Comparative Phylogeography of North American *Diporeia hoyi* and *Gammarus lacustris* (order: Amphipoda)

by

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Biology

Waterloo, Ontario, Canada, 2009

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

This thesis examines the phylogeographic distribution of both *Diporeia hoyi* and North American populations of *Gammarus lacustris*. The first section of this thesis investigates the genetic distribution and species composition of actively dispersed *Diporeia* within the Laurentian Great Lakes. A phylogeographic analysis of *Diporeia* across glaciated North America concluded that there was no evidence to suggest the existence of two or more phylogenetic species and that all contemporary populations were colonized from either the Missourian or Mississippian refugia (average of 1.75% mtDNA divergence between the two). Investigations into the phylogenetic history of *Diporeia* revealed that it represents an ancient genetic lineage that began to independently evolve from *Monoporeia affinis* approximately 15.4 Mya and that the two diverged from *Pontoporeia femorata* during the early Miocene. Populations of *P. femorata* consist of two markedly divergent genetic lineages and represent a cryptic species complex. Low levels of mtDNA sequence divergence (1.41% average) was detected between *G. lacustris* haplotypes and is a result of its recent invasion into North America from Asia. *Gammarus lacustris* in unglaciated regions of the USA contained a greater level of genetic divergence between geographically close populations then those examined in glaciated regions, which showed widespread distributions of a few haplotype groups. Southern populations were initially established by random colonization events, whereas in glaciated regions *G. lacustris* could also actively disperse via the proglacial lakes.

Acknowledgements

I would first like to thank Jonathan Witt for providing me with the amazing opportunity to work in his lab. I am deeply grateful for all the patients and guidance you have given me over the past three years.

Thank you to my committee members Kirsten Müller and David Barton for all their support and valuable contributions to the completion of my thesis. I also would like to thank Dragana Miskovic, Linda Zepf, Vivian Dayeh and Michael Lynch for all the times I sat in your offices and you provided words of encouragement when I couldn't find any.

I am most appreciative to my lab-mates for all there assistance and friendship: Matthew Hrycyshyn, Sarah Adamowicz, Janice Wong, Eric Dunford, Daniel Allen and Jeff Martin. I am especially thankful to Matt for all the coffee's, conversations and laughs that you have provided over the years.

Thanks to all my family and friends who took time out of there busy schedules to share a coffee and lend an ear. Finally, I want to acknowledge my mother, Snezana Usjak. I could not have made it here without your encouragement, love and support. Thank you.

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Introduction

Biogeographers seek to understand the principles that govern the geographic distribution of species. However, this endeavor requires that species and lineage diversity are adequately characterized. Until the last quarter of a century, the identification of species in biogeographical studies has been based on the morphological and biological species concepts. The morphological species concept (MSC) defines species on the basis of phenotypic traits, while the biological species concept (BSC) identifies species on the criteria of reproductive isolation (Mayr, 1942). There are several inherent difficulties with both of these concepts when applied to populations. Convergent and parallel evolution can select for, and maintain similar phenotypes in different species, reducing the utility of the MSC (Bickford *et al*., 2006). A major problem with the BSC is that it can only be applied to sexually reproducing populations, rendering it useless for a large portion of Earth's taxa which reproduce asexually (de Meeûs *et al*., 2003). Furthermore, it has been revealed that these concepts underestimate the underlying genetic diversity (Avise, 2000; Bickford *et al*., 2006; Goodman *et al.*, 2009; Moussalli *et al.*, 2009).

Traditionally, morphological comparisons grouped organisms together according to their overall similarity, but the introduction of cladistics in the 1950's (Hennig, 1950, 1966) provided a new measure for morphological classification. Cladistics, also referred to as phylogenetic systematics, groups taxa together on the basis of shared derived traits, or synapomorphies rather than overall similarity (Wiley *et al.*, 1991). Advances in the ability to easily acquire protein and sequence data for a wide range of taxa have allowed the comparison of many species on the molecular level. The development of the phylogenetic

species concept (PSC) by Cracraft (1983) introduced a new method to identify species based on cladistics and molecular data. The PSC defines a species as the smallest diagnosable, well supported monophyletic cluster that share a pattern of common ancestry and descent. It was not long until this concept was applied to biogeographical problems, with focus on the geographical distribution of distinct evolutionary lineages at both the inter and intraspecific levels, resulting in the emergence of the sub-discipline of phylogeography (Avise *et al*., 1987). The tools for phylogeographic analysis continued to expand as new methods allowed for the testing of gene flow between populations based on the distribution of allelic and haplotypic frequencies, providing a more accurate understanding of the genetic structuring within species (Charlesworth *et al*., 2003). Eventually the discipline became an integral part of conservation efforts, with authorities attempting to preserve evolutionary distinct populations and lineages across entire ranges, rather than focusing on single locations that may not accurately represent the species genetic composition in its entirety (Moritz, 1994; Barker, 2002; Pearse and Crandall, 2004; Elderkin *et al.*, 2008).

The majority of phylogenetic and phylogeographic studies among animal taxa have been completed with the use of mitochondrial DNA (mtDNA) because of its unique properties as a consequence of its matrilineal mode of inheritance. The arrangement of the genes in animal mtDNA is highly conserved, allowing for easy amplification of target sequences as universal primers will work on a broad range of taxa (Folmer *et al.*, 1994; Pereira, 2000; Sheffield *et al.*, 2008). In most animals, mtDNA is primarily maternally inherited, transmitted from mother to daughter. Paternal leakage of mtDNA however has been documented in some species including mussels, where it is common for individuals to receive copies from both

parents (Cogswell *et al*., 2006; Kmiec *et al*., 2006; White *et al.*, 2008). The effective population size of mtDNA within a given gene pool is smaller in comparison to nuclear genes because it is haploid and primarily maternally inherited. As a result, it is influenced by the effects of genetic drift to a greater degree (Neigel and Avise, 1986; Hare, 2001). Coupled with its low effective population size and an increased rate of mutation (Brown *et al*., 1979; Brown *et al*., 1982), mtDNA is a powerful tool for studying recent evolutionary events as it will accumulate fixed mutations between populations faster than nuclear genes (Avise, 2000).

Even with higher rates of mutations in mtDNA, it still requires extended periods of time for distinct sequences to evolve in isolated populations and for those populations to become reciprocally monophyletic (Avise, 2004). Recently derived populations separated for only a few thousand years often exhibit low levels of sequence divergence, making it difficult to tease out recent historical events from contemporary interactions using phylogenies alone. The application of population genetic analysis permits the identification of small, but significant molecular differences within species by examining not only the relationships between haplotypes, but also their distributions and frequencies among populations (Avise, 2004; Pearse and Crandall, 2004). By combining knowledge of population structure, phylogenetic relationships and species distributions with geological data, phylogeographic studies can provide insights into the evolutionary history of species that previous biogeographical studies based on morphological data could not.

