites (Fig. 4) between July 2000 and April 2003 throughout New Zealand from a variety of aquatic habitats, which included lakes, ponds, rivers, ditches, streams and seepages. Habitats were sampled using either sieves, small hand nets or larger nets on long handled poles with a (mesh size of 1mm). Samples were sorted immediately in white trays, and amphipods were preserved in liquid nitrogen for genetic analysis or 70% ethanol for morphological identification.

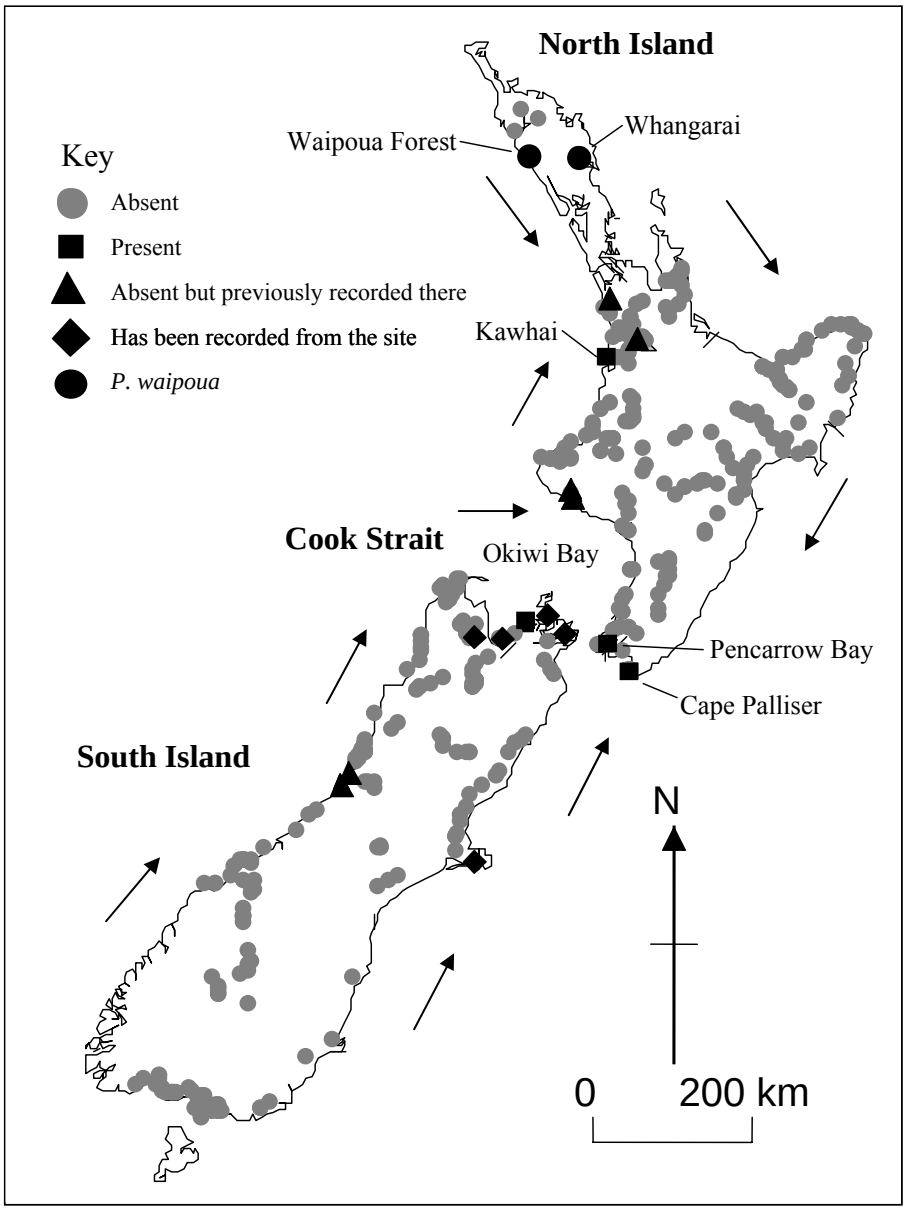

Figure 4. Map of New Zealand with prevailing oceanic currents shown which may affect the dispersal of *Phreatogammarus helmsii*. The location of study sites where: *Phreatogammarus* was absent (grey circles), where *P. helmsii* was found (black squares), where *P. helmsii* has been recorded before but not found by us (black triangles), other locations where *P. helmsii* has been found (black star) and where *P. waipoua* was recorded (black circles).

Allozyme electrophoresis

I used the genetic technique cellulose acetate gel electrophoresis to examine genetic variability within and among populations of *Phreatogammarus*. We initially tried 20 enzymes, however, eight enzymes did not display sufficient levels of activity and were not used in the analysis: hexokinase (HK: EC 2.7.1.1); glyceraldehyde-3-phosphate (G3PDH: EC 1.2.1.12); glucose-6-phosphate dehydrogenase (G6PDH: EC 1.1.1.49); glycerol-3-phosphate dehydrogenase (GPDH EC 1.1.1.8); 6-phosphogluconate dehydrogenase (6GPDH: EC 1.1.1.44); aconitate hydratase (ACON: EC 4.2.1.3); alcohol dehydrogenase (ADH: EC 1.1.1.1); adenylate kinase (AK: EC 2.7.4.3); leucine aminopeptidase (LAP: EC 3.4.11.1). Twelve enzymes did exhibit sufficient electrophoretic activity and resolution to be scored reliably were: aldehyde dehydrogenase (AD: EC 1.2.1.5), aldehyde oxidase (AO: EC 1.2.3.1); malate dehydrogenase NADP+ (ME: EC 1.1.1.40); isocitrate dehydrogenase (IDH: EC 1.1.1.42), peptidase (PEP: EC 3.4.11/13); arginine kinase (ARK: EC 2.7.3.3), glucose-6-phosphate isomerase (GPI: EC 5.3.1.9), malate dehydrogenase (MDH: EC 1.1.1.37); fumarate hydratase (FUM: EC 4.2.1.2), lactate dehydrogenase (LDH: EC 1.1.1.27); phosphoglucomutase (PGM: EC 2.7.5.1) and mannose phosphate isomerase (MPI: EC 5.3.1.8). Two enzymes (*AD* and *AO*) were coded by two loci and were designated by increasing electrophoretic activity (e.g. *AD-1*, *AD-2*). Two individuals from previous runs were re-run to control for any variation in mobility between gel plates. Allelic designations were verified using gel line-ups (sensu Richardson et al. 1986). Stain recipes, buffers and running conditions were adapted from Hebert and Beaton (1993) and Richardson et al. (1986).

Data analyses

The computer program BIOSYS-1 (Swofford and Selander 1981) was used to calculate population statistics and to construct a dendrogram using the unweighted pair group method with arithmetic means (UPGMA) algorithm (Sneath and Sokal 1973) calculated using Nei's (1978) unbiased genetic distance (D) values. Timing of divergence was estimated by using a standard alloyzme clock (Nei's genetic distance assumes 0.2 D MY^{-1}, Nei 1987). This was done to examine relationships among populations. To determine the levels of genetic variability within populations the following statistics were produced: mean sample size per locus, mean number of alleles per locus, percentage of polymorphic loci and the observed and expected levels of heterozygosity. *F* statistics for each locus were tested for significance using the formulas given by Waples (1987) $F_{IS,}\ \chi^2 = F_{IS}{}^2 N\ (k-1)$, d.f. $= k(k-1)/2$ and for $F_{ST,}\ \chi^2 = 2NF_{ST}\ (k-1)$, d.f. $= (k-1)(s-1)$ where N = total number of individuals, k = number of alleles at the locus, and s is the number of populations.

Results

We found six sites that had *Phreatogammarus* present, four sites contained *P. helmsii* and the other two *P. waipoua* (Fig. 4). One *P. waipoua* site was the type locality in the Waipoua Forest and the other a new recording of *P. waipoua* in Whangarei. At five sites, previously reported to contain *P. helmsii*, we were unable to find any animals. In some cases (e.g. Greymouth), this was possibly due to considerable habitat degradation (e.g. vegetation changes and agriculture runoff, pers. obs). The four sites containing *P. helmsii* and two sites containing *P.*

waipoua were relatively small streams and other amphipod species were usually present (Table 1).

Table 1. Allele frequencies of 14 loci for four populations of *Phreatogammarus helmsii* and one of *P. waipoua*.

Locus	Allele	Kawhia	Pencarrow Bay	Cape Palliser	Okiwi Bay	*P. waipoua*
N		10	12	10	5	15
AD-1	A	1.000	1.000	1.000	1.000	0.533
	B					0.467
N		10	10	10	3	15
AD-2	A					0.533
	B	0.400	1.000	1.000	0.333	0.433
	C	0.600			0.333	0.033
	D				0.333	
N		10	12	10	5	13
AO-1	A	1.000	1.000	1.000	0.800	0.423
	B				0.200	0.577
N						
AO-2	A	0.400				
	B	0.600	1.000	1.000	1.000	1.000
N		10	12	10	5	15
ME	A	0.500	0.125		0.700	
	B	0.500	0.875	1.000	0.300	1.000
N		10	12	10	5	15
IDH	A		1.000	1.000	1.000	
	B	1.000				
	C					1.000
N		10	12	10	5	15
LDH	A	1.000	1.000	1.000	1.000	1.000
N		10	12	10	5	15
MPI	A	0.050				1.000
	B	0.550				
	C	0.400				
	D		1.000	1.000	1.000	
N		10	12	10	5	15

Table 1. continued,

PEP	A	0.300		0.300		
	B	0.700	0.333	0.700		
	C		0.667		1.000	1.000
N		10	12	10	5	15
PGM	A					0.033
	B	1.000		1.000	1.000	0.967
	C		1.000			
N		10	12	10	5	15
ARK	A	1.000	1.000	1.000	1.000	
	B					1.000
N		10	12	10	5	15
GPI	A	0.050	0.125		1.000	1.000
	B	0.800	0.875	0.850		
	C	0.150		0.150		
N		10	12	10	5	15
MDH	A	1.000	1.000	1.000	1.000	
	B					1.000
N		10	12	10	5	15
FUM	A	1.000		0.900	1.000	
	B		1.000	0.100		1.000

Allozyme variation and population genetic structure

Of the 14 scorable loci examined, 4 loci (*AD-1*, *MDH*, *ARK* and *LDH*) did not show any variability within *P. helmsii* although another allele was detected at three loci (*AD-1*, *MDH* and *ARK*) for *P. waipoua* (Table 2). The percentage of polymorphic loci (95% criterion) ranged from 7.1% to 65.3% with the mean number of alleles per polymorphic locus ranging from 1.2 to 1.6 (Table 3). Heterozygote deficiencies were found for *P. helmsii* and in all cases were the result of heterozygote deficiencies (Table 3). Significant ($P < 0.05$) departures from Hardy-Weinberg expectations found at some loci: *AD-1* for *P. waipoua*; *AD-2* for Kawhia, Okiwi Bay, *P. waipoua*; *AO-1* for *P. waipoua*; *AO-2* for Kawhai,

Okiwi Bay; *PEP* for Kawhia, Pencarrow Bay, Cape Palliser; *ME* for Pencarrow Bay; and *FUM* for Cape Palliser.

Table 2. Genetic variability at 14 loci for all populations of *Pheatogammarus helmsii* and *Phreatogammarus waipoua*. N = mean sample size per locus, A = mean number of alleles per locus, P = percentage of polymorphic loci, H_{obs} = observed heterozygosity (direct count) and H_{exp}= expected heterozygosity (Standard errors in parentheses).

Location	N	A	P	H_{obs}	H_{exp}
Kawhia	11.9 (0.0)	1.6 (0.2)	64.3	0.114 (0.065)	0.264 (0.067)
Pencarrow Bay	11.1 (0.1)	1.3 (0.1)	28.6	0.030 (0.19)	0.072 (0.037)
Cape Palliser	10.0 (0.0)	1.2 (0.1)	21.4	0.021 (0.021)	0.064 (0.037)
Okiwi Bay	4.9 (0.1)	1.3 (0.2)	21.4	0.043 (0.043)	0.116 (0.066)
P. waipoua	14.9 (0.1)	1.4 (0.2)	7.1	0.077 (0.066)	0.117 (0.059)

Wright's (1978) F_{ST} for all populations was (F_{ST} = 0.71) and for populations of *P. helmsii* only was (F_{ST} = 0.62), indicating very great (*sensu* Wright 1978) levels of genetic differentiation among populations, and suggesting little or no present-day gene flow (Table 3). A high level of intraspecific structuring was also found for all populations (F_{IS} = 0.47) and *P. helmsii* populations only (F_{IS} = 0.51). The UPGMA analysis showed that *P. waipoua* was highly distinct from *P. helmsii* (D = 0.81; divergence time = 4.05 MY^{-1}) and that the Kawhia population was the most basal *P. helmsii* population (D = 0.35; divergence time = 1.75 MY^{-1}) and had a fixed allelic difference (non-shared alleles) for *IDH* indicating no recent gene flow between it and the other Cook Strait populations (Fig. 5). The three

populations around the Cook Strait were the most geographically proximate still significantly genetically differentiated: Okiwi Bay and Pencarrow - Cape Palliser cluster (D = 0.26; divergence time = 1.3 MY^{-1}) and Pencarrow – Cape Palliser (D = 0.18; divergence time = 0.9 MY^{-1}) (Fig. 5).

Table 3. Wright's (1978) F_{IS} and F_{ST} values for the four populations of *Phreatogammarus helmsii* and one population of *P. waipoua*.

Locus	F_{IS}	*P* value	F_{ST}	*P* value
AD-1	1.000	*P*<0.001	0.412	
AD-2	0.442	*P*<0.001	0.387	*P*<0.001
AO-1	0.905	*P*<0.001	0.384	*P*<0.001
AO-2	1.000	*P*<0.001	0.348	*P*<0.001
ME	-0.303	*P*<0.05	0.415	*P*<0.001
IDH			1.000	*P*<0.001
LDH				
MPI	0.252		0.815	*P*<0.001
PEP	1.000	*P*<0.001	0.558	*P*<0.001
PGM	-0.034		0.961	*P*<0.001
ARK			1.000	*P*<0.001
GPI	-0.175		0.707	*P*<0.001
MDH	-0.043		0.952	*P*<0.001
FUM	1.000	*P*<0.001	0.926	*P*<0.001
Mean	0.417	*P*<0.001	0.712	*P*<0.001

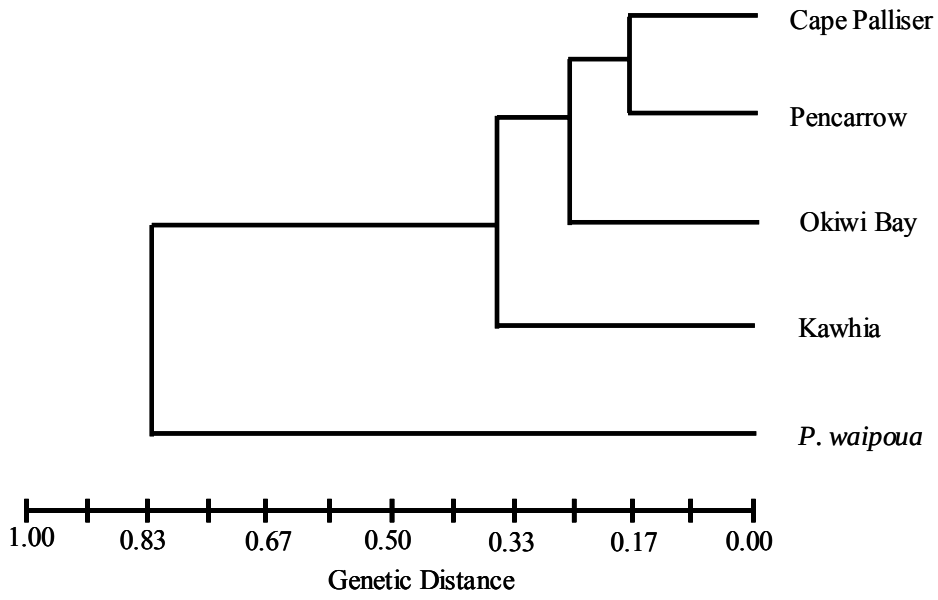

Figure 5. UPGMA dendrogram (Nei's (1978) using unbiased genetic distance) of four populations of *Phreatogammarus helmsii* and one of *P. waipoua* from the type locality in the Waipoua forest.

Discussion

We found only four populations of *P. helmsii* and two of *P. waipoua* suggesting that neither is abundant, even in known habitats. Based on Nijboer and Verdonschot's (2004) abundance classifications *P. helmsii* is uncommon (0.5-1.5%) and *P. waipoua* rare (0.15-0.5%). Our inability to collect specimens from some locations where *Phreatogammarus* has been previously found may be due to low numbers of animals present at some localities (e.g. the Waikato River, Chapman 2003). Chapman (2003) also reported the absence of animals from the type locality at Greymouth and it seems possible that in sites where low numbers were previously found that local extinctions may have occurred. Little is known about the genus (Chapman 2004) and the effect human activities have on its current distribution and abundance. Other native New Zealand stream fauna have had populations declines attributed to habitat degradation, especially in developed areas (e.g. the fish *Galaxias maculatus*; Jowett 2002) and deforestation and landuse change over the last 150 years have been extensive in New Zealand (Stevens 1995). Therefore, anthropogenic disturbances may have caused population reductions or possibly extinctions in *Phreatogammarus* species resulting in their restricted distribution.

Genetic differentiation between *P. helmsii* and *P. waipoua* was very high (D = 0.81) which supports Chapman (2003) creation of the species *P. waipoua* which was based on morphological characters. There were high levels of genetic differentiation between the four *P. helmsii* populations (D = 0.18-0.35) and a fixed allelic (*IDH*) difference existed between the Kawhia and Cook Strait

populations suggesting that no recent gene flow has occurred. Therefore, the Kawhia population may be a cryptic species, though no morphological differences have been detected (Chapman 2003). Further data, e.g. behavioural or ecological, is required to establish whether the Kawhia population is a genetically distinct population or a cryptic species. The high levels of genetic differentiation found among the *P. helmsii* populations would probably be due to low levels of gene flow, presumably because of low levels of migration among populations. However, there are other scenarios explaining the large allelic differences, especially among the Cook Strait populations which had no fixed allelic differences. Firstly, there could be selection against migrants from other genetically distinct populations, which has been found in the New Zealand amphipod *Paracalliope fluviatilis* (D. Sutherland unpubl. data). Secondly, rapid population turnover may radically alter allele frequencies via genetic drift, or thirdly a founder effect could result in a new population with a substantially different allelic representation than the source population.

Although the species is considered to be a freshwater and not brackish water taxon, it is usually found very near the coast in and around the high tide mark (Chilton 1918). Accordingly, salinity tolerances would be expected to be reasonably high and coastal dispersal is the most likely method of inter-catchment migration. Therefore, oceanic currents (Fig. 6) may play an important role in the dispersal and distribution of *P. helmsii* with only limited dispersal likely against prevailing currents. A similar distributional pattern based on oceanic currents has been suggested for other New Zealand amphipod species, e.g. the estuarine *Paracorophium excavatum* and *P. lucasi* (Schnabel et al. 2000, Stevens and Hogg 2004). This may explain why no North Island east coast and lower South Island

populations have been found as dispersal would be against the prevailing currents. Small-scale dispersal seems to have occurred relatively recently. During the last glacial maxima approximately 17,000 years ago (Stevens 1995) the Okiwi Bay site was a significant distance inland. Given *P. helmsii's* strong coastal affiliations it is likely that during the Holocene when sea levels rose to there present position, a migrant from one of the other Cook Strait populations founded the Okiwi Bay population. This suggests that where suitable habitat is available *P. helmsii* can successfully disperse there indicating that habitat availability and not dispersal may be the cause of their current restricted distribution.

We suggest that *P. helmsii* populations have been separated for a significant length of time (e.g. Kawhia diverged 1.75 MY^{-1}) with little gene flow occurring among current populations resulting in high levels of genetic differentiation. Lack of dispersal may be as a consequence of limited habitat availability and this has likely been caused or further exacerbated by human activities. We suggest monitoring of sites where *P. helmsii* occurs is prudent to determine if populations are in decline and more future research should be focused on the habitat requirements of both *P. helmsii* and *P. waipoua*.

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CHAPTER II

PHYLOGEOGRAPHY OF THE AMPHIPOD GENUS *PARALEPTAMPHOPUS* (EUSIRIDAE): EVIDENCE OF MULTIPLE MIOCENE- PLEISTOCENE RADIATIONS INSTIGATED BY DISPERSAL AND ADAPTIVE RADIATION

Keywords: speciation, Crustacea, phylogenetics, biogeography, New Zealand

To be submitted under the same title as: Sutherland, D.L., Hogg, I.D., and Chapman, M.A.

Abstract

The isolated archipelago of New Zealand has undergone a number of geological and climatic events that would have created interesting evolutionary processes and lead to high levels of biodiversity. We examined the phylogeography of the New Zealand freshwater amphipod genus *Paraleptamphopus* to assess patterns of diversity and how they relate to geography and past natural events. Sequence data from the mitochondrial cytochrome oxidase c subunit I (COI) gene revealed 28 distinct haplotypes (genetic distances > 2%) split into four main clades (inter-clade genetic distances > 20%). Examination of the nuclear gene 28S rDNA gene showed less genetic variation and revealed only two main clades but was still generally congruent with the mtDNA data. Morphological analysis suggested the presence of three broad ecotypes: surface, benthic and subterranean with the surface type having evolved twice, possibly three times. However, morphological differences were not congruent with genetic differences with morphological stasis occurring among some genetic lineages whereas morphological differences were found within other lineages and did not correspond to any discernible genetic differences. Molecular estimates of divergence times indicated that most lineages arose during the Late Miocene (12-7 Mya), a period of increasing land area and potentially greater habitat diversity, with subsequently fewer radiations during the Pliocene and Pleistocene. Both allopatric speciation, caused by multiple range expansions over millions of years, and adaptive radiation, facilitated by changes in micro and macro-habitat preferences are likely to have resulted in the observed levels of genetic and species diversity within the genus.

Introduction

Phylogeographic studies have been increasing in the genetics literature (e.g. Avise 1998; Masta 2000). Combining phylogenetics and biogeography has allowed the examination of the evolutionary processes that shape and maintain genetic diversity (Bermingham and Moritz, 1998). New Zealand has undergone a number of geological (volcanism, mountain building) and climatic (glaciation, marine inundation) events (Fleming, 1979; Stevens, 1995) that would have produced significant levels of biodiversity and evolutionary processes (Chinn and Gemmell, 2004; Trewick and Morgan-Richards 2005). Furthermore, New Zealand has been isolated from other landmasses since the break up of Gondwana approximately 75 million years ago (Stevens, 1995) and during this long period of isolation, an extensive endemic fauna and flora has evolved (Cooper and Millener, 1993). However, despite the unique nature of the biota and the natural processes that have shaped it, only limited attention has been directed to the freshwater invertebrate fauna.

The diversity of freshwater invertebrate species may be underestimated because some taxa exhibit limited morphological variation and are often undetected (Taylor et al., 1998; Witt and Hebert, 2000). Crustacea in particular have been shown to have significant degrees of genetic variation with limited morphological variation (Colbourne and Hebert, 1996; Hogg, et al. 1998; Lee, 2000) This underestimation of biodiversity has been found for the amphipod genera *Paracorophium* and *Paracalliope* in New Zealand where potentially cryptic species were found (Stevens and Hogg, 2004, Hogg et al. 2006). Accordingly, New Zealand amphipods may be suitable models to study

phylogeographic processes because they do not have an active dispersal stage and hence there may be high levels of genetic diversity among populations, possibly coupled with allopatric speciation.

The amphipod genus *Paraleptamphopus* (Family Eusiridae) currently consists of two described freshwater species endemic to New Zealand. They are morphologically and ecologically distinct: *P. caeruleus* has black body pigmentation, possesses eyes and inhabits surface waters (usually small streams), while *P. subterraneus* is pale, blind and subterranean (Chapman and Lewis, 1976). Non-typical *P. subterraneus* have been reported from surface waters (Watson, 1972) and additional species have long been suspected (Chapman and Lewis, 1976; Fenwick, 2000). As the genus only inhabits freshwater waterbodies its dispersal capabilities are primarily limited to within catchment dispersal. Combined with its habitat preferences for small streams, seepages and subterranean waters, populations should show very low levels of dispersal. However, *Paraleptamphopus* has been reported throughout the New Zealand landmass (Hurley, 1975), suggesting that the genus has had a relatively long time to disperse. Amphipod morphology is often highly conserved relative to other taxa. The presence of large morphological and ecological differences between the two described species suggests that speciation events may predate the Pleistocene era. In New Zealand this period is considered to have produced the majority of invertebrate speciation events (Chinn and Gemmell, 2004; Stevens and Hogg, 2004; Neiman and Lively, 2004).

The mitochondrial cytochrome c oxidase subunit I (COI) gene, the nuclear 28S rDNA (28S) gene and morphological characteristics were used to determine levels

of *Paraleptamphopus* species diversity, their phylogeographic patterns and relationships as well as potential and divergence times among taxa.

Methods

Field sites and animal collections

Between July 2000 and April 2003 we sampled 421 sites from a variety of freshwater habitats throughout New Zealand for *Paraleptamphopus* spp. (Fig. 6). Sites were usually sampled in summer to avoid sampling temporary waterbodies that were unlikely to contain aquatic amphipods. In some regions (e.g. portions of the east coast South Island), there were a lack of suitable sampling sites due to arid conditions. Animals were collected using 1 mm mesh size sieves, small hand nets or large nets with long handled poles. Samples were sorted on site and amphipods preserved in either 95% ethanol or liquid nitrogen for use in genetic analyses while additional animals were kept in 70% ethanol for a preliminary morphological examination. Laboratory samples preserved in ethanol were kept at – 20 °C while all others were kept at – 76 °C.

Three morphological features were recorded to assess physical characters of the genus. These were: (1) body and eye pigment (or lack of an eye); (2) gnathopod 2 dactyl morphology (important in mating); and (3) the position of setae on uropod 3 (a highly variable feature). Gnathopod 2 and uropod 3 variables were examined under a compound microscope at 400 x magnification (Fig. 7).

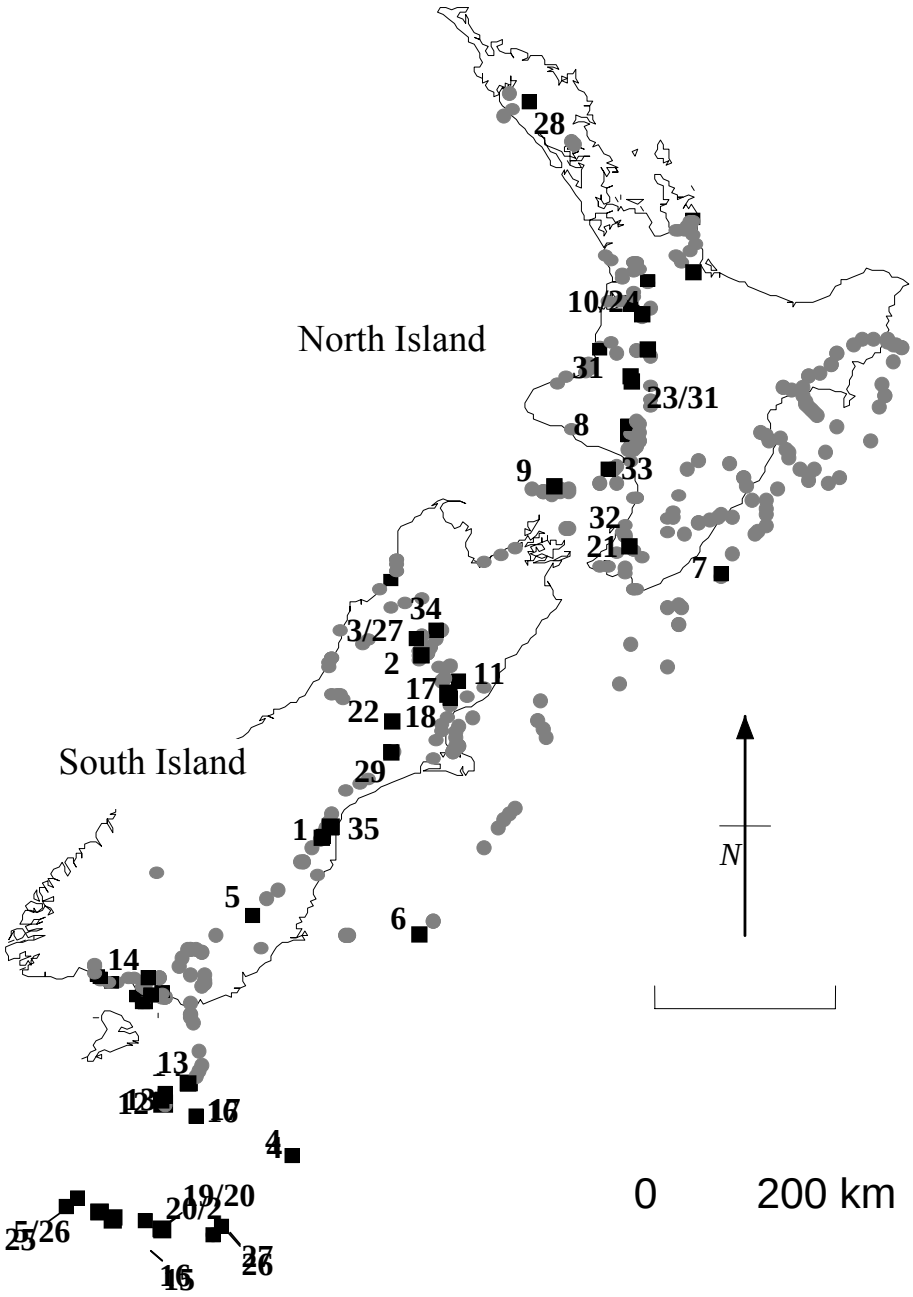

Figure. 6. Map of New Zealand showing study sites with black squares indicating presence of *Paraleptamphopus* and grey circles apparent absence. Numbers refer to sites where haplotypes were sequenced.

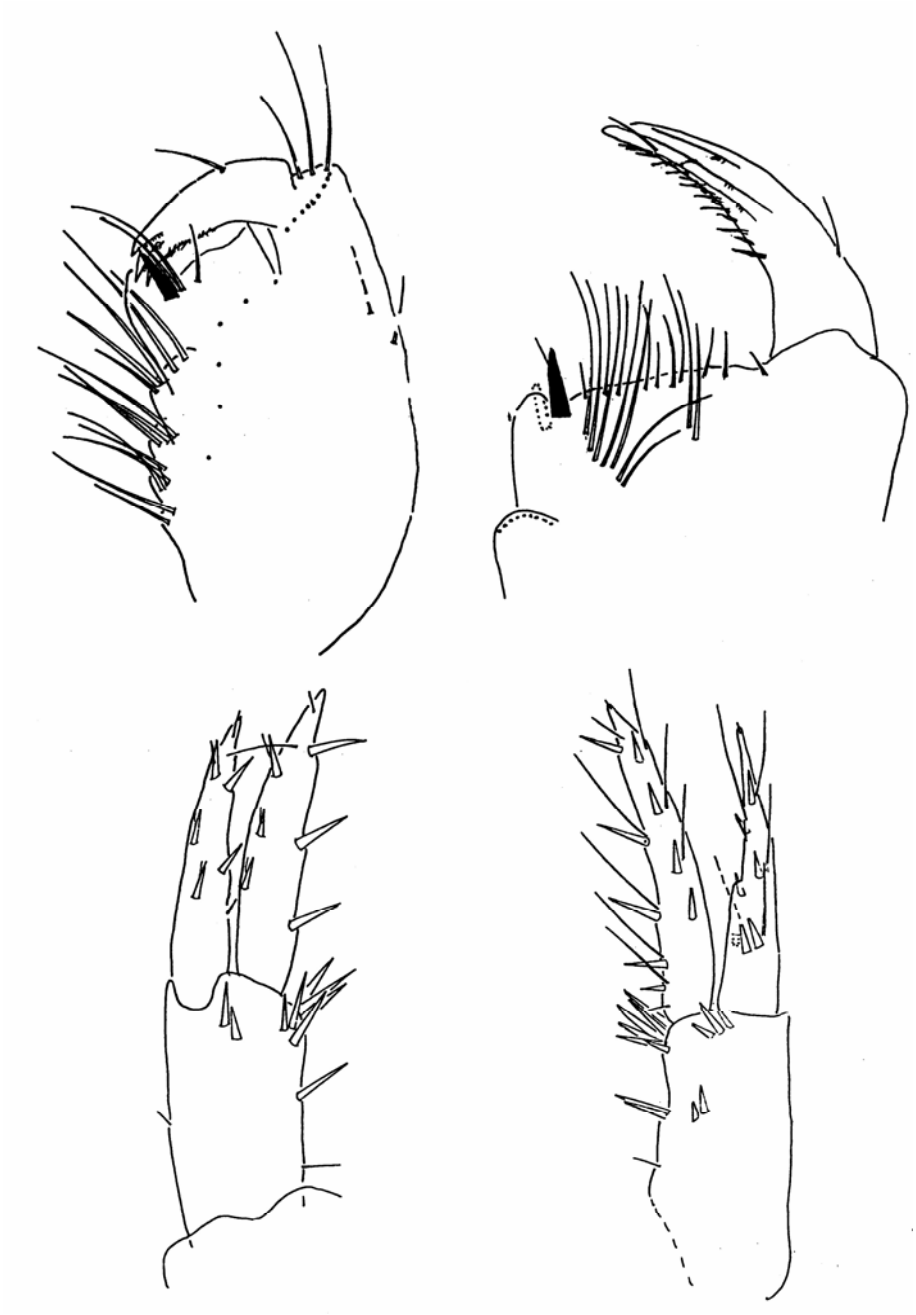

Fig 7. Drawings of two amphipod appendages. Gnathopd 2 (top) from a Waikato population of Paraleptamphopus showing a rough claw tip (right) and a Brooklyn population (left). showing a smooth claw tip. Uropod 3 (bottom) from a Waikato population of Paraleptamphopus showing marginal setae (right) and a Brooklyn population (left) showing sub apical setae. (pictures drawn by M.A. Chapman).

DNA-sequence analysis

DNA was extracted from an entire individuals (1-3 individuals from each suspected species per site) using the DNeasy Tissue kit (Qiagen Inc) as per the manufacturer's instructions, with the exception that we incubated the sample at 56°C for 24 hours and used 60 µl of H_2O to elute the DNA. PCR amplification was carried out using a 50 µl reaction volume consisting of 2 µl of DNA, 10×PCR buffer + $MgCl_2$ (Roche), 2.2 mM $MgCl_2$, 0.2 mM of each dNTP (Boehringer Mannheim), 1.0 µM of each primer, and 1.0 unit of *Taq* DNA polymerase (Roche) on a Eppendorf Mastercycler gradient thermocycler. A 710 base pair fragment of the mitochondrial gene cytochrome *c* oxidase I (COI) gene was amplified using the universal primers LCO1490 (5'- ggt caa caa atc ata aag ata ttg g -3') and HCO2198 (5'- taa act tca ggg tga cca aaa aat ca -3') (Folmer *et al*. 1994). The thermal cycling conditions were: 94°C for 1 min followed by 5 cycles of denaturation and polymerase amplification (94°C for 1 min, 45°C for 1.5 min and then 1 min at 72°C) and followed by 35 cycles of 94°C for 1 min, 51°C for 1.5 min and then 1 min at 72°C, followed by 5 min at 72°C. For nuclear DNA a 1200 base pair fragment of the nuclear gene 28S rDNA was amplified using the primers 28F (5'-ccagctatcctgagggaacttcg-3') and 28R (5'-gggactacccccctgaatttaagcat-3') (Schnabel and Hebert, 2003). The thermal cycling conditions were: 94°C for 1 min followed by 5 cycles of denaturation and polymerase amplification (94°C for 1 min, 51°C for 1.5 min and then 1 min at 72°C) and followed by 35 cycles of 94°C for 1 min, 55°C for 1.5 min and then 1 min at 72°C, followed by 5 min at 72°C. For both mitochondrial and nuclear DNA were purified using the QIAquick PCR purification kit (Qiagen Inc).

Sequencing was performed using the same primers as those used for PCR amplification on an ICM version 3.1 automated sequencer ((MegaBace) at the University of Waikato DNA sequencing facility. The forward direction was always sequenced with the reverse used in approximately 10% of samples to ensure sequence consistency.

Sequences were aligned using Sequencher (Gene Codes ver. 4.1.2 for Macintosh) sequence editor and verified as being derived from amphipod DNA using the GenBank BLAST algorithm. A Mantel test was performed for pairwise sequence divergences (based on the COI gene) and geographic distances using GenAlEx V5 (Peakall and Smouse, 2001) to determine if a significant relationship existed between genetic and geographic distance. Degree of mutational saturation was estimated by examining the correlation between ts/tv ratio and pairwise sequence divergence. If saturation occurred then a decrease in ts/tv ratio is expected as sequence divergence increases (Kocher et al. 1995). We used χ^2-tests, as implemented in PAUP* 4.0b10 (Swofford 2002) to determine whether the assumption of equal base frequencies among sequences was violated on all sites, parsimony-informative sites only and with the third codon position only. The presence of stop codons was analysed in MacClade 4.03 PPC using the *Drosophila* amino acid model. We then constructed phylogenies using PAUP* 4.0b10 (Swofford 2002). A neighbour-joining (NJ) phylogram was constructed using the TrN+I+G model (selected using the program Modeltest 3.7, Posada & Crandall 1998). The estimated parameters under this model were: ts/tv ratio = 14.51; proportion of invariable sites = 0.2646; variable sites (Gamma distribution shape parameter) = 0.4114. A maximum likelihood (ML) search was conducted using the above model parameters. A maximum parsimony (MP) analysis using

the heuristic search option with unweighted characters was also implemented. This was repeated for the 28S gene except for a GTR + G model was used with the following parameters: ts/tv ratio = 1.323; variable sites (Gamma distribution shape parameter) = 0.3434). Two COI and two 28S sequences were obtained from GenBank for use as the outgroup. These were from two distinct populations of the amphipod *Paramphithoe hystrix* (Schnabel and Hebert, 2003) and were selected based on an analysis of a range of amphipod species which suggested that they were the most closely related to *Paraleptamphopus* from sequences held by GenBank. We used three tree construction methods in order to minimise the potential for error that may arise from assumptions inherent in phylogeny reconstruction methods. Confidence in the cladistic analyses was assessed by estimation of the g_1 skewness statistic from 100,000 random tree length distributions (Hillis & Huelsenbeck 1992), and by bootstrap analysis with 1000 pseudoreplicates for the NJ and MP trees (Felsenstein 1976). A Kishino-Hasegawa test was conducted for NJ, MP and ML trees to determine whether significant differences existed. To examine whether lineages within trees were evolving at a similar rate, a two-cluster test was employed (Takezaki et al. 1995). To test whether sequences were diverging in a clock-like manner, a log-likelihood ratio test was carried out in PAUP* 4.0b10 that compared ML trees generated with the molecular clock option enforced with unconstrained trees (Felsenstein 1988). Divergence times were then estimated using a molecular clock approximation for CO1 of 2.4% nucleotide sequence divergence per million years (Knowlton 1993). This rate was derived from the study of several malacostracan crustaceans whose divergence resulted from a distinct geological event, the formation of the Isthmus of Panama.

Results

Morphology

Body colour and eye type (e.g. unpigmented, pigmented) were correlated with each other in all haplotypes except for *P. subterraneus*, which was eyeless. Twenty-three *Paraleptamphopus* populations collected were of a pale colour with unpigmented eyes and a further eleven had black body colour with black pigmented eyes. Gnathopod 2 morphology and uropod 3 setae position were often not congruent with each other or with body colour and eye type (Table 4).

Mitochondrial Genetic Diversity

A 610 bp fragment of the COI mitochondrial gene was analysed from a total of 79 *Paraleptamphopus* individuals from 32 sites. Consensus sequences of individuals from the same population that shared the same haplotype were made. There were 350 informative sites, 38 variable but parsimony uninformative sites, and 222 constant sites. No insertions, deletions and stop codons were detected suggesting no numts (add brief definition) were present. A total of 27 different haplotypes were found with pairwise sequence differences ranging from 2% to 45%. Between one and three distinct haplotypes were found per site. All unique sequences will be deposited to GenBank. The nucleotide composition of all sequences was biased for A and T (A = 27%, T = 32%, C = 23%, G = 18%), a common feature of arthropod mitochondrial DNA (Frati *et al.* 2001). Heterogeneity of base frequencies was detected for all codon positions (χ^2_{105}= 261.67, $P < 0.001$),

Table 4. Four morphological variables for each *Paraleptamphopus* population: Body colour, eye type, gnathopod 2 dactyl morphology and uropod 3 setae position (data on gnathopod 2 and uropod 3 collected by A. Chapman as part of a larger study describing *Paraleptamphopus* species).

No.	Population	Body colour	Eye type	Gnathopod 2	Uropod 3 setae
		Black, Pale	None, Black, Unpigmented,	Rough, Smooth	Marginal, subapical, none
1	Awatuna	Pale	Unpigmented	Smooth	Marginal
2	Brown Hut	Pale	Unpigmented	Rough	Subapical
3	Anatori (Sp 1)	Pale	Unpigmented	NA	NA
4	Mt Cargill	Pale	Unpigmented	Smooth	Subapical
5	Fox	Pale	Unpigmented	Smooth	Subapical + Marginal
6	*P. subterraneus*	Pale	None	Smooth	Marginal
7	Mangatewai River	Pale	NA	NA	NA
8	Awaikino Gorge	Pale	Unpigmented	Rough	Subapical
9	Mt Egmont	Pale	Unpigmented	Rough	Subapical
10	Lake Waikare (Sp 2)	Pale	Unpigmented	Rough	Subapical
11	Brooklyn	Black	Black	NA	NA
12	Queenstown	Black	Black	Smooth	Subapical
13	Cromwell	Black	Black	Smooth	Marginal
14	Jackson Bay	Black	Black	Rough	None
15	Bluff	Black	Black	Rough	None
16	*P. caeruleus*	Black	Black	Rough	None
17	Lee Valley	Black	Black	Smooth	Marginal
18	Pearce Valley	Black	Black	Smooth	Marginal
19	Waituna (Sp 2)	Pale	Unpigmented	Rough	Marginal
20	Waituna (Sp 1)	Pale	Unpigmented	Smooth	Subapical
21	Whanganui NP	Pale	Unpigmented	Rough	Marginal
22	Karamea Bight	Black	Black	Smooth	Marginal
23	Ngutunui (Sp 2)	Pale	Unpigmented	NA	NA
24	Lake Waikare (Sp 1)	Pale	Unpigmented	NA	NA
25	Port Craig	Black	Black	Rough	Marginal
26	Horseshoe Falls	Black	Black	Rough	Marginal
27	Anatori Sp 2	Black	Black	Smooth	Subapical
28	Lake Omapere	Pale	Unpigmented	Rough	Subapical
29	Murchison	Pale	Unpigmented	Smooth	Marginal
30	Pirongia	Pale	Unpigmented	Rough	Subapical
31	Ngutunui (Sp 1)	Pale	Unpigmented	Rough	Subapical
32	Atene	Pale	Unpigmented	Rough	Subapical
33	Taumarunui Gorge	Pale	Unpigmented	Rough	Subapical
34	Whanganui Inlet	Pale	Unpigmented	Rough	Marginal
35	Shantytown	Pale	Unpigmented	Smooth	Marginal

parsimony-informative sites only (χ^2_{105}= 469.81, $P < 0.001$), and for the third codon position only (χ^2_{105}= 486.60, $P < 0.001$). The plot of tv/ts ratio versus sequence divergence showed a negative slope (y = -3.7619x + 2.2041, R^2 = 0.0993) but this was small suggesting very limited mutational saturation). The Mantel test indicated that there was no significant correlation between genetic and geographic distance (y=2E-05x+0.02577, R^2=0.0035, P=0.180). This was expected as highly divergent clades were located in close proximity to each other (Figures 8 and 9).

Nuclear Genetic Diversity

A 448 bp fragment of nuclear 28S rDNA was used from a total of 21 *Paraleptamphopus* spp. individuals from 18 sites. Consensus sequences of individuals from the same population that shared the same haplotype were made. There were 122 informative sites, 22 variable but parsimony uninformative sites and 304 constant sites. There were three major (25-100 bp) insertions detected and these were deleted for analysis as the size and composition of the insertions were highly variable and produced few informative sites. A total of 18 different haplotypes was found with pairwise sequence differences ranging from 0.002% to 10.916%. Between one and two distinct haplotypes were found per site. The nucleotide composition of all sequences deviated from 25% for A and T (A = 21%, T = 32%, C = 25%, G = 22%). The assumption of homogeneity of base frequencies was supported using all codon positions (χ^2_{57} = 7.85, P =1.000), parsi mony-informative sites only (χ^2_{57}= 8.617, P =1.000) and when the third codon position was excluded (χ^2_{57}= 20.559, P = 0.999). The Mantel test indicated that there was no significant correlation between genetic and geographic distance (y=-

2E-06x+0.0514, R^2=0.0003, *P*=0.500). There was no substantial resolution within 28S for the white morph epigean populations but there was concordance between the nuclear and mtDNA data sets for the relationships among the black morph epigean populations. The two data sets were also concordant in distinguishing between black and white morphs -- the exception was the two Anatori mtDNA lineages (one black the other white) that possessed identical nuclear sequences.

Phylogeny Reconstruction

The maximum parsimony analysis produced 18 most parsimonious trees (tree length = 1907, C.I. = 0.357, R.I. = 0.651; Fig. 8) with good phylogenetic signal (g_1 = -1.17, g_{crit} = -0.09, *P*<0.01). The 18 trees differed only in the arrangement of seven closely related populations within clade B5 (morphological analyses suggest that five of the six populations belong to the same species, M.A. Chapman, unpubl. data), along with a basal seventh population (pairwise sequence divergence among the seven populations 0 - 0.04%). Both the MP and NJ trees (Fig. 9) tree had good bootstrap support within clades but poor support among clades. A Kishino-Hasegawa test examining the similarity of trees showed no significant differences between the MP and NJ tree (*P*=0.473) but revealed significant differences between the MP and ML trees (*P*=0.003) and NJ and ML trees (*P*=0.008). This result is reflected in the NJ and MP tree topologies being relatively congruent with only five differences between the two trees. These were: (1) the placement of the Mt Cargill haplotype in clade 2 (MP) instead of clade 3; (2) clade two in the MP tree is basal to clade three in the NJ tree; (3) the Lake Omapere group being placed in the first major grouping (A) in the NJ tree; (4) Anatori (Sp 2) shifted from clade 7 to 5; and (5) the splitting up of group B. For

the 28S gene a ML tree (Fig. 10) was produced. This tree had the best overall score using the NJ, MP and ML tree reconstruction methods although there were no significant differences between trees when using a Kishino-Hasegawa test (P>0.05).

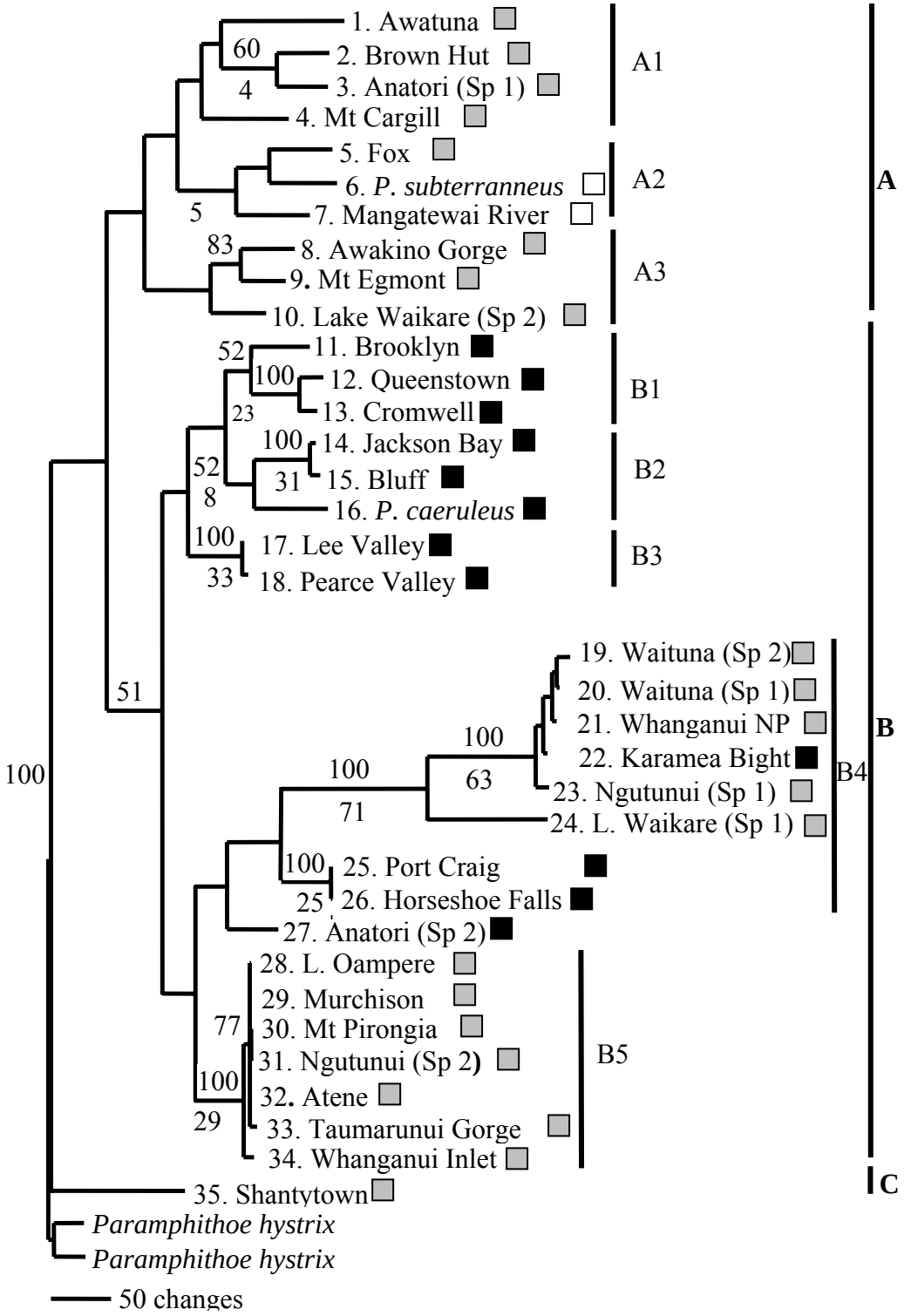

Figure 8. Phylogram of the mitochondrial gene CO1 constructed using Maximum Parsimony. Bootstrap values are above nodes and decay indices below nodes. Squares indicate major morphotypes for each lineage: subterranean □, surface epigean ▢, and benethic epigean ■.

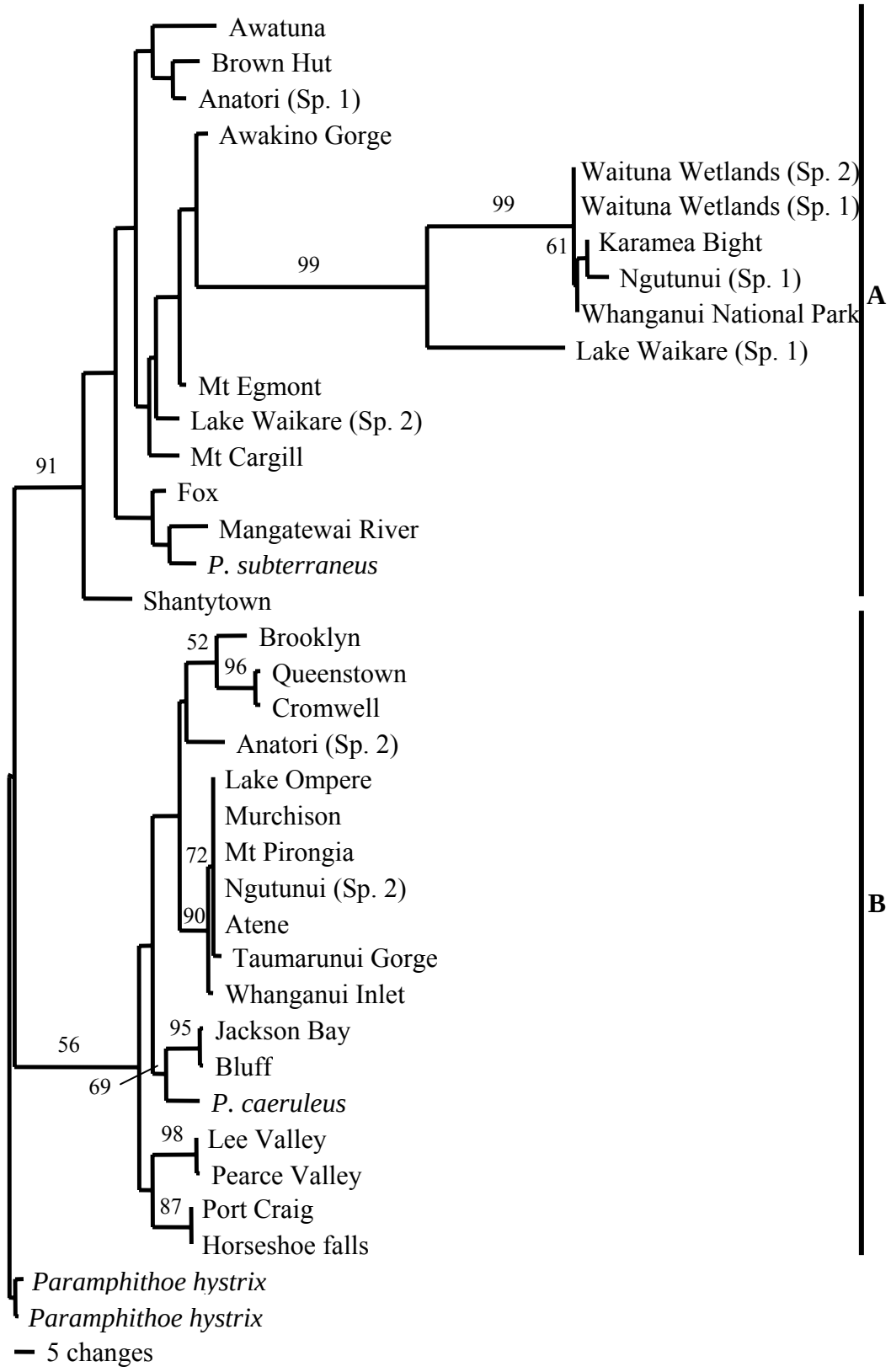

Figure 9. Phylogram of the mitochondrial gene CO1 constructed using Maximum Likelihood and displaying bootstrap values (100 replicates).

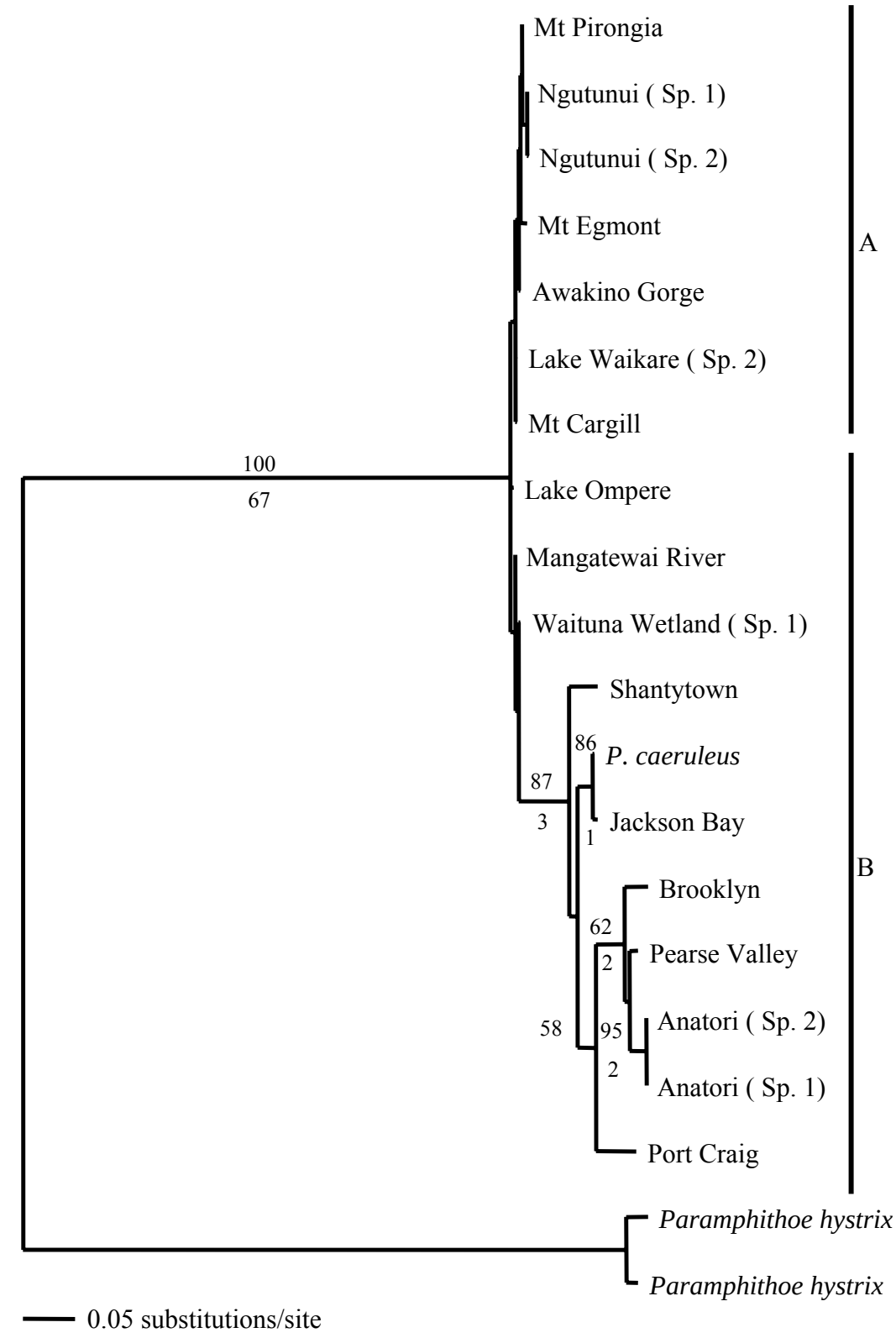

Figure 10. Phylogram of the nuclear gene 28S rDNA constructed using Maximum Likelihood with bootstrap support (100 replicates) above nodes and decay indices below nodes.

Molecular Clock and Divergence Times

The two-cluster test using COI showed that there was strong evidence (P<0.001) for rate heterogeneity between the six most divergent haplotypes from clade B4 and the other haplotypes with these six haplotypes possessing considerable rate acceleration compared with the other lineages. Removal of the six haplotypes eliminated any rate heterogeneity. A log-likelihood ratio test could not reject the hypothesis that existing lineages were evolving according to a clock-like model of evolution (-ln L = 7974.81 without molecular clock enforced and -ln L = 8001.17 with molecular clock enforced (difference = 26.36), χ^2 = 44.903, d.f = 34, P > 0.10). However, given that COI has two rate differences for the sequences analysed and there is no molecular clock calibration for *Paraleptamphopus*, caution should be applied when inferring timing of divergence events. Using the molecular clock rate of 2.4% nucleotide sequence divergence per million years, differences between clades A1-B3 range from 8 - 12 million years ago during the Late Miocene. Interestingly, within-clade ranges also fall mostly in the Late Miocene era with only clades B2, B3, B4 and B5 having haplotypes diverging less than 5 million years ago. Of these, only the Queenstown – Cromwell haplotypes and five haplotypes of clade B4 appear to be distinct species with the other clades representing morphologically similar populations. A two-cluster test using the 28S gene showed that there was strong evidence (P<0.01) for rate heterogeneity between clade A and B, Fox and Mangatewai versus the rest of B and Fox, Mangatewai and Waituna versus the rest of B.

Discussion

There were several genetically distinct lineages within *Paraleptamphopus*. The CO1 gene showed 28 separate haplotypes with genetic distances of over 2%, and 21 of those haplotypes had distances of over 20%. This suggests the possibility of different species based on genetic diversity levels found in other invertebrates (Hebert et al., 2003). Similarly deep divergences using COI have been found in other amphipod taxa (e.g. *Hyalella azteca* (20% divergence) Witt and Hebert 2000; *Paracalliope fluviatilis* (>20% divergence), Hogg et al. 2006, Chapter IV). In both studies, highly divergent lineages have been suggested to be cryptic species. The 28S nuclear gene showed smaller genetic divergences among haplotypes which was expected as it has been shown to be considerably slower evolving in related taxa (Schnabel and Hebert, 2003). The overall phylogenetic patterns between mtDNA and 28S were similar with most haplotypes belonging to the same major clades. There were two sites (Ngutunui and Anatori) that had distinct mitochondrial lineages (genetic distances of above 20%) but identical 28S sequences. This suggests two possibilities. Highly distinct mitochondrial lineages may occur within species, and therefore few, highly genetically variable (CO1) species exist. The more probable explanation is that some sites contain hybrids from two distinct species. Natural hybrid zones caused by overlapping congeneric species ranges are common and have been reported for a diverse range of fauna (newts *Triturus vulgaris* x *T. montandoni*, Babik et al. 2003; mussels *Mytilus edulis* x *Mytilus trossulus*, Riginos and Cunningham, 2005; waterfleas *Daphnia laevis* complex, Taylor et al. 2005).

Species Diversity

The geographic area containing the greatest genetic (and potentially species) diversity was the upper West Coast of the South Island, with nearly every clade having a representative from there. This suggests that this region represents the source of the genus in New Zealand from where it has dispersed throughout the landmass via several separate radiations. No strong geographic partitioning remains between haplotypes which can be explained by the old age of lineages in combination with the presence of multiple radiations within the genus resulting in members of different clades being in close proximity to, or existing together.

The high number of potentially new species found (21 species based on mtDNA genetic distances of over 20%) is probably due to two reasons: firstly, most amphipod species have poor inter-habitat dispersal capabilities - hence have limited levels of gene flow and secondly, the majority of populations occurred in isolated habitat types. Most sites (65%) containing *Paraleptamphopus* were in isolated, first degree streams and spring fed seepages well away from larger waterbodies connecting to other parts of the river catchment. However some populations (27%) were found in second and third degree streams suggesting some tolerance for medium sized waterbodies. Dispersal within river catchments is probably due to flood events when individuals are swept downstream and then migrate upstream to other suitable small habitats. Significant flood events may also be responsible for inter-catchment dispersal when whole catchments are flooded, as suggested for the Australian freshwater crayfish *Cherax destructor* (Hughes and Hillyer, 2003). River catchment changes and river capture would also enable inter-catchment dispersal, with this method of dispersal evident in New Zealand (e.g. *Galaxias vulgaris* complex; Waters and Wallis, 2000; Waters

et al. 2001) and Australian native fish (e.g. *Mogurnda adspersa*, Hurwood and Hughes 1998). Low levels of gene flow would therefore be expected between *Paraleptamphopus* populations which could result in high levels of allopatric speciation.

Between catchment dispersal is probably rare. New Zealand has a high level of diadromous (mostly fish species but also the shrimp *Paratya curvirostris*; McDowall, 1998) and saltwater tolerant freshwater taxa (e.g. Crustacea; Chapman and Lewis, 1976). Some freshwater species may have dispersed and maintained gene flow between populations in different river catchments via the sea. The ancestral *Paraleptamphopus* species may have originated from a freshwater form that has had a long enough period to subsequently disperse throughout New Zealand allowing significant speciation to occur. It may also have gradually evolved from a marine form until it become fully adapted to a freshwater environment. Isolated habitats and low dispersal capabilities explain why most sites contain such divergent mitochondrial haplotypes as some haplotypes may be limited to a small number of catchments. However, in two instances closely related haplotypes were found at sites over 700 km apart and on separate islands.

The existence of multiple haplotypes within the same site suggests niche partitioning as partly shown by the presence of the three distinct morphotypes within the genus: surface epigean (black colour and black pigmented eyes), benthic epigean (pale white colour and unpigmented eyes) and hypogean (pale colour and no eyes). However, evidence of niche partitioning for surface lineages was based on anecdotal evidence. During collecting, black morphs were observed to be more likely at the surface/ middle portion of the water column amongst macrophytes while white morphs were found at the base/ among the roots of

macrophytes. There also appears to be further niche separation within the benthic epigean forms as the Lake Waikare, Ngutunui and Waituna sites have two different benthic epigean haplotypes. No morphologically or genetically distinct black types were ever found together suggesting they occupy the same niche. Clade B is solely comprised of black haplotypes with the four other black haplotypes belonging to clade B4 suggesting that the black morphotype evolved at least twice, and possibly three times (twice within clade B4 as Karamea Bight haplotype most likely evolved from a pale type given its position on the phylogram trees).

There appears to be morphological stasis occurring as *P. caeruleus* appears identical to the Jackson Bay and Bluff haplotypes (19% genetic difference) and some members of the B5 clade had identical features to the A3 clade haplotypes (20% genetic difference) and Whanganui NP (42% genetic difference) (M.A. Chapman, unpubl. data) yet large mitochondrial differences exist between these groups. In addition, significant morphological differences exist between genetically similar populations e.g. Murchison versus Atene (gnathopod 2 and uropod 3 differences). This suggests rapid evolution of morphological traits may have occurred in some lineages, indicating adaptive radiation. This pattern of morphological stasis between lineages coupled with morphological differences within lineages seems common in Crustacea (Colbourne and Hebert, 1996; Lee, 2000, Witt and Hebert, 2000; Witt et al., 2003) and will lead to incongruence between taxonomic units depending on the data, morphological or genetical, used to distinguish them. It has been suggested by Witt et al. (2003) for the amphipod genus *Hyalella* that the presence of fish may promote morphological stasis while genetic divergence continues and that fishless habitats may therefore promote

morphologically diversification. *Paraleptamphopus* are predominately found in fishless habitats, which may explain their rapid morphological divergence in some cases. However, some populations were found in streams where fish undoubtedly occur which may explain why some lineages are genetically distinct yet appear morphologically similar.

Divergences Times in Relation toHistorical Events and Dispersal

Based on a molecular clock rate of 2.4% divergence per million years it appears that the majority of the haplotypes diverged approximately 8 - 12 million years ago during the Late Miocene. The Miocene era in New Zealand was a period in which rapid cooling produced by an accumulation of ice in Antarctica, resulted in a gradual loss of tropical organisms (Stevens, 1995). For *Paraleptamphopus* this may have reduced competition. The gradual cooling also increased the available land area and possibly increased habitat diversity, potentially producing new niches to exploit. However, speciation in other New Zealand arthropod groups during this time has been attributed to allopatric rather than adaptive speciation (e.g. weta genera *Deinacrida* and *Hemideina*, Trewick and Morgan-Richards, 2005). The existence of deeply divergent mitochondrial lineages exhibiting apparent morphological stasis within *Paraletamphopus* suggests that allopatric speciation was important in some *Paraleptmaphopus* lineages.

Only two divergences equate to Pliocene (5 – 2 m. y. a.) speciation events, that of the Cromwell- Queenstown and Ngutunui – Karamea Bight haplotypes. The Pliocene was a period in New Zealand where the emergence of mountains in the South Island is thought to have caused the diversification of a number of invertebrate groups (Trewick and Wallis, 2001, Chinn and Gemmell, 2004).

Mountain building may have separated the Cromwell- Queenstown haplotypes, as they are located across several mountain ranges. However, divergence between the Ngutunui – Karamea Bight haplotypes was most likely due to long distance dispersal given the large geographic distances separating them, though during the Pliocene both sites were isolated from one another by a large marine strait. Divergence may have occurred during the Pliocene with further dispersal in the Pleistocene when New Zealand was a single landmass.

The Pleistocene was a period of climate cycling with cooler periods inter-mixed with more temperate periods. Two haplotypes that diverged during the Pleistocene occur at the same site (Waituna) and may have evolved in sympatry. Both haplotypes had distinct morphological features and differences in microhabitat preference or diet that may explain their ability to co-exist. Whanganui Inlet versus the other Clade B4 haplotypes had genetic distances between 2-3% and some different morphological features indicating that Whanganui Inlet is a distinct species from the rest of the clade. There were also large geographic distances between some of these haplotypes (e.g. 1200 km). This suggests that substantial dispersal occurred during the last million years, possibly during cooler periods when land bridges could enable inter-island access (Stevens, 1995).

We found a number of distinct mitochondrial haplotypes that may correspond to up to 21 species although nuclear gene variation was substantially less. Morphological and genetic differences were not always congruent. Morphologically cryptic lineages may have been produced by dispersal into similar habitats followed by long term isolation between populations while rapid morphological change within lineages may have been caused by adaptation to

new niches. We suggest that both allopatric speciation, followed by multiple dispersal events, and adaptive radiation, facilitated by changes in micro and macro-habitat preferences have created high levels of genetic and species diversity within the genus.

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CHAPTER III

NEGLECTED SPECIES IN NEGLECTED HABITATS: THE IMPORTANCE OF SEEPAGE HABITATS AND CATCHMENT VEGETATION FOR THE NEW ZEALAND AMPHIPOD GENUS *PARALEPTAMPHOPUS*

Keywords

Conservation, Landuse, Biodiversity, Amphipoda, Streams, Freshwater ecology

To be submitted under the same title as: Sutherland, D.L., Hogg, I.D., and Chapman, M.A.

Abstract

Seepage (Holocrene spring) habitats and their faunas are understudied globally. In New Zealand, the endemic amphipod genus *Paraleptamphopus* is thought to occur mainly in seepages and thus may be representative of such habitats. Two species are currently described although several others are thought to exist, suggesting a possible underestimation of species diversity. In order to more accurately assess the potential species richness, distribution and habitat preferences of the genus *Paraleptamphopus*, we sampled 419 freshwater habitats throughout New Zealand. *Paraleptamphopus* were found at 49 widely distributed sites, mostly in small (<50 cm wide) seepages but also in small (2^{nd} order) streams and roadside ditches. Only 14 sites contained a presently described species; all others contained undescribed species. *Paraleptamphopus* spp. were associated with forested and scrubland sites composed of native vegetation, particularly shaded sites. Accordingly, *Paraleptamphopus* may have been negatively affected by the conversion of natural vegetation to the pastoral farmland that now dominates lowland New Zealand. Furthermore, the continuing degradation of water quality in parts of pastoral New Zealand may threaten remaining populations. We conclude that seepage habitats and other small waterbodies such as drainage ditches can provide an important habitat for rare taxa, which may not be found in larger waterbodies. Accordingly, they merit attention as potential hotspots of biodiversity relative to more disturbed, larger, lowland habitats. The potential for overlooking such habitats is high. We suggest conservation efforts be targeted towards inventorying and protecting such habitats.

Introduction

The adequate conservation of biological diversity requires accurate identification, and knowledge of species' distributions, abundances, and ecology (Dayton 2003). Inadequate knowledge of such factors can lead to a loss of genetic and/or species resources through a combination of anthropogenic disturbance and lack of appropriate protective conservation strategies (Daugherty et al. 1990; Hogg et al. 1998). In the freshwater arena, public attention, and hence the focus of biodiversity surveys and conservation priorities is often on larger habitats such as lakes and rivers. Accordingly, our understanding of the biodiversity of smaller waterbodies is often limited (Armitage et al. 2003).