A failure to recognizes lineage diversity, distinct populations and the interactions of individuals between groups within a species have resulted in poor conservation practices in

the past that did not adequately protect species diversity (Buhay *et al.*, 2002; Mace, 2004; Dalen *et al.*, 2006; Ludwig, 2006; Allentoft *et al.*, 2009). The identification of genetically differentiated populations within a species and their spatial distribution has become an important part of phylogeographic studies. The term 'evolutionary significant unit' (ESU) has been used to describe populations within a species that are reciprocally monophyletic for mtDNA, but not nuclear DNA (Ryder, 1986; Moritz, 1994). The term 'management unit' (MU) is used to describe populations that vary in allelic frequencies at either mitochondrial or nuclear DNA without regard to the phylogenetic relationship of the alleles (Moritz, 1994). By classifying populations into management units, conservation biologists can focus their efforts on distinct groups and maximize the amount of diversity protected. The concept of ESU's and MU's have been widely accepted and applied in many genetic studies (Dawe *et al.*, 2009; Koumoundouros *et al.*, 2009; Munoz-Fuentes *et al.*, 2009).

Repeated glaciations in the in North Hemisphere have had a profound impact on the geographic distribution of species. Climate is subject to changes due to periodical shifts in the Earth's axial wobble, axial tilt and solar orbit which occur every 21, 41 and 100 thousand years respectively. These variations adjust the total solar radiation that reaches the Earth's surface, causing intermittent periods of glaciation (Ruddiman, 2006). The last 2.4 million years of the Earth's history is known as the Quaternary Period, which is noted by the establishment of the arctic ice cap and repeated glaciations (Hewitt, 2000). The Quaternary is divided into two Epochs, the Pleistocene and the Holocene, however Pielou (1991) argued that the latter is simply an interglacial period that started 10,000 years ago, and should not be formally recognized. There were several major glaciations in the Pleistocene, all of which

had dramatic effects on the biota as the ice both advanced and retreated. The Wisconsinan glaciation was the last major advance to impact North America, and reached its maximum approximately 18,000 years ago. Two ice sheets, the Laurentide and the Cordilleran covered most of Canada as well as parts of Alaska, and extended as far south as Northern Wisconsin (Dyke and Prest, 1987). Species that were displaced by the ice and survived in unglaciated regions were reduced to a fraction of their original population size, causing genetic bottle necks that facilitated the rapid divergence of populations (Pielou, 1991; Hewitt, 2004; Soltis *et al.*, 2006).

The retreat of the glaciers exposed new habitat that could be colonized by species without the difficulty of competing with locally established populations. This provides a unique opportunity to examine the impact of dispersal abilities on the distribution of genetic variation within species. The phylogeographic comparison of numerous species across both glaciated and unglaciated North America has confirmed the existence of key refugia and dispersal routs as well as distinct genetic patterns in numerous species (Avise 1987; Bernatchez and Wilson 1998; Avise 2000; Cox and Hebert 2001; Swenson and Howard 2005; Soltis *et al.* 2006).

Objectives

This thesis examines the impact of the Pleistocene glaciations on the phylogeography of the two amphipod species, *Diporeia hoyi* and *Gammarus lacustris* in North America using the mitochondrial cytochrome c oxidase subunit I (COI) gene. Phylogenetic analyses have identified high levels of morphologically cryptic species within the order Amphipoda (Witt

and Hebert, 2000; Gervasio et al., 2004; Hogg et al., 2006 Witt et al., 2006; Murphy et al., 2009) suggesting that both these species may possess a substantial amount of unrecognized genetic diversity. This thesis will explore the possibility that North American populations of *Diporeia* and *G. lacustris* represent species complexes using the phylogenetic species concept.

Diporeia occurs in deep, cold, freshwater lakes located in formally glaciated North America and is restricted to active dispersal. Previous studies have revealed that actively dispersing species possess low levels of intraspecific genetic divergences (Audzijonytė and Väinölä, 2006; Dooh *et al.*, 2006; Kawamura *et al.*, 2009) and it is therefore expected that *Diporeia* populations will also exhibit low levels of genetic divergence. The glacial melt waters played a substantial role in the distribution of *Diporeia*, allowing populations to disperse across the continent (Dadswell, 1974). However, it is not know from which refugia *Diporeia* dispersed from, and several active dispersers that share its geographic distribution do not appear to have colonized North America from the same refugia (Kontula and Väinölä, 2003; Dooh *et al.,* 2006; Sheldon *et al*., 2008). Comparison of the phylogeographic analysis with the distribution of the former proglacial lakes will indicate the most probable refugia that contemporary populations dispersed from. If *Diporeia* dispersed from multiple refugia then each group will be represented by genetically distinct populations. The possibility that *Diporeia* is a species complex does not imply that they all dispersed from the same refugia and each species may show a unique phylogeography.

Currently there are as many as eight distinct male morphotypes of *D. hoyi* identified across North America (Bousfield, 1989); however it is unclear if any of these warrants species

recognition. Three male *Diporeia* morphotypes, *Diporeia hoyi filicornis* and *Diporeia hoyi brevicornis* sampled from Lake Superior, and *Diporeia hoyi erythrophthalma* from Lake Washington are examined to determine if they represent independent phylogenetic species. If *Diporeia* represents a species complex, than two or more genetically divergent monophyletic lineages will be identified in the phylogenetic analysis. Any morphotype that does represent a distinct phylogenetic species should also be represented as an independent lineage.

Gammarus lacustris is capable of both active and passive dispersal and has been identified in parts of Europe, Asia and North America. As a result of its ability to passively disperse, *G. lacustris* is located in both glaciated and unglaciated regions of North America and unlike *Diporeia*, was not reliant on the glacial melt waters to disperse. Passively dispersed species have been shown to possess greater levels of intraspecific genetic divergences than active dispersers (Witt and Hebert, 2000; Gomez *et al.*, 2002; Adamowicz *et al.*, 2009). Populations of *G. lacustris* are therefore expected to show a greater level of genetic divergence than *Diporeia*. High levels of cryptic speciation and genetic divergences have been detected in populations of passively dispersed species located in unglaciated regions (Cox and Hebert, 2001; Witt et al., 2006; Adamowicz *et al.*, 2009). If *G. lacustris* does represent a cryptic species complex then it is expected that populations in unglaciated regions will contain substantially more endemic species and greater levels of intraspecific divergences in comparison to their conspecific populations in formerly glaciated habitats.

Chapter 1 - The Mitochondrial Genetics of *Diporeia* in the Laurentian Great Lakes: What is disappearing?

1.1 Overview

Over the past two decades, populations of *Diporeia hoyi* within the Laurentian Great Lakes have declined, resulting in serious ecological concerns. Despite these concerns, the taxonomic status of *D. hoyi* remains a source of confusion. In this study, mitochondrial cytochrome *c* oxidase I (COI) gene sequence data are used to determine if *D. hoyi* populations in Lakes Huron, Michigan, Ontario and Superior represent a species complex. The phylogenetic species concept, as well as a species screening threshold (SST) are utilized to discriminate potential species. An analysis of molecular variance (AMOVA) is also conducted to estimate the partitioning of genetic variation among and within lakes. There is no evidence to suggest that Great Lakes populations of *Diporeia* consist of multiple species, and two commonly encountered morphotypes, *Diporeia hoyi filicornis* and *Diporeia hoyi brevicornis* do not merit recognition as separate species. Haplotypes derived from individuals within Lake Superior form a distinct cluster that is paraphyletic with respect to a cluster containing haplotypes derived from individuals within the remaining Great Lakes. The average nucleotide sequence divergence between the two haplotypic groups is 1.8%, and the differences between L. Superior and the other lakes account for 58% of the overall genetic variation, while populations within the other Great Lakes are very similar to each other. The results suggest that the Great Lakes may have been colonized by two separate refugial lineages during the last Pleistocene glacial retreat.