Seepages, or holocrine springs, are small lotic habitats usually 20-50 cm wide and less than 5 cm deep that result from groundwater issuing through unsaturated or saturated soil. Because of their small size, they are often overlooked in habitat surveys and studies of invertebrate communities. However, other small waterbodies such as ditches, and other spring-types (e.g. rheocrenes, limnocrenes) have been found to be important freshwater habitats as they may provide refugia for rare or habitat-restricted species (Williams and Hogg 1988; Painter 1999; Williams et al. 2003) and increase the overall biodiversity of catchments (Armitage et al. 2003). Seepages are often less disturbed than larger downstream habitats and therefore have the potential to harbour unique fauna, and one that is perhaps unable to cope with substantial anthropogenic disturbance. This is certainly true of small headwater streams that often contain disturbance-intolerant taxa not found further downstream (Cole et al. 2003).

Small lotic waterbodies have been shown to be important habitats for a wide range of taxa in a number of different environments. Specific examples include, water beetles in Spain (Sanchez-Fernandez et al. 2004), invertebrates in Switzerland (Ilg et al. 2001), caddisfly *Rhyacophila viquaea* in Canada (Cole et al. 2003), and a new genus and species of water beetle (*Boongurrus rivulus*) in Australia (Larson, 1994). Seepages may also harbour rare plant communities (e.g. shale band seepage communities in South Africa, Sieben et al. 2004) and small headwater streams can contain a large percentage of a country's entire freshwater flora (e.g. 12% for a stream in Denmark, Baattrup-Pedersen et al. 2000). Small lotic waterbodies can therefore be an important component in freshwater landscapes by harbouring a range of taxa and increasing the total biodiversity present.

One taxon that may be representative of small lotic habitats in New Zealand is the freshwater amphipod genus *Paraleptamphopus* which is endemic to New Zealand. It contains only two currently described species, the epigean *P. caeruleus* and the hypogean *P. subterraneus* (Chapman and Lewis 1976), although it is thought to contain a number of undescribed species (Bousfield 1983; Fenwick 2000). Accordingly, the genus is in need of taxonomic revision. This situation is at least partly due to the limited sampling of seepages and other small waterbodies where putative epigean species of *Paraleptamphopus* are thought to occur. *Paraleptamphopus* spp. may also prove to be useful indicators of groundwater and/or seepage habitats as they may be stenotopic and therefore ideal as an assessment tool (Schindler et al. 2003). Similar indicator species have been shown to be very useful in characterising other aquatic habitats (e.g. caprellid

amphipods for coastal sites, Guerra-Garcia and Garcia-Gomez, 2001; and Ostracoda for a range of freshwater aquatic ecosystems, Kulkoyluoglu, 2004).

In order to characterise more accurately the species richness distribution and habitat affiliations of *Paraleptamphopus*, we sampled a variety of freshwater habitats throughout New Zealand. Specifically, we tested the hypothesis that *Paraleptamphopus* spp. are representative of smaller, seepage habitats. Further, we examined the presence or absence of *Paraleptamphopus* relative to aquatic habitat features (e.g. catchment vegetation, habitat type)

Methods

Field sites and sampling protocol

Between July 2000 and April 2003 we sampled 419 sites throughout New Zealand (Fig. 11). At each site we qualitatively assessed: 1) habitat-type (as per Table 5); 2) catchment vegetation (native, exotic, or mixed vegetation); 3) vegetation type (trees, scrub or grass); 4) within-stream vegetation (terrestrial, macrophytes, macro-algae, leafpacks and wood/ woody debris); 5) stream width/depth; 6) presence/absence of shade; and 7) macroinvertebrates present (order, family or genus level).

Macroinvertebrates were sampled using 1mm mesh-size sieves, small hand nets or larger dip nets with long handled poles. Samples were sorted on site and amphipods preserved in either 95% ethanol or in liquid nitrogen for ongoing genetic analyses while additional animals were kept in 70% ethanol for a parallel morphological study (A. Chapman, unpubl. data).

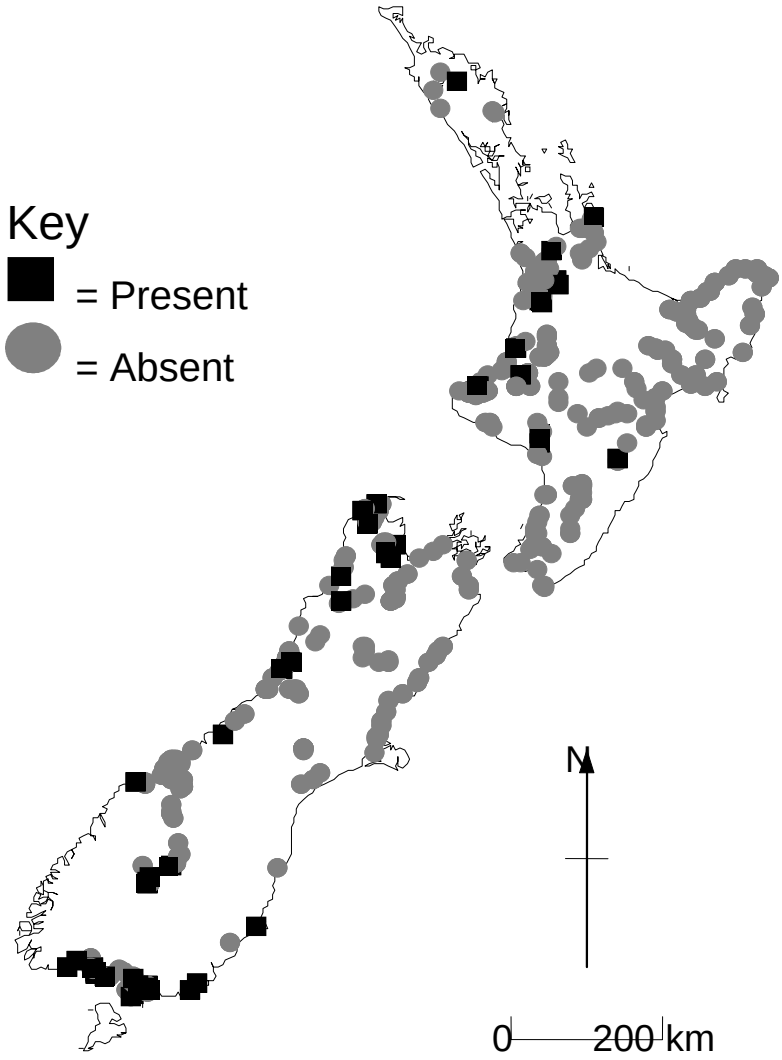

Figure 11. Map of New Zealand showing the location of study sites.

Table 5: Types and number of habitats sampled and definitions used to identify them

Waterbody	No. sampled	Definition
Lakes	17	Lentic body of water >1 ha
Ponds	7	Lentic body of water < 1 ha
Rivers	57	Lotic waterbodies > 5 m wide
Streams	175	Lotic waterbodies < 5 m wide but > 50 cm
Seepages	147	Small 1st order streams ≤ 50 cm wide
Ditches	14	Artificial channels created to drain water, typically these were roadside ditches < 100 cm wide and 50 cm deep

Statistical analysis

One-way ANOVAs were used to determine significant differences between the presence or absence of *Paraleptamphopus* versus shade type, waterbody type, catchment vegetation type, within waterbody vegetation types, macroinvertebrate groups, and depth and width (except lentic sites were excluded due to the highly variable width and depth values and none contained *Paraleptamphopus* spp.). For within-waterbody vegetation types and macroinvertebrate groups individual analysis were conducted for each separate type or group). A Tukey's post-hoc test was performed to determine which factors were significantly different. A non-metric multi-dimensional scaling (MDS) analysis using Bray-Curtis similarity data was plotted to examine relationships with other macroinvertebrate taxa.

Results

Paraleptamphopus were found at 49 sites throughout the country (Fig. 11). Of these, *P. caeruleus* occurred at 14 sites and no *P. subterraneus* were found. All other sites contained presently undescribed species and five of the aforementioned sites with *P. caeruleus* also had other undescribed species present. Of the undescribed species, at least six morphologically distinct taxa were recognised and ongoing genetic analyses suggest that perhaps as many as 28 genetically distinct taxa may exist (D. Sutherland, I. Hogg, A. Chapman unpubl. data).

Paraleptamphopus were more likely to be found in roadside ditches and seepages than in streams, rivers, lakes and ponds (F= 5.44, P> 0.001; Fig. 12). Habitat width and depth were inversely related to the presence of

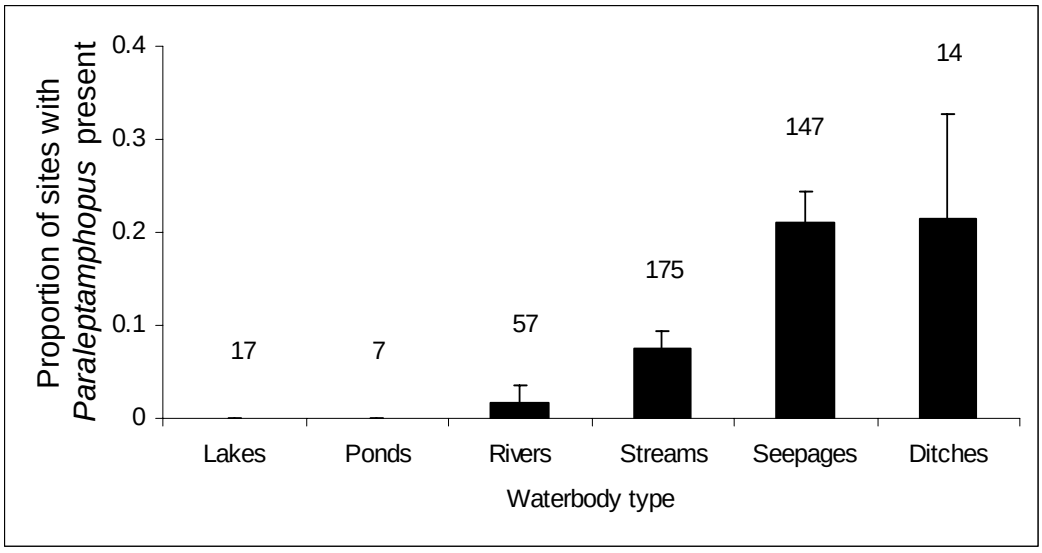

Figure 12. The proportion of *Paraleptamphopus* present in various waterbodies with total numbers of each category sampled listed above each respective bar.

Paraleptamphopus in lotic water bodies (F= 62.91, P< 0.001; F= 15.16, P< 0.001; respectively), demonstrating an affiliation with smaller waterbodies.

Paraleptamphopus were more often found at sites that had native vegetation in the surrounding catchment areas, either with complete coverage or mixed native and exotic vegetation (F= 10.12, P< 0.001; Fig. 13). They also were more common at shaded than at non-shaded sites (F= 12.20, P= 0.001; Fig. 13), and similarly shade producing forested and scrubland sites were more likely to contain *Paraleptamphopus* than grassland sites (F= 4.95, P= 0.001; Fig 14).

There were no significant ($p<0.05$) differences for the presence or absence of *Paraleptamphopus* versus within-waterbody vegetation types with leaf packs (F=1.93, P= 0.166) and woody debris (F=2.12, P= 0.146) and P >0.50 for all other types. Individual macroinvertebrate groupings also showed no significant relationships to *Paraleptamphopus* presence or absence.

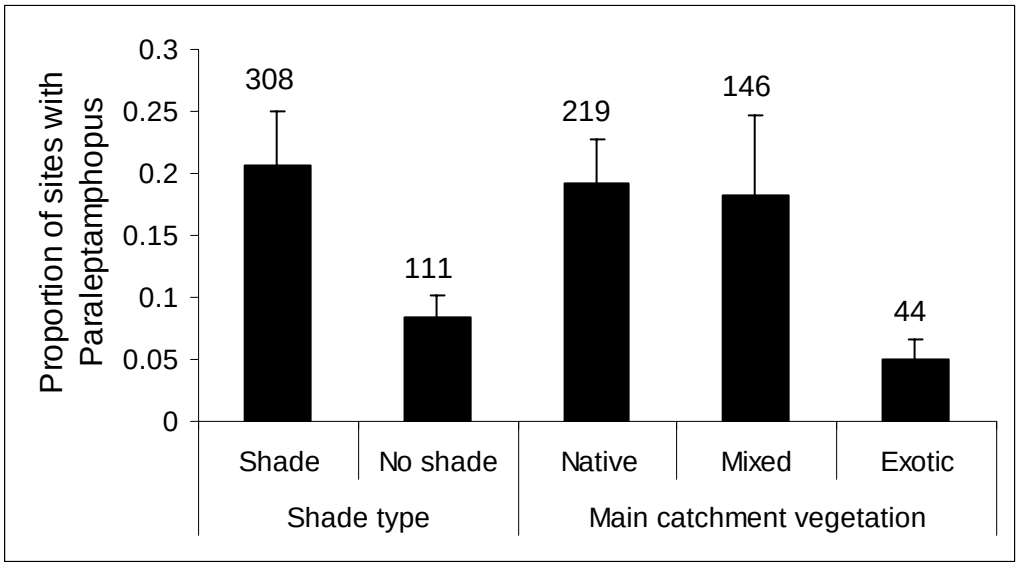

Figure 13. The proportion of *Paraleptamphopus* present in relation to shade type and the naturalness of catchment vegetation with total numbers of each category sampled listed above each respective bar.

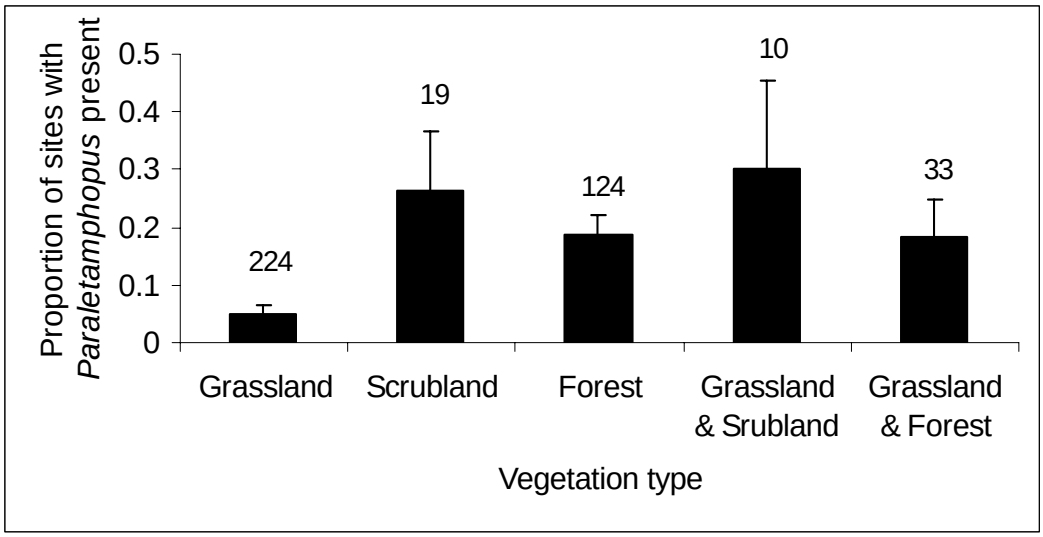

Figure 14. The proportion of *Paraleptamphopus* present in relation to catchment vegetation with total numbers of each category sampled listed above each respective bar.

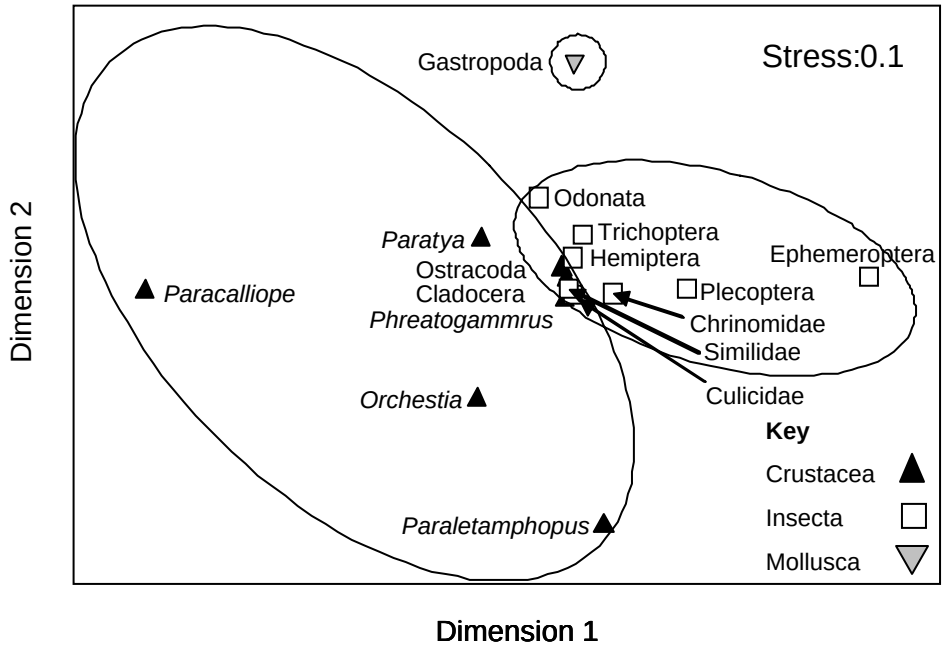

Figure 15. Non-metric multi-dimensional scaling (MDS) ordination plot using Bray Curtis distances for square root transformed presence/ absence data showing the relationships between the macroinvertebrate groupings.

At sites containing *Paraleptamphopus*, 20 other taxa were collected (Table 6). An MDS plot showed that *Paraleptamphopus* grouped well out from other macroinvertebrate taxa with the closest taxa being the amphipod family Talitridae. In general, the three major taxonomic groupings (Crustacea, Insecta and Mollusca) separate out from each other with a small band of overlap (Fig. 15).

Table 6. The broad taxonomic groups collected around New Zealand while sampling for amphipods from the genus *Paraleptamphop*us.

Taxonomic group		Taxon
Crustacea	Amphipoda	Paracalliope *sp.*
		Phreatogammarus sp.
		Orchestia sp.
		Chiltoni sp.
		Milata sp.
		Paracorophium sp.
	Isopoda	
	Mysidacea	Tenagomysis *sp.*
	Decapoda	Paratya *sp.*
	Cladocera	
	Ostracoda	
Mollusca	Gastropoda	
Insecta	Ephemeroptera	
	Plecoptera	
	Culicidae	
	Simuliidae	*Austrosimulium* sp.
	Chirnomidae	
	Trichoptera	
	Odonata	
	Hemiptera	*Anisops* sp., *Sigara* sp.

Discussion

Our sampling of 419 aquatic habitats throughout New Zealand indicated that *Paraleptamphopus* were concentrated in the North, West and South coasts of the South Island and the West Coast of the North Island. A lack of permanent small waterbodies on the East Coast of the South Island which could inhibit epigean *Paraleptamphopus* dispersal and distribution caused by highly regionalized rainfall patterns (Tait and Fitzharris 1998). Seepages were the most common habitat type for *Paraleptamphopus* spp. Very few river (2%) and stream (8%) sites contained *Paraleptamphopus* and they were not found in any lentic habitats although they have been previously reported at 160m depth in one lake (Hurley 1975). Seepages are often fishless habitats due to their small size (no fish were ever seen or caught in seepages we sampled) and this is likely an advantage for large, conspicuous amphipods such as *Paraleptamphopus.* In an evolutionary context, fishless habitats may also allow for greater species diversification by releasing amphipods from morphological constraints caused by predators (Witt and Hebert 2000, Witt et al. 2003). Accordingly, seepages may be just as important in terms of biodiversity than larger habitats such as streams and rivers.

Roadside ditches also frequently contained *Paraleptamphopus*. Previous studies have shown ditches to contain rare taxa (Painter 1999; Williams et al. 2003), and harbour species that other parts of catchment do not possess (Armitage et al. 2003). Roadside ditches and seepages may therefore provide a good indication of the surrounding seepage biota and are often more convenient to sample (e.g. near roads). Sampling of ditches and seepages may thus provide a

useful strategy for quickly assessing local taxa. They may also serve as potential conservation areas in their own right. Roadside habitats have been shown to support native invertebrate fauna and more natural habitats generally have higher abundances of target taxa (e.g. butterflies in the United States, Ries et al. 2001). Roadside habitats in areas with modified landscapes can also provide useful corridors (e.g. beetles, Vermeulen 1994). Roadside ditches and seepages with sufficient riparian vegetation may therefore be useful in providing corridors for dispersal and maintenance of seepage and small waterbody communities in highly modified habitats.

The significant relationship between catchment vegetation and *Paraleptamphopus* may be attributed to deforestation of native forest and subsequent conversion to pastoral grassland as few sites with only exotic vegetation, typically pastoral grassland, supported *Paraleptamphopus*. A change in macroinvertebrate communities due to landuse effects such as pastoral farming is well documented in New Zealand (Quinn 2000) as well as overseas (e.g. Harding et al. 1998). Forest and scrubland vegetation produces shading which was also positively correlated with the presence of *Paraleptamphopus*. The main benefit from shading is most likely a reduction in water temperature which has been shown to be beneficial for New Zealand stream macroinvertebrates (Parkyn et al. 2003).

Having a mixture of vegetation types (i.e. exotic and native) appears to provide suitable habitat. In most cases this was typically a forest remnant within pasture grassland (e.g. in a gully) or a site within pasture grassland just below forest. Other studies have shown forest remnants can offset the negative effects of pastoral farming over relatively short spaces (e.g. 50 metres; Scarsbrook and

Halliday 1999). Also, agricultural sites near forested habitats can support higher invertebrate numbers than sites further away (e.g. moths; Ricketts et al. 2001). This may explain the suitability of mixed vegetation types for *Paraleptamphopus*, which appears to be sensitive to waterbody degradation. Mild nutrient enrichment has shown to increase arthropod abundance in some cases (Zrum and Hann 2002; Scarsbrook and Fenwick, 2003) and if other potentially negative factors (e.g. sedimentation, pesticides) are minor than this may partially offset sub-pristine conditions. The presence of the genus in roadside ditches, artificial habitats that may be prone to pollution, also indicates that the genus may be tolerable to low levels of pollution.

No significant correlations were found between any of the within-site vegetation types and *Paraleptamphopus.* This may be due to the analysis being too coarse as several species exist and this may confound results (i.e. within a genus different species may have different vegetation preferences). However, a number of putative species were found in only one location and therefore clumping all the populations together allowed for a larger sample size to detect biologically significant trends. Leafpacks and woody debris had the closest association with *Paraleptamphopus* and may be biologically significant as some putative species were closely associated with one or both vegetation types. Furthermore, one species shreds and consume leaves (pers. obs.), an unusual characteristic for New Zealand aquatic macroinvertebtates (Quinn 2000), which suggests that the presence of decaying leaves would be important to it. Therefore, identification down to species, rather than genus, level is important in some ecological situations (Giangrande 2003).

Paraleptamphopus had no close associations with other macroinvertebrate groups which appears to be largely due to other groups associating with larger habitats (e.g. streams and rivers). The three major groupings: Crustacea, Insecta and Mollusca, separated out from each with a small band of overlap and *Paraleptamphopus* was more closely associated with other Crustacea than Insecta or Mollsuca. In general, individual seepages may have fewer macroinvertebrate taxa which may be due to their small size (i.e. island biogeography theory, the smaller the habitat, the fewer niches it will support and subsequently have lower biodiversity, Begon et al. 1990). However, collectively, seepages across landscape scales are likely to have high levels of diversity and sites close to the outpouring of springs may have hypogean fauna present (Gunn et al. 2000). Furthermore, they may contain species not present in other waterbodies and therefore would increase the overall biodiversity of catchments.

Our study shows that *Paraleptamphopus* is relatively widespread throughout New Zealand and predominately occupies small freshwater seepages and other small waterbodies such as roadside ditches and is rarely found in larger waterbodies such as rivers and lakes. Several undescribed species were collected and this is probably due to the lack of sampling effort and study on seepages and roadside ditches where they most often occur. The genus appears vulnerable to anthropogenic disturbance, although at least some species appear to be able to tolerant minor landuse changes. We conclude that seepage habitats and other small waterbodies such as drainage ditches can provide an important habitat for rare taxa, and are therefore critical in maintaining overall levels of biodiversity within catchments and consequently more attention should be focused on such habitats.

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CHAPTER IV

PHYLOGEOGRAPHY AND SPECIES RECOGNITION IN THE *PARACALLIOPE FLUVIATILIS* SPECIES COMPLEX (CRUSTACEA; AMPHIPODA): CAN MORPHOLOGICALLY SIMILAR HETEROSPECIFICS DISCRIMINATE POTENTIAL MATES?

Key words, *Paracalliope fluviatilis*, outbreeding, phylogeography, cryptic species, Amphipod

To be submitted under the same title as: Sutherland, D.L., Hogg, I.D., and Waas, J.R.

Abstract

The amphipod *Paracalliope fluviatilis* appears morphologically uniform throughout its range but has CO1 sequence divergence rates as high as 24%, suggesting the existence of one or more cryptic species. Species recognition and discrimination may contribute to or maintain divergence by preventing random mating between individuals from different populations. We examined the phylogeographic patterns of *P. fluviatilis* over its entire range and the prevalence of mate discrimination in laboratory mate choice tests using genetically distinct populations. We hypothesised that the level of genetic divergence between populations would be positively correlated with mate discrimination. Individuals were collected from seven populations. Males from a reference population were presented with "local" (same population) or "foreign" (genetically divergent) females. Males were more likely to pair with local than foreign females – the more genetically divergent the foreign female, the greater the preference for local females. However, the response was not gradual - an abrupt shift in preference occurred when genetic divergence among populations exceeded c.20%. The abrupt shift may be attributable to the evolution of distinctive cues (e.g. behaviour) that enable discrimination and/or improvements in the species' recognition system. This suggests that evolutionary history rather than genetic isolation alone is responsible for the patterns of discrimination we observed.

Introduction

Traditional taxonomy based on morphology is gradually being enhanced by direct measures of molecular variation (Tautz, et al. 2003). In some cases, species can be identified on the basis of one or two gene sequence fragments (e.g. barcoding species using CO1; Hebert et al. 2003a; Hebert et al. 2003b). Many of these species are "cryptic species", morphologically indistinguishable but genetically distinct from their sibling species (e.g. Witt and Hebert 2000). However, there are concerns about employing species identification techniques based on genetics alone, especially without considering morphological or behavioural information (e.g. Mallet and Willmot, 2003; Will and Rubinoff, 2004). For example, genetically similar sibling species may be erroneously grouped together as a single species while genetically distant populations may be wrongly assigned species status.

In order to delineate species boundaries using gene sequences, knowledge of the relationship between genetic distances and speciation is required (Edmands, 2002). If the relationship varies widely between taxa, the utility of molecular techniques may be limited and rates of gene mutation will then have to be calibrated for each particular group of interest. Differences in mutation rate for CO1, relative to other invertebrate groups, have already been found for cnidarian species which appear to have low rates of CO1 mutation (Hebert et al. 2003b) and therefore congeneric species could conceivably be grouped together as single species if identified solely on the basis of the CO1 gene. In morphologically distinct species, genetic misdiagnoses may be confirmed by visual inspection.

However, this is not possible for morphologically indistinct, cryptic species and alternative means of identification are required.

The ability to recognise, and hence mate with, members of the same species is an important component of reproductive success and ultimately speciation processes (Mayr 1982; Colgan 1983). Deviation from random mating between different populations may indicate the existence of different species or processes initiating speciation. Accordingly, behavioural studies of species recognition are needed to identify or validate cryptic species, which cannot be elucidated using traditional taxonomic means. Additionally, experiments involving multiple, inter-population comparisons may assist in a better understanding of how genetic distances correspond with species-level boundaries. For example, is there a level of divergence where mate recognition, and correspondingly speciation processes potentially establish?

One of the most speciose aquatic groups is the order Amphipoda (Ruppert and Barnes, 1994), and recent studies have indicated that species diversity may actually be underestimated, primarily due to the existence of morphologically similar, but genetically distinct (cryptic), species (Hogg et al. 1998; Witt and Hebert 2000). Amphipods are also known to display pre-copulatory mate guarding whereby males of some species guard females by remaining in close proximity, or physically attaching themselves, to females. This behaviour has proved useful for investigations of mate selection (Jormalainen**,** 1998). For example, Dick and Elwood (1992) studied the morphologically similar congenerics *Gammarus pulex* and *Gammarus duebeni*, and showed that *G. pulex* males distinguish between, select and preferentially guard *G. pulex* females over the closely related *G. duebeni* females. Accordingly, mate guarding amphipods

may provide a useful model for investigations of cryptic species as well as factors that may promote behavioural and genetic differentiation in the absence of morphological differences.

The amphipod *Paracalliope fluviatilis* (Family Paracalliopiidae) is endemic to New Zealand where it is the most abundant and widespread freshwater amphipod species. It is commonly found amongst macrophytes in the slow flowing margins of rivers and on the edges of lakes. A high degree of genetic variation occurs within the species throughout its range, with sequence divergences of up to 24% for the most divergent populations (Hogg et al., 2006). The species is also the only known New Zealand freshwater amphipod to display pre-copulatory (physical attachment) mate guarding (Chapman & Lewis 1976).

Here, we assessed phylogeographic patterns to determine the relationships among populations as well as existing levels of genetic divergence and the timing of divergence events. Secondly, we conducted mate choice experiments to determine whether males would discriminate against genetically-divergent females and if so at what level of divergence discrimination would occur. These data will allow us to assess the use of genetic divergences as a method for detecting cryptic species, as well as determine the role of evolutionary history on mate recognition systems and/or discrimination patterns.

Materials and Methods

Field Sampling

Between July 2000 and April 2003 we sampled for *Paracalliope fluviatilis* at 419 sites in a variety of freshwater habitats throughout New Zealand (Fig. 16).

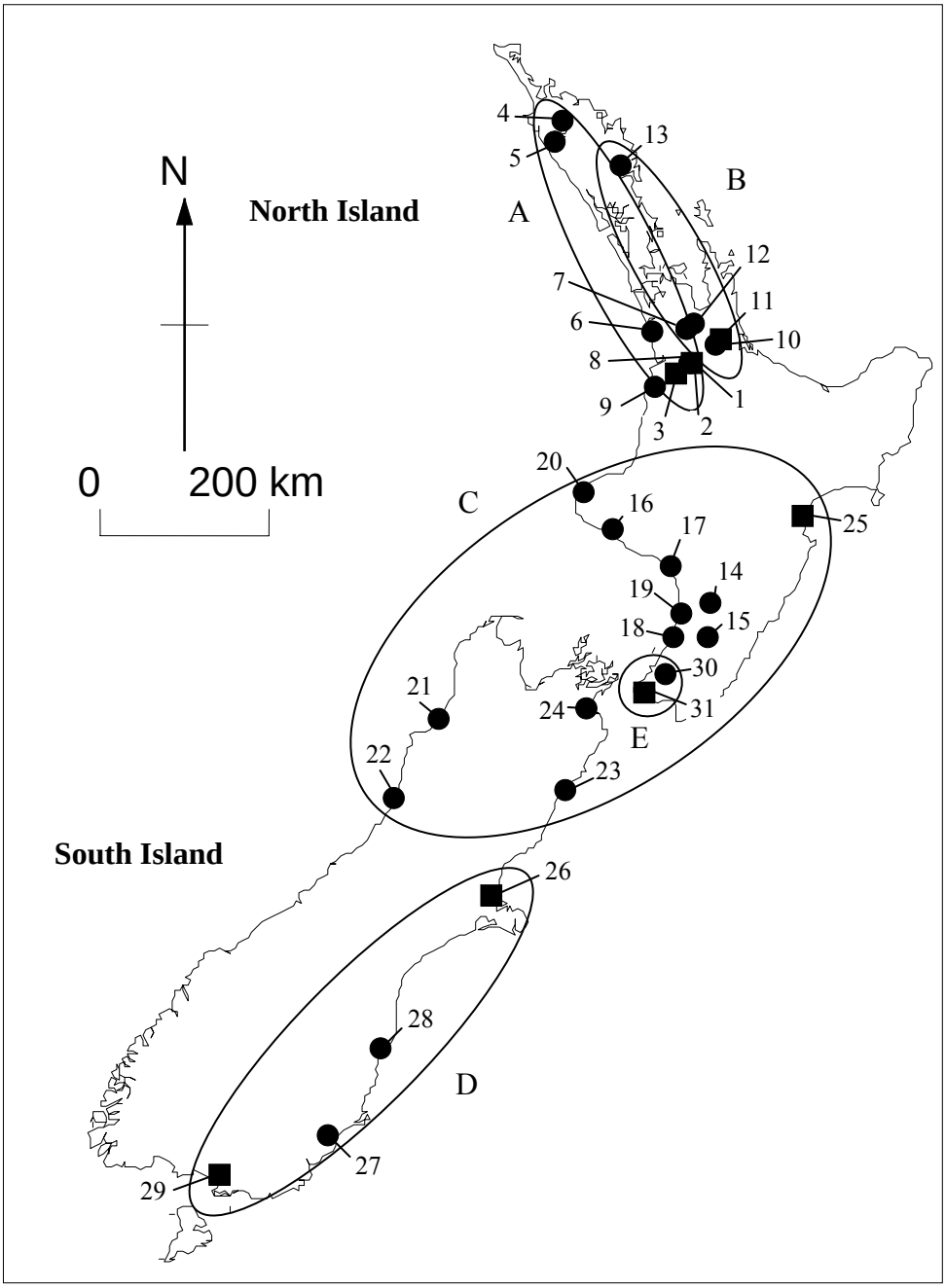

Figure 16. Map of New Zealand showing collection sites. Sites with squares indicate populations used in the mate choice experiment; numbers next to collection sites correspond to those used in the Maximum Likelihood Tree (Fig. 17).

Animals were collected using 1 mm mesh size sieves, small hand nets or large nets with long handled poles. Samples were live sorted on site in trays, and amphipods preserved in either 95% ethanol or in liquid nitrogen. Laboratory samples preserved in ethanol were kept at – 20 °C while all others were kept at – 76 °C until needed for DNA analysis. Samples were also obtained from GenBank (accession numbers DQ285299-DQ285343; Hogg et al. 2006).

DNA Sequence Analysis

DNA was extracted from entire individuals (1-2 individuals per site) using the DNeasy Tissue kit (Qiagen Inc) as per the manufacturer's instructions, with the exception that we incubated the sample at 56°C for 24 hours and used 60 µl to elute the DNA. PCR amplification was carried out using a 50 µl reaction volume consisting of 2 µl of DNA, 1×PCR buffer plus $MgCl_2$ (Roche), 2.2 mM $MgCl_2$, 0.2 mM of each dNTP (Boehringer Mannheim), 1.0 µM of each primer, and 1.0 unit of *Taq* DNA polymerase (Roche) on a Eppendorf Mastercycler gradient thermocycler. A 710 base pair fragment of the mitochondrial gene cytochrome *c* oxidase I (COI) gene was amplified using the universal primers LCO1490 (5'- ggt caa caa atc ata aag ata ttg g -3') and HCO2198 (5'- taa act tca ggg tga cca aaa aat ca -3') (Folmer *et al*. 1994). The thermal cycling conditions were: 94°C for 1 min followed by 5 cycles of denaturation and polymerase amplification (94°C for 1 min, 45°C for 1.5 min and then 1 min at 72°C) and followed by 35 cycles at 94°C for 1 min, 51°C for 1.5 min and then 1 min at 72°C, followed by 5 min at 72°C. Sequencing was performed using the same primers as those used for PCR amplification on an ICM version 3.1 automated sequencer (MegaBace) at the

University of Waikato DNA sequencing facility. The forward direction was always sequenced while the reverse was also sequenced in approximately 10% of samples to verify sequence accuracy.