1.2 Introduction

Over the last few decades, numerous invasive species have become established within the Laurentian Great Lakes, altering ecosystem dynamics and impacting the native biota (Dermott and Kerec, 1997; Dermott et al. 1998; Madenjian *et al*., 2002; Vanderploeg *et al.,* 2002; Simon and Townsend, 2003). Exotic taxa have entered the Great Lakes through several vectors, but the most important has been transoceanic shipping and ballast water discharges (MacIsaac *et al*., 2002; Bailey *et al*., 2004; Drake and Lodge, 2007). Many invasive species within the Great Lakes originated from the Ponto-Caspian region -including the mussels, *Dreissena bugensis* (May and Marsden, 1992) and *Dreissena polymorpha* (Hebert *et al*. 1989), the cladocerans *Bythotrephes* (Lehman, 1987) and *Cercopagis* (MacIsaac *et al*., 1999), the amphipod *Echinogammarus* (Witt *et al*., 1997), and the gobies *Apollonia melanostoma* and *Proterorhinus semilunaris* (Jude *et al.,* 1992; Vanderploeg *et al*., 2002).

Ecological changes within the Great Lakes have resulted in species composition and abundance shifts within the benthic invertebrate community (Dermott and Kerec, 1997; Nalepa *et al.,* 1998; McNickle *et at.,* 2006; Nalepa *et al*., 2007). The amphipod crustacean *Diporeia hoyi* formerly occurred throughout the Great Lakes system at depths greater than 10 meters, but began to disappear at an alarming rate during the 1990's. A recent study revealed that the lake wide densities of *Diporeia* in Lake Huron had decreased from 5365/m^2 to 329/m^2 from 1994 to 2005 and it was virtually absent from locations shallower than 90m by 2007 (Nalepa *et al*., 2009a). Similar declines in *Diporeia* densities have been documented in Lake Erie (Dermott and Kerec, 1997), Lake Huron (Nalepa *et al.,* 2003; Nalepa *et al*., 2007; French, 2009), Lake Michigan (Nalepa *et al*., 1998; Nalepa *et al*., 2009a) and Lake Ontario

(Dermott, 2001; Watkins *et al*., 2007). Populations in Lake Superior appear to be stable, and there is no indication of large scale declines such as those observed in the other lakes (Scharold *et al*., 2004). The disappearance of *Diporeia* may have negative impacts on fish populations because it provides an important link in the transfer of carbon and energy from lower trophic levels to the fish community (Gardner *et al*., 1990). The decline of *Diporeia* populations within the Great Lakes coincided with the invasion of *Dreissena*, but the link between the appearance of *Dreissena* and the disappearance of *Diporeia* is unclear. Several mechanisms by which *Dreissena* could potentially impact *Diporeia* have been proposed including competition for resources, toxicity of pseudofeces, as well as a possible reservoir and vector for pathogens (Watkins *et al*., 2007). The exact mechanism causing *Dreissena* pseudofeces to be toxic to *Diporeia* is unknown, but it has been suggested that selective filter feeding by *Dreissena* on algae results in the expulsion and accumulation of toxic algae strains in the local environment (Vanderploeg *et al*., 2001).

Despite their important ecological role and declining numbers within the Great Lakes, the taxonomic composition of *Diporeia* remains a source of confusion. As many as 8 distinct adult male morphotypes have been identified across North America, with the majority of them purportedly present in the Great Lakes basin (Bousfield, 1989). It has been implied that these variants represent distinct species (Bousfield, 1989), but there is currently little evidence to support these claims, and *Diporeia* is often referred to as *Diporeia spp*. in the literature (e.g. Kiziewicz and Nalepa, 2008; Bunnell *et al*., 2009; Messick, 2009; Nalepa *et al.,* 2009b). *Diporeia* was first recorded in Lake Superior by S. I. Smith (1871) who identified it as *Pontoporeia affinis*, a related European amphipod. Following more rigorous

morphological comparisons, North American populations were designated *Pontoporeia hoyi* (Smith, 1874), although the name did not enter common use until over a century later. Additional morphological analyses resulted in the assignment of *Pontoporeia hoyi* to the genus *Diporeia* (Bousfield, 1989). A molecular study based on allozyme electrophoresis further supported this assignment, as deep genetic divergences were identified between these genera (Väinölä and Varvio, 1989).

The two most widely distributed morphotypes, *D.h. filicornis* and *D.h. brevicornis,* both occur within the Great Lakes, sometimes in the same location. Allozyme comparisons between the two suggest that they are not genetically differentiated, and do not support the hypothesis that the morphotypes represent distinct species (Väinölä and Varvio, 1989). There was, however, a difference at one locus (Mannose Phosphate Isomerase) in Lake Mazinaw, where a statistically significant Hardy-Weinberg deviation was detected. The authors acknowledged the possibility that this could be reflective of reproductive isolation between the two morphotypes, but other factors can account for this, and the authors argued that they do not warrant species recognition (Väinölä and Varvio, 1989).

This study investigates the genetic structuring and phylogenetic species composition of *Diporeia* populations in Lakes Huron, Michigan, Ontario and Superior. The mitochondrial cytocrome *c* oxidase I (COI) gene is employed to construct a phylogeny of individuals sampled throughout these lakes, and to assess the level of genetic differentiation among populations. This gene has been successfully utilized to investigate amphipod population structure and species composition in numerous studies (Witt and Hebert, 2000; Hogg *et al.,* 2006; Witt *et al.,* 2006; Browne *et al.,* 2007; Lefébure *et al.,* 2007; Henzler and Ingolfsson,

2008; Carlini *et al.,* 2009). The phylogenetic species concept (PSC), which classifies a species as the smallest well supported monophyletic lineage, has become an important tool in species identification among taxonomists. A major concern with the PSC is the potential recognition of weakly divergent monophyletic clusters within a species as separate taxa, which has previously resulted in the designation of species that are inconsistent with the biological and morphological species concepts (Harrison, 1998; Avise, 2004). The implementation of a universal barcoding system using the COI region has resulted in thousands of sequenced individuals. Comparison of highly detailed taxonomic groups has revealed that the level of COI sequence divergence between sister species is substantially larger than the divergence observed within species (Hebert *et al*., 2003; Hebert *et al.*, 2004). However, when investigating poorly described groups it can be difficult to determine what level of genetic divergence is significant at the species level. The application of a standard species screening threshold (SST) has been suggested to flag genetically distinct lineages as provisional species for further investigation (Hebert *et al.*, 2004; Witt *et al*. 2006). In a case study using birds, the application of a SST based on 10 times the intraspecific variation discriminated over 90% of all species examined (Hebert *et al.*, 2004). Poor species characterization in *Diporeia* prevents the development of an SST using intraspecific divergences. However, a similar approach was proposed by Witt *et al.,* (2006) using the mean within-population pairwise haplotype divergence instead and the authors justify its replacement by arguing that the two values are likely similar within small geographic ranges.

The phylogenetic relationship of *Diporeia* specimens from Lakes Huron, Michigan, Ontario and Superior, as well as the two male morphotypes *D.h filicornis* and *D.h.*

brevicornis are investigated to help clarify there taxonomic status. The application of an SST will prevent the recognition of intraspecific variants as independent phylogenetic species and flag divergent lineages that may represent provisional species. If *Diporeia* populations contain two or more phylogenetic species, than each will be represented by a well supported monophyletic lineage with divergence levels greater than the estimated SST.

Recent population subdivision among *Diporeia* populations will not likely be reflected in the phylogenetic analysis and can be more adequately characterized by examining the geographic distribution and frequency of haplotypes throughout Lakes Huron, Michigan, Ontario and Superior. Pairwise population comparisons and a hierarchical analysis of molecular variance (AMOVA) are employed to determine if there are significant genetic differentiations among *Diporeia* populations both within and between the four lakes.

1.3 Materials and Methods

1.3.1 Collections

Diporeia specimens were collected from 17 locations within Lakes Huron, Michigan, Ontario and Superior between April and October, 1996 (Fig. 1.1). The specimens were collected at depths ranging between 29 and 100 meters using a benthic drag and identified as sub-adults, adult female's, *D. h brevicornis* or *D.h. filicornis* males (Segerstråle, 1971a). Samples of *D.h. brevicornis* and *D.h. filicornis* type males were acquired from a single population in Lake Superior. A specimen of *Monoporeia affinis* was obtained from the Beaufort Sea, off Tuktoyaktuk (NWT, Canada) for use as an outgroup in the phylogenetic analyses. All individuals were preserved and stored in absolute ethanol.

1.3.2 DNA sequence analyses

Total DNA was extracted from 15-16 individuals from each population, as well as 10 *D.h. filicornis* and 9 *D.h. brevicornis* males from Lake Superior (Table 1.1) by grinding a leg in 50 µL of proteinase K extraction buffer (Schwenk, 1996). A 680 base pair fragment of the mitochondrial cytochrome *c* oxidase I (COI) gene was amplified using the primers LCO1490 and HCO2198 (Folmer *et al.,* 1994). The 50 µL PCR reactions contained 2.0 µL of DNA template, 5.0µL 10x buffer, 0.2 mM of each primer, 0.2 mM of each dNTP and 0.5 units of Taq DNA polymerase. The PCR conditions consisted of 60 s at 94°C followed by 5 cycles of 60 s at 94°C, 90 s at 45°C, 60 s at 72°C; followed by 35 cycles of 60 s at 94°C, 90 s at 51°C, 60 s at 72°C; followed by 5 min at 72°C. The PCR products were gel purified using the Qiaex kit (Qiagen Inc.). Products were sequenced in one direction using primer LCO1490 on an ABI 3730 automated sequencer (Applied Biosystems).

The sequences were aligned by eye, and haplotypes identified by constructing a distance tree using the unweighted pair group method with arithmetic averages (UPGMA) using a matrix of nucleotide differences between all pairwise sequence comparisons in MEGA 4.1 (Tamura *et al.*, 2007). Nucleotide composition, mean pairwise transition/transversion ratios, nucleotide (p-distances) distances matrices, and amino acid translations (invertebrate mitochondrial code) for all haplotypes were also calculated using MEGA 4.1.

1.3.3 Population level analyses

Haplotype diversity (h) and nucleotide diversity ($\prod$) for each population was estimated in DnaSP (Rozas *et al*., 2003). An analysis of molecular variance (AMOVA) was conducted

using *Diporeia* haplotypes and their population frequencies in the program ARLEQUIN version 3.01 (Excoffier *et al*., 2005). *Diporeia h. brevicornis* and *D. h. filicornis* were excluded from the AMOVA analysis. The analysis was hierarchically structured to determine the contribution of three genetic covariance components to the total genetic variation: among lakes, among populations within lakes and within populations. Pairwise exact tests of population differentiation and F_{ST}s (0.05 level of significance) between all population pairs were conducted using ARLEQUIN version 3.01 (Excoffier *et al*., 2005).

Unique COI haplotypes from each population were used to estimate the mean within-population pairwise sequence divergences using the Jukes and Cantor model (JC) (Jukes and Cantor 1969) of nucleotide substitution for all populations that possessed 2 or more haplotypes. The species-screening threshold (SST) was set at 10 times the average of the within-population estimates (Witt *et al*., 2006). The resulting value was then used as the minimum level of sequence divergence required to recognize groups indentified in the phylogenetic analysis as species.

1.3.4 Phylogenetic analyses

A phylogeny of all 88 haplotypes, was constructed using the Neighbour-Joining (NJ) distance method (Saitou and Nei, 1987) using the JC model of nucleotide substitution in MEGA 4.1. Confidence in the NJ analysis was assessed using the bootstrap method and interior branch test with 1000 replicates each.

As a result of computational constraints, the data set was reduced to 31 haplotypes and the program MODELTEST version 3.0 (Posada and Crandall, 1998) was used to estimate the

best fit model of nucleotide substitution using the Akaike information criterion (Posada and Buckley, 2004). A maximum likelihood analysis (ML) was conduced in PAUP 4.0b10 (Swofford, 2001) using the model and parameters estimated by MODELTEST. The ML analysis was executed using a heuristic search with the starting tree obtained using the NJ method, and the tree bisection reconnection (TBR) branch swapping algorithm. Confidence in the tree was assessed using the bootstrap method with 500 pseudoreplicates.

A Maximum Parsimony (MP) analysis was conducted using this data set in PAUP 4.0b10. The MP analysis was carried out using a heuristic search with 1000 replicates with each starting from a random tree, and the TBR branch swapping method. Confidence in the MP analysis was determined using 1000 bootstrap psuedoreplicates, with each pseudoreplicate consisting of 4 heuristic search replicates, and replicates were started with a random tree, using the TBR branch swapping method.

Since the relationships among haplotypes in phylogenetic trees are assumed to have a bifurcating branching pattern, most phylogenetic methods do not accommodate the presence of ancestral haplotypes in the data set. As a result, the evolutionary relationships among closely related sequences are better resolved using evolutionary networks as opposed to phylogenetic trees (Posada and Crandall, 2001; Morrison, 2005). Evolutionary networks represent the relationship of haplotypes within a population without assuming a hierarchical pattern of decent or direction of change. A statistical parsimony network with 95% connection limits was constructed with all 88 *Diporeia* COI haplotypes using the program TCS version 1.21 (Clement *et al.* 2000). Each connection between haplotypes in a network represents a mutational step, or a single basepair difference between them. In the statistical

parsimony network intermediate haplotypes that are not represented in the data set are indicated by circle. A haplotypes age and relationship to other sequences can be inferred from its position and connections within the network. Older haplotypes are located in the center of the network and contain many connections, where as a newly derived haplotype are often characterized by a single connection. Loops or cycles present in the network indicates uncertainty in the relationship between haplotype and suggests parallel, convergent, or back mutations (Posada and Crandall, 2001; Morrison, 2005). Within sexually reproducing organisms loops may also represent recombination or lineage hybridization events (Posada and Crandall, 2001), however since mitochondrial DNA is primarily maternally inherited and haploid, recombination is an unlikely source of ambiguity within the network.

1.3.5 Morphotype analysis

Two adult male morphotypes, *D.h. brevicornis* and *D.h. filicornis* were collected from a single population in Lake Superior and identified on the basis of variations in the segmentation and length of the second antenna, as described by Segersträle (1971a). Nine *D.h. brevicornis* and 10 *D.h. filicornis* adult males were sequenced and analyzed. An exact test of population differentiation and F_{ST} was estimated between the two morphotypes using ARLEQUIN version 3.01.