Sequences were aligned using Sequencher (Gene Codes ver. 4.1.2 for Macintosh) sequence editor and verified as being derived from amphipod DNA using the GenBank BLAST algorithm. A Mantel test was performed on pairwise sequence divergence and geographic distances using GenAlEx V5 (Peakall and Smouse, 2001) to determine whether there was a significant relationship between genetic versus geographic distance. The degree of mutational saturation was estimated by examining the correlation between the ts/tv ratio and the pairwise sequence divergence. If saturation occurred, then a decrease in ts/tv ratio is expected as sequence divergence increases (Kocher et al. 1995). We used χ^2-tests, as implemented in PAUP* 4.0b10 (Swofford 2002), to determine whether the assumption of equal base frequencies among sequences was violated on: (1) all sites; (2) parsimony-informative sites only; and (3) the third codon position only. We then constructed phylogenetic trees using PAUP* 4.0b10 (Swofford 2002). A neighbour-joining (NJ) phylogram was constructed using the TrN+G model (selected using the program Modeltest 3.5, Posada & Crandall 1998). The estimated parameters under this model were: ts/tv ratio = 5.056; and Gamma distribution shape parameter = 0.1972. A maximum likelihood (ML) search was conducted using the above model parameters. A maximum parsimony (MP) analysis using the heuristic search option with unweighted characters was also implemented. Two amphipod sequences were obtained from GenBank for use as outgroups. These were *Epimeria geogiana* and *Eusirus perdentatus*, and were selected on the basis of an analysis (MP tree, data not shown) of a range of

amphipod species which suggested that from amphipod sequences held by GenBank they were the most closely related to *P. fluviatilis*. We used three tree construction methods in order to minimise the potential for error that may arise from assumptions inherent in phylogenetic reconstruction methods. Confidence in the cladistic analyses was assessed by estimation of the g_1 skewness statistic from 100,000 random tree length distributions (Hillis & Huelsenbeck 1992), and by bootstrap analysis with 1000 pseudoreplicates for the NJ tree (Felsenstein 1976). Node support for the NJ and ML trees was assessed using decay indices calculated in PAUP* 4.0b10 using command lines computed by MacClade 4.03. A Templeton (Wilcoxon signed-ranks) test was used to determine whether significant differences existed between the trees. To examine whether lineages within trees were evolving at a similar rate, a two-cluster test was employed (Takezaki et al. 1995). To test whether sequences were diverging in a clock-like manner, a log-likelihood ratio test was carried out in PAUP* 4.0b10 that compared ML trees generated with the molecular clock option enforced with unconstrained trees (Felsenstein 1988). Divergence times were then estimated by using a molecular clock approximation for CO1 of 2.2-2.6% nucleotide sequence divergence per million years (Knowlton et al. 1993). This rate was derived from the study of several malacostracan crustaceans whose divergence resulted from a distinct geological event, the formation of the Isthmus of Panama.

Mate Choice Experiments

We collected live specimens of *P. fluvatilis* for laboratory experiments from seven locations throughout New Zealand: 1) Hamilton (S 37.80°, E 175.30°); 2) Pirongia (S 37.82°, E 174.90°); 3) Te Aroha (S 37.55°, E 175.71°); 4) Napier (S

39.39°, E 176.87°); 5) Wellington (S 41.12°, E 175.06°); 6) Christchurch (S 43.69°, E 172.57°); and 7) Invercargill (S 46.39°, E 168.38°) (Fig. 16). Animals from each population were kept in separate two litre holding tanks containing macrophytes (*Elodea canadensis*) to enrich the environment and to provide oxygen. Fish food flakes (Nutrafin Staplefood™) were ground to a paste and added by a pipette to supplement grazing on epiphytic algae. Water was changed weekly and excess food pipetted off every second day. Containers were maintained at 18 ˚C under a 15:9 light regime to simulate summer conditions to enhance pairing. All animals were held at least three days in holding containers before experiments began to acclimatise animals to laboratory conditions.

Choice tests were conducted in clear plastic containers (62 x 62 x 110 mm high) filled with 200 ml of water, an eight cm piece of *E. canadensis* and a drop of fish food. Choice tests consisted of placing three single individuals, a male and two females, together. For half of the tests a Hamilton male was presented with a Hamilton female and a female from a "foreign" population while for the other tests a male from the same foreign population was used instead of the Hamilton male. Individuals from six different populations were tested with the Hamilton reference animals resulting in six interpopulation comparisons and 64 individual choice tests. Female size was kept constant (e.g. a small female was used with another small female), as female size has been shown to influence male choice in other amphipod species (Jormalainen, 1998). Males prefer large females and those about to moult (D. Sutherland unpubl. data); therefore, males do not simply pair with the first female encountered and are likely to be selective during tests. As individuals from different populations were morphologically indistinguishable, females were identified in choice tests by the placement of a single white spot of

fast drying correction fluid (Bic Wite Out™ Milford, USA) on a unique position on each of their backs. Subjects were checked every 24 hours for up to eight days by which time the majority of animals had paired.

When pairing was first observed (i.e. a male was physically attached to the female consistent with mate guarding) pairs were observed for at least 30 seconds to ensure that the male had a maintained grasp on the female. The time to the first detected pairing was recorded, as well as the population the paired female came from. Pairs were left undisturbed until they separated (usually 1-4 days in this species); the duration of pairing was then recorded and the animals placed in 70% ethanol. At the end of the experiment the number of eggs produced by each female was recorded. Chi-square tests were used to determine whether males preferred pairing with females from their own population or paired randomly. To assess discrimination, we used the proportion of males selecting local (versus foreign) females in a linear regression with: (1) pairwise sequence divergence; and (2) geographic distance. One-way ANOVAs were performed on: (1) the length of time taken for pairing to be detected; (2) the duration of pairing (to determine if males would invest more time guarding local females); and (3) female egg number (to determine if post-zygotic isolating barriers could prevent egg production).

Results

Genetic Diversity and Phylogeny Reconstruction

A 578 base pair fragment of the COI mitochondrial gene was used for our analyses of 48 *P. fluviatilis* from 31 sites. Consensus sequences of individuals

from the same population that shared the same genotype were made. There were 239 informative sites, 32 variable but parsimony uninformative sites and 307 constant sites; no insertions or deletions were detected. A total of 30 different haplotypes were found with pairwise sequence differences ranging from 1% to 27%. Each site was represented by a single haplotype. All sequences have been deposited with GenBank (Accession numbers to be added in proofs). The nucleotide composition across all sequences was: A = 26%, T = 28%, C = 27% and G = 19%. The assumption of homogeneity of base frequencies was supported using all codon positions ($\chi^2_{90} = 77.27$, $P = 0.828$), and using the third codon position only ($\chi^2_{90} = 6.67$, $P = 1.000$), but not for parsimony informative sites only ($\chi^2_{90} = 204.32$, $P < 0.001$). The graph of ts/tv ratio versus sequence divergence revealed only a slight negative slope ($y = -20.028x + 5.6256$; $R^2 = 0.3835$; Appendix IV.1) indicating limited mutational saturation (Kocher et al. 1995). The Mantel test indicated that there was a significant correlation between genetic and geographic distance ($y = 0.0001x + 0.1119$; $R^2 = 0.332$, $P = 0.01$; Appendix IV.2).

The maximum parsimony analysis produced 34 most parsimonious trees (tree length = 1080, C.I. = 0.4343, R.I. = 0.7907) with good phylogenetic signal ($g_1 = -0.495$, $g_{crit} = -0.09$, $P < 0.01$). A Templeton (Wilcoxon signed-ranks) test examining tree similarity showed there was a significant difference between the ML (Fig. 17) and MP (not shown) trees ($P < 0.05$). No significant differences occurred between the NJ (Fig. 18) and strict consensus MP trees ($P = 0.52$) and the NJ and ML trees ($P = 0.06$). For the NJ tree (Fig. 18), Clades A and B had good bootstrap support but clades C, D and E were not well supported, though haplotypes of clades D and E were always basal to those of clades A, B and C. Clade C had five distinct groups with haplotypes of each individual group well

supported but the relationships between the five groups were not. This result was congruent with that of the MP and ML trees, which were also unable to adequately resolve clades C, D and E.

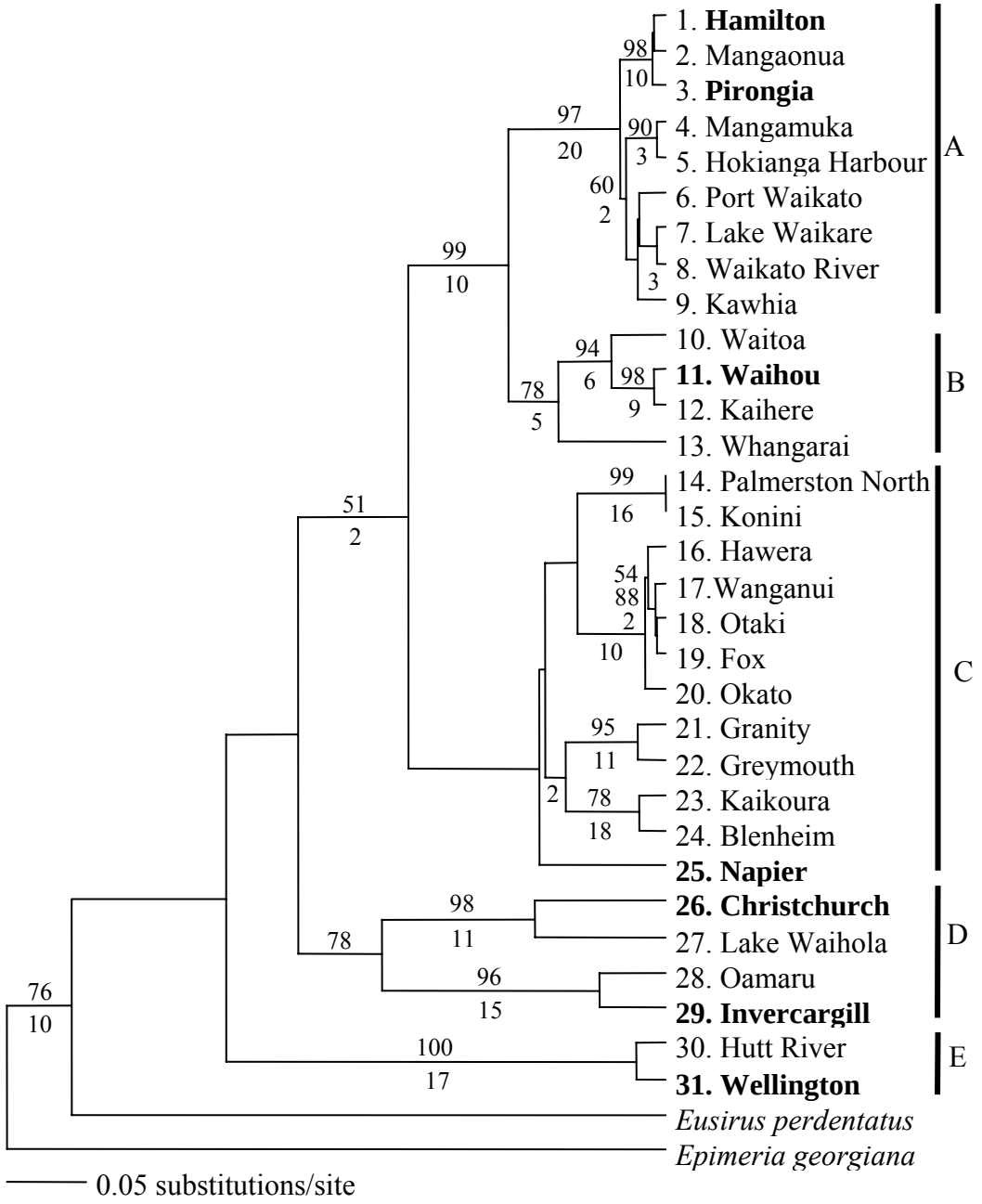

Figure 17. Maximum Likelihood tree for *Paracalliope fluviatilis* with sites used in mate choice experiment highlighted in bold with bootstrap values (> 50) above nodes and decay indices (>0) below nodes.

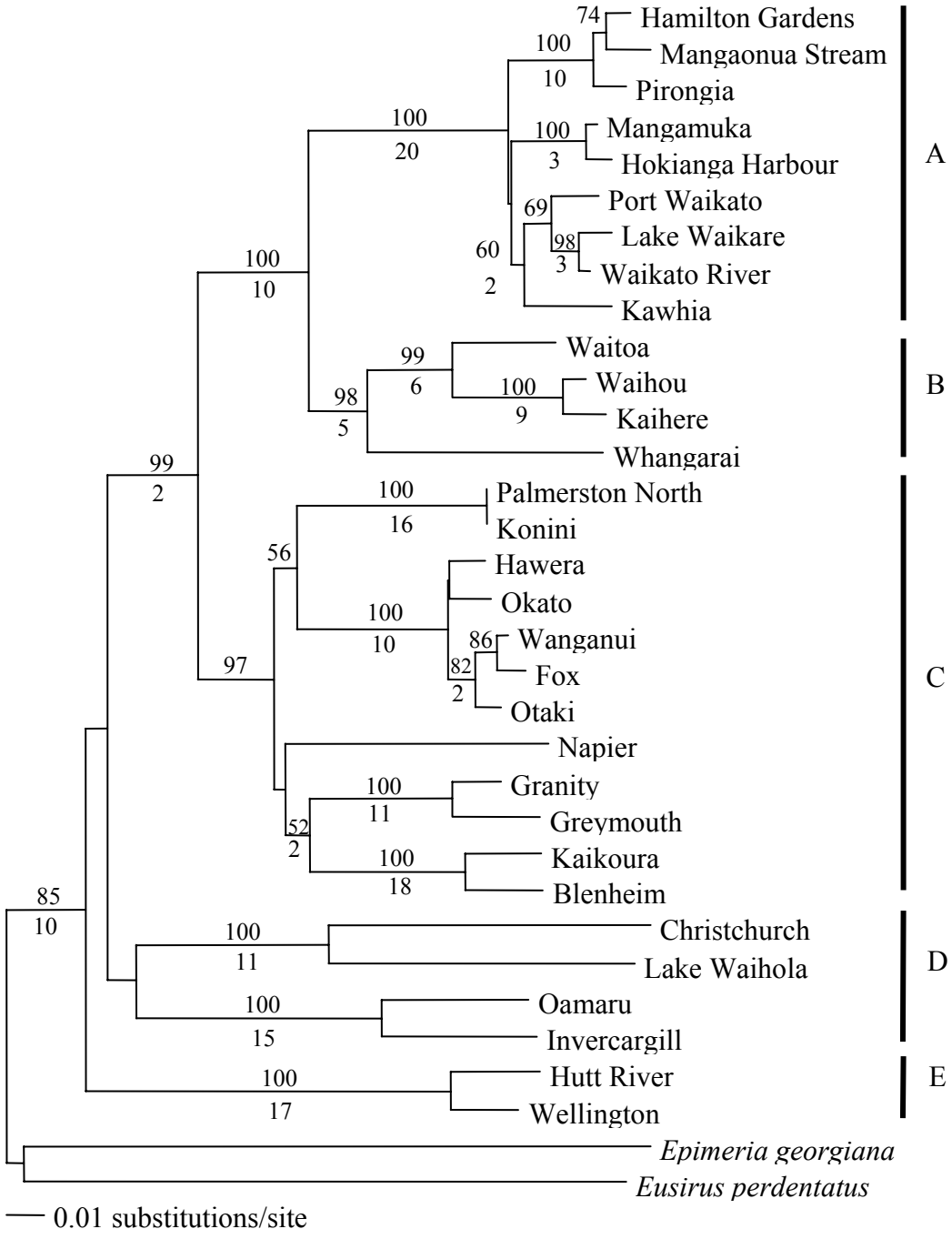

Figure 18. Neighbour Joining tree for *Paracalliope fluviatilis* with bootstrap values (> 50) above nodes and decay indices (>0) below nodes.

The two-cluster test showed that there was evidence for rate heterogeneity between three different clades ($P<0.05$) indicating differential rates of evolution. These differences were between: (1) Oamaru – Invercargill versus Christchurch – Lake Waihola; (2) clade C versus clades A and B and (3) the Mangamuka - Hokianga Harbour clade versus the Pirongia, Hamilton and Mangaonua Stream clade. A log-likelihood ratio test supported the hypothesis that lineages were evolving according to a clock-like model of evolution (-ln L = 5161.34 without molecular clock enforced and -ln L = 5170.33 with molecular clock enforced [difference = 8.99]; χ^2 = 15.655, d.f = 31, $P > 0.99$). Using a molecular clock rate of 2.4% nucleotide sequence divergence per million years (Knowlton et al. 1993), clade E diverged approximately 11 million years ago and clade D diverged approximately 10 million years ago during the Miocene. The differences between clades A-C were smaller and ranged from 6 - 7.5 million years ago, during the late Miocene/ early Pliocene boundary. Within-clade ranges for clades A-C were between 0 – 5 million years ago (i.e. from the Pliocene onwards).

Mate Choice Experiments

Males had a preference for females from the same population (Table 7). There was also a significant correlation between geographic distance and the local: foreign ratio (R^2= 0.904, $T=6.15$, P = 0.004) but not for sequence divergence and the local: foreign ratio (R^2= 0.373, $T=1.54$, P = 0.198). There was no difference between the six populations in the time taken to form pairs (P = 0.09-0.90) or in how long pairing lasted (P = 0.30-0.77). There was also no difference in mean egg

number per female or the proportion of females producing eggs when the number of pairings was taken into account (P = 0.22-0.87). However, there was a significant difference for total number of females producing eggs when the Hamilton reference population was compared to Wellington (Intra:Inter-population 11:2, F= 8.63, P = 0.005), Christchurch 6:0, F= 4.14, P = 0.046), and Invercargill 5:0, F= 5.74, P = 0.020; Table 8.).

Table 7. Table showing the ratio of local to foreign pairs formed from six intra and inter-population crosses with significance values and their geographic (km) and pairwise genetic (uncorrected p) distances.

Hamilton	Local: Foreign	X^2	P value	Geographic Distance	Genetic Divergence
Pirongia	25:24 (1.04:1)	0.083	0.773	15	0.019
Waihou	25: 23 (1.09:1)	0.191	0.662	25	0.140
Napier	22: 20 (1.10:1)	0.095	0.758	243	0.195
Wellington	34: 7 (4.86:1)	17.780	<0.001	416	0.215
Christchurch	20: 4 (5.00:1)	10.667	0.001	689	0.235
Invercargill	26: 2 (13.00:1)	20.571	<0.001	1120	0.215

Table 8. Comparisons of time taken to pair (TTP), time paired (TP), the number of females who produced eggs, and the mean egg number per female for six intra and inter-population crosses.

Hamilton	Local: Foreign	TTP	TP	Females with eggs	Mean Egg No.
Pirongia	25	1.84	1.84	4	3.75
	24	1.88	2.36	6	4.17
Waihou	25	1.68	1.00	7	3.86
	23	2.17	1.04	6	3.17
Napier	20	2.27	1.64	3	3.33
	20	1.50	1.50	3	2.00
Wellington	34	2.41	2.11	11	3.36
	7	2.29	1.63	2	3.50
Christchurch	20	2.60	2.25	6	4.83
	4	3.25	1.00	0	0
Invercargill	26	4.08	1.75	5	3.20
	2	5.00	1.00	0	0

Discussion

Phylogeography

There were considerable genetic divergences among populations of *P. fluviatilis* with values ranging up to 23.5% suggesting that cryptic species may exist (e.g. Hebert et al., 2003b). The greatest sequence divergences occurred between some of the South Island populations (e.g. Invercargill and Lake Waihola) while generally smaller differences occurred between North Island populations. Despite the large genetic divergences among some lineages, no morphological differences have yet been found (Hogg et al. 2006; M.A. Chapman unpubl. data). This pattern of morphological conservatism coupled with large genetic divergences has been previously reported among other crustaceans (Colbourne and Hebert, 1996; Lee, 2000, Witt and Hebert, 2000).

The genus *Paracalliope* was likely introduced to New Zealand from Australia via the Tasman current (Barnard 1972). This current has been present since the Miocene (Field et al. 2002), and is thought to be responsible for the dispersal of several other aquatic species into New Zealand (e.g. Mudfish genus *Neochanna*; Waters and White 1997; the Galaxiid fish, *Galaxias maculatus*; Waters et al. 2000). Based on divergences we detected in *P. fluviatilis* (<23.5%), dispersal to New Zealand may have occurred during the late Miocene (11-15 MYA). The ancestral form may have undergone an estuarine phase before becoming fully adapted to the freshwater environment. Genetic divergences among populations would then have resulted from a reduction in dispersal between river catchments as populations became more restricted to freshwater habitats.

The beginning of the Pliocene (5 MYA) was characterised by rising sea levels that resulted in marine inundation in New Zealand creating two major and several minor islands (Stevens and Ridge 1995; Fig. 19). The resulting fragmentation of the New Zealand landmass has been attributed to patterns of genetic divergence seen in insects (e.g. *Hemideina thoracica*; Morgan-Richards 2001) as well as speciation events in land snails (e.g. *Lissotes*; Fleming 1979). The separation of clades A and B and the fragmentation of clade C correspond to the Pliocene, suggesting that they may have become isolated on different islands. Clade A appears to have become isolated on the upper North Island and clade B in the small islands above the North Island (Fig. 19). Clade C includes populations from the east and west coasts of the upper South Island, the lower North Island (Fig. 19). The marine inundation may have facilitated dispersal to the West Coast of the South Island, previously blocked by mountain ranges and prevailing ocean currents.

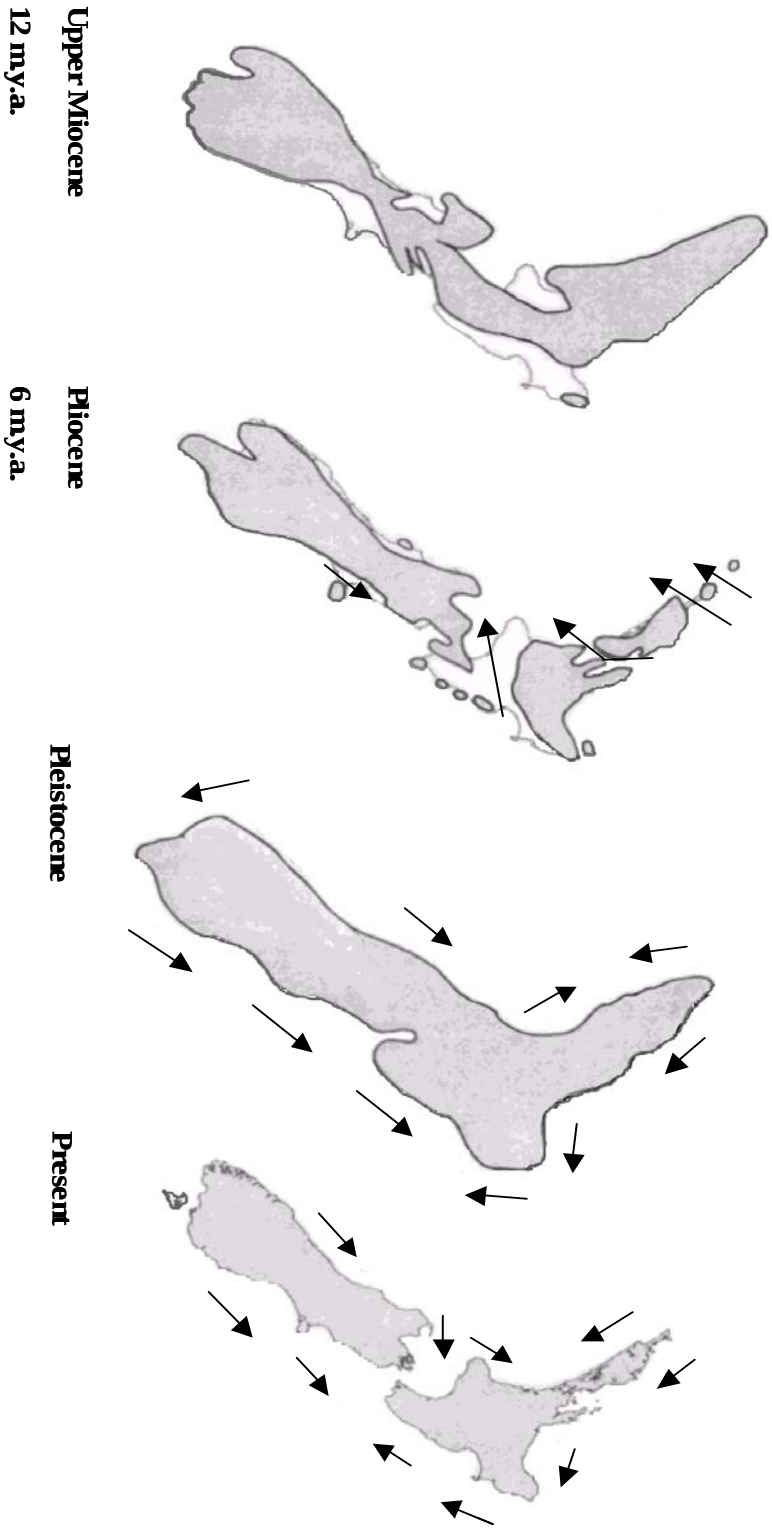

Figure 19. Time series of New Zealand during the last 12 million years showing land above sea-level (grey) superimposed on the current New Zealand coastline. Arrows indicate prevailing currents. Figures adapted from Fleming (1979) and Stevens and Hogg (2004).

The Pleistocene glacial cycles caused the recession of the sea and unification of New Zealand's major islands as sea levels fell as a result of the global cooling and subsequent build up of ice (Stevens and Ridge 1995). The existence of continuous land would have allowed the re-colonisation of previously inundated habitat by *P. fluviatilis* and it appears that individuals from clade A may have dispersed southward from the top of the North Island to at least as far south as the central North Island (Fig. 16) during this period while clade B appears to have spread north to the top of the North Island. The interesting geographic split between clades A and B corresponds to east and west flowing watersheds and suggests that *P. fluviatilis* are only capable of dispersing between parallel catchments via the sea. Hence, the east and west coast populations have not come into contact (see also Hogg et al. 2006).

Mate Choice Experiments

There was evidence for non-random (assortative) mating between the Hamilton reference population and the three populations that were the most genetically divergent (i.e. Wellington, Christchurch and Invercargill). This finding in conjunction with large genetic divergences (≥ 21.5%), and the lack of eggs produced from foreign/local pairings suggests that individuals from the three populations are indeed a separate species from the Hamilton population. During Hamilton/Napier tests (19.5% genetic divergence from Hamilton), males showed little preference for local versus foreign females – suggesting that discrimination is not gradual but abrupt. Accordingly, identifying species on the basis of genetic divergence alone may be problematic for this taxon. This may reflect an inability

of different taxa to distinguish more closely related individuals (i.e. within the same clade) even when genetic divergences are high – evolutionary history rather than genetic divergence *per se* may be a more accurate determinate of species relations. Furthermore, significant discrimination was only apparent when genetic divergences were above 21.5%, nearly double the mean divergence of 11.3% for most congeneric pairs found by Hebert et al. (2003a). This suggests that *P. fluviatilis*, and perhaps Amphipoda in general, may be more genetically diverse than other animal taxa.

Individuals from morphologically similar species may need to use behavioural cues or biochemical products such as pheromones to discriminate potential mates. Conspecific recognition via behavioural displays is common within some taxa (Colgan, 1983; Johnson, 2000; Wrens, 2000). However, no obvious behavioural differences were observed among populations of *P. fluviatilis*. In some amphipod species, females release hormones when their exoskeleton is shed and this can attract males as it indicates that the female is ready to be fertilised (Jormalainen, 1998). Accordingly, it is possible that differences in pre-moulting hormones are used to identify conspecific mates (e.g. crayfish *Procambarus clarkii*, Corotto et al. 1999).

Although strong bias in mate selection existed for the three most divergent crosses, some local/foreign pairings resulted in eggs being produced. This raises the possibility that viable hybrids could form between the putative sibling species. This could be particularly problematic for allopatric species when isolating barriers are removed as lower levels of pre-zygotic reinforcement may exist (Coyne and Orr, 1997). Furthermore, in some crustacean taxa (e.g. *Tigriopus californicus*), F_1 hybrids show hybrid vigour and as such may be

disproportionately represented in subsequent generations, potentially disrupting any co-adapted gene complexes (Edmands 1999).

The large genetic divergences we found suggest that *P. fluviatilis* is a species complex. There appears to be limited gene flow among river catchments suggesting that geographically isolated populations may be undergoing speciation. Mate choice tests suggest that discrimination is occurring among the most divergent populations. However, the changes are not gradual but abrupt perhaps reflecting the inability of individuals to distinguish mates of common ancestry (i.e. within clades). From a conservation perspective, the accidental translocation of genetically divergent individuals could potentially disrupt evolutionary trajectories, species boundaries and/or the overall fitness of populations.

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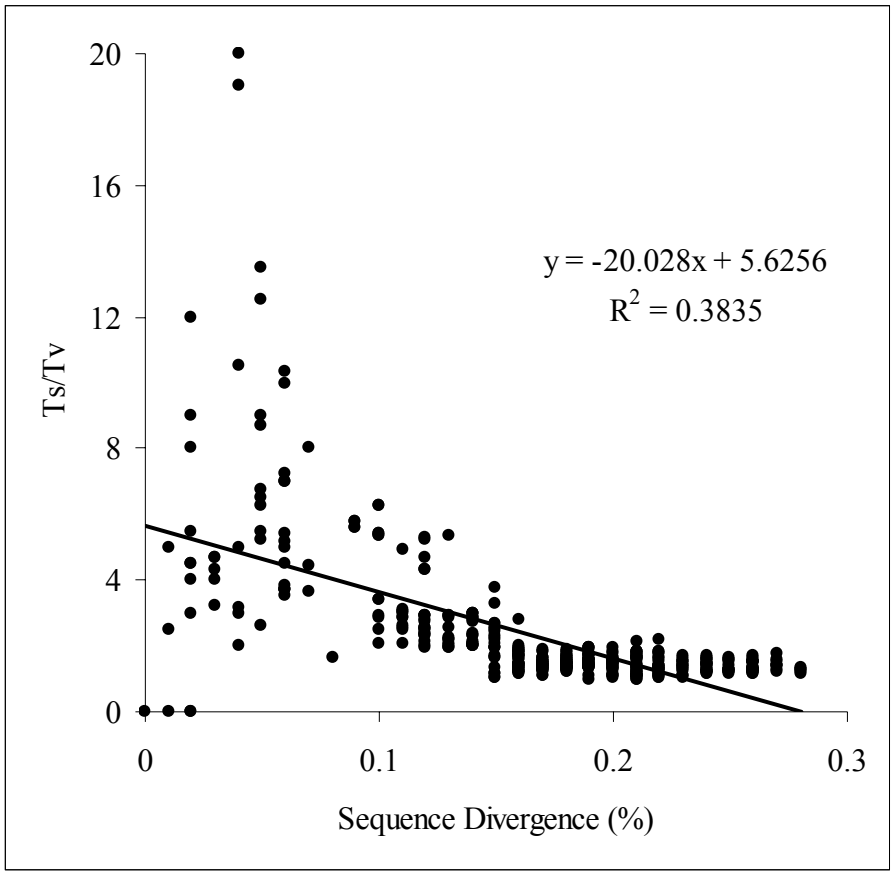
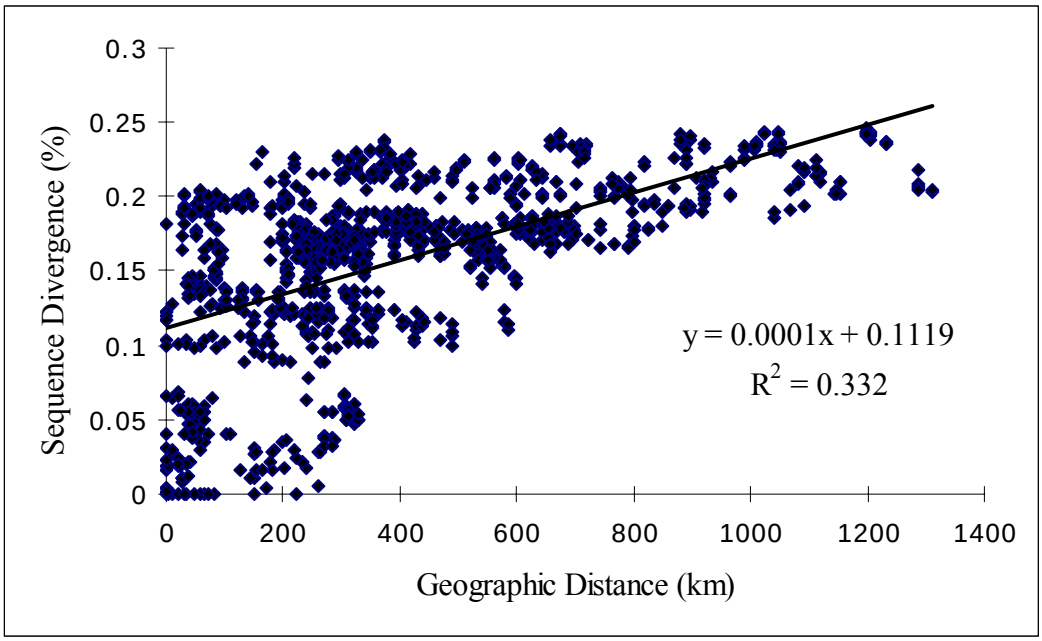
APPENDIX IV.1. Ts/tv ratio versus CO1 sequence divergence for *Paracalliope fluviatilis*.



APPENDIX IV.2. Pairwise comparisons between CO1 sequence divergence and geographic distances for *Paracalliope fluviatilis*.



CHAPTER V

IS SIZE ASSORTATIVE MATING IN *PARACALLIOPE FLUVIATILIS* (CRUSTACEA; AMPHIPODA) EXPLAINED BY MALE-MALE COMPETITION OR FEMALE CHOICE?

Keywords: amplexus, size assortative mating, sexual selection, *Paracalliope fluviatilis*, pre-copulatory mate guarding.

Submitted under the same title as: Sutherland, D.L., Hogg, I.D., and Waas, J.R.

ABSTRACT

Field and laboratory studies were used to assess: (a) if size assortative mating occurred in the New Zealand amphipod *Paracalliope fluviatilis* and (b) hypotheses developed to explain size assortative mating. We found that assortative mating occurred and that larger females carried more eggs, suggesting they may be more valuable as mates. Laboratory experiments were then used to determine if: (1) male size influenced the size of the female selected (mechanical constraints hypothesis); (2) male size influenced pairing success in the presence of competition (intra-sexual selection hypothesis); (3) take-overs of females occurred and whether large males were more successful (intra-sexual selection hypothesis); (4) guard duration varied relative to male and female size (guard duration hypothesis); and (5) females had control over pairing success and guard duration (inter-sexual selection hypothesis). Although there was evidence to suggest intra-sexual competition for mates (i.e. both small and large males preferred large females), there was no evidence of overt competition (i.e. takeovers of paired females). There was also no difference in how long small and large males guarded females, but large females were guarded longer by both male size classes. Females handicapped by having their mobility reduced were guarded for the same duration as control females but males were more likely to pair with handicapped females suggesting that they were easier to amplex. Given the lack of evidence for direct male-male competition or female choice, we suggest assortative mating may be the result of indirect competition (e.g. *in situ* large males may be better able to access and amplex the largest females), or female resistance to small males in combination with higher costs small males may incur in securing large females.

INTRODUCTION

Pre-copulatory mate guarding is common in species when mating is confined to a short period in the female reproductive cycle. For example, guarding is common in crustaceans where fertilisation takes place during a brief interval after the moulting of the female's hard exoskeleton (reviewed by Ridley 1983). Amphipods exhibit two types of mate guarding: "attending" occurs when males guard females by simply staying in close proximity to them, while "amplexus" occurs when males physically attach themselves to the female (the most common strategy; Conlan 1991).

Studies on amphipod species displaying amplexus, such as *Gammarus pulex,* have demonstrated positive size-assortative mating (Elwood & Dick 1990). This situation may occur because larger females are generally more fecund and large males are better able to compete for them (Birkhead & Clarkson 1980; Ward 1988). Competition among males is expected to be strong as the operational sex ratio is likely to be male biased (Trivers 1972). Although the amphipod mating system has been extensively studied, no single explanation satisfies all cases of size-assortative mating (Jormalainen 1998).