1.4 Results

1.4.1 Collections

The *Diporeia* samples contained only female and/or sub-adult individuals at 16 of the 17 locations sampled. Specimens of *D.h brevicornis* and *D.h. filicornis* were collected from a single population in Lake Superior.

1.4.2 DNA sequence analyses

Two-hundred and sixty *Diporeia* COI sequences were obtained, and the final alignment was 637 base pairs in length. Among these sequences, 88 unique haplotypes were identified. Eighty-two variable sites were identified among the 88 haplotypes, with 44 sites being phylogenetically informative using the parsimony criterion. The average nucleotide frequencies among the 88 haplotypes are T: 0.36, C: 0.19, A: 0.25 and G: 0.20, and the mean pairwise haplotype divergence is 0.011 (SE=0.002). The overall mean pairwise transition/transversion ratio is 2.63 (SE =1.76). Amino acid sequence translations were unambiguous, and no gaps or nonsense codons were detected in the data set.

1.4.3 Population level analyses

The haplotype diversities in populations are high, and range from 0.5333 to 0.9619 (average h = 0.8261, SE = 0.1093) and nucleotide diversities are low, with Π being less than 0.01 in all populations (Table 1.1).

The hierarchical AMOVA conducted on populations within Lakes Huron, Michigan, Ontario and Superior indicated that the contribution of the covariance components among lakes and within populations contributed the greatest to the total genetic variation, but among

populations within lakes accounted for only 6.18% (Table 1.2). The values of F_{ST}, F_{SC}, and F_{CT} were respectively estimated as 0.6425, 0.1474 and 0.5807, and all are significant ($p < 0.05$).

The pairwise exact tests of population genetic differentiation indicated that all populations in a given lake were significantly differentiated from populations in the other three lakes with the exception of Lake Huron, Goderich, which was not significantly different from three Lake Ontario populations: Niagara on the Lake, Station 41, and 50 Point ($p = 0.092$, 0.240, and 0.160 respectively). Differences between populations in Lake Superior and those in Lakes Huron, Michigan and Ontario account for majority of the variation among the four lakes; the average pairwise estimate of F_{ST} between populations in L. Superior and populations within Lakes Huron, Michigan and Ontario is 0.7501. In contrast, the average pairwise F_{ST} estimate between populations among the latter three lakes is 0.2337 (Table 1.3).

Four to 12 haplotypes were detected in the 16 *Diporeia* populations (Table 1.1). The mean within population pairwise haplotype divergence was 0.503% (SE = 0.074%), resulting in a species screening threshold (SST) of 5.03%.

1.4.4 Phylogenetic analyses

The preliminary NJ analysis of all 88 haplotypes resolved two clusters (Fig 2). Haplotypes 1 to 63 form cluster 1, and occurred within Lakes Huron, Michigan, and Ontario (Fig 2), with the exception of H33, which was indentified in Lake Superior. Cluster 2 consists of haplotypes 64 to 88, which occurred in Lake Superior with the exception of H71, which was identified in 3 individuals sampled from Lake Huron, Goderich (Table 1.1). The NJ analysis

revealed that cluster 2 is paraphyletic with respect to cluster 1. The average pairwise haplotype divergence between the two clusters is 1.8% (SE = 0.4%) while the average pairwise haplotype divergence within cluster 1 and cluster 2 is 0.61% (SE = 0.094%) and 0.58% (SE 0.013%) respectively.

The reduced data set composed of 32 sequences (31 *Diporeia* and outgroup) possessed 145 variable sites (52 in the ingroup alone) with 31 being informative based on the parsimony criteria. The overall mean pairwise (± SE) transition/transversion ratio for the sequences was 3.32 (SE=1.89). The mean base pair frequencies were (T) 0.36, (C) 0.19, (A) 0.25, and (G) 0.20 and there was no evidence for heterogeneous nucleotide composition (homogeneity $\chi 2$ = 8.078, d.f. = 252, P > 0.999).

The Akaike information criterion indicated that out of the 56 DNA sequence substitution models, the data were best explained by the HKY+I+G model of sequence substitution with α =0.5345, proportion of invariable sites = 0.3756 and Ts/Tv = 4.7913. The ML analysis resolved the same clusters as the NJ analysis, with cluster 2 again paraphyletic with respect to cluster 1 (Fig. 1.3). The MP analysis identified 471 equally parsimonious trees (length = 58 steps, consistency index = 0.5690, retention index = 0.8311). This analysis also resolved the same haplotype clusters, placing cluster 2 paraphyletic with respect to cluster 1.

The statistical parsimony analysis of all 88 mitochondrial haplotypes discriminated the two haplotype clusters identified in the phylogenetic analyses (Fig. 1.4). The two groups are connected by a loop which indicates uncertainty, with each evolutionary path consisting of 6 mutational steps. The first of the two paths connects clusters 1 and 2 through H40 to H68,

while the second path connects either H52 or H63 to H71. Several loops have been identified within cluster 1 and the majority of haplotypes in this group are derived from either H17 or H52 by a single mutational step (Fig. 1.4).These two haplotypes are also the most widespread in Lakes Huron, Michigan and Ontario, and account for 9.7% and 6.4% of all individuals sequenced in the Great Lakes respectively. The majority of haplotypes in cluster 2 were derived from H71 by a single mutational step (Fig. 1.4) and H71 accounted for 32.9 % of all individuals sequenced in Lake Superior (7.2% of all individuals sequenced from the Great Lakes) (Table 1.1).

1.4.5 Morphotype analysis

The pairwise exact tests of population differentiation between the sympatric *D.h. filicornis* and *D.h. brevicornis* morphotypes from Lake Superior revealed that they are significantly differentiated (P =0.00095, SE=0.0008) and the pairwise estimate of F_{ST} between the two was 0.167. Four haplotypes were identified from each morphotype, and both variants possess a single copy of H71 (Table 1.1). The haplotypes from both *D.h brevicornis* and *D.h. filicornis* did not form independent monophyletic clusters, but instead were distributed within cluster 2 identified in Lake Superior in the phylogenetic analyses (Fig. 1.2) and the haplotype network (Fig. 1.4).

1.5 Discussion

This study has identified the presence of distinct levels of genetic variation within *Diporeia* populations among Lakes Huron, Michigan, Ontario and Superior. Multiple COI haplotypes and high levels of intra-population genetic variation were revealed at all locations. Despite

high haplotypic diversity, all haplotypes identified within populations were closely related, which is reflected in low population nucleotide diversities (Table 1.1). *Diporeia* populations within the same lake are not significantly differentiated from one another, and divergences between them only contributed 6.18% of the total variation observed. The divergences among the four lakes accounted for 58.07% of the total genetic variation within the Great Lakes. This is primarily attributed to the haplotypes sequenced from individuals in Lake Superior, which are genetically distinct from those identified from Lakes Huron, Michigan and Ontario.

The phylogenetic analyses revealed two haplotype clusters (Fig. 1.3). The first cluster consists of *Diporeia* individuals sequenced from Lakes Huron, Michigan and Ontario, while the second group of haplotypes was identified in Lake Superior. The statistical parsimony analysis also identified these two clusters, separating them by 6 mutational steps, but could not resolve the evolutionary path connecting the two (Fig. 1.4).

There is no evidence to support the existence of multiple *Diporeia* species in the Great Lakes using the phylogenetic species concept. The two clusters of haplotypes identified did not form reciprocally monophyletic clades, but instead cluster 2 is paraphyletic with respect to cluster 1 (Fig. 1.3), with an average nucleotide sequence divergence of 1.8% between the two groups. This level of sequence divergence falls below the calculated SST of 5.03%. Haplotypes sequenced from the two morphotypes *D.h. filicornis* and *D.h. brevicornis* did not form monophyletic clusters and therefore do warrant the recognition of separate phylogenetic species.

1.5.1 *Diporeia* species composition in the Great Lakes

This study did not identify phylogenetically distinct species of *Diporeia,* but it does not exclude the possibility of reproductively isolated biological species existing within the Great Lakes. It does imply however, that if two or more species do exist in the Great Lakes, their divergences are the result of recent evolutionary events. Over time, single haplotypes and their descendents will alternately become fixed in reproductively isolated populations. Once this occurs the populations are considered to be reciprocally monophyletic. The rate at which a new mutation will become fixed in a population is directly related to the generation time and effective population size (N_e) (Caballero, 1994; Neiman and Taylor, 2009) of the species. The average density of *Diporeia* within Lake Michigan has been estimated at 3,800/m^2 (Hondorp *et al.,* 2005) and as high as 13,686/m^2 (McDonald *et al.,* 1990), which equates to potentially millions of individuals in a kilometer radius. With an average life cycle of two years, it could potentially take on the order of hundreds of thousands of years for a single mutation to become fixed in a newly diverged species. This is assuming the effective population size is equivalent to the census size, but the N_e is usually revealed to be substantially lower (Frankham, 1995).

Several distinct morphotypes of fully developed *Diporeia* males have been identified in the Great Lakes basin and it has been suggested that they represent separate species (Bousfield 1989), although there are no distinguishable characteristics among females and juveniles, and only two (*filicornis/brevicornis*) have been adequately characterized. The most common morphotype, *D.h. filicornis,* is in fact a normal adult *D. hoyi* male and received its name as a result of its elongated antenna (Smith 1874; Segersträle 1971a). *Diporeia h. filicornis* should

not be considered a separate species, but rather the name should be used as a descriptive term for a normal adult male.

Diporeia h. brevicornis males are widely distributed throughout the Great Lakes Basin and other parts of North America, and often coexist in the same populations and *D.h. filicornis* (Henson 1954; Segersträle 1971a). These males are easily distinguished by their smaller size and the reduction of the second pair of antenna, which only reaches half of their body length as a result of fewer and shorter segments. Interestingly, the morphological characteristics of *D.h. brevicornis* strongly resemble the penultimate stage of *D. h. filicornis* males, and it is the last molt that produces much of the phenotypic modifications observed in *filicornis* males (Segersträle 1937; 1971a). Individuals of *D. h. brevicornis* are regularly present in warmer, shallower waters, often at or above the thermocline (Bousfield 1989; Väinölä and Varvio 1989). It is possible that due to increased exposure to light and higher temperatures, *D.h. brevicornis* males mature more quickly, not allowing sufficient time for all secondary sexual characteristics to develop, and represent a neotenic form.

Although the phylogenetic analysis did not support the recognition of *D.h. filicornis* and *D.h. brevicornis* as separate phylogenetic species, the exact test of population differentiation did reveal that the two are significantly differentiated. A single haplotype (H71) was sequenced from both morphotypes, but H71 is likely an ancestral haplotype (Fig. 1.4) and was identified in all Lake Superior populations (Table 1.1). It is important to note that the sample sizes for both morphotypes were small and that they may share more haplotypes that were not sampled in this analysis. High haplotype diversities were revealed in all populations

and it is possible that the difference between the two morphotypes is simply an artifact of insufficient sampling (Table 1.1) and does not necessarily reflect isolation between them.

An allozyme analysis conducted on the two morphotypes in Lake Mazinaw identified a statistically significant Hardy-Weinberg deviation at one locus (Mannose Phosphate Isomerase), but the authors concluded that the two morphotypes do not represent independent species (Väinölä and Varvio, 1989). The differentiation was observed when 71 *D.h. brevicornis* males, collected at 25m were compared to all samples collected at 100m (10 *filicornis* and 1 *brevicornis* males). Caution should be applied when interpreting this result, as the sample size of *D.h filicornis* at 100m was small, and the two morphotypes were not sympatric. Even with this in consideration, the genetic variations detected between *D.h. filicornis* and *D.h. brevicornis* could be a result of a recent speciation event between the two that is not reflected in the molecular data. If they do represent distinct species, it is likely that the two morphotypes diverged from one another in a single speciation event and dispersed across North America during the last glacial retreat. However, it is possible that *D.h. filicornis* and *D.h. brevicornis* have independently evolved at multiple locations through parallel sympatric speciation. During last decade, several studies have suggested that similar environmental conditions can trigger the independent evolution of phenotypically similar forms or "species" at multiple locations (Johannesson, 2001; Rico *et al*., 2003; Adams *et al.*, 2008). To test this hypothesis, extensive genetic surveying of multiple sympatric populations would need to be conducted using fast evolving molecular markers such as microsatellites.

The phylogenetic analysis did not identify *D.h. filicornis* and *D.h. brevicornis* as separate phylogenetic species, despite the population differentiation detected between the two. The

results of this analysis support the conclusions of previous studies (Segersträle 1937; 1971a; Väinölä and Varvio, 1989) that there is insufficient evidence to classify *D.h. filicornis* and *D.h. brevicornis* as independent species.

1.5.2 Phylogeographical distribution in the Great Lakes

The phylogographic patterns of several fish species in the Great Lakes were documented to have similar genetic distributions as *Diporeia*. Populations of Brown bullhead (Murdoch and Hebert, 1997), small mouth bass (Stepien *et al.*, 2007), walleye (Stepien and Faber, 1998) and yellow perch (Sepulveda-Villet *et al.*, 2009) in Lake Superior were all revealed to be genetically divergent from their conspecific populations in Lakes Erie, Huron, Michigan and Ontario. The pronounced genetic divergences between the fish populations were all attributed to independent colonization events into Lake Superior from separate refugia than those that colonized the remaining Great Lakes. Approximately 11,000 years ago the Laurentide ice sheet permanently retreated from what is present day Lakes Erie, Huron, Michigan and Ontario, providing the opportunity for colonization by aquatic organisms from the Mississippian or Atlantic refugia (Eschman and Karrow, 1985; Hansel et al., 1985; Muller and Prest, 1985; Larson and Schaetzl, 2001). However, the Superior basin was still covered by the Superior Lobe of the Laurentide ice sheet so these populations would have been prevented from entering the basin. When the Superior lobe did retreat, separate colonization events presumably occurred from the west via Lake Agàssiz (Dyke and Prest 1987; Dadswell, 1974) from the Missourian or Beringian. Based on the results of this study, I suggest that the strong genetic divergence observed between Lake Superior *Diporeia*

populations and those from Lakes Huron, Michigan and Ontario are the result of two independent colonization events from separate refugial populations.