Five main hypotheses have been developed to explain size assortative mating: (1) small males may be physically unable to handle large females due to the mechanical features of the clasping process (mechanical constraints hypothesis; Crozier and Snyder 1923); (2) smaller females may be less of a burden for smaller males when swimming in strong currents and therefore preferred (loading hypothesis; Adams & Greenwood 1983); (3) a correlation between size and microhabitat may exist (habitat segregation hypothesis; Birkenhead & Clarkson

1980; Ward and Porter 1993); (4) large males may be better able to takeover females in amplexus (sexual selection hypothesis; Ridley 1983 (referred to here as the intra-sexual selection hypothesis)); and (5) size related variation in amplexus duration may result in large males guarding large females longer than small males (guard duration hypothesis; Elwood et al. 1987).

Most of these hypotheses relate to male mate choice or "handling" abilities and do not take into consideration female choice, specifically in the form of female resistance. While guarding has obvious benefits for males, it may sometimes be disadvantageous for females. For example, the risk of predation may increase (Strong 1973; Ward 1986) or foraging may be inhibited (Jormalainen & Merilaita 1993). Hatcher & Dunn (1997) reported female resistance to male guarding in *G. duebeni* and Jormalainen & Merilaita (1993) reported female resistance in the isopod *Idotea baltica*. As a result, we hypothesized that females may have some control over whom they mate with or the length of time they are guarded (i.e. inter-sexual selection hypothesis). However, little work has specifically investigated the role females play in determining guarding duration and mate choice, especially in amphipods (Jormalainen 1998). Plaistow et al. (2003) found the energetic cost of amplexus in *G. pulex* occurred only at formation suggesting that females resist male attempts at amplexing. Females may provide less resistance to large males if, for example, offspring sired by large males are more valuable. Alternatively, resistance could result in assortative mating if large males are more successful in overcoming large resisting females, and incur lower energetic costs while doing so.

I investigated the occurrence of, and possible mechanisms for, size-assortative mating in the amphipod *Paracalliope fluviatilis* (Thomson 1879), super family

Paracalliopiidae, previously unrecognized as possessing mate guarding species (Conlan 1991). *P. fluviatilis* is endemic to New Zealand and is the most abundant and widespread freshwater amphipod; it is found amongst macrophytes in the slow flowing margins of lentic systems and in lotic habitats. The species exhibits sexual dimorphism with males on average 30% larger than females and it is also the only New Zealand freshwater amphipod species that displays amplexus (Chapman & Lewis 1976).

P. fluviatilis is a particularly suitable model organism because it allows hypotheses based on well studied northern hemisphere genera to be tested on a species from a novel group where mate guarding and associated phenomena have evolved independently. In order to determine whether assortative mating occurred and examine whether female choice (exhibited by active female resistance) might influence amplexus duration and mate choice, we used a combined field and laboratory approach. The field study demonstrated that the species exhibited positive size assortative mating, and that larger females may be superior mates (i.e. they carried more eggs). A series of laboratory experiments were then conducted. Experiment 1 assessed whether male size influenced their choice of female size (mechanical constraints hypothesis) and whether male size influenced pairing success in the presence of a potential competitor (intra-sexual selection hypothesis). In experiment 2, we determined if "take-overs" of guarded females occurred and if large males were more successful in this capacity (intra-sexual selection hypothesis). Experiment 3 was conducted to determine if male body size influenced the duration of guard episodes (guard duration hypothesis). In experiment 4, we used muscle relaxants or added extra weight to females to discover how much control females have on amplexus success and duration (inter-

sexual selection hypothesis). The loading hypothesis was not tested because it requires a current when *P. fluviatilis* populations were found in very low flow microhabitats and individuals did not appear to swim much (pers. obs.). The habitat segregation hypothesis was not tested because populations were always located in macrophytes that did not provide a variety of small spaces where small males could enter and large males could not (pers. obs.).

METHODS

Field Study

Fifty amplexed pairs and 50 single individuals of each sex were sampled from a single seepage habitat in Hamilton, New Zealand (S37.80°, E175.30°). Head length measurements were taken from all individuals and full body length measurements were taken from all paired animals. Head and body lengths were highly correlated for the 50 males (R^2 =0.728, P<0.001; Appendix V.1) and 50 females (R^2 =0.762, P<0.001; Appendix V.2). Head lengths were less prone to measurement error (e.g. telescoping of the animal) and therefore were used to estimate size. An additional 52 amplexed pairs were later collected in roughly equal proportions from the above site and from three others: New Memorial Park, Hamilton situated four km from Hamilton Gardens (S37.80°, E175.30°), the Waipa River site was 14 km from Hamilton (S37.80°, E175.15°) and an unnamed creek was 37 km from Hamilton (S37.82°, E174.90°). Linear regressions were used to determine: 1) the occurrence and direction of size-assortative mating using data combined from all four sites and analysed individually, 2) relationships between size of paired and single males and females, and 3) the relationship

between egg number and female size using log transformed values (Ln + 1) to adjust for the allometric relationship between body size and egg number. A Spearman correlation coefficient was used to gauge the strength of size assortative mating in order to make comparisons with other amphipod species (e.g. Bollache and Cezilly, 2004a). Two-tailed two-sample t-tests were used to determine if paired males and females were significantly larger than their single counterparts.

Animal collections and maintenance for laboratory experiments

In June 2002, we collected samples of *P. fluvatilis* from the four sites mentioned above as part of our laboratory experiments. The four sites were used to ensure that the patterns observed were indicative of the species and not population specific as spatial heterogeneity may result in artificially inflated estimates of size-assortative mating (Bollache et al. 2000). The micro-habitat of *P. fluviatilis* at all four sites was similar, with submerged aquatic vegetation rooted in a soft silt substrate. Accordingly, animals of all sizes would have had equal access to all parts of the habitat (i.e. a visual inspection of sites showed that no structures or spaces were present that would allow small males and females access but would prohibit entry by large males and females). Animals from each population were kept in separate two litre holding tanks containing macrophytes (*Elodea canadensis*) to mimic the natural environment and help aerate the water. Fish food flakes (Nutrafin Staplefood™, Auckland, NZ) ground to a paste were pippetted into containers to supplement food obtained by grazing on epiphytic algae. Water was changed weekly and excess food pipetted off every second day. Containers were maintained at 18˚C under a 15:9 light regime to simulate summer conditions

to enhance pairing. All animals had at least a three-day settling period in holding containers before the experiments began.

Experiment 1

Four types of choice tests were conducted to determine if male size influenced pairing rates with females. If the mechanical constraints hypothesis is correct, small males should have difficulty pairing with large females. The intra-sexual selection hypothesis was examined by measuring pairing success of small and large males when presented with a single female of either small or large size. For tests of each hypothesis, three single individuals were placed together; tested males (M) and females (F) were of two size classes, large (L) and small (S). The four combinations we examined were: a LM with a SF and LF, a SM with a SF and LF, a SM and LM with a SF and a SM and LM with a LF. Size classes were established using head length measurements based on the field study and equated to approximately the 1st and 4th quartiles: LM≥500µm, LF≥375µm, SM≤425µm and SF≤325µm. There were 32 replicates per test.

Females of each size class were held in containers with males not otherwise included in the experiment while males used in the experiment were held in uni-sex groups in the same type of holding container. Once 128 pairs had formed (within 2-3 days), females were immediately separated from males and used in the experiment. By doing this, we ensured that only females close to moulting were used thus reducing the confounding effect of time to moult which may affect female selection by males (Thomas et al. 1998; Bollache and Cezilly 2004b). However, we caution that other size-correlated traits (e.g. endurance) may also influence size selection. Pairs were separated by being temporarily removed from

water, which caused males to release females. Even numbers of animals from each of the four sites (see previous section) were used.

Choice tests were completed in clear plastic containers (62x62x110mm high) filled with 200ml of water. Containers included a piece of *E. canadensis* and a drop of fish food. A preliminary study of 15 males and 15 females showed that 13 of 14 pairs formed within four hours of the start of the experiment suggesting that four hours was an appropriate, initial observation period. Accordingly, animals were added to the containers and observed at two, four and six hours. When pairing had occurred, pairs were observed for at least 30s to ensure that the male had a maintained grasp on the female. Chi-square tests using Yates' correction for continuity (Zar 1996) were used to determine: 1) if small and large individuals of each sex paired differentially with small and large individuals of the opposite sex; and 2) overall levels of pairing success for small versus large individuals of each sex.

Experiment 2

We determined whether males physically take over paired females from other males and if so whether larger males were more successful at doing this than small males (intra-sexual selection hypothesis). Animals were collected from the four sites described above. Females were again allowed to amplex prior to the experiment but this time they were paired with males of a known size class (large or small). Amplexed pairs were placed in small, white 25ml containers with one drop of fish food added. Once a pair had been placed in the container, a single male was introduced immediately. Four combinations of animals were used: 1) SF paired with a SM, with a LM introduced, 2) LF paired with a SM, with a LM

introduced, 3) SF paired with a LM, with a SM introduced and 4) LF paired with a LM, with a SM introduced. A total of 32 tests were completed for each combination. Combinations of animals were observed continuously for four hours to assess if the single males attempted to takeover the paired female.

Experiment 3

To assess the guard duration hypothesis, sixteen pairs were collected from each of the four sites described above; each pair was placed in a separate 200mL container as described in previous experiments. Animals were checked every 24h until all the animals had separated. Significant variation in guard length between pairs may have existed at the time of collection. However, randomly selected pairs of amphipods in an asynchronous breeding population will on average have paired for 50% of the duration of their guard length. Accordingly, no bias should occur between animals allocated to different categories (Dick & Elwood 1996). Once a pair had separated, the duration of amplexus was recorded to the day and the animals placed in 70% ethanol. However, data were discarded if an individual from a pair was found dead or if a female had not moulted (and therefore not copulated). At the end of the experiment, the head sizes of animals were measured and a Kruskal-Wallis test employed to verify that a site effect for male and female size was absent before results were pooled. A Spearman Rank Correlations test was then performed by plotting male and female size against the duration of amplexus. Additionally, differences in length of guard duration were detected using a Kruskal-Wallis test to compare animals from the largest and smallest size quartiles.

Experiment 4

On occasion, we observed females to actively resist males in laboratory conditions by swimming away or flexing their abdomen vigorously when males attempted to attach to them. Accordingly, we examined how female behaviour might influence amplexus duration. Individuals were handicapped using a 10g/L solution of magnesium sulphate ($MgSO_4$) mixed with water as a muscle relaxant. $MgSO_4$ is a known narcotizing agent for freshwater animals (Pantin, 1962); it causes a dissociation of actomysosin, preventing muscle contraction and thus leaves muscles in a relaxed state (Wilson, 1979). A range of concentrations between 1-20g/L were initially trialed before 10g/L was selected as the final concentration as this was found to inhibit movement of animals while not causing any mortality. To quantify the effect of the relaxant and to determine if males and females responded differently to the solution, the effect of four treatments on swimming activity (20 individuals per treatment) was examined: control male, handicapped male, control female and handicapped female. Animals were placed either in the solution (treatment) or aged tap water (control) for one hour before being placed in a second container filled with water for at least 10 minutes. This allowed any chemical residual to be washed off before being placed in a third container, at which point swimming activity was recorded. We noted if an animal moved in each of 30 consecutive ten-second periods, and the total number of active periods was recorded for each individual. The muscle relaxant had a significant effect on activity (ANOVA: $F_{1, 76}$=6.93, P=0.01; control χ±SE=11.93±1.97, handicapped =5.5±1.44) with no difference between males and females (ANOVA: $F_{1, 76}$ =1.75, P=0.19 males = 7.1±1.76, females = 10.33±1.81, and there was no significant interaction between treatment and sex (ANOVA: $F_{1, 76}$=0.50, P=0.48).

Three types of pairs were used in the experiment: 1) a handicapped female with a control male, 2) a control female with a handicapped male and 3) a control female with a control male. If conflict over amplexus duration exists between sexes, with females preferring a shorter time in amplexus than males (due to possible fitness (Strong 1973; Ward 1986) or foraging consequences (Jormalainen & Merilaita 1993)), then test type 1 should lead to the longest amplexus duration and type 2 the shortest. We also examined the size of males to determine if the time in amplexus for large males differed from those of small males.

A total of seventy-two male and seventy-two female amphipods were collected from the Hamilton Gardens site (N=24 for each treatment) and one of each sex was randomly assigned to a 200 mL container. Animals to be handicapped with the $MgSO_4$ were pipetted into a small container filled with the solution for one hour at 1100 h. Once treated with the solution, animals were placed in a second container filled with water for 10 minutes to wash off any solution residue before being placed into the experimental container. The same was done with control animals except that aged tap water was used instead of the $MgSO_4$. The application of the $MgSO_4$ (treatment) or water (control) was repeated daily using the above protocol until the animals had formed pairs. Animals were checked twice daily at 1000 and 2200 hours to examine their status (i.e. whether they had formed pairs or had separated from pairs). Data were discarded if an individual from a pair was found dead or if a female had not moulted (and therefore not copulated). A one-way ANOVA was used to detect if any differences occurred in the time taken to amplex, the duration of amplexus and pair success (analysed by comparing the number of pairs formed for each of the three treatments).

Experiment 5

In order to further define the role of female choice in determining the duration of amplexus, females were handicapped by adding extra weight to their bodies to increase the energy required for swimming. Weight was added by applying a spot of quick drying solution (Bic Wite Out™ Milford, USA) to the back of a female. The mean weight for untreated females was 395±15 µg (*N*=10) and the mean weight of the dried solution was 81±7 µg (*N*=10), creating a mean weight increase of 21%. Weighted females were given 24 hours to adapt to the extra weight before the experiment began. No obvious deterioration of the applied substance was observed over the course of the experiment. Males were given the choice between an untreated female and a female with the added weight. Males were unlikely to detect the additional weight as male-female encounters occurred on the substrate and not in the water column (where males could potentially lift / attach themselves to females, allowing them to judge weight). Animals were examined at 24-hour intervals (1000 h) for six days or until paired. A chi-square test, using Yates' correction for continuity, was used to test if males were more likely to pair with weighted versus untreated females. A two-tailed t-test was used to determine if weighted and untreated females differed in the length of time it took to form pairs.

RESULTS

Field study

A regression analysis of male and female size using data combined from all four sites showed positive size-assortative pairing (N=102, R^2=0.354, P<0.001; Fig. 20); the magnitude of the relationship was high (Spearman correlation coefficient, N=102, r_s, P<0.01). A significant relationship also occurred for the Hamilton Gardens site only (N=66, R^2=0.374, P<0.001) while individually the other three sites showed a trend towards positive size assortment but these were non-significant, probably due to low sample sizes (N=12-15) (calculations not shown). There was a slight trend for paired males to be larger than single males (paired male χ±SE=463±3.59µm, N=50; single male 455±2.51µm, N=50; t=1.46, P=0.15); for females, no difference was found (paired female 349±4.63µm, N=50; single female 346±3.22µm, N=50; t=1.40, P=0.49). There was a positive relationship between female size and egg number (N=72, R^2=0.08, P=0.05; Appendix V.3).

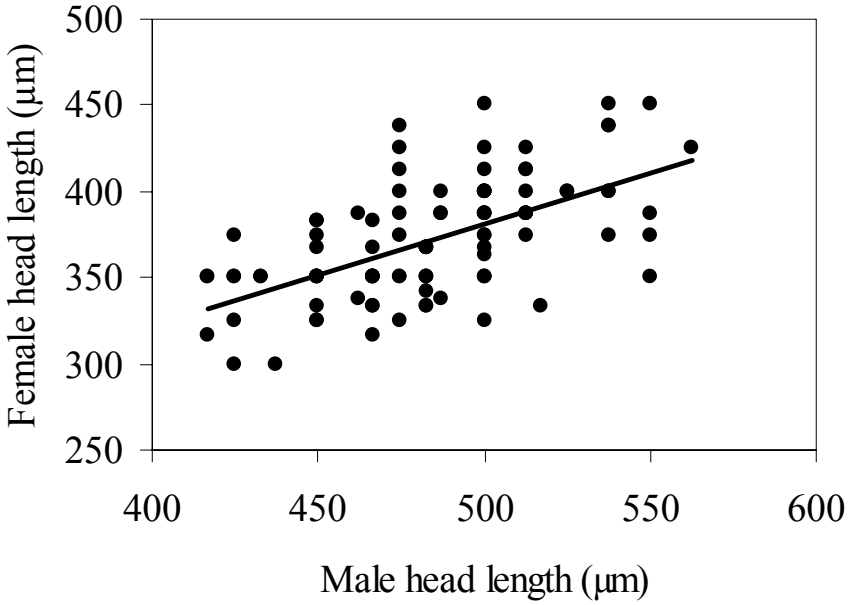


Figure 20. The relationship between male and female head length in 102 amplexed pairs of *Paracalliope fluviatilis*. Line of best fit is also shown.

Experiment 1

When offered a choice, large males were more likely to pair with large females than small females (χ^2_1 =9.09, N =11, P =0.01); the same trend occurred for small males but the effect was not statistically significant (χ^2_1 =0.50, N=10, P=0.11; Fig. 21). However, there was no difference in the number of pairings that large and small males achieved when they were placed together in a container with either a single large female (χ^2_1 =0.31, N=13, P=0.53) or single small female (χ^2_1 =0.08, N=12, P=0.82; Fig. 21). Both male size classes had the same level of overall pairing success (χ^2_1 =0, N=21, P=1.0), as did the female size classes (χ^2_1 =0.64, N=25, P=0.48), with between 10-13 pairs formed in each treatment group from the initial 32 tests per group.

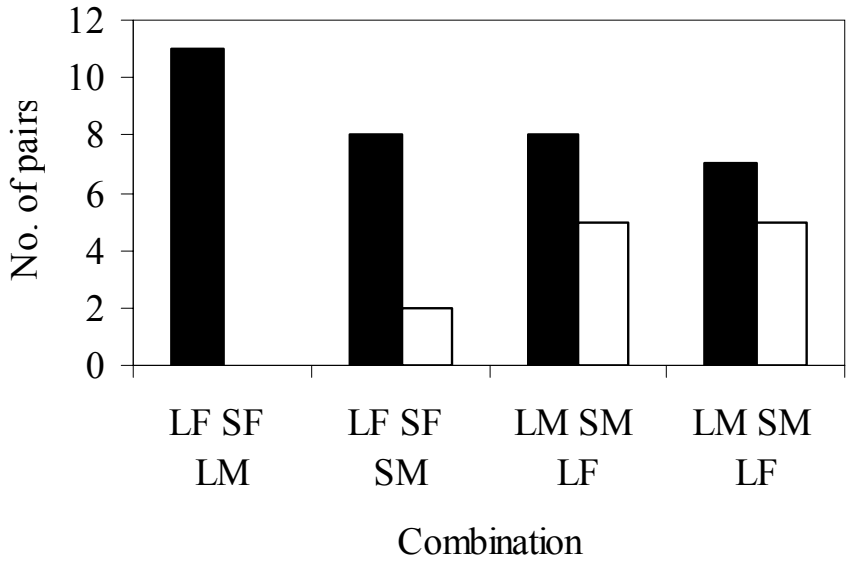


Figure 21. The relationship between pair formation success and size. The four cross types were: a large male, a large female and a small female (LF/ SF, LM), a small male with a large female and a small female (LF/ SF, SM), a large female with a large male and a small male (LM/ SM, LF), a small female with a large male and a small male (LM/ SM, LF).

Experiment 2

We observed only two attempts of a single male to pair with a female already in amplexus over the course of 128 tests, both unsuccessful. In both cases, a large male attempted to pair with a female in amplexus with a small male. One failed to dislodge the incumbent male while in the second attempt the single male caused the incumbent male to release the female but then attempted to amplex with the male.

Experiment 3

Of the 64 pairs collected, 12 pairs had individuals that died during the experiment and were therefore not included in the analysis. We found no site effects for male (*N*=52, *H*=1.75, *P*=0.63) or female (*N*=52, *H*=0.36, *P*= 0.95) size and therefore pooled our data. There was no influence of male size on amplexus duration (*N*=52, r_s=0.22 *P*>0.1; Fig. 22), nor any difference between large and small males (*N*=26, *H* =2.14, *P*=0.14). However, larger females on average were amplexed longer than smaller females (*N*=52, r_s=0.41, *P*<0.01; Fig. 22) with a highly significant difference between large and small females (*N*=26, *H*=6.11, *P*=0.01).

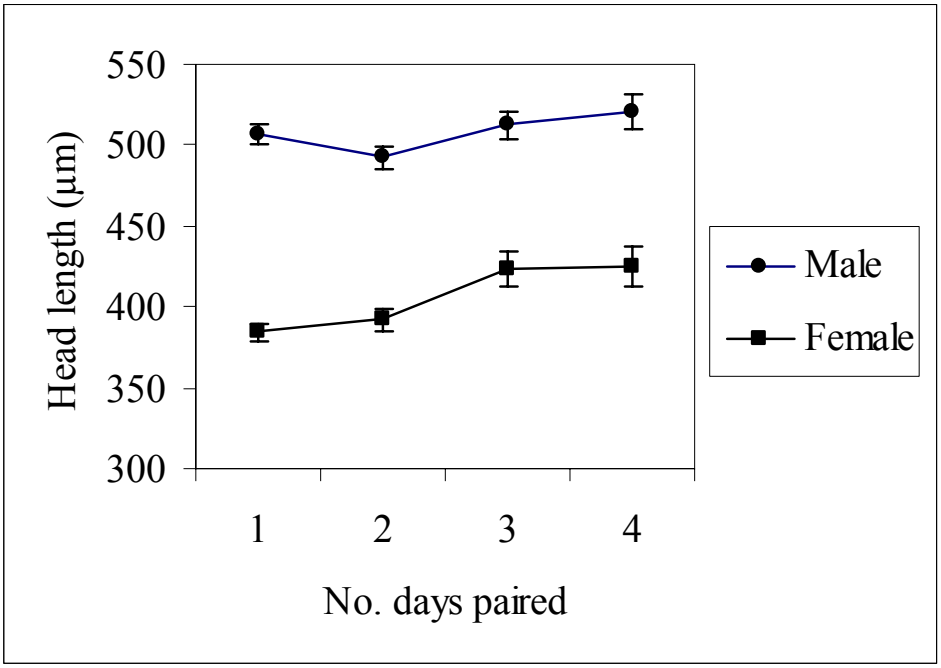


Figure 22. The relationship between animal size and amplexus duration for 64 pairs of *Paracalliope fluviatilis* caught in the field and then maintained in captivity until the female moult (+/- 1S.E.)

Experiment 4

There were no significant differences between muscle relaxed and untreated individuals for: 1) time taken to amplex (ANOVA: $F_{2, 69}$=2.60, P=0.082; Fig. 23); 2) amplexus duration (ANOVA: $F_{2, 69}$=0.19, P=0.823; Fig. 23); or 22) pairing success (ANOVA: $F_{2, 69}$=1.50, P=0.229). Furthermore, there was no evidence to suggest that large males differed from small males in the time to amplex or in the duration of amplexus (size classes were evenly distributed across the range of data for each treatment group).

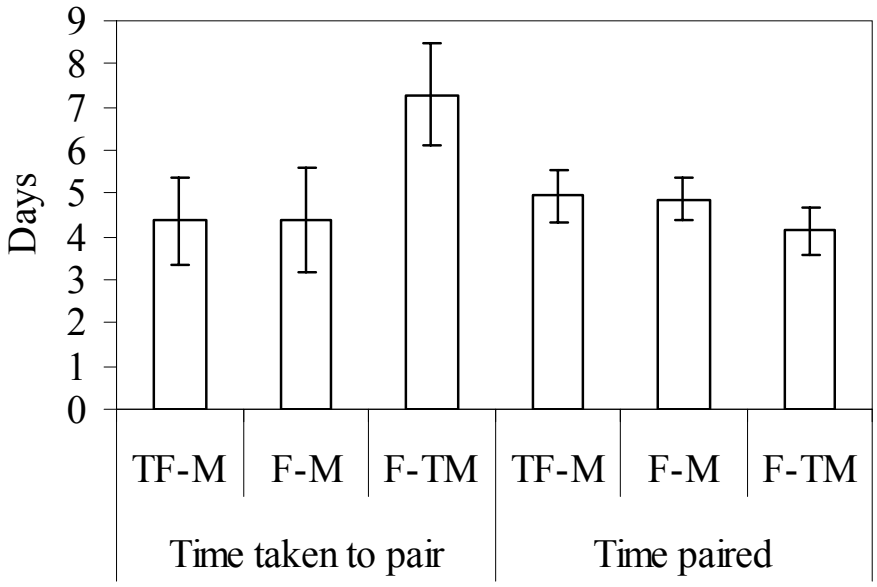


Figure 23. The time taken to form pairs and the total time of pair duration for three crosses examining female resistance. Animals were handicapped with a $MgSO_4$ solution. The three crosses were: a handicapped female with an untreated male (TF-M), an untreated female and male (F-M) and an untreated female with a handicapped male (F-TM) (+/- 1S.E.).

Experiment 5

There was a trend for males to be more likely to amplex with weighted females (19 pairings) than untreated females (9 pairings) (χ^2_1=2.89, *N*=28, *P*=0.09). However, there was no significant difference in amount of time taken to amplex (*t*=0.28, *P*=0.781) for untreated (χ±SE=2.16±0.44 days, *N*=28) and weighted females (2.00±0.34 days, *N*=28).

DISCUSSION

We found clear evidence for positive size-assortative mating from our field collections of *P. fluviatilis.* The strength of assortative mating was similar to that found in other amphipod species (e.g *Gammarus pulex*; Bollache and Cezilly 2004b) and may reflect an equilibrium between males attempting to mate with large females and their availability and/or ability to secure such females. Positive size-assortative mating in other amphipods, and for the related Isopoda, has been explained by: 1) male preference for larger, and hence usually more fecund, females (Birkhead & Clarkson 1980); 2) larger males being better competitors than smaller males (see review by Jormalainen 1998), or 3) larger males being better detectors of females by having larger antennae relative to smaller males (e.g. as in the isopod *Asellus aquaticus*; Bertin and Cezilly, 2003; Bertin and Cezilly, 2005). In support of the first explanation, we found that large females carry more eggs that smaller females; in support of the second explanation, we found a male preference for large females and that small males were less likely to be found pairing with the largest females in field samples, which may support the mechanical constraints hypothesis (i.e. that small males have difficulty amplexing

with the largest females; Crozier and Synder 1923). Indeed, paired males were marginally larger than single males. By contrast, paired and single females collected from the field were very similar in size. This may occur because, at any given time, there are more available males relative to the small subset of potentially fertile females (i.e. those close to moulting). This would exaggerate an already male-biased sex ratio.

However, based on the field data alone we were unable to determine conclusively if size assortative pairing was due to mechanical, energetic or ecological constraints or other possible mechanisms. Sexual dimorphism for size was evident with an approximately 1.3:1 (male: female) ratio found, similar to that found in *G. pulex* (Adam & Greenwood 1983). Under experimental conditions we found large females were able to be amplexed by both large and small males; this indicates that small males are physically capable of carrying large females. Accordingly, the mechanical constraints hypothesis may not be applicable to *P. fluviatilis*. Birkhead & Clarkson (1980) also suggest the hypothesis is not valid for *G. pulex*. Large males were not significantly more successful than small males in pairing with females in any of our choice tests ($P>0.53$ for large females and $P>0.82$ for small females), suggesting that there is no size advantage for large males when competing for single, "close to moult" females.

When we combined single males with amplexed pairs there was little evidence to suggest that single males regularly attempt to take over already paired females. Therefore, the intra-sexual selection hypothesis does not seem to apply to size-assortative mating in *P. fluviatilis*. Hatcher & Dunn (1997) reported no attempted takeovers in already paired females of *G. duebeni* and considered male-male

competition an insufficient explanation for size-assortative mating. Birkhead & Clarkson (1980) and Dick & Elwood (1990) also found takeovers to be rare in *G. pulex*, and that small males were capable of securing large females. Some studies have reported male takeovers to be common, so male-male competition may promote positive size assortative mating in some species (e.g. *Eogammarus oclairi*; Iribarne et al. 1996). The occurrence of takeovers has also been thought to influence amplexus duration for small males of *G. pulex* (Elwood & Dick 1990). Small males may not guard for as long because they may be removed from females by larger males. Our results suggest male-male competition (e.g. in the form of aggressive takeovers of paired females) does not play an important role in the *P. fluviatilis* mating system.

We found that amplexus duration was positively correlated with female size but not with male size and therefore could not be the mechanism that produced size assortative mating. All sizes of males appear to be willing to invest more time in amplexus with larger females, probably because they carry more eggs. In contrast, Elwood & Dick (1990) found a positive correlation for both male and female size in relation to guarding duration for *G. pulex*; they suggested that large males guard larger females for longer because they can endure the increased costs of guarding (guard duration hypothesis). However, Plaistow et al. (2003) who also studied *G. pulex* found that the species exhibited the same pattern that we found for *P. fluviatilis* (i.e. a positive relationship for females but not for males). They also showed that males' stored energy reserves were independent of guarding duration. Furthermore, *G. pulex* still demonstrated size assortative mating even when all available females were close to moult (Hume et al. 2002) suggesting that it is not an energetic limitation of small males but a preference for large females.

Accordingly, we conclude that small male *P. fluviatilis* do not endure significantly higher costs in amplexus than large males and therefore will on average remain amplexed as long as larger males.

We found little evidence of female choice in relation to guard duration in *P. fluviatilis*. Handicapped females (i.e. those less able to resist male attempts at guarding) did not differ from control females in terms of the length of time they remained single or spent in amplexus. There was a trend for handicapped males to take longer to amplex than untreated males which may reflect a lack of muscle control. Furthermore, other studies have shown that stressed males remain in amplexus for shorter durations relative to unstressed males (Jormalainen & Merilaita 1993). Jormalainen & Merilaita (1995) used either osmotic stress or a neuromuscular blocking agent to reduce the female's ability to resist male attempts at guarding in three species of Peracarida (two isopod and one amphipod species). They found longer amplexus durations for the isopod *I. baltica* but not for *Asellus aquaticus* and the amphipod *G. zaddachi*. They considered the longer amplexus duration in *I. baltica* as evidence of female resistance and therefore inter-sexual conflict.

Males were more likely to amplex with females handicapped with extra weight though the effect was not statistically significant (P=0.09), possibly due to a small sample size. The result may suggest that female resistance occurs in *P. fluviatilis*. However, if resisting behaviour does occur it does not seem to alter the amount of time taken for a male to enter into amplexus. Accordingly, size assortative mating in *P. fluviatilis* could occur if large females were more resistant to, or better able to, resist small males; this effect would have to occur in association with a size-related differential in a male's ability to amplex large females and/or endure the

associated energetic costs of amplexing with large females. Small males then would have to make a tradeoff between mating with large females and enduring the higher cost of overcoming such females. Accordingly, they may not always choose to, or succeed in, amplexing with large females. One of our experiments showed there were no differences in the numbers of small and large males selecting large females.

We found evidence for size assortative mating and a positive relationship between female size and egg number in the amphipod *P. fluviatilis*. The results of our experiments did not support: (1) the mechanical constraints hypothesis as small males were capable of pairing with large females; (2) the intra-sexual selection hypothesis as no successful takeovers of paired females occurred and there was no evidence of overt male-male contests; (3) the guard duration hypothesis as no differences existed in guarding duration between small and large males; or (4) the inter-sexual selection hypothesis as we found little evidence of female resistance. We conclude that it is unlikely that any of the existing hypotheses adequately explain positive size assortative mating in *P. fluviatilis*. However, assortative mating may result from a combination of female resistance and size-related variation in a male's capacity to amplex larger females or a form of indirect intra-sexual competition – i.e. large males may be capable of swimming faster, or better able to detect and respond to moulting females. Future work should focus on investigating possible interactive effects between intra- and inter-sexual selection, as well as indirect competition between males.

Acknowledgments

We thank Barry O'Brien for helpful advice on muscle relaxants in crustaceans, Gavin Reynolds for technical support, Ann Chapman and Dave West for useful comments on the manuscript, and members of the biodiversity and ecology research group for helpful discussion. Financial support was provided through the Department of Biological Sciences and a University of Waikato Doctoral Fellowship to DLS.

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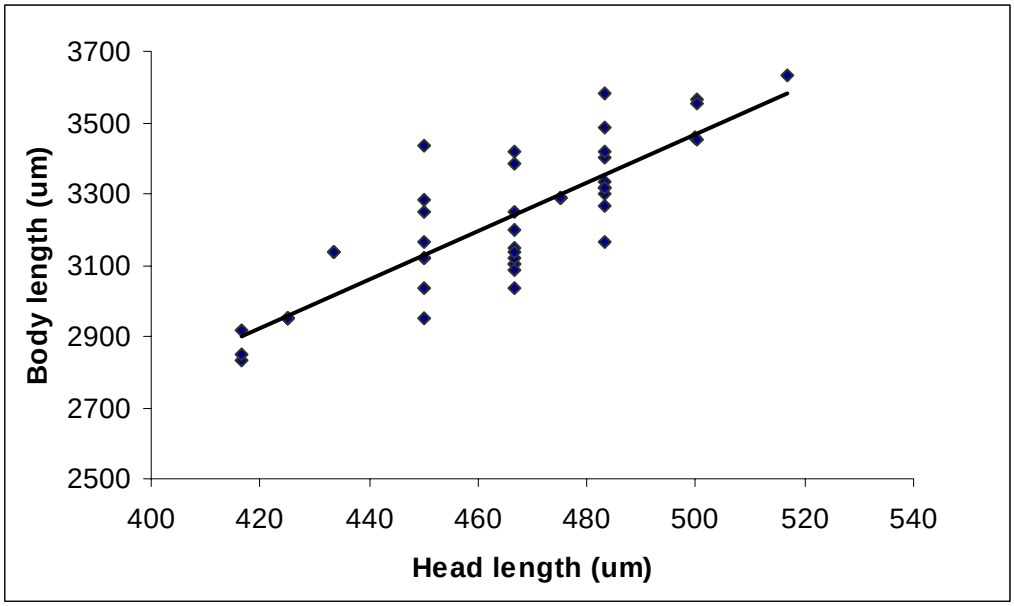
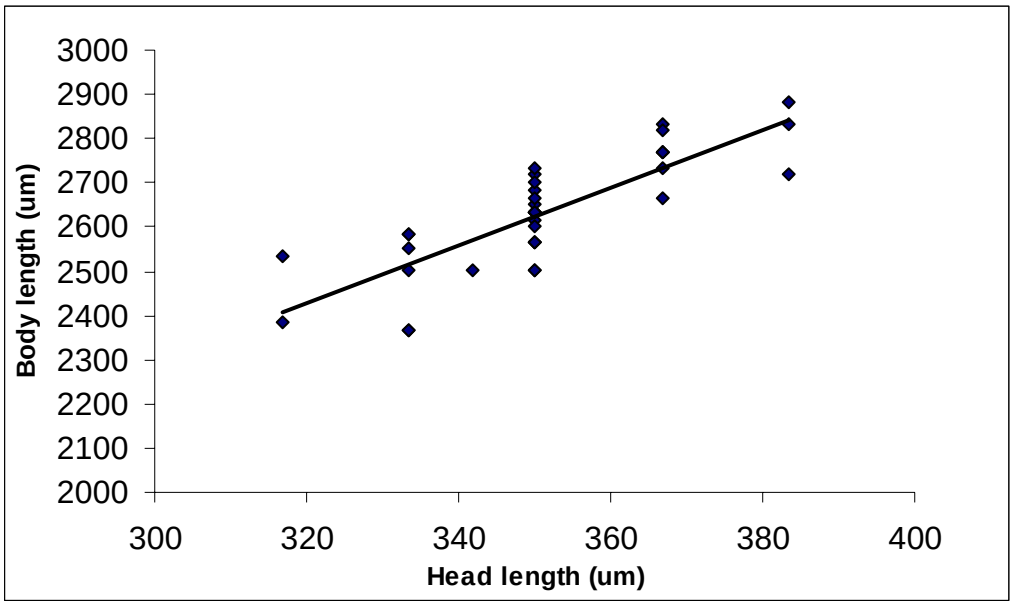
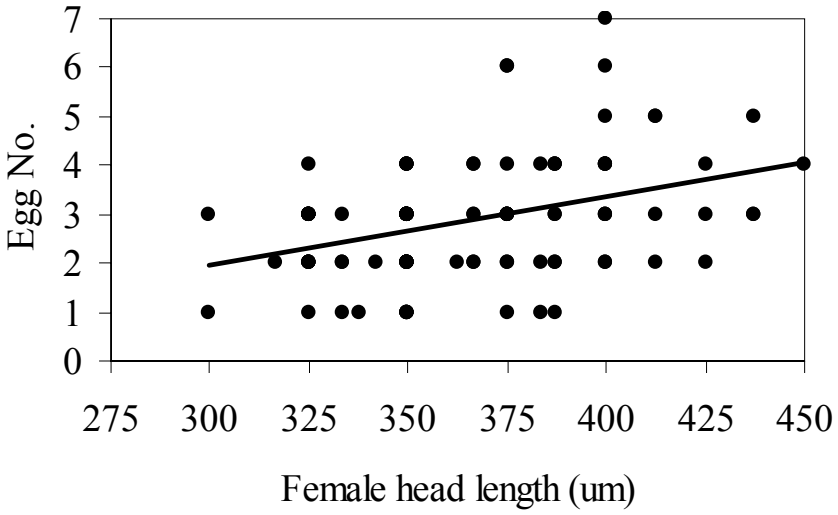
APPENDIX V.1. Body versus head length for 50 male *Paracalliope fluviatilis* collected from Hamilton Gardens.