1.5.3 Implications for conservation practices

The two distinct groups of *Diporeia* should both be treated separately when conservation management practices are considered. The unique genetic diversity within Lake Superior may result in different biological responses to stressors in comparison to *Diporeia* populations within Lakes Huron, Michigan and Ontario. The genetic distinction between the two groups, though low, may be reflective of significant biological differentiation. Studies on the metabolic responses of various chemical stressors in *Diporeia* specimens taken from Lake Superior and Lake Michigan revealed different metabolic profiles between the two (Ralston-Hooper *et al.*, 2008). The variations observed in the metabolic reactions of the two groups may reflect different responses to diseases and resource competition. To date, there has been no documented decline in *Diporeia* populations within Lake Superior (Scharold *et al.*, 2004) despite the presence of *Dreissena* species in the lake (Griffiths *et al.*, 1991; Grigorovich *et al.,* 2008).

The *Diporeia* specimens used in this study were collected in 1996, prior to the major declines in population densities. The continued survival of *Diporeia* in Lakes Huron, Michigan and Ontario, despite their reduction in numbers, and in Lake Superior suggests that both genetic lineages are still present. As a result of populations within a lake being highly similar, decreasing *Diporeia* population size may not have substantially impacted their genetic diversity. The true impact of the decline can not be assessed until current populations

are examined. *Diporeia* specimens from Lake Erie were not included in this study, but it is almost certain that COI sequences from those populations would fall into cluster 1 of the phylogenetic analysis (Fig. 1.2). The fish species walleye, brown bullhead, yellow perch and small mouth bass were all revealed to possess similar genetic distributions within the Great Lakes as *Diporeia* and the genetic composition of the four species in Lake Erie resembled those identified in Lakes Huron and Ontario. Therefore, despite the substantial decline of *Diporeia* within Lake Erie, it is unlikely that a genetically distinct lineage became extinct. Similarly of the populations between these lakes also indicates that there is a strong potential for reintroduction of *Diporeia* back into Lake Erie if favorable conditions return. Currently, nothing can be done about the invasion of *Dreissena* since they have established themselves in the lakes. However, *Diporeia's* high genetic diversity warrants mild optimism about their ability to recover, as high genetic variation is correlated with a population's capability to adapt to new stressors (Frankham, 1995; Wise *et al.*, 2002; Pertoldi *et al.*, 2007).

1.6 Conclusions

This study has significant implications for conservational management of *Diporeia* in the Great Lake basin. There was no phylogenetic evidence to suggest the existence of two or more *Diporeia* species present within Lakes Huron, Michigan, Ontario and Superior. The examination of *D.h filicornis* and *D.h. brevicornis* specimens collected from Lake Superior did not support their recognition as separate species.

High levels of genetic variation have been identified in all *Diporeia* populations, but populations within the same lake are very similar to each other. *Diporeia* haplotypes derived

from individuals in Lake Superior are genetically distinct from those present in Lakes Huron, Michigan and Ontario, and may be the result of colonization from different Pleistocene refugia lineages. The marked genetic differentiation observed between Lake Superior and the remaining lakes underscores the importance of understanding the evolutionary history of a species. It is recommended that conservation efforts to maintain *Diporeia* populations within the Great Lakes address the two lineages and treat them as evolutionary significant units.

Figure 1.1. Map showing sample locations of *Diporeia* in the Laurentian Great Lakes. Population names indicate sample locations and follow Table 1.

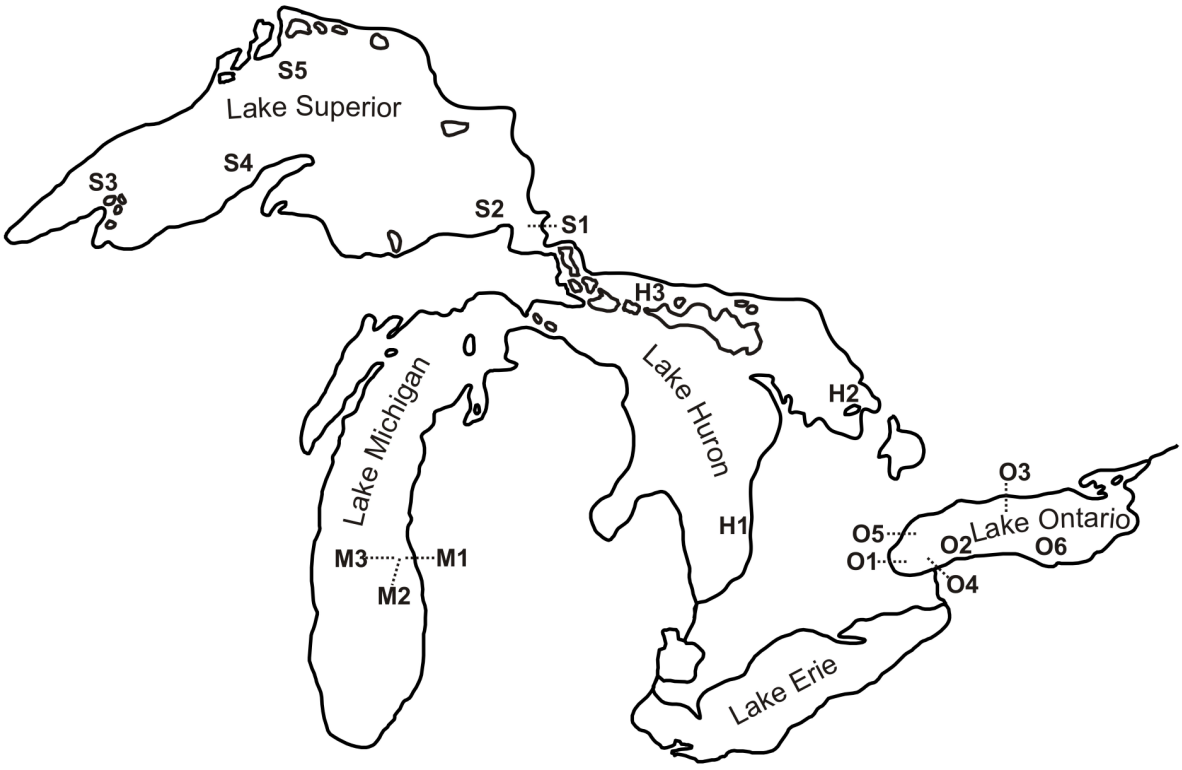
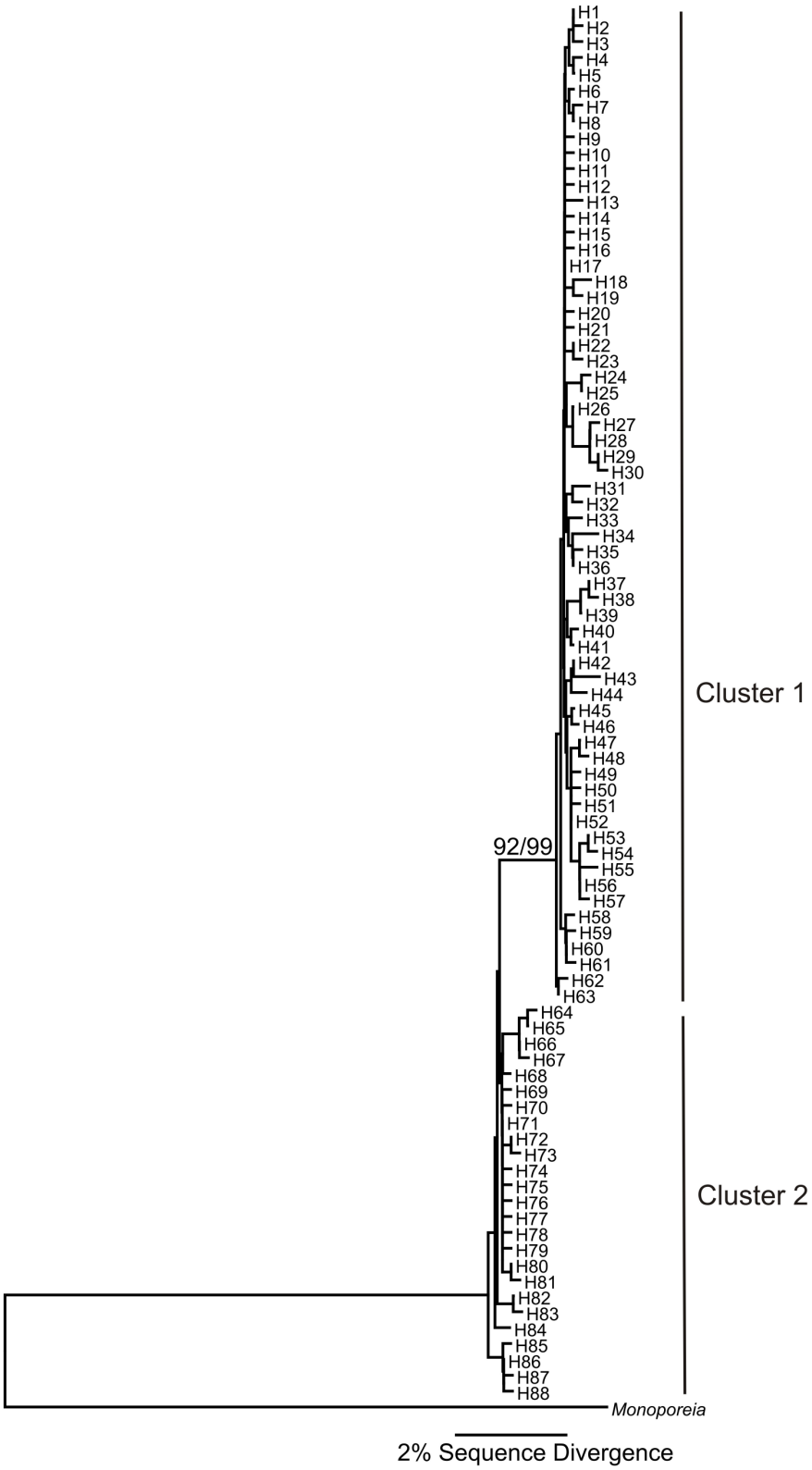