APPENDIX V.2. Body versus head length for 50 female *Paracalliope fluviatilis* collected from Hamilton Gardens.



APPENDIX V.3. Egg number versus head length for 50 female *Paracalliope fluviatilis* collected from Hamilton Gardens.



THESIS CONCLUSION

The discipline of phylogeography has lead to a better understanding of phylogenetics and how it relates to geographical distributions and genetic distances among evolutionary lineages of animals (Bermingham and Moritz, 1998). It highlights the historical aspects of current phylogenies (Avise 1996) and because of its integrative approach, incorporating molecular genetics, population genetics, phylogenetics, demography, ethology, and historical geography (Avise, 1998) enables a comprehensive evaluation of the influences that have shaped taxa. My research used a phylogenetic approach examining patterns of genetic diversity in three amphipod taxa in New Zealand and looked at recognition behaviour in one of these to determine if assortative mating occurred for size and genetic divergence.

I found evidence for positive size assortative mating and a positive relationship between female size and egg number in the amphipod *Paracalliope fluviatilis* using a field study (Chapter I). The underlying reason behind assortative mating may be because larger females are generally more fecund and large males are better able to compete for them (Birkhead & Clarkson 1980; Ward 1988). My finding mirrors that of northern hemisphere studies that have shown that a number of amphipod species display size assortative mating (e.g. *Gammarus minus*; Elwood & Dick, 1990) though the mechanism producing it appears to differ between species and is not well understood (Jormalainen 1998). Laboratory experiments investigated four hypotheses relating to how size assortative mating may be produced in crustaceans. The results of the experiments did not support: (1) the mechanical constraints hypothesis as small males were capable of pairing

with large females; (2) the intra-sexual selection hypothesis as no successful takeovers of paired females occurred and there was no evidence of overt male-male contests; (3) the guard duration hypothesis as no differences existed in guarding duration between small and large males; or (4) the inter-sexual selection hypothesis as we found little evidence of female resistance. Existing hypotheses do not appear to adequately explain positive size assortative mating in *P. fluviatilis*. I suggest that size assortative mating may result from a combination of female resistance and size-related variation in a male's capacity to amplex larger females or a form of indirect intra-sexual competition (e.g. large males may be capable of swimming faster, or better able to detect and respond to moulting females).

Paracalliope fluviatilis is a widespread freshwater species whose current distribution and genetic patterns should reflect the historical events affecting freshwater taxa in New Zealand. My research showed that *P. fluviatilis* populations were divided into five clades with the most genetically divergent clades having been separated for approximately 10 million years, as indicated by a molecular clock (Chapter II). The large genetic divergence between some populations shows that populations are relatively isolated from each other with little dispersal among different catchments. This geographical isolation may allow allopatric speciation to occur and based on the large genetic divergences found, *P. fluviatilis* may potentially be a species complex. The species recognition experiments suggest that this may be true, as there is a large bias in mate selection with same population females favoured over genetically distinct females for crosses involving the largest genetic distances. Given the lack of morphological differences I suggest that species recognition could be due to inter-population

variation in mating habits (e.g. sexual cues) or biochemical differences (e.g. pre-moulting hormone).

Geography is an important component of phylogeography and to complement genetic work I examined the distribution and ecology of the genus *Paraleptamphopus* (Chapter III). As expected *Paraleptamphopus* populations preferred smaller waterbodies such as seepages and few were found in larger waterbodies such as rivers. There is a lack of sampling effort in such habitats in New Zealand and globally (Cole et al. 2003) which explains why New Zealand's potentially more specious freshwater amphipod genus (Chapter IV) currently has only two described species. The genus preferred catchment vegetation with at least some native vegetation in it suggesting that the genus could be affected by anthropogenic disturbances.

I examined the phylogeography of the amphipod genus *Paraleptamphopus* to assess patterns of diversity and how they relate to geography and past natural events (Chapter IV). There were 28 mitochondrial haplotypes found with genetic divergences above 2% suggesting that a large number of species exist. Using a molecular clock rate of 2.4% most haplotypes diverged approximately 8-12 million years ago during the Miocene era, possibly as a result of greater land availability increasing habitat diversity or by allopatric speciation. Nuclear DNA variation showed substantially less variation but overall patterns were similar to the mtDNA. Morphological and genetic differences were not always congruent with some morphologically similar taxa appearing among lineages while genetically similar taxa had obvious morphological differences showing that rates of genetic and morphologically change can be different among congeneric species (Colbourne and Hebert, 1996; Lee, 2000, Witt and Hebert, 2000).

A study on the species *Phreatogammarus helmsii* (Chapter V) revealed that populations had high levels of genetic differentiation among populations. This suggests that populations have been separated for a reasonably length of time with little dispersal occurring between current populations. This lack of dispersal may be one reason why few sites containing *P. helmsii* exist compared with other comparable New Zealand amphipod species that have numerous populations (e.g. *Paracoroupium excavatum* and *Paracalliope fluviatilis*; Hurley, 1975).

Future research

A number of potentially interesting lines of investigation could be followed up from the thesis which include:

1. The mtDNA and nuclear sequence data did not offer strong support among the more divergent clades for the genus *Paraleptamphopus* study. Here I suggest using a marker with an intermediate rate of evolution to try and resolve the relationships among clades. In addition, sequencing the whole CO1 gene would also give better resolution.
2. There was incongruence between the morphological and genetic characters for the genus *Paraleptamphopus*. Some taxa appear morphologically similar yet genetically distinct from each other while other taxa are nearly genetically identically yet have differences in their morphologically characters. This presents an interesting opportunity to conduct breeding trails to see what features, if any, correspond to actual species distinctions.
3. Crossing Wellington with Christchurch, Wellington with Invercargill and Christchurch with Invercargill populations of *P. fluviatilis* would potentially resolve the issue of wether two or three species exist in the complex.
4. For the *Phreatogammarus* study using mtDNA sequence data would strengthen the hypothesis that populations are relatively isolated from one another if distinct mitochondrial lineages could be found for each population.

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APPENDIX 1. Table listing the location of study sites used in the PhD. Waterbody type, width and depth are also given.

Date	General Location	Latitude	Longitude	Waterbody type	Width (cm)	Depth (cm)
7/10/00	Near Pirongia	37.92	175.11	Creek	150	40
7/10/00	Walter Scott Reserve	38.06	175.09	Creek	70	20
7/10/00	Between Pirongia & Kawhia	38.04	174.99	Seepage	20	3
7/10/00	East of Kawhia	38.05	174.82	Creek	200	20
7/10/00	South of Pirongia	38.04	174.94	Creek	100	15
7/11/00	Near L. Areare	37.66	175.20	Creek	200	40
7/11/00	L. Waahi	37.58	175.13	Lake	300000	>150
7/11/00	Creek near L. Waahi	37.58	175.13	Creek	100	15
7/11/00	Near Huntly Power Station	37.56	175.15	River	600	100
7/11/00	Waikato R. at Huntly	37.56	175.15	River	5000	>150
7/11/00	Taupiri	37.60	175.15	Creek	40	10
7/11/00	Waignaro Rd	37.67	175.12	Seepage	20	2
7/11/00	East of Waingaro	37.70	174.99	Creek	200	40
7/11/00	South of Waingaro	37.76	174.99	River	500	80
1/08/00	Waihou River at Te Aroha	37.55	175.71	River	1500	>150
1/08/00	Between Te Aroha & Paeroa	37.50	175.68	Creek	200	40
1/08/00	Between Te Aroha & Paeroa	37.46	175.66	Creek	150	20
1/08/00	Between Thames & Tairua	37.16	175.64	Seepage	50	5
1/08/00	Between Thames & Tairua	37.15	175.67	Seepage	30	3
1/08/00	SH 25A (Tairua)	37.15	175.73	Stream	400	80
1/08/00	SH 25A (Tairua)	37.10	175.77	Stream	350	60
1/08/00	Tairua	37.01	175.84	Seepage	30	5
1/08/00	SH 25A (Tairua)	37.06	175.81	Stream	400	80
1/08/00	SH 25A (Tairua)	37.03	175.83	Stream	400	80
1/08/00	Wharekawa	37.11	175.82	Stream	250	40
1/08/00	Whangamata	37.20	175.85	Creek	200	30
1/08/00	Waihi	37.40	175.81	Seepage	30	5
1/08/00	Whiritoa	37.32	175.89	Creek	50	10
1/09/00	Mahoenui	38˚30	174˚40	Seepage	30	5
1/09/00	Mahoenui	38˚37.603	174˚43.912	Seepage	NA	NA
1/09/00	Awakino	38˚38.614	174˚43.742	Seepage	30	1
1/09/00	Tongaporutu	38˚48.854	174˚35.574	Seepage	40	3
1/09/00	Tongaporutu	38˚49.370	174˚35.809	Seepage	80	8
1/09/00	Mt Messager	38˚53.581	174˚35.832	Seepage	40	8
1/09/00	Mt Messager	38˚53.722	174˚35.752	Seepage	30	8
1/09/00	Uruti	38˚50	174˚20	Stream	600	>150
1/09/00	Motuaui	38˚59.692	174˚18.271	Drainage ditch	90	30
1/09/00	Stratford	39˚05.425	174˚12.270	Stream	200	50
1/09/00	Hawera	39˚38.510	174˚22.632	Stream	500	>150
1/09/00	Hawera	39˚38.510	174˚22.632	Seepage	NA	NA
2/09/00	Wanganui	40˚00.279	175˚10.017	River	3000	>150
2/09/00	Foxton Beach	40˚28.212	175˚15.060	Drainage ditch	60	3
2/09/00	Foxton Beach	40˚28.100	175˚14.495	Creek	200	30
2/09/00	Foxton Beach	40˚20	175˚10	Creek	200	60
2/09/00	Waikanae	40˚49.217	175˚06.294	Creek	100	15
2/09/00	Waikanae	40˚54.417	175.05.226	Creek	200	40
2/09/00	Akatarawai	40˚55.931	175˚06.166	Seepage	50	3
2/09/00	Akatarawai	40˚56.556	175˚06.571	Seepage	30	1
2/09/00	Akatarawai	40˚56.679	175˚06.642	Seepage	40	5
2/09/00	Wellington	41.15	174.99	River	3000	>150
2/09/00	Wellington	40˚18.682	174˚52.973	Seepage	90	10
3/09/00	Rimutaka	41˚06.784	175˚13.084	Seepage	30	5
3/09/00	Rimutaka	41˚06.949	175˚13.818	Seepage	60	15
3/09/00	Featherstone	41˚11.540	175˚21.593	Drainage ditch	200	30
3/09/00	Cape Palliser	41˚36.504	175˚16.359	Seepage	30	1
3/09/00	Cape Palliser	41˚36.175	175˚15.195	Creek	40	8
3/09/00	Cape Palliser	41˚35.371	175˚14.003	Creek	25	1
3/09/00	Lake Ferry	41˚23.777	175˚08.697	Pond	1500	60
3/09/00	Masterton	40˚53.352	175˚39.615	Stream	800	80
3/09/00	Masterton	40˚42.655	175˚38.668	Creek	150	30
3/09/00	Eketahuna	40˚38.207	175˚42.639	Creek	120	25
3/09/00	Eketahuna	40˚26.072	175˚48.257	Creek	100	10
4/09/00	Pungarehu	39˚49.267	175˚08.797	Creek	100	8
4/09/00	Atene	39˚43.232	175˚07.691	Seepage	50	2

APPENDIX 1. continued

4/09/00	Atene	39˚41.599	175˚09.314	Creek	60	15
4/09/00	Rahana	39˚34.561	175˚05.465	Seepage	30	30
4/09/00	National Park	39˚19.760	175˚.22.931	Stream	300	60
4/09/00	National Park	39˚15.835	175˚23.950	Drainage ditch	30	15
4/09/00	National Park	39˚04.398	175˚22.590	Drainage ditch	30	10
19/9/00	Pirongia	37.99	175.12	Stream	150	40
19/9/00	Pirongia	38.09	175.10	Seepage	30	2
26/10/00	Hamilton	37.79	175.29	Pond	300	40
26/10/00	Hamilton Gardens	37.80	175.30	Seepage	40	3
26/10/00	Waikato University	37.79	175.31	Seepage	40	3
17/11/00	Wellington	41.32	174.77	Stream	170	15
18/11/00	Nelson	41.27	173.26	Creek	10	2
18/11/00	Okiwi Bay	41.11	173.66	Stream	10	2
18/11/00	Near Okiwi Bay	41.19	173.48	Seepage	20	2
18/11/00	Murchison	41.72	172.41	Seepage	40	5
18/11/00	Murchison	41.78	172.22	Seepage	15	3
19/11/00	Karamea Bluff	41.50	172.03	Seepage	40	5
19/11/00	Karamea	41.25	172.11	Stream	300	60
19/11/00	Karamea	41.24	172.11	Stream	200	80
19/11/00	Karamea	41.29	172.10	River	400	>150
19/11/00	Liitle Wanganui	41.37	172.08	Creek	40	10
19/11/00	Liitle Wanganui	41.38	172.08	Seepage	20	
19/11/00	Hector	41.61	171.87	Seepage	40	8
19/11/00	Punakaiki	41.99	171.34	River	1500	>150
20/10/00	Greymouth	42.45	171.20	River	500	50
20/10/00	Greymouth	42.45	171.19	Creek	50	10
20/10/00	Greymouth	42.45	171.19	Creek	50	10
20/10/00	New River	42.50	171.17	River	800	>150
20/10/00	New River	42.55	171.14	River	1200	>150
20/10/00	Saltwater creek	42.51	171.17	Creek	400	50
20/10/00	Reefton	42.29	171.62	Creek	150	30
20/10/00	Reefton	42.23	171.70	Creek	150	30
20/10/00	Lewis Pass	42.37	172.36	Seepage	50	10
20/10/00	Lewis Pass	42.38	172.39	Seepage	30	5
20/10/00	Lewis Pass	42.43	172.40	Stream	200	15
20/10/00	Lewis Pass	42.50	172.38	Seepage	40	5
20/10/00	Near Gynn Wye	42.58	172.64	River	00	>150
20/10/00	Lochiel	42.58	172.77	Creek (flooded)	3000	10
20/10/00	Hanmer Springs	42.55	172.80	Creek	150	20
20/10/00	Hanmer Springs	42.57	172.79	River	800	120
20/10/00	Avon River	43.50	172.56	River	500	60
15/12/00	Near Styx River	42.89	171.19	Seepage	50	20
15/12/00	Near Styx River	42.89	171.25	Seepage	30	5
16/12/00	Styx Saddle	42.90	171.32	Seepage	30	5
16/12/00	Near Lake browning	42.96	171.33	Pond	60	20
27/12/00	Bluff golf course	46.54	168.28	Stream	150	80
27/12/00	Bluff golf course	46.54	168.28	Seepage	40	5
1/01/01	Bluff walkway	46.62	168.36	Drainage ditch	40	20
1/01/01	Bluff walkway	46.62	168.36	Drainage ditch	40	20
1/01/01	Bluff walkway	46.62	168.35	Seepage	35	10
1/01/01	Bluff walkway	46.62	168.35	Seepage	80	8
1/01/01	Bluff walkway	46.63	168.34	Drainage ditch	40	20
1/02/01	Waituna wetlands	46.50	168.61	Stream	120	40
1/02/01	Waituna creek	46.56	168.55	Stream	150	60
1/02/01	Toetoes Bay	46.57	168.68	Stream	100	40
1/02/01	Waituna lagoon	46.57	168.67	Lake	600000	>150
1/02/01	Waituna lagoon	46.58	168.65	Lake	600000	>150
1/02/01	Waituna lagoon	46.58	168.64	Pond	50	60
1/02/01	Waituna wetlands	46.55	168.66	Stream	60	30
1/02/01	Waituna wetlands	46.55	168.65	Stream	60	30
1/02/01	Waituna wetlands	46.53	168.64	Stream	80	60
1/02/01	Waituna wetlands	46.51	168.64	Stream	120	60
1/04/01	Kingswell Creek	46.44	168.37	Creek	300	60
1/04/01	Otepuni Stream	46.42	168.38	Stream	400	60
1/04/01	Waihopai River	46.39	168.38	River	500	100
1/04/01	Invercargill airport	46.42	168.34	Creek	120	60
1/09/01	Woodend	46.47	168.38	River	500	>150
1/09/01	Tiwai Rd	46.51	168.45	Creek	150	60
1/09/01	Tiwai Rd	46.53	168.45	Creek	140	60
1/09/01	Mokotua Ceek	46.55	168.45	Creek	180	80
1/11/01	Tomoporakau Creek	46.31	168.22	Creek	150	30
1/11/01	Waianiua Stream	46.30	168.17	Stream	150	30

APPENDIX 1. continued

1/11/01	Riverton	46.35	167.99	Seepage	50	40
1/11/01	Colac Bay	46.36	167.92	Seepage	50	20
1/11/01	Lake George Creek	46.35	167.86	Creek	250	60
1/11/01	Pahia	46.32	167.77	Seepage	40	30
1/11/01	MaCrakens Point	46.23	167.66	Seepage	30	10
1/11/01	Waiu River	46.13	167.69	River	3000	>150
1/11/01	Boundary Creek	46.13	167.69	Creek	300	80
1/11/01	Camp Creek	46.15	167.69	Creek	300	80
1/11/01	Waihoaka	46.25	167.71	Seepage	30	10
1/11/01	Waimeamea River	46.26	167.72	River	500	120
1/11/01	Kenny Creek	46.29	167.73	Creek	150	30
11/04/01	Waikorea	37.53	174.83	Creek	150	30
11/04/01	Kaawa Stream	37.48	174.77	Stream	6000	>150
5/07/01	Queenstown	45.03	168.66	Creek	150	30
10/07/01	Lake Waihola	46.01	170.10	Lake	>100000	>150
12/07/01	Awamoa Creek	45.11	170.92	Creek	200	30
12/07/01	Looker Rd	44.08	171.36	Creek	150	30
12/07/01	Hinds	44.01	171.56	Stream	300	100
12/07/01	Winslow	43.95	171.66	Creek	150	30
13/07/01	Halswell River	43.69	172.56	River	500	100
14/07/01	Styx River	43.47	172.62	River	400	120
14/07/01	Cam River	43.35	172.66	River	500	>150
14/07/01	Waikuku	43.29	172.69	Creek	150	30
14/07/01	Liethfield	43.19	172.75	Creek	150	30
14/07/01	Omihi Stream	43.04	172.79	Stream	150	30
14/07/01	Wyllies Stream	42.96	173.01	Stream	150	30
14/07/01	Jed River	42.83	173.25	Stream	400	80
14/07/01	McPhreasons Stream	42.78	173.28	River	300	50
14/07/01	Limestone Creek	42.57	173.43	Stream	150	30
14/07/01	Goose Bay	42.48	173.53	Stream	400	60
14/07/01	Kahutara River	42.43	173.59	Stream	500	40
14/07/01	Lyall Creek, Kaikoura	42.37	173.68	River	600	100
14/07/01	Seddon	41.67	174.07	Creek	150	15
14/07/01	Dashwood	41.60	174.05	Stream	200	15
14/07/01	Blenheim	41.51	173.56	River	800	>150
16/07/01	Picton	41.29	174.00	Stream	300	50
16/07/01	Wellington	41.15	174.99	River	3000	>150
20/09/01	Near Hamilton	37.84	175.35	Roadside ditch	60	40
17/11/01	SH 4 (Near Aratoro)	38.49	175.19	Seepage	60	30
17/11/01	SH 4 (Near Aratoro)	38.51	175.20	Creek	90	30
17/11/01	Ongarue River	38.61	175.21	River	1500	>150
17/11/01	Taumarunui	38.70	175.23	River	1500	>150
17/11/01	Pokonaruru Creek	38.71	175.22	Creek	50	200
17/11/01	Near Te Whakarae	38.73	175.20	Seepage	NA	NA
17/11/01		38.75	175.17	Seepage	NA	NA
17/11/01	Opetea stream	38.77	175.15	Stream	350	50
17/11/01	Turnoff to Ohura	38.77	175.09	Creek	120	30
17/11/01		38˚57.793	174˚55.923	Seepage	NA	NA
17/11/01		35˚58.177	174˚54.979	Seepage	NA	NA
17/11/01		38˚58.728	174˚53.868	Seepage	NA	NA
17/11/01		38˚58.850	174˚51.806	Seepage	NA	NA
17/11/01		38˚58.433	174˚49.662	Seepage	130	10
17/11/01		39˚07.734	174˚94.667	Stream	NA	NA
17/11/01		39˚07.736	174˚94.667	Seepage	NA	NA
18/11/01	Mt Egmont	39.25	174.13	Stream	80	20
18/11/01	Whiskey Creek	39˚09.081	174˚10.468	Creek	90	40
18/11/01	Maketehinu Stream	39˚12.137	174˚13.786	Strean	400	50
18/11/01	Near Waituku-iti Stream	39˚12.859	174˚14.588	Creek	80	40
18/11/01	Lake Ratapiko	39˚12.275	174˚19.664	Lake	NA	NA
18/11/01	Near Lake Ratapiko	39˚12.134	174˚19.549	Seepage	100	30
18/11/01	Tawhiti Stream	39˚35.464	174˚18.229	Stream	350	50
18/11/01	Tangahoe River	39˚34.510	174˚21.560	River	450	60
18/11/01	Near Wanganui	39˚57.535	175˚05.377	Drainage ditch	100	30
19/11/01	Konini	40˚30.321	175˚47.611	Drainage ditch	60	20
19/11/01	Rock Road	40˚30.458	175˚47.816	River	400	60
19/11/01	Manawatu Gorge	40.33	175.82	Seepage	100	10
19/11/01	Manawatu Gorge	40˚19.802	175˚47.690	Seepage	50	10
4/12/01	Lake Rotoiti	41.81	172.84	Seepage	40	5
4/12/01	Lake Rotoiti	41.81	172.84	Lake	300000	> 150
4/12/01	St Arnaud	41.81	172.85	Seepage	80	20
4/12/01	Near St Arnaud	41˚47.465	172˚52.132	Seepage	80	15
4/12/01	Near St Arnaud	41˚46.366	172˚53.509	Seepage	60	10

APPENDIX 1. continued

4/12/01	Near St Arnaud	41˚45.578	172˚53.932	Seepage	80	15
4/12/01	Near St Arnaud	41˚45.685	172º53.827	Seepage	50	10
4/12/01	Near St Arnaud	41˚45.392	172˚53.851	Seepage	60	15
4/12/01	Near St Arnaud	41˚44.932	172˚53.793	Seepage	50	15
4/12/01	Near Kikiwa	41˚40.070	172˚52.454	Creek	200	NA
4/12/01	Near Kikiwa	41˚37.100	172˚52.386	Creek (pool)	400	80
4/12/01	Near Golden Downs	41˚31.516	172˚55.785	Creek	200	30
4/01/02	McLean Falls	46.57	169.36	Seepage	30	5
4/01/02	McLean Falls	46.57	169.36	Seepage	100	5
4/01/02	Horseshoe Falls	46.51	169.47	Seepage	30	5
6/01/02	Hump Ridge Track	46.15	167.44	Seepage	30	10
7/01/02	Hump Ridge Track	46.21	167.28	Stream	80	10
11/01/02	Okeover Stream	43.51	172.58	Stream	200	30
21/01/02	Lake Waitawa	40.73	175.17	Lake	100000	> 150
21/01/02	Masterton	40.95	175.66	Creek	150	30
22/01/02	Makaretu River	40.02	176.32	River	800	90
22/01/02	Napier	39.50	176.89	River	800	60
22/01/02	Esk River	39.39	176.87	River	1200	> 150
22/01/02	Te Ngaru Stream	39.31	176.88	Stream	250	60
22/01/02	Lake Tutira	39.23	176.89	Lake	3 km	> 150
22/01/02	Wairoa River	39.03	177.42	River	1600	> 150
22/01/02	Mangaruha River	38.97	177.41	River	350	100
22/01/02	Waikaretaheke	38.91	177.26	River	500	> 150
22/01/02	Lake Waikaremoana	38.76	177.14	Lake	700000	> 150
22/01/02	Managakakaho Stream	38.64	176.89	Stream	300	40
23/04/02	SH 38	38.56	176.75	Seepage	100	10
23/04/02	Ngaputahi	38.60	176.84	Stream	350	50
23/04/02	Te Waiti	38.61	177.00	Seepage	100	50
23/04/02	Aniwaniwa Stream	38.74	176.16	Stream	1200	100
23/04/02	Lake Waikaremoana	38.80	177.12	Lake	700000	> 150
23/04/02	Waikari River	39.13	177.00	River	800	70
23/04/02	Esk River	39.39	176.87	River	1200	> 150
23/04/02	Ngaruroro River	39.59	176.76	River	1200	> 150
23/04/02	Mangatewai River	39.99	176.33	River	400	60
24/04/02	Hutt River	41.13	175.06	River	3000	> 150
24/04/02	Lake Waitawa	40.73	175.17	Lake	100000	> 150
24/04/02	Manatu River	40.35	175.64	River	00	> 150
1/6/02	Hamilton Gardens	37.80	175.30	Seepage	40	3
1/6/02	New Memorial reserve	37.80	175.30	Seepage	40	30
1/6/02	Waipa River	37.80	175.15	River	1000	> 150
1/6/02	Raglan	37.82	174.90	Creek	300	60
8/10/02	Pearse Stream	41.22	172.75	Stream	100	20
8/10/02		NA	NA	Stream	NA	NA
8/10/02	Wairoa Gorge Rd	NA	NA	Stream	NA	NA
8/10/02	Wairoa Gorge Rd	41.47	173.10	Stream	70	20
8/10/02	Wairoa Gorge Rd	NA	NA	Stream	NA	NA
8/10/02	Wairoa Gorge Rd	NA	NA	Stream	NA	NA
8/10/02	Wairoa Gorge Rd	NA	NA	Stream	NA	NA
9/10/02	L. Rotoiti	NA	NA	Lake	NA	NA
9/10/02	L. Rotoiti	NA	NA	Lake	NA	NA
9/10/02	L. Rotoiti	NA	NA	Lake	NA	NA
9/10/02	Tapuwera	NA	NA	Seepage	NA	NA
9/10/02	Kaiteriteri-Marahau	NA	NA	NA	NA	NA
10/10/02		NA	NA	Stream	NA	NA
10/10/02	Brooklyn Stream	41.10	172.91	Seepage	70	10
10/10/02	Pearse V. Near Woodstock	41.27	172.82	Stream	NA	NA
26/10/02	Mt. Egmont	39.20	174.01	Seepage	50	5
26/10/02	Mt. Egmont	39.22	174.01	Seepage	50	5
26/10/02	Mt. Egmont	39.23	174.02	Seepage	50	5
31/10/02	Waimana River	38.10	177.04	River	6000	>150
31/10/02	Near Waimana River	38.10	177.04	Seepage	20	3
31/10/02	Nukuhou North	38.13	177.12	Stream	150	60
31/10/02	Opotiki	37.99	177.34	Seepage	80	10
31/10/02	Tirohanga	37.99	177.36	Seepage	100	5
31/10/02	Torere stream	37.96	177.48	Stream	500	100
31/10/02	Houpoto	37.87	177.60	Stream	150	5
31/10/02	Houpoto	37.86	177.63	Seepage	80	15
31/10/02	Te Kaha	37.74	177.68	Stream	100	10
31/10/02	Puremutahuri Stream	37.75	177.68	Stream	400	80
31/10/02	Wairuru Stream	37.65	177.87	Stream	400	130
31/10/02	Whangaparoa	37.58	178.00	Stream	220	60
31/10/02	Potaka	37.58	178.14	Stream	120	20

APPENDIX 1. continued.

31/10/02	Hicks Bay	37.61	178.31	Stream	220	50
31/10/02	Hicks Bay	37.57	178.29	Seepage	50	5
31/10/02	Hicks Bay	37.57	178.29	Seepage	50	10
1/11/02	Horoera	37.66	178.49	Stream	100	30
1/11/02	Horoera	37.66	178.50	Stream	500	110
1/11/02	Te Araroa	37.63	178.41	Seepage	80	20
1/11/02	Te Araroa	37.63	178.40	Seepage	80	5
1/11/02	Poroporo River	37.78	178.39	River	7000	>150
1/11/02	Te Puia Springs	38.02	178.27	Streams	70	10
1/11/02	Tokomaru Bay	38.13	178.32	Drainage ditch	100	5
1/11/02	Tokomaru Bay	38.13	178.32	Drainage ditch	30	2
1/11/02	Makokomuto	38.25	178.25	Stream	100	20
1/11/02	Pouawa River	38.61	178.18	River	400	>150
1/11/02	Morere	38.98	177.79	Seepage	50	5
1/11/02	Waikatuka Stream	39.05	177.64	Stream	400	>100
1/11/02	Marumaru	38.90	177.48	Seepage	10	2
1/11/02	Marumaru	38.90	177.48	Stream	120	50
1/11/02	Hangoroa River	38.72	177.60	Stream	200	50
2/11/02	Waihuka River	38.46	177.74	River	300	80
2/11/02	Matawai	38.36	177.46	Seepage	50	3
2/11/02	Matawai	38.34	177.45	Seepage	40	5
2/11/02	Matawai	38.32	177.39	Stream	200	30
2/11/02	Gorge	38.29	177.35	Seepage	30	2
2/11/02	Gorge	38.27	177.30	Seepage	60	8
2/11/02	Gorge	38.23	177.31	Seepage	50	3
2/11/02	Gorge	38.17	177.27	Seepage	60	5
2/11/02	Gorge	38.10	177.29	Seepage	50	5
3/01/03	Mt Cargill	45.82	170.55	NA	NA	NA
8/11/02	Devil's Staircase	45.21	168.74	Seepage	60	8
8/11/02	Devil's Staircase	45.24	168.74	Seepage	80	10
8/11/02	Devil's Staircase	45.25	168.75	Stream	400	80
8/11/02	Devil's Staircase	45.17	168.75	Seepage	70	2
8/11/02	Devil's Staircase	45.15	168.75	Seepage	70	10
9/11/02	Near Cromwell	45.04	169.12	Seepage	60	5
9/11/02	Lake Dunstan	45.00	169.23	Lake	1000	000
9/11/02		44.93	169.27	Drainage ditch	80	50
9/11/02		44.90	169.29	Stream	300	40
9/11/02		44.75	169.27	Seepage	30	5
9/11/02	Hawea	44.45	169.21	Seepage	80	10
9/11/02	Wanaka	44.40	169.19	Seepage	40	5
9/11/02	Wanaka	44.38	169.18	Seepages	50	5
9/11/02	Wanaka	44.36	169.18	Seepage	80	10
9/11/02	Makarara	44.27	169.20	Stream	100	70
9/11/02	Haast Pass	44.19	169.36	Stream	300	40
9/11/02	Haast Pass	44.07	169.38	Seepage	80	30
9/11/02	Haast Pass	44.06	169.38	Seepage	50	5
9/11/02	Haast Pass	44.04	169.38	Stream	100	10
9/11/02	Haast Pass	43.97	169.41	Seepage	30	5
9/11/02	Haast Pass	43.97	169.21	Seepage	80	10
9/11/02	Haast	43.90	169.06	Stream	400	100
10/11/02	Near Jackson's Bay	43.99	168.79	Seepage	150	80
10/11/02	Hindly Creek	44.00	168.68	River	600	>150
10/11/02	Near Jackson's Bay	43.99	168.62	Seepage	70	8
10/11/02	Near Haast	43.81	169.09	Stream	200	80
10/11/02		43.74	169.20	Seepage	40	4
10/11/02		43.74	169.20	Seepage	40	4
10/11/02		43.72	169.23	Seepage	30	4
10/11/02	Near Knights Lookout	43.73	169.24	Seepage	20	3
10/11/02	Near Lake Maeraki	43.73	169.28	Seepage	50	5
10/11/02	Near Lake Maeraki	43.73	169.28	Seepage	80	10
10/11/02	Lake Maeraki	43.73	169.28	Lake	NA	NA
10/11/02	Windbag Creek	43.77	169.38	River	1000	120
10/11/02		43.75	169.39	Stream	200	50
10/11/02	Bruce Bay	43.60	169.59	Stream	200	80
10/11/02	Bruce Bay	43.60	169.59	Stream	80	40
10/11/02	Near Fox	43.43	170.09	Seepage	50	10
10/11/02		43.26	170.28	Stream	150	80
10/11/02	Near Harihari	43.18	170.45	Seepage	50	5
12/11/02	Saltwater creek	42.51	171.17	Stream	400	50
12/11/02	Infants creek	42.53	171.18	Stream	300	50
12/11/02	Shantytown	42.54	171.20	Seepage	60	3
12/11/02	Shantytown	42.56	171.21	Stream	50	40

APPENDIX 1. continued

13/11/02	Kumara Junction	42.58	171.11	Stream	200	20
13/11/02	Kumara Junction	42.57	171.12	Pond	5000	00
13/11/02	Serpentine River	42.58	171.12	River	500	40
13/11/02	Arce creek	42.60	171.11	Stream	400	20
13/11/02	Kapitea creek	42.61	171.09	Stream	800	50
13/11/02	Awatuna	42.63	171.08	Seepage	30	2
13/11/02	Flowery creek	42.66	171.04	Stream	40	5
13/11/02	Greymouth	42.42	171.21	Stream	140	60
14/11/02	Ross	42.89	170.82	Stream	400	50
14/11/02	Ross	42.89	170.80	Seepage	40	5
14/11/02	Clear Creek	42.90	170.79	Stream	400	30
14/11/02	Mananui	42.75	170.93	River	700	>100
14/11/02	Inangahua	41.80	172.03	Seepage	120	10
14/11/02	Inangahua	41.80	172.03	Seepage	30	8
14/11/02	Inangahua	41.81	172.04	Seepage	40	6
15/11/02	Collingwood	40.77	172.56	Stream	150	60
15/11/02	Near Heapy Track	40.82	172.50	Seepage	30	3
15/11/02	Near Heapy Track	40.82	172.50	Stream	200	40
15/11/02	Brown Hut	40.85	172.45	Seepage	45	12
15/11/02	Near Heapy Track	40.83	172.47	Seepage	60	8
15/11/02	Near Heapy Track	40.82	172.52	Stream	150	10
15/11/02	Quartz Rd	40.83	172.52	Seepage	40	2
15/11/02	Quartz Rd	40.83	172.51	Stream	300	80
15/11/02	Quartz Rd	40.83	172.51	Seepage	30	2
15/11/02	Collingwood	40.70	172.63	Stream	200	25
15/11/02	Pakawau	40.59	172.69	Stream	300	40
15/11/02	Whanganui Inlet	40.59	172.62	Seepage	80	10
15/11/02	Paturau River	40.65	172.44	Seepage	40	5
15/11/02	NWSI	40.67	172.40	River	700	>100
15/11/02	NWSI	40.70	172.37	Seepage	50	10
16/11/02	Cobb Vally Rd	41.08	172.74	Seepage	30	3
16/11/02	Cobb Vally Rd	41.08	172.74	Seepage	50	6
16/11/02	Cobb Vally Rd	41.11	172.68	Seepage	25	3
21/12/02	Mt Somers	43.63	171.4	River	500	60
21/12/02	Mt Somers	43.62	171.4	Seepage	30	5
21/12/02	Mt Somers	43.63	171.38	Seepage	30	5
21/12/02	Mt Somers	43.64	171.38	Pond	100	40
8/01/03	Otepuni Stream	46.41	168.39	Stream	400	35
7/02/03	Mangamuka River	35.24	173.54	River	NA	NA
7/02/03	Whirinaki	35.47	173.46	Creek	120	10
7/02/03	Hatea River	35.73	174.35	River	1200	?
7/02/03	Waiaorahia Stream	35.7	174.32	Stream	300	60
31/03/03	Okato	39.19	173.88	Stream	NA	NA
3/04/03	Near Lake Omapere	35.33	173.78	Seepage	40	5
4/04/03	Waipehe Stream	38.88	175.97	Stream	200	25
4/04/03	Mangakoura Stream	38.96	175.85	Stream	250	50
4/04/03	Waitangi Stream	39.45	175.68	Stream	100	40
4/04/03	Near Moawhango River	39.6	175.85	Stream	150	20
4/04/03	Near Mangamaraqha River	39.5	176.01	Creek	40	10
4/04/03	Near Mangamaraqha River	39.5	176.01	Seepage	20	3
4/04/03	Near Taruarau River	39.45	176.15	Stream	20	60
4/04/03		39.43	176.27	Seepage	20	3
4/04/03	Near Ngarururoro River	39.4	176.31	Seepage	30	10
4/04/03		39.43	176.46	Stream	100	20
5/04/03	Omahu	39.65	176.76	Stream	300	100
5/04/03	Ngarururoro River	39.59	176.76	River	1200	> 150
5/04/03	Mangatewai River	39.99	176.33	River	400	60
5/04/03	Makaretu River	40.02	176.32	River	800	90
5/04/03	Mangamate Stream	39.78	176.47	Stream	200	20
6/04/03	SH 5	39.25	176.68	Stream	300	20
6/04/03	SH 5	39.12	176.6	Stream	50	10
6/04/03	SH 5	39.02	176.56	Seepage	40	3
6/04/03	SH 5	39.02	176.56	Seepage	40	3
6/04/03	SH 5	38.88	176.37	Stream	300	60
31/04/03	Near L. Waikare	37.44	175.23	Stream	60	20
31/04/03	L. Waikare	37.44	175.23	Lake	10000+	> 150
31/04/03		37.37	175.41	Stream	300	40
11/05/03	Near Lake Tawawera	36.28	176.38	Seepage	20	5
11/05/03	Lake Okareka	36.32	176.27	Lake	30000+	> 150
11/05/03		36.16	176.46	Stream	50	5

APPENDIX 2. Sequence data for the COI gene amplified from individuals from the genus *Paraleptamphopus* used in chapter II.

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Awatuna                  -----TT-TT-AAG---AC-G-----T--T-----T--G-----------C----T--C----------C--------
Awakino Gorge            -----C--GA-AAG------T--C--G--C--G--T--T-----A--G-----------C-----C-----C-----C--
Brooklyn                 --A--------T-----A--T-----------T-----A-A--A--T--------------C--C-----C--C-----
Mt Egmont                -----C--CA-AAG------T--C--C--------T--C-----A--A--------T--G----------C-----C--
Mt Cargill               -----CT-T---AG---CC--------------T----------G--A--CC----T-----C--C-----C-----C--
Fox                      -----TT----TAG---C--------------G-----CT----C------C----------C-----G-----C-----
Brown Hut                -----CT-C---AG---C--T-----G--------T-----A--C--T-----------A-------G--C--C--C--
Lake Waikare(Sp. 2)      -----CT-T--AA----A--T-----C-----G--CG-CT-T-----A--------T--G--C--------C--------
Anatori (Sp. 1)          -----TT-T---AG-----------G--------C--G-----G------C-------------C-----C--------
Queenstown               --A------T-A-----A----------C--------GA----C--T--C--------------C--------C-----
Cromwell                 --AG-------------A-----C-----C---------A-T--C--T----------------C----------CC-
Jackson Bay              --------C--A-----------------C---T-----A-T--C--T----------------------C--C--C--
Bluff                    ---G----C--A--------------G--C---T-----A-T--C--TGG--------------------C--C--C--
Shantytown               -----CT-TT-AAG----G-G--C--------G----------A----------------C--C---G-A--C--C--
Lee Valley               --A-----C--A-----A-----C-----C--G------A----------------------C--C--G----------
Waituna Wetlands (Sp. 1 TTATTTT-TG-TATTT---GGC-AGA-TT--C-A-GCCC-GTGGACAT-TAC--GGA-CTA--T-----C-A------GT
Waituna Wetlands (Sp. 2)TTATTTT-TG-TATTT---GGC-AGA-TTG-C-A-GCCC-GTGGACAT-TAC--GGA-CTA--T-----C-A------GT
Karamea Bight            TTATTTT-TG-TATTT---GGT-AGA-TT--C-A-GCCC-GTGGACAT-TAC--GGA-CTA--T-----C-A------GT
Whanganui National Park TTATTTT-TG-TATTT---GGC-AGA-TTG-C-A-GCCC-GTGGACAT-TAC--GGA-CTA--T-----C-A------GT
Ngutunui (Sp. 1)         --GTA-TT--C-GT-T---GGT-AGA-TT--C-A-GCCC-GTGGACAT-TAC--GGA-CTA-CT-----C-A------GT

Consensus Sequence       GGCACAGCACTCTCAGTTATCATTCGAACAGAACTAAGAGCCCCTGGCAATTTAATCGGAGATGATCAAATTTATAATAC
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Anatori (Sp. 2) --A-T---CT-A-----CG----------T--GT----------C--A--------TA-------C-------T---C--
Lake Ompere --G-T---------T--------C-----T--GT----T--A--------C------A----C-----------C-----
Murchison --G-T---------T--------C-----T--GT----T--A--------C------A----C-----------C-----
Mt Pirongia --G-T---------T--------C-----T--GT----T--A--------C------A----C-----------C-----
Atene --G-T---------T--------C-----T--GT----T--A--------C------A----C-----------C-----
Lake Waikare (Sp. 1) T-T-T--TC--G-T---A-----C-GT--GC-CATATA-AT-TA---CATTCTA--T-----T-A-T---CGT
Port Craig (Sp. 2) --T--C-----T-----C--T--------T---T----CA----------C-----T------------G-A--C-----
Horseshoe falls --T--C-----T-----C--T--------T---T----CA----------C-----T------------G-A--C-----
Ngutunui (Sp. 2) --G-T---------T--------C-----T--GT----T--A--------C------A----C-----------C-----
Whanganui Inlet --G-T------------------C-----T--G-----T--A--------C----------C----------C-----
Taumarunui Gorge --G-T---------T-CC-----C-T---T--GT----T--A--------C------A----C-----------C-----
Pearce Valley ----C--T--C--AC----C-----C--G------A----G----------------C--C--G----------
P. caeruleus --A--------T--G--A--------G--------T---AGA-----T---C----T--------C-----C--C--C--
Mangatewai River -----T--C---AG------T-----G--------T--G--------G----------G--C--C-----C--------
P. subterraneus ---G--G-------A-----G--------------C--
Paraphithoe hystrix. -----CT-CT-AAG---CC----C--G-----G--------T--------CA----------C--
Paraphithoe hystrix. -----CT-CT-AAG---CC-------------G--------T--G--T--CA-------------C----------C--
Consensus Sequence GGCACAGCACTCTCAGTTATCATTCGAACAGAACTAAGAGCCCCTGGCAATTTAATCGGAGATGATCAAATTTATAATAC

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Awatuna                 T--------G--T-----T-----A----------C-----G--------------------T-----A-----AC---
Awakino Gorge           C--T--T--A----------C--A----------C-----A-----A--C-----T--C--------A-----GC---
Brooklyn                T--G-----A-----C--T--C--------------------A-----C----------G--------A-----AC--G
Mt Egmont               T--C--C--A--T-----T-----A----------C-----C--G--A----------C--G-----A-----GC-T-
Mt Cargill              ------T--A--------T--CA----------------G--T-----A----------------C--A-----AC---
Fox                     ---------------C--------A--------C--------A-----C--C--------C--G-----C-----AC-GC
Brown Hut               C---T----------C--------G----------C-----A-----C--------T--C-----C--A--T--G--G-
Lake Waikare (Sp. 2)    T--G--C--G--------A-----A----------C-----A-----A--C-----T--C-------------A----
Anatori (Sp. 1)         C----------T----------A----------------G----------------C--G-----A--T--GC---
Queenstown              G--------A--A-----T-----A----------C-----A-----A--------T--T--C-----A-----AC-GC
Cromwell                T-----G--A--A-----T-----A----------C-----A-----A----------T--C-----A-----AC--C
Jackson Bay             T--------A-----C--A--C--A-----C--C--------T---------------------------T--A----
Bluff                   T--------A-----C--A--C--A-----C--C--------T------------------------G-----A----
Shantytown              T--G--T--A--------A-----A----------------A-------------T-----G-----C-----A----
Lee Valley              C-----G-----T--C------A----G--C----------A--G----------T--C-------------AC---
Waituna Wetlands (Sp. 1)-T--A-T--C--T-----GA-AA----GG-G-----C--GA-T--G---GC-T--T-T--T----------T-AC--TG
Waituna Wetlands (Sp. 2)-T--A-T-----T-----GA-AA----GG-A-----C--GA-T--G---GC-T--T-T--T----------T-AC--CG
Karamea Bight           -T--A-T-----T-----GA-AA----GG-A-----C--GA-T--G---GC-T--T-T--T----------T-AC--TG
Whanganui National Park -T--A-T-----T-----GA-AA----GG-A-----C--GA-T--G---GC-T--T-T--T----------T-AC--CG
Ngutunui (Sp. 1)        -T--A-T-----T-----GA-AA----GG-A-----C--GA-T--G---GC-T--T-T--T----------T-AC—-TG

Consensus Sequence      AATAGTAACTGCCCATGCCTTTGTTATAATTTTTTTTATAGTKATACCTATTATAATCGGAGGATTTGGTAACTGRTTAA
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Anatori (Sp. 2)          T-----C-----T----------------C----------G-----A----------------------T--AC-T-
Lake Ompere              ----------------------------------------G-----C--------------G--C--------G----
Murchison                ----------------------------------------G-----C--------------G--C--------G----
Mt Pirongia              ------------------------------A----------G-----C--------------G--C--------G----
Atene                    ----------------------------------------G-----C--------------G--C--------G----
Lake Waikare (Sp. 1)     GT-T-----A-GA-----TG-AA-A--GG-A----A---GA-G--G---GCAT----A--T-C---------T--G--TG
Port Craig (Sp. 2)       T--T--G-----T-----A--CA-------------------T--G--AG-------T--------C--C-----GC---
Horseshoe falls          T--G--G-----T-----A--CA-------------------T--G--AG-------T--------C--C-----GC---
Ngutunui (Sp. 2)         ------------------------------A----------G-----C--------------G--C--------G----
Whanganui Inlet          ----------------------C--G----------------G-----C--------------G--C--------G----
Taumarunui Gorge         ----------------------------------------G-----C--------------G--C--------G----
Pearce Valley            C-----G-----T--C------A----G--C----------A--G----------T--C--------------AC---
P. caeruleus             ------T--A-----C--A-----A----------C-----A-----A--C--------T--C----------G----
Mangatewai River         C-----G--C--------T-----A-----C----------C-----A--C----------C-----A--T--GC-CC
P. subterraneus          C--------AC----C--------C----------C-----G--G----------T--G--C-----C-----AC-GC
Paraphithoe hystrix      ------T--A-----C--A-----A--------C--------T-----G--C--G--------T--C-----T--AC-TG
Paraphithoe hystrix      T-----T--A--------A-----A--------C-----G-AT--------------------T--C-----T--AC-TG

Consensus Sequence       AATAGTAACTGCCCATGCCTTTGTTATAATTTTTTTTATAGTKATACCTATTATAATCGGAGGATTTGGTAACTGRTTAA
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Awatuna                  ----TC-T--G-----T--C--A--C-----A------------------------------T-----C--C-----A
Awakino Gorge            ----CT-----------GCG--A--------T--T--G--C-----C----------------C-----C--CG-C--A
Brooklyn                 -A---T-----T----A-----A--------A--T-------------T-------------T--A--C--C---T-A
Mt Egmont                ----CC----------A-----A--------T--T--G-------------------------T--A--C--CG-C--G
Mt Cargill               -----C-----T-------------C-----C--T-----------------G-----C--------A--C--G------
Fox                      ----CC-----T----T-----C--C--G--G-----G-------------G-----C-----CT----G--C--C--C
Brown Hut                ----CT-------C--T--C-----C-----G--T-----------C----------------C-----G--C--A--A
Lake Waikare (Sp. 2)     ----CC-------C--A-------------C--T-----C--------------------G--TT-A--C--CG-C--C
Anatori (Sp. 1)          -----C-------T--A--C--C--C-----A--T-----------------------------C-----C-----G--G
Queenstown               -A---T-----T----T-------------T-----G-------------------------TT-A--G-----AT-G
Cromwell                 -A--GT-----T----T-----A--C-----T-----A----------T-------------TT-A--G-----AT-A
Jackson Bay              ----CC-----A-C--A-----A-----G--C-----A----------------T--C------T-A--G------T-A
Bluff                    ----CC-----A-C--A-----C-----G--T-----A----------------T--C------T-A--G------T-A
Shantytown               ----TC-T--GT-------C--C--C-----T--T-------------T-------------T--C--T-----A---
Lee Valley               ----CC-----T-------G----------C--T----------C----------C--G--T--A--T---------
Waituna Wetlands (Sp. 1)-A---A-TT--A----TGC---A--C--G--T--T--AA-GC-------T-----T-----G-----TGTT---G--T--
Waituna Wetlands (Sp. 2)-A---A-TT--A----TGC---G-----G--T--T--AA-GC-------T-----T-----G-----TGTC---G--T--
Karamea Bight1           -A---A-TT--A----TGC---G-----G--T--T--AA-GC-------T-----T-----G-----TGTC---G--T--
Whanganui National Park -A---A-TT--A----TGC---G-----G--T--T--AA-GC-------T-----T-----G-----TGTC---G--T--
Ngutunui (Sp. 1)         -A---A-TT--A----T-C---G-----G--T--T--AA-GC-------T-----T-----G-----TGTC---G--T—-
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Consensus Sequence TCCCAYTAATACTAGGGAGACCTGATATAGCYTTCCCTCGAATAAATAACATAAGATTTTGACTACTGCCYCCTTCTCTT

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Anatori (Sp. 2)          -----C----------A-----C--------C--T--C--T-----C--T-----------GT----T--T-----A--A
Lake Ompere              -----T----------------------G--C--------T-----------------C------T----T--------G
Murchison                -----T----------------------G--C--------T-----------------C------T----T--------G
Mt Pirongia              -----T----------------------G--C--------T-----------------C------T----T--------G
Atene                    -----T----------------------G--C--------T-----------------C------T----T--------G
Lake Waikare (Sp. 1)     -T--TT--C--A----TGCT--A-----G--T--T--A---T-------T-----T-----G---A-ATTTG----AT-A
Port Craig (Sp. 2)       -----C-------------T--C-----G--T-----------------T-----------G-----T--T--GG-C--C
Horseshoe falls          -----C-------------T--C-----G--T-----------------T-----------G-----T--T--GG-C--C
Ngutunui (Sp. 2)         -----T----------------------G--C--------T-----------------C------T----T--------G
Whanganui Inlet          -----T----------------------G--C--------T-----------------C------T----T--------G
Taumarunui Gorge         -----T----------------------G--C--------T-----------------C------T----T--------G
Pearce Valley            ----CC-----T-------G-----------C--T-----------C-----------C--G--T--A--T---------
P. caeruleus             ----CC-T---T----A--C--G--C-----C--T-----------C--------C--C---T----T--G--R--A---
Mangatewai River         ----CT----G--C--CT-C--C--------C-----A--------------------C-----C-----C-----CT-A
P. subterraneus          ----GC-----T----CT-C--A--------C-----C--C----------------------T--T--C--C--C--C
Paraphithoe hystrix.     -T--TT-----T-------C--A--C-----T-----A--------C--T--------C-----T--C--C-----C--C
Paraphithoe hystrix.     ----TT-----T----A--C-----C-----T-----C--------C--T--------C-----T--T--C-----C---

Consensus Sequence       TCCCAYTAATACTAGGGAGACCTGATATAGCYTTCCCTCGAATAAATAACATAAGATTTTGACTACTGCCYCCTTCTCTT
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Awatuna                  --T------T--TW----------A-T--------------------C--------------T---T----A--GGGCG-
Awakino Gorge            -TA---C-T-----------G---A-T-----------G-----C--C--------T--T------T----C---GG-GC
Brooklyn                 -----C-----T--G---A-A--------G-----------A-----A--------T-----G--AT----A-----TA-
Mt Egmont                GTA---C-T--------C--G---A-T--G-----G--C-----C--C--------T-----G------------G-G-
Mt Cargill               T----CC-T--T--------G---A-C--------G--T--T-----A--------------T--A-----A----G-G-
Fox                      ------C-T--T--G--G---C--A-T-----C--G--C-----C--C--------A--T--G--C-----A---GG-G-
Brown Hut                --A---C-T-----------A---A-C--------G--C--------C-----------------CT---------G-G-
LakeWaikare(Sp. 2)       --A---C-T--G--------A--GA-C--G-----G--T-----C--C-----C--T-----T--C-----C----G-GC
Anatori (Sp. 1)          ------C-CT----------AC--A-C----------G--T--C--------C-----T------T----A----G-G-
Queenstown               --A--------T--------G-----T----------T--A----------T--A-----A---T-------CG-TC-
Cromwell                 --A--------T--------G-----T----------T--A----------T--A--------CT--------G-TC-
JacksonBay               --A--CC-G----CC-----T---A-C----------------C--------T-----------T----------CC-
Bluff                    --A--CC-G----CC-----T---A-C----------------C--------T-----------T----------CC-
Shantytown               --T--CC-T--------------GA-T--------G--G--G--T--------C--T--------------C---GGGA-
Lee Valley               --T---C-G----------------------------------G-----C-----T--G-----G---T----C---G-TC-
Waituna Wetlands (Sp. 1)-TG--A---A-GC-TTC--CT--T--T--TG-T----C---T--T--C--G---C-T--T---------AG---CCT-G-
Waituna Wetlands (Sp. 2)-TG--A---A-GC-TTC--CT--T--T--TG-T----C---T--T--C--G---C-T--T---------AG---CCT-G-
Karamea Bight            -TG--A---A-GC-TTC--CT--T--T--TG-T----G---T--T--C--G---C-T--T---------AG---CCT-G-
Whanganui National Park -TG--A---A-GC-TTC--CT--T--T--TG-T----C---T--T--C--G---C-T--T---------AG---CCT-G-
Ngutunui (Sp. 1)         -TG--A---A-GC-TTC--CT--T--T--TG-T----C—--T--T--C--G---C-T-----T------AG---TTT-G-

Consensus Sequence       ACCTTTTTACTAATAAGAGGCTTAGTAGAAAGAGGAGTAGGCACAGGTTGAACAGTCTACCCCCCTCTAGCTAGAACART
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Anatori (Sp. 2)          --A--------CG-------A----------------------T--A--------A--T--G--AT----A------A-
Lake Ompere              ---------T--G----------------------G--G-------------------------C-----A------A-
Murchison                ---------T--G----------------------G--G--------------C--------T--C-----A------A-
Mt Pirongia              ---------T--G----------------------G--G-------------------------C-----A------A-
Atene                    ---------T--G----------------------G--G-------------------------C-----A------A-
Lake Waikare (Sp. 1)     -TT--A---TGT--TTCT-CTG-TAC--CTGA----CCT--A-TT--A--G----CT-----A-----TT--TC--T-CA
Port Craig (Sp. 2)       --T--------T-----G-------------------------T-----------A-----G------T-------TA-
Horseshoe falls          --T--------T-----G-------------------------T-----------A-----G------T-------TA-
Ngutunui (Sp. 2)         ---------T--G----------------------G--G-------------------------C-----A------A-
Whanganui Inlet          ---------T--G----------------------G--G-----------------------------A------A-
Taumarunui Gorge         -------A-T--G----------------------G--G-------------------------C-----A------A-
Pearce Valley            --T---C-G-------------------------------G-----C-----T--G-----G---T----C---G-TC-
P. caeruleus             --T------T---C------T---A-C--------G-----A--T--------C--T-----T--CT----------CA-
Mangatewai River         --T---C-T--------G--GC-CA-T--G-----T--C-----G--C-----C--------A-----T--G--GGGGGC
P. subterraneus          -----CC-CA-----------C-TA-C--G--G--G--C--G--------G-----A-------------G--GGGGG-
Paraphithoe hystrix      --A--CC--T----C------C----------------C-----T--------------------AT-----TCGG-TAC
Paraphithoe hystrix      --G--CC-CT----C------C--A--N-G--G-----CN----T-----G--------T--T--AT-G---TC-G-CAC

Consensus Sequence       ACCTTTTTACTAATAAGAGGCTTAGTAGAAAGAGGAGTAGGCACAGGTTGAACAGTCTACCCCCCTCTAGCTAGAACART
```

```
Awatuna                  G--G--------T-----T--C---A-C--------C--C--C--CT-------A-----C------T----T--T----
Awakino Gorge            ------------G--C--C-----CA-T--------C--C-----------G-----------C-----C-----T----
Brooklyn                 ------------CA-TG-------CT----C--C--C--A--T-------------TT--A--A---T----T-------
Mt Egmont                A--------G--T--C--T------A-C-----------C-----C-----C-----T-----------G-----T----
Mt Cargill               -----C-----C--C--G------A-C-----C--C--A--T---CT----C--------C--A--C--T--G-------
Fox                      ---G--C--C--C--T-----G-----C--C-----C--C--T--C--G-----T-----G--G--C--T-----C--C-
Brown Hut                T-----------G--T--C------A-C--C-----C-----T---T-------A------------T-------T--C-
Lake Waikare (Sp. 2)     ---A--C-----G--C--T--G---A-C--C-----------C---T-------C-----G--------C-----C--C-
Anatori (Sp. 1)          T-----C-----G--C--C--G---A-T--C--C--C--C--T---T----G--------C-----C--T-----T----
Queenstown               T--A--------CA-----------T----C--------G------T-G-----A-----A--A--------A--C----
Cromwell                 T--A--------CA----C--G---T----C-----C--A------T----C--A--T--A--A--------G--T----
Jackson Bay              ---G---------A-----------T-------------C-----CT-G-----C-----A------T-------T----
Bluff                    ---G---------A-----------T-------------C-----CT-G-----C-----A------T-------T----
Shantytown               T-----C--------G--G------T-------C------T-G--C--G--------T--C-----------GG------
Lee Valley               -------------A-------T------------------T-------C--C--C-----A--------T-----T--C-
Waituna Wetlands (Sp. 1)A-GA---CCT--G-C-G-T--C---A-G--------AAGCT-------TA----ACTT--C--------T----------
Waituna Wetlands (Sp. 2)A-GA---CCT--G-CGG-T--C---A-G--------AAGCT-------TA-----CTT--C--------T----------
Karamea Bight            A-GG---CCT--G-CGG-T--C---A-G--------AAGCT-------TA-----CTT--C--------T--T-------
Whanganui                A-GA---CCT--G-CGG-T--C---A-G--------AAGCT-------TA-----CTT--C--------T--T-------
Ngutunui(Sp. 1)          A-GA---CCT--G-CGG-T--C---A-G--------AAGCT-------TA-----CTT--C--------T--T-------

Consensus Sequence       CGCCCATAGAGGAGGATCAGTAGATCTAGCTATTTTTTCTCTACATCTAGCAGGGGCCTCTTCTATTCTAGGCTCAATTA
```

```
Anatori (Sp. 2)         A--T--C-----TA----------------------C--AT------------T--T--------CT----T--C----
Lake Ompere             ------C--------------G--C--G--A--C--------------T--------------A---T----T-------
Murchison               ------C--------------G--C--G--A--C--------------T--------------A---T----T-------
Mt Pirongia             ------C--------------G--C--G--A--C--------------T--------------A---T----T-------
Atene                   ------C--------------G--C--G--A--C--------------T--------------A---T----T-------
Lake Waikare (Sp. 1)    ATTT---CCTA-T-CTG-T--T---A----A------AG-T-----T--------TTG---------T-T--AG------
Port Craig (Sp. 2)      ---G-----T--GA----C-----------A---------T----CA----C--A--T-----------------T----
Horseshoe falls         ---G-----T--GA----T-----------A---------T----CA----C--A--T-----------------T----
Ngutunui (Sp. 2)        ------C--------------G--C--G--A--C--------------T--------------A---T----T-------
Whanganui Inlet         ------C--------------G--C--G--A--C--------------T--------------A---T----T-------
Taumarunui Gorge        ------C--------------G--C--G--A--C--------------T--------------A---T----T-------
Pearce Valley           -------------A-------T-----------------T-------C--C--C-----A--------T-----T--C-
P. caeruleus            T-----C------A-G--C-----C--------------CT-----T-------C-----C--A--------G-------
Mangatewai River        G--A--C-----------G--------C--C-----C--CT-G--C--G--T-----T--------CT-------C----
P. subterraneus         ------C--G--C--C--T-----C--C--A--C--C--C--T--CT-G--G--------C--G-----G--G--T----
Paraphithoe hystrix     T-----CG----C--GG--------ACT--------C-----C---T-G--C---------------T-----G-C--C-
Paraphithoe hystrix     A-----CG-G--C---G-T-----CA----------C-----C---A----C--A--A---------T-----G-C----

Consensus Sequence      CGCCCATAGAGGAGGATCAGTAGATCTAGCTATTTTTTCTCTACATCTAGCAGGGGCCTCTTCTATTCTAGGCTCAATTA
```

```
Awatuna                  -T--------A-----T----------G------G-------C---------G-A----------C-----T--C---
Awakino Gorge            ----C-----A--C--------------A-G--AG------G------C-----A--AC-------------T--A---
Brooklyn                 -T---T----C--------C--T-----G-----A-T----A----------------------A-C------A-----
Mt Egmont                ----C--------G--------------------AG-------------C-----A--C--------C--------A---
Mt Cargill               -T--C--------A--------------T--A--AG-C-----------C---G-A--C--------A--G-----C---
Fox                      -------------G-----------G--------GG-T-----C-----C--G--G--CC----C-----G-----A---
Brown Hut                -T-----G--C-----G--C--------------AG------GC-----C---G-G--------C--C-----T------
Lake Waikare (Sp. 2)     -T--C-----C--A--------------C--G---G-G----G------C--------C--------G--G--T------
Anatori (Sp. 1)          -------G--C-----T-----------------AG------GC--------GG-G----------C-----C------
Queenstown               -T---T-------------C--T--------A----T----A-------C----------------------T--A---
Cromwell                 -T---T----A--------C--T--------A----T----A-------C--------A--------C-----T--A---
Jackson Bay              ----C--T--------------T-----C--A----A------------C-----T---C-------A-----T-CC---
Bluff                    ----C--T--------------T-----C--A----A------------C-----T---C-------G-----T-CC---
Shantytown               -------C--------C--C--T------------G-C---AGC--------G--T-----------------T------
Lee Valley               -----C-C-----------------G---T----A-T------------C-----------------G-----T------
Waituna Wetlands (Sp. 1)--A-G--TGTC---A-CT----T--GA--A-TGA-G-T--GGGGT-GTT-G----G--C--G---A----G---A----A
Waituna Wetlands (Sp. 2)-TA-G--TGTC---A-CT----T--GA--A-TGA-G-T--GGGGT-GTT-G----G--C--G---A----G---A----G
Karamea Bight            --A-G--TGTC---A-CT----T--GA--A-TGA-G-T--GGGGT-GTT-G----G--CC-----A----G---A-C--G
Whanganui National Park -TA-G--TGTC---A-CT----T--GA--A-TGA-G-T--GGG-T-GTT-G----G--CC-----A----G---A----A
Ngutunui (Sp. 1)         --A-G--TGTC---A-CT----T--GA--A-TGA-G-T—-GGGGT-GTT-GC---G--CC-G---A----G---A--C-G

Consensus Sequence       ACTTTATATCTACTGTAATTAACATACGAGCCCCTAGAATACAAATAGATCAAATCCCTTTATTTGTTTGATCAGTTTTT
```

```
Anatori (Sp. 2)          -T---T----G--C-----C--T-----CA----A-T----A---------------------------------A---
Lake Ompere              -----TA-----------------------------------A-------------T--------C-----------G---
Murchison                -----T------------------------------------A-------------T--------C-----------G---
Mt Pirongia              -----T------------------------------------A-------------T--------C-----------G---
Atene                    -----T------------------------------------A-------------T--------C-----------G---
Lake Waikare (Sp. 1)     -T-----TA-A--AA-T--------G--T--A--AG--G---G-T-TAG-A-T--G--G--G-----A--GG--A-A---
Port Craig (Sp. 2)       ----------A-----T-----T-----T--G--G------TT----AC---G--A--C--------A-----T--C---
Horseshoe falls          ----------A-----T-----T-----T--G--G------TT----AC---G--A--C--------A-----T--C---
Ngutunui (Sp. 2)         -----T------------------------------------A-------------T--------C-----------G---
Whanganui Inlet          -----T-----------------------------A----A-T-----------T--------C-----------G---
Taumarunui Gorge         ----AT-------------------------------------T----------T--------C-----------G---
Pearce Valley            -----C-C-----------------G---T----A-T------------C-----------------G-----T------
P. caeruleus             ----C--C--C--------C--------C-----A-AC--G--C--G--C-----A--GC-------C--G--G-CC--C
Mangatewai River         -T--------A--GA-C-----T-----G--G--GG-GC--GCC-----C--G-----CC-T-----------C--C---
P. subterraneus          -------C--C--AA-T--------------T--AG-C---G-C-----C---G----CC-------C--G--C--A--C
Paraphithoe hystrix      -T-----CG----AA-T-----T------A--GTA-A-----G-T----C--G-C----C----------G--T--CC--
Paraphithoe hystrix      -T-----TG----AA-TG----T------A--GTA-A-----GGT----C--G-C----C----------G--T--AC--

Consensus Sequence       ACTTTATATCTACTGTAATTAACATACGAGCCCCTAGAATACAAATAGATCAAATCCCTTTATTTGTTTGATCAGTTTTT
```

```
Awatuna                  --T-----A---T-----TA------C--G-----TC-T-------------C--------C--AA----T--T-----
Awakino Gorge            ------------C-C-----TT----TT----T--C-----C--------T--------C-----C-----G--------
Brooklyn                 --T--C--G---C-T--------C--T-----T--T-----------A--T--T--G--AT----A---------T----
Mt Egmont                ------------C-------TT----CT-G--T--GC-------G--C--T--------AT----C--T--T--T-----
Mt Cargill               --T--------CC-G-----T-----G--------C-----C-----C--T--------------A--------------
Fox                      --------A---C-G-----C--G--C--G-----T-----G--G--A--T--T-----A--G--G--------------
Brown Hut                -----------CT----C--CT----G--C--A--C-----C--G--C-----T--G-----C--A--T-----TT----
Lake Waikare (Sp. 2)     -----------CT----C--C-----C--C-----TC----------A--T-----G--------G--------------
Anatori (Sp. 1)          --T--------CT----C--CT-G--C--G--A--T-----C-----A-----C--------T--A-----C--------
Queenstown               --T--T-----CT--------T----G--------GC--------------------T-AT-------T-----T--T--
Cromwell                 --T--T-----CT--------T-------------GC--------------------T-AT-------T-----T--T--
Jackson Bay              --T--C--A--CC-C--A--------CT----T---C----G--C--A--T--C--G--------A--T-----TT----
Bluff                    --T--C--A--CC-G--A--------CT----T--------G--C--A--T--C--G--------A--T-----TT----
Shantytown               --T---GT----C-T-----T--C--T-----T--TC----T--------------------T--A--T------T----
Lee Valley               ------------T--T-A--TT----TT--------C----------------T-----C-----A--------TA-T--
Waituna Wetlands (Sp. 1)G----TG--T-CT-GT-AA----GG--A-T--G-------GC--T--A--A--C--GT-AT-------------TT-TGG
Waituna Wetlands (Sp. 2)G----TG--T-CT-GT-AA-----G--A-T--G-------GT--T--A--A--C--GT-AT-------------TT-TGG
Karamea Bight            G----TG-CT--T-G--AA-----G--A-T--G-------GT--T--A--A--C--GT-AT-------------TT-TGG
Whanganui National Park G----TG-CT-CT-GT-GA----GG--A-T--G-------GT--T--A--A--C--GT-AT-------------TT-TGG
Ngutunui (Sp. 1)         G----TG-CT-CT-G--GA-----G--A-T--G-------GT--T--A--A--C--GT-AT-------------TT-TGG

Consensus Sequence       ATCACAACTATTYTACTTCTACTATCACTACCCGTATTAGCAGGAGCTATCACAATACTTCTAACTGACCGAAACCTAAA
```

```
Anatori(Sp2             --T-----A--CC-TT-A--G------T----T--TC-------G-----T----------------------------
Lake Ompere             -----C--AG--C-T---TAGT-------------------G-------------------------T--------T--
Murchison               -----C--AG--C-T---T-GT-------------------G-------------------------T--------T--
Mt Pirongia             -----C--AG--C-T--AT-GT-------------------G-------------------------T--------T--
Atene                   -----C--AG--C-T---T-GT-------------------G-------------------------T--------T--
Lake Waikare (Sp. 1)    G-T---T-AT--G-TA--A-TT-T-GTA-T--A--T--T--T--G--A--A-----GA-A--T-----TA----TT-TGG
Port Craig (Sp. 2)      -----------CT----A-----C--T--T--A---C-G--------------C--------------------------
Horseshoe falls,C       -----------CT----A-----C--T--T--A---C-G--------------C--------------------------
Ngutunui (Sp. 2)        -----C--AG--C-T--AT-GT-------------------G-------------------------T--------T--
Whanganui Inlet         -----C-AAG--C-T---TCGT-------------------G-------------------------T--------T--
Taumarunui Gorge        -----C--AG--C-T---T-GT-------------------G-------------------------T--------T--
Pearce Valley           ------------T--T-A--TT----TT--------C----------------T-----C-----A--------TA-T--
P. caeruleus            -----C--A---C-TT-A--------T--G----------R-----A-----CW-GT-A-----C--------------
Mangatewai River        --T--G---G-AC----AT-G--T-----G-----C-----T-----------------AT----A-----C--------
P. subterraneus         -----------CT-------T--G--C--C--A--GC-------G--------T-----G--G--A--------------
Paraphithoe hystrix     ------G-----T----C--G-----T-----A---C----C--C--------T--------T--A-----T--TT----
Paraphithoe hystrix     ------G-----T----C--------T--G------C----C--C--------T--------T--A-----Y---T----

Consensus Sequence      ATCACAACTATTYTACTTCTACTATCACTACCCGTATTAGCAGGAGCTATCACAATACTTCTAACTGACCGAAACCTAAA
```

```
Awatuna                 C-
Awakino Gorge           C--T-----C-----T--C-----G--C--------------T----
Brooklyn                ---T--C-----------A-----------------T---
Mt Egmont               ---T-----C--C-----------G----------------------
Mt Cargill              C--------C--C--------C--G----------------------
Fox                     ---G-----------------G--T-----------W----------
Brown Hut               ---A-----C--C--
Lake Waikare(Sp. 2)     ---------C--C-----C--G--------------T----------
Anatori (Sp. 1)         C--A-----C--C-----C----------------------A-----
Queenstown              ---G-----C-------------------------------------
Cromwell                ---A-----C--------A--------------------------
Jackson Bay             ------C-----------A-----C------------------C--
Bluff                   ------C-----------A-----C-----------T-------C--
Shantytown              C-----------C-----C--TT-----------------------
Lee Valley              C--T--A-----------C--T--G----------------G-----
Waituna Wetlands (Sp. 1)----A----C---A-A---GAG--T--C---------G-A----TT-
Waituna Wetlands (Sp. 2)----A----C---A-A---GAG--T--T-----T---G-G----TT-
Karamea Bight           ----A----C---A-A---GAG--T--C-----T---G-G----TT-
Whanganui National Park C--TA----C---A-A---GAG--G--C---------G-G----TT-
Ngutunui (Sp. 1)        ----A----C---A-A---GAG--G-----------G-A-----T-

Consensus Sequence      TACCTCTTTTTTTGACCCTAGAGGAGGAGGAGACCCAATTTTATACC
```

```
Anatori(Sp. 2)          ------A--C--------A---------------
Lake Ompere             C-----A--------------C------------------
Murchison               C-----A--------------C
Mt Pirongia             C-----A--------------C------------------------
Atene                   C-----A--------------CC-G
Lake Waikare Sp. 1)     A--AA-A--C---A-T--ATCT-----------T--TC--C-T-TT-
Port Craig (Sp. 2)      ---G--A-----C--T--C--C------------------------
Horseshoe falls,        ---G--A-----C--T--C--C-----G-----------C-----T-
Ngutunui (Sp. 2)        C-----A--------------C------------------------
Whanganui Inlet         C--------C-----------G------
Taumarunui Gorge        C-----A----------
Pearce Valley           C--T--A-----------C--T--G---
P. caeruleus            C--T--C-----C--------G--T----------------------
Mangatewai River        ------C--C-----T--C--C--G-----------TT---AT----
P. subterraneus         ------C-----------GTCG--C----------------------
Paraphithoe hystrix     -----TC--------------------T--------T----------
Paraphithoe hystrix     C-----N------------------------T--T-----G----

Consensus Sequence      TACCTCTTTTTTTGACCCTAGAGGAGGAGGAGACCCAATTTTATACC
```

APPENDIX 3. Sequence data for the 28S gene amplified from individuals from the genus *Paraleptamphopus* used in chapter II.

```
Mt Pirongia             ----------------------------------------------------------------------------
Ngutunui (Sp. 1)        ----------------------------------------------------------------------------
Ngutunui (Sp. 2)        ----------------------------------------------------------------------------
Lake Ompere             ----------------------------------------------------------------------------
Lake Waikare (Sp. 2)    ----------------------------------------------------------------------------
Mt Egmont               ----------------------------------------------------------------------------
Awakino Gorge           ----------------------------------------------------------------------------
Mt Cargill              ----------------------------------------------------------------------------
Mangatewai River        ----------------------------------------------------------------------------
Waituna Wetland (Sp. 1)                               ------------------------------------------------
Shantytown              ----------------------------------------------------------------------------
P. caeruleus            ----------------------------------------------------------------------------
Jackson Bay             ----------------------------------------------------------------------------
Brooklyn                ----------------------------------------------------------------------------
Pearse Valley           ----------------------------------------------------------------------------
Anatori (Sp. 2)         ----------------------------------------------------------------------------
Anatori( Sp. 1)         ----------------------------------------------------------------------------
Port Craig (Sp. 1)      ----------------------------------------------------------------------------
Paramphithoe hystrix    ----------------------------------------------------------------------------
Paramphithoe hystrix    ----------------------------------------A-----------------------------------

Consensus Sequence      ACAACGGCTACGGGCCTCCACCCCAGTTTCCTGGGGCTTCGCCCTCGCCAGGCATAGTTCACTATCTTTCGGGTCATAGC
```

```
Mt Pirongia             ------------------------------------------------------------------------------
Ngutunui( Sp1)          ------------------------------------------------------------------------------
Ngutunui (Sp2)          ------------------------------------------------------------------------------
Lake Ompere             ------------------------------------------------------------------------------
Lake Waikare (Sp2)      ----------------------------------------------T-------------------------------
Mt Egmont               ------------------------------------------------------------------------------
Awakino Gorge           ------------------------------------------------------------------------------
Mt Cargill              ------------------------------------------------------------------------------
Mangatewai River        ----------------------------------------------T-------------------------------
Waituna Wetland(Sp. 1)  -------------------------T------------------T-------------------------------
Shantytown              ----------------------------GAGCGC-----------------------------------G---------
P. caeruleus            ------------------------G----GAGCGC-------G-------------------------G-----------
Jackson Bay             ------------------------G----GAGCGC-------G-------------------------G-----------
Brooklyn                ----------G-------------------C-C-C-----T-G-CG---------------------G-----------
Pearse Valley           ----------G-------------------CACGC-----T-G-C----------------------G-----------
Anatori (Sp. 2)         ----------G-------------------CACGC-----T-G-C----------------------G-----------
Anatori (Sp. 1)         ----------G-------------------CACGC-----T-G-C----------------------G-----------
Port Craig (Sp. 2)      ----------G-------------T----TCGCGT-----T-G-T-T--------------------G----------:
Paramphithoe hystrix    ---------CC-----------ACTC--TTCCG---:AAG-G----T--A--------A-C-----C-T-C-T-GCG---
Paramphithoe hystrix    ---------CC-----------ACTC--TTCCG---:AAG-G----T--A--------A-C-----C---C-T-GCG---

Consensus Sequence      ATGCACGCT:AACAGTGCTCCCCGAGACAAGTAAAGTGCCCCATGACGGGGAGCCTGGGGTTTGCG:CACAGATAAACGA
```

```
Mt Pirongia             ----------------------------------------------------------------------------
Ngutunui (Sp. 1)        ----------------------------------------------------------------------------
Ngutunui (Sp. 2)        ----------------------------------------------------------------------------
Lake Ompere             -------------------A--------------------------------------------------------
Lake Waikare (Sp. 2)    ----------------------------------------------------------------------------
Mt Egmont               ----------------------------------------------------------------------------
Awakino Gorge           ----------------------------------------------------------------------------
Mt Cargill              ----------------------------------------------------------------------------
Mangatewai River        ----------------------------------------------------------------------------
Waituna Wetland (Sp. 1) ----C-----------------------------------------------------------------------
Shantytown              ----C--------------A----------------C----T-----------A--------------T-----------
P. caeruleus            -G--C--------------A--------------------T-----------C--------------A-----------
Jackson Bay             -G--C--------------A--------------------T-----------C--------------A-----------
Brooklyn                -A--T--C-------------------------------------------G-C---G----------A-----------
Pearse Valley           -A--C--C-----------A-------------A-----------------G-C--------------A-----------
Anatori (Sp. 2)         -A--C--C-----------A--------------------T---------G-C--------------A-----------
Anatori (Sp. 1)         -A--C--C-----------A--------------------T---------G-C--------------A-----------
Port Craig (Sp. 2)      ::--CTA------------A--------------------T---------G-C----T---------A-------G---
Paramphithoe hystrix    GGAC-:-T-G--A--GG-G-ATC----C----TAA-C---AGA---C--A--TCCG--TC---G--AG-G-AAA--GG-A
Paramphithoe hystrix    GGAC-:-T-G--A--GGT--ATC----C----TAA-C---AGA---C--A--TCCG--TC---G--AG---AAA--GG-A

Consensus Sequence      ACTTGACGTTCTTACAACAGCCGAGCTGGGTA:GCGTCGG:CTGCA:AT:CAGTGATCAACCC:TGTCGAGCTGCAA:AG
```

```
Mt Pirongia             ----------A---A-----------------------------------------------------------------
Ngutunui (Sp. 1)        ----------T---A-----------------------------------------------------------------
Ngutunui (Sp. 2)        ----------T---A-----------------------------------------------------------------
Lake Ompere             ----------------------------------------------------------------------------------
Lake Waikare (Sp. 2)    ----------------------------------------------------------------------------------
Mt Egmont               ----------A---A-----------------------------------------------------------------
Awakino Gorge           ----------A-------G-------------------------------------------------------------
Mt Cargill              ------------------------------------------------------------------------T--------
Mangatewai River        -A----------------------------------------------------------------------------------
Waituna Wetland (Sp. 1) -A----------------------------------------------------------------------------------
Shantytown              -A--G---A------A-C--T---------------------A--------------A--G-----------C-------
P. caeruleus            -A------G-A----A-C------------------------------------------------------------------
Jackson Bay             -A------G-A----AAC--T---------------------------------------------------------------
Brooklyn                -A--G----------AAC------G-----------------------------------G--A-::-A---C---A---
Pearse Valley           -A--G----------A-C----T-G-----------------------------------A--G--A-AG-A---C-------
Anatori (Sp. 2)         -A--G----------A-C--T---G--------------------------G----A--G--A-AG-A---C-------
Anatori (Sp. 1)         -A--G----------A-C--T---G--------------------------G----A--G--A-AG-A---C-------
Port Craig (Sp. 2)      TG--G----------A-C-----------------------------------G-------G---------------A---
Paramphithoe hystrix    T-G---CAA-TA-----:-TG---:-CCT-C-C-AC-:GG-TA---GCT-AA--G-A-A--C:--:::A:--:::::::::
Paramphithoe hystrix    T-G---TAA-T------:-TG---:-CCT-C-C-AC--GG--A---GC-AAA--G-A-A--C:--:::A:--:::::::::

Consensus Sequence      CTACACATTCGTCCGGGTCGACCCAGGAATTG:CGGCA:CAATGCA:TACGGACTGCCTCAGC:CTTGCGACTGAAGCAC
```

```
Mt Pirongia             ------------------------------------------------------------------------------
Ngutunui (Sp. 1)        ------------------------------------------------------------------------------
Ngutunui (Sp. 2)        ------------------------------------------------------------------------------
Lake Ompere             ------------------------------------------------------------------------------
Lake Waikare (Sp. 2)    ------------------------------------A-------------------------
Mt Egmont               ------------------------------------A-------------------------------------------
Awakino Gorge           ------------------------------------A-------------------------------------------
Mt Cargill              ------------------------------------A----------------------
Mangatewai River        ------------------------------------------------------------------------------
Waituna Wetland (Sp. 1) ------------------------------------------------------------------------------
Shantytown              ----------C-----------------------------------------T--------------------------
P. caeruleus            ----------C-------------------A-------------------T--------------------------
Jackson Bay             ----------C-----------------------------------------T--------------------------
Brooklyn                -T---------C-----------------------------------------T--------------------------
Pearse Valley           -T---------C-------------------------------------------------------------------
Anatori (Sp. 2)         ----------C----------T---------------------------------------------------------
Anatori (Sp. 1)         ----------C----------T---------------------------------------------------------
Port Craig (Sp. 2)      ----------C----------------G----------------------T--------------------------
Paramphithoe hystrix    ::::::::::----------------G--------AG-------C----T-------------G------------
Paramphithoe hystrix    ::::::::::----------------G--------AG-------T----T-------------G------------

Consensus Sequence      GCCCTTACCGTTTGCTTTCACTTCGCCTCCAGGTTTTGTTAGACCCTTAGACTCGCGCACATGCTATACTCCTTGGCCCG
```

```
Mt Pirongia             ----------------------------------------------T
Ngutunui (Sp. 1)        ----------------------------------------------T
Ngutunui (Sp. 2)        --G-------------------------------------------T
Lake Ompere             ----------------------------------------------
Lake Waikare (Sp. 2)
Mt Egmont               ---AA------------------------------------------T
Awakino Gorge           ----------------------------------------------T
Mt Cargill
Mangatewai River        ----------------------------------------------
Waituna Wetland (Sp. 1) ----------------------------------------------
Shantytown              -----------------
P. caeruleus            ----------------------A------------------A------
Jackson Bay             ----------------------A------------------A------
Brooklyn                ----------------------A-------------------G-----
Pearse Valley           ----------------------A-------------------G-----
Anatori (Sp. 2)         ----------------------A-------------------T-----
Anatori (Sp. 1)         ----------------------A-------------------T-----
Port Craig (Sp. 2)      ----------------------A------------------
Paramphithoe hystrix.   -----------------A-----C-C-G-ATT-G-:-----AT-----
Paramphithoe hystrix.   -----------------A-----C-C-G-ATTAG-:-----AT-----

Consensus Sequence      TGTTTCGAGACGTGACCGATGGCTCGGAACAGTTTCCACCACACATCC
```

APPENDIX 4. Sequence data for the COI gene amplified from individuals from the species complex *Paracalliope fluviatilis* used in chapter IV.

```
Hamilton Gardens       -----C--AT------------------------G-----------C--------T--TA----C--------------
Palmerston North       ---------------------------------------T--G-----A-----G----------------------T--
Napier                 --T----------------------G--G--G-----T--T-----A---T-A-----------------C--------
Hawera                 --------------------------------G-----T--A-----A-----A--------------------------
Wanganui               ---------------------------------------T--A-----A-----A--------------------------
Otaki                  ---------------------------------------TT-A-----A-----A------A------------------
Mangamuka              -----C---T-----------------------------------------C--------T--C-----C-----------T--
Hokianga Harbour       -----C---T-----------------------------------------C--------T--C-----C-----------T--
Port Waikato           -----C---T-G---------------------------------------C--------T--C--------------------
Waitoa                 --------T---------C-------------------C-----G--C--------T--------C--------------
Whangarai              --------TT--------C-------------------C--------C--------T--T--T--C--------------
Waihou                 --T--C--TA--------C-------------------C--------C--------T--------C--------------
Hutt river             -----A-----------G--T--------T-----G-----------A--T--A--------T--C-----C--------
Granity                -----------------T--------------------T--G-----A-----G--------------G--------T--
Kaikoura               --------A--G-----G--------------------C--A-----A-----A--------------------------
Blenheim               --------A-----------------------------CA-A-----A-----A--------------------------
Pirongia               --------AT------------------------G-----------C--------T--TA----C--------------
Kawhia                 -----C---T-------------G-----------------------C--------T--C--------------------

Consensus Sequence     GGCACTTCCCTAAGAGTAATCATTCGAACAGAACTAAGGGCCCCAGGMAACCTTATCGGAGACGATCAAATTTACAACAC
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Lake Waikare           -----C---T--GA---------------------------------C--------T--CA-------------------
Waikato River          -----C---T-G-----------------------------------C--------T--C--------------------
Mangaonua Stream       -----C--AT--------C----------------G-----------C--------T--TA----C--------------
Kaihere                --T-----TA--------C----C--------------C--------C--------T--------C--------------
Okato                  ---------------------------------------T--G-----A-----A--------------------------
Fox                    ---------------------------------------TT-A-----A-----A--------------------------
Greymouth              ------------G-----C--------------------T--A-----A-----G--------------G-----------
Wellington             -----A-----------G--T--------T-----G-----------A--T--C--------T--C-----C--------
Christchurch           --------A--C--G--G--T--------C--G-----A-----G--C--TT-G--T---A------------T-----
Lake Waihola           --------A--C-----------C-----C--GT-G--------T--C--TT-A-----G--------------T-----
Oamaru                 -----------C-----T-----------G--GT-------A--C--A-----A-----------C-----C--------
Invercargill           -----C--------G--G-----C--C-----------------G--C--T--G-----------C--------------
Konini                 ---------------------------------------T--G-----A-----G----------------------T--
Eusirus perdentatus    --T--C--T--------T--T-----T------T----A--A-----G---T-A-----------C---G-G--T-----
Epimeria georgiana     --T--C-----T--T-----T--------C---T----A--A--T--T--T------TC-A-T--C-----------T--

Consensus Sequence     GGCACTTCCCTAAGAGTAATCATTCGAACAGAACTAAGGGCCCCAGGMAACCTTATCGGAGACGATCAAATTTACAACAC
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Hamilton Gardens       ----------A-----T----------C--------------G--------------A--------G----------
Palmerston North       ------G-----T--T--------T-----T-------------------------------------T----------
Napier                 ------------T--T------G-------T----------C--G-------C---T-----G-----T----------
Hawera                 ---------------T------G-T-----T----------C--------------T--------C--T----------
Wanganui               ---------------T------G-T-----T--------------------------T--------C--T----------
Otaki                  ---------------T------G-T-----T----------C--------------T--------C--T----------
Mangamuka              ------G-----A-----T----------C--------------G--T-----------A--------A----------
Hokianga Harbour       ------------A-----T----------C--------------G--TA----------A--------A----------
Port Waikato           ------------A-----T----------C--------------G--------------A--------A----------
Waitoa                 -A---------------------------T-----C--G--C-----G-------------------A----------
Whangarai              ------------G--T------G-------C-----C-----C--------------------G-----A----------
Waihou                 CA---------------------------C-----C--G--C-----A-------------------A----------
Hutt river             --CA-----C--G--------T--T-----C-----C-----C-----A--------T----------C--------C-
Granity                ------------T--T--T-----T-----T--------G--------------------------T----------
Kaikoura               ------------T--T--T--TG-------T--------G-----------------T-----G-----C----------
Blenheim               ------------T-----T--TG-------T--------G-----------------T-----G-----C----------
Pirongia               ------------A-----T----------C--------------G--------------A--------G----------
Kawhia                 CA----------------T----------C----------C--G--------------A--------A----------
Lake Waikare           ------------A-----T----------C-----------------------------A--------A----------
Waikato River          ------------A-----T----------C-----------------------------A--------A----------
Mangaonua Stream       ------------A-----T----------C--------------G--------------A--------G----------
Kaihere                CA---------------------------C-----C--G--C-----A-------------------A----------
Okato                  ---------------T------G-T-----T--------------------------T--------C--T----------
Fox                    ---------------T--------T-----T--------------------------T--------C--T----------
Greymouth              ------------T--T--T-----T-----T--------G----------T----------------T----------
Wellington             --CA-----C--G--------T--T-----C-----C-----C-----G--------T----------C--------C-
Christchurch           TA----------T-----A--T--T--G--T-----C--G-----G----------T-----G--C--C--------G-
Lake Waihola           T-----G-----T-----A--T--T--G--T-----C--G-----G-----G-----T-----G-----T--------AA
Oamaru                 C-------------------T--T-----C--C--C--G--G--G-------------G--C--C--C-----G--C-
Invercargill           C-----G--G--------------------C--C--C-----G--G----------------C-----C-----G--C-
Konini                 ------G-----T--T--------T-----T-------------------------------------T----------
Eusirus perdentatus    CA-G-----G--------A--TG-G-----T-----C-----C-----TAT------------G--C--C-----GT-A-
Epimeria georgiana     T--A--T-----T--------TG-A-----C-----C-----A-----TATT--------T--T-----A--T---T-A-

Consensus Sequence     AGTTGTAACAGCCCACGCCTTCATCATAATYTTTTTTATAGTTATACCCGCCATAATCGGCGGATTTGGWAACTGACTTG
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Hamilton Gardens       -------C---T-------C--------A--------A--C--------T-----------G--A---------------
Palmerston North       ----A------C-G--A-----------------------G-----T-------------G-----A--G-----T---
Napier                 ----A------C-G--A--------------------------G---------------------C----T--T--T---
Hawera                 ----T------T-G--A-----G--C--------------------T-----G-----C-----------------TT--
Wanganui               ----T------T-G--A--------C--------T-----------T-----G-----C-----------------TT--
Otaki                  ----T------T----A-----------------T-----------T-----G-----C-----------------TT--
Mangamuka              -------C---C-------T--------------------C--------T-----------G--A---------------
Hokianga Harbour       -------C---C-------T--------------------C--------T-----------G--A---------------
Port Waikato           -------C---T-------T--------------------C--------T-----------G--A---------------
Waitoa                 ----T--G--GC----------------A--------------------T-------------GC-C-----T-----T
Whangarai              ----T------C----------C-----------T--T----------T-------------GC-C-----T-----G
Waihou                 -------G---C----------------A--------T----------T------------T-AC-A-----T-----T
Huttriver              ----G--A--GC----G--C--C--C-----T-----G-----------------A---------C----------CT-G
Granity                ----A------T-G--A--------------------A-----------------------T--A--G-----T---
Kaikoura               -A--A--A---C-G--G-----C-------------A----------------A--------------------C---
Blenheim               -A--A--A---C-G--G-----C--------T-----A----------------A--------------------C---
Pirongia               -------C---T-------C--------A--------A--T--------T-----------G--A---------------
Kawhia                 -T-----C---T-------T-----C--A----------C--------T-----------G--A---------------
Lake Waikare           -------C---T-------T--------A----------C--------T-----------G--A--------T------
Waikato River          -------C---T-------T--------A----------C--------T-----------G--A--------T------
Mangaonua Stream       -------C---T-------C--------A--------A--C--------T-----------G--A----------G---
Kaihere                ----A--G---C----------------A--------T----------T------------T-AC-A-----T-----T
Okato                  ----T------T-G--A--------C--------------------T-----G-----C-----------------TT--
Fox                    -T--T------T-G--A--------C--------T----------T-----G-----C-----------------TT--
Greymouth              -A--A------T-G--A-------------A-----A-------------------------T--A--A-----T---
Wellington             ----A--A--GC-G--GGCC--C--C-----T-----G----------------A---------C----------TT-G
Christchurch           ----G--A---T-G-----C--------------------G-----T--------G-----G--G--------T-----G
Lake Waihola           ----G--A---T-G-----A--T--C--A--T--T--T--T-----T-----G--A-----GT-G---------------
Oamaru                 -------A---C-G--A-----T--C-------------G-------------A--------TC-------G--GT--
Invercargill           -G-----G--GC-------C--C--C-----G--------------------G--A--C--G---C----------CT--
Konini                 ----A------C-G--A----------------------G-----T-------------G-----A--G-----T---
Eusirus perdentatus    ----TT-A--GC----T--A--T-----A--T-----T-----------------A--C--G--TC-C--T------T--
Epimeria georgiana     ----TA----GT-------A--------A--------T--C-------------A--C------C--------G-T--C

Consensus Sequence     TCCCCCTTATAYTAGGCAGGCCAGATATGGCCTTCCCCCGAATAAACAACATAAGCTTTTGACTCTTGCCCCCCTCACTA
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Hamilton Gardens      --------------C---A-------------G--G-----G-----------T-----------T--G--G--A-----
Palmerston North      ---------C---TG--G-----------------T--G----------------------A--C-----G--------
Napier                -----G--GC---TG------T----------------G--------T-------------G--T--GG-G--------
Hawera                ------------------------------------G--------C-----------------G--C---G----T-----
Wanganui              ------------------------------------G--------G-----------A-----G--CT--G----T-----
Otaki                 ------------------------------------G--------G-----------------G--CT--G----T-----
Mangamuka             --------------C--G-----------------------A-----------T-----------T-----G--------
Hokianga Harbour      -----G--------C--G-----------------------A-----------T--A--------T-----G--------
Port Waikato          --------------C--------------------------T-----------T-----------T--G-----------
Waitoa                --------------C--G---T-------------T--C--------------------------T--------------
Whangarai             ---T----G-----T--G-----------G-----G--C-----C-----------A--------C--------------
Waihou                -----G--------C------T-G--------------C-------------------------C-----G--------
Huttriver             --C--T--CC-G-T---G--GT----------G--C--G-----G-----------C-----G--CT--G-G--A--C--
Granity               --T--G---C---T-------T-G-------------------------------T--------C--G-----T-----
Kaikoura              --G-----GC---T-------------------------------------G-----A--------G--G---A-T-----
Blenheim              --G-----GC---T------G--------------------T--------------A--------G------A-T-----
Pirongia              --------G-----C-----------------G--------A-----------T-----------T-----G--A-----
Kawhia                --------------C--------------------G-----A-----------T--------T--T--G-----------
Lake Waikare          --------------C-------------------------A-----------T-----------T--G-----------
Waikato River         --------------C-------------------------A-----------T-----------T--G-----------
Mangaonua Stream      --------------C-----------------G--G-----G-----------T-----------T--G--G--A-----
Kaihere               --------------C------T-G----------G--C-------------------------C-----G--T-----
Okato                 -------------T------G-------------G--------G-----------------G--C---G----T-----
Fox                   ------T-----------------------------G--G-----G-----------------G--CT--G----T-----
Greymouth             --T--G---C---T-------T-----------------------------------T--------C--G-----T---C-
Wellington            --C--T--CC---T---G--G-----G--------T--G-----------------C-----G--C---G-G--A--C--
Christchurch          --CT--T--C---T------CT-------G--------G-----G--T-----A--------G--TT--G-GA-A-----
Lake Waihola          --CT---------T---T--TT-------------C--------------G--G--------G--TT-GG-CA-A-----
Oamaru                --C--T---C-C-T------C--C-----G--G--G--C--------------T--------G--C--GG-C-----C--
Invercargill          --C--C--GC---T------C--G--C--G-----G--C--------------G--------G--T--GG-C-----C--
Konini                ---------C---TG--G-----------------T--G-----------------------A--C-----G--------
Eusirus perdentatus   ---T-C--TC-T-TT--C--GT-------G-C------G-----C--------A--A--------T---G--A--GCG-C
Epimeria georgiana    -T-T-TA-GC----------C---A-C--------------T-----------A--A-----T--TT-----A-AG-A--

Consensus Sequence    ACACTACTATTAACAAGAGGACTAGTAGAAAGAGGAGTAGGCACAGGCTGAACCGTGTACCCCCCYCTATCAGGCAATAT
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Hamilton Gardens      ---------C--C-----A------T-A---------AG---A--T--------T--A---------T-G--T-------
Palmerston North      ---------A----------G-----A------------------------A-------------G-----A----
Napier                ------------------------------A-----C--A-----T--G-------T---C-------------A----
Hawera                ------T-----A-----------------------------------------------C----------------A----
Wanganui              ------T-----------------------------------------G--------C----------------A----
Otaki                 ------T-----------------------------------------G-----A--C----------------A----
Mangamuka             ---------T--C-----A------T-A---------AG---A----------T-------------G--T-------
Hokianga Harbour      ---------T--C-----A------T-A---------AG---A----------T-------------G--T-------
Port Waikato          ---------T--T-----A------T-A---------AG---A--T--------T-----C--------G--C-------
Waitoa                T--------C--C-----A--------C---------AG------T--G--G--A-----C-----C-----C--T----
Whangarai             T--------A--C-----A--------A--C------AG------T-----G-----C--C-----C--G--C-------
Waihou                T--------C--C-----A-----T-----------AG------T--G--A-----A--C-----C-----T-------
Hutt river            ---------C----------------T--A--C-----GT-G---C-T-----C--C-----A--C----------T-
Granity               ------T--A-------------------------------G--T-----G----------------------A----
Kaikoura              ---G----------A--------------------------A--------T----------------------G--T-
Blenheim              ---G----------G--------------------------G-------------A-----A--------A--G--T-
Pirongia              ---------T--C-----A------T-A---------AG---A--T--------T--A---------T-G--T-------
Kawhia                ---------T--A-----A------T-A---------AG---A----------T-----C--------G--T-------
Lake Waikare          ---------T--T-----A------T-A---------AG---A--T-------------C--------G----------
Waikato River         ---------T--T-----A------T-A---------AG---A--T-------------C--------G----------
Mangaonua Stream      ---------T--C-----A------T-A---------AG---A--T--------T--A---------T-G--C-----T-
Kaihere               T--------C--C-----A-----T-----------AG------T--G--A-----A--C-----C-----C--T----
Okato                 T-----T----------------------------------------G--------C----------------A----
Fox                   ------T----------------------------------------G--------C----------------A----
Greymouth             ------T--A-------------------------------A--T-------------------C--------A----
Wellington            ---------T----------------C--A--C-----A--G---C-T-----C--C-----A--C----------T-
Christchurch          ---T-------------A--T--TT----A--------G--------G--A--C--C--G--T---T-------T--T-
Lake Waihola          ---G----------A--A--T--------G--------G--C-----G--A--A--C--G--T-------------T-
Oamaru                --------------------T--------C--------TT-G--------A-----T--A-----C--G-----A----
Invercargill          --------------------T--------C--------TT-G--------A-----T--A-----C--G-----A----
Konini                ---------A----------G-----A------------------------A-------------G-----A----
Eusirus perdentatus   T--------A--T-----C-----TA-C--A--------TT-A--TC-T--T--T-----C--A----------G--T-
Epimeria georgiana    ---A--T-------GG--A--G-----T-----C--C---T-A---C-------CATC--C--A---T-------T--T-

Consensus Sequence    CGCCCACAGGGGGGCCTCTGTAGACCTGGCTATTTTTTCCCTTCACTTAGCCGGGGCGTCTTCCATTCTAGGGGCCATCA
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Hamilton Gardens      -------C--------------------T--G-------------G--------T--C--G--------------------
Palmerston North      ----------G--------------G--G-----------G--------------T--------C-----------G-----
Napier                --------------------------------A-----------CT-------------T-----C--------G--------
Hawera                ----------------------------------T-----G--------------T--------------------G-----
Wanganui              ----------------------------------T-----G-----------------------------------G-----
Otaki                 ----------------------------------T-----G-----------------------------------G-----
Mangamuka             -------C-----------------G--T--G-------------G-----------C--G-----T--------------
Hokianga Harbour      -------C-----------------G--T--G-------------G-----------C--G-----T--G-----------
Port Waikato          -------C-----------------G--T--G-------------G--------T--C--G-----T--G-----------
Waitoa                ---------------------C--------------------C-----T--G-----C--------T--------------
Whangarai             -------------------C--C-----G--G-----G--------------------TT----C--T-----G--------
Waihou                ----C-----------------------------------G--C-----T----------------T--------------
Hutt river            -------C--A-----C--C--C--G--G--G---G-C---A-A-------------------------------C-----
Granity               --------------------------------G--T-----G--C--G-----G-----C-----C--------G--G-----
Kaikoura              ------------------------G-----A--T------A-C--------------------C--------G--------
Blenheim              -------C----------------------A--T------G-C--------------------C-----------G-----
Pirongia              -------C--------------------C-TG-------------G--------T--C--G--------------------
Kawhia                -------C-----------------G--T--G----------------------T--C-----------G-----------
Lake Waikare          -------C--------------------C--G-------------G--------T--C--G-----A--G-----------
Waikato River         -------C--------------------C--G-------------G--------T--C--G-----T--G-----------
Mangaonua Stream      -------C--------------------C--A-------------G--------T--C--G------------------G-
Kaihere               ----C-----------------------------------G--C-----T----------------T--------------
Okato                 ----------------------------------T-----G-------------T--------------------G-----
Fox                   ----------------------------------T-----G-----------------------------------G-----
Greymouth             ------------------------G-----A--T-----G--C--G-----G-C---C-----C--------G--G--A--
Wellington            -------C--A-----C-CC--C--G-----G---------A-A--G-------------------------------------
Christchurch          -T-----C--G-----------C--G--G--G---GC---G--CC-G---AT-GC---TT-------T-----T--T-----
Lake Waihola          -T--------A-------------G--G------GCT--------------GGCT--TT----------G--T--T-----
Oamaru                ----------A-----------C--G--G------GCC---C----------GGC---T--C--C--T--G--T--T-----
Invercargill          ----------A-----------C--G--G------GCC---C----------GGC---T--C--C--T--G--T--T-----
Konini                ----------G-------------G--G-----------G-------------T--------C-----------G-----
Eusirus perdentatus   ----C--------TA-T--------G-----G--T-AT---A-AT-C--------A--TT-------T--G--TA-T-----
Epimeria georgiana    -T-----C--A--TA-TC---------A-A----AG--GC-AAA--T-------C---TT-------------T--T--A--

Consensus Sequence    ACTTTATTTCCACAGTAATTAATATACGAGCCCCCAGAATATCTATAGACCAAATCCCACTATTTGTCTGATCAGTATTTAT
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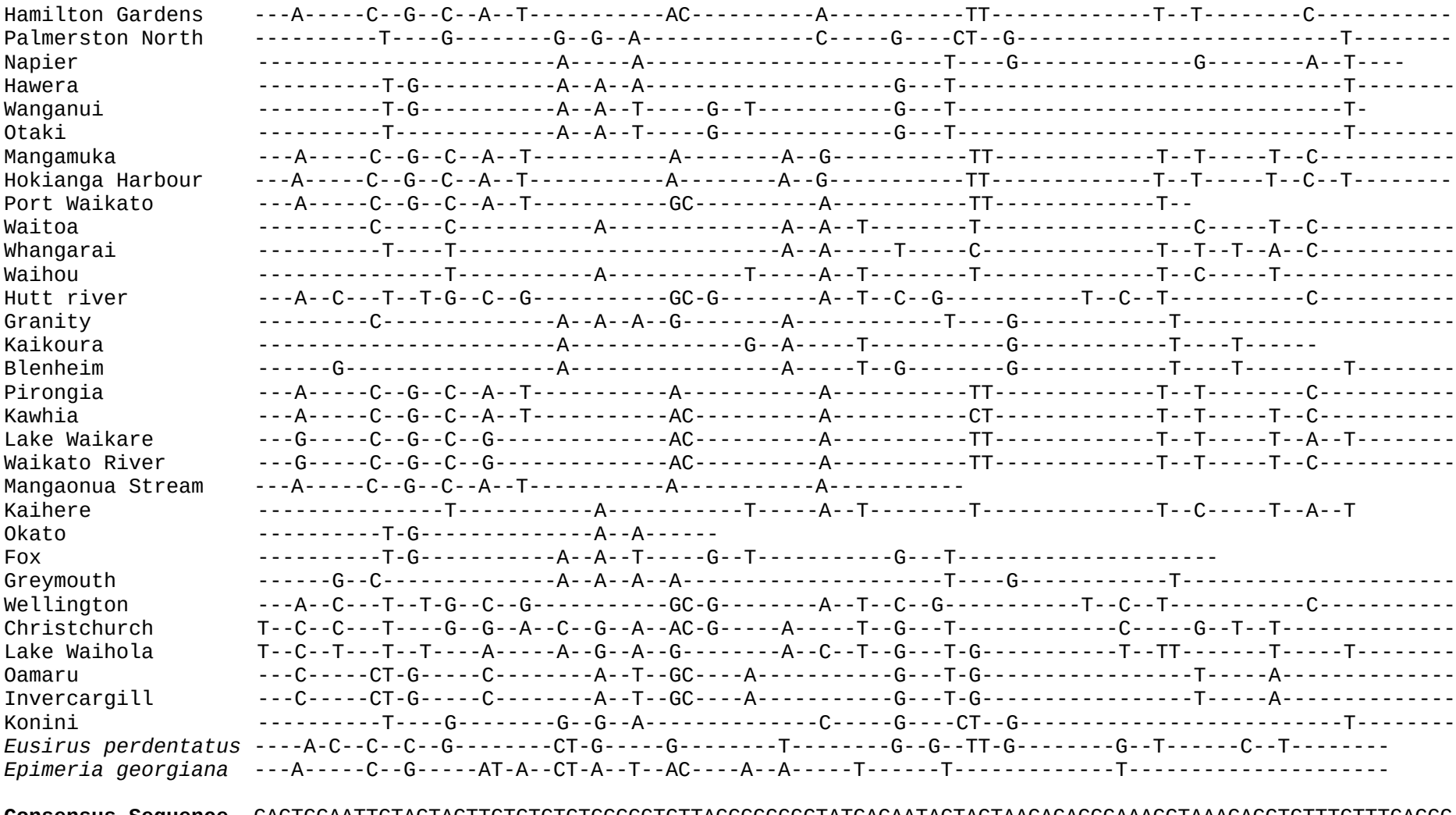

Consensus Sequence CACTGCAATTCTACTACTTCTCTCTCTCCCCGTCTTAGCCGGGGCTATCACAATACTACTAACAGACCGAAACCTAAACACCTCTTTCTTTGACCC