Figure 1.2. NJ phenogram showing relationships among 88 cytochrome *c* oxidase I (COI) haplotypes. Numbers above nodes indicate interior branch test and NJ bootstrap percentages respectively.


H1
H2
H3
H4
H5
H6
H7
H8
H9
H10
H11
H12
H13
H14
H15
H16
H17
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H67
H68
H69
H70
H71
H72
H73
H74
H75
H76
H77
H78
H79
H80
H81
H82
H83
H84
H85
H86
H87
H88
92/99
Cluster 1
Cluster 2
Monoporeia
2% Sequence Divergence

Figure 1.3. NJ tree showing the relationships among 32 cytochrome *c* oxidase I (COI) haplotypes. Numbers above the nodes give NJ bootstrap percentages, interior branch test percentages, ML bootstrap percentages, MP bootstrap percentages, and partition percentages among the 471 equally parsimonious trees respectively.

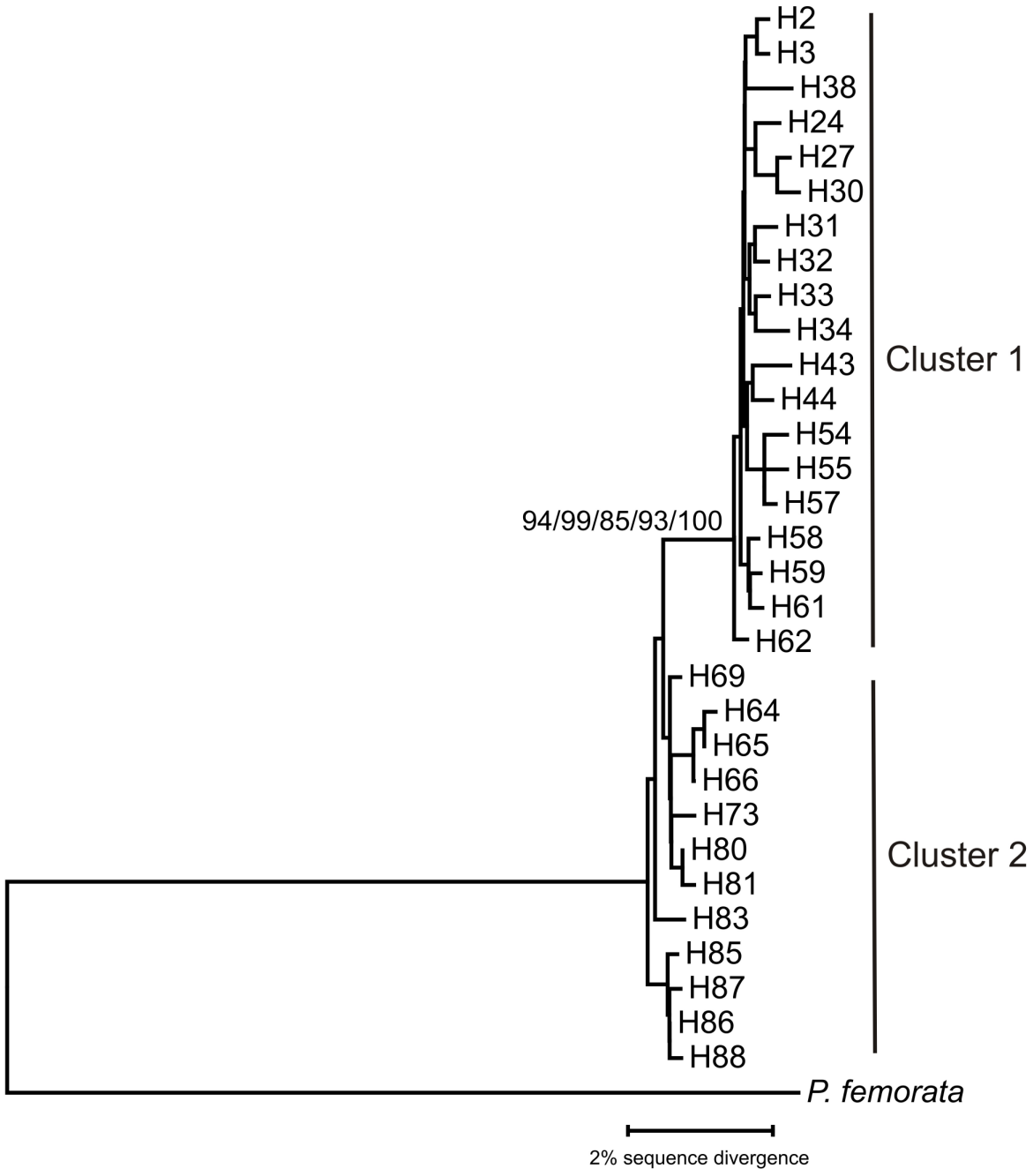


Figure 1.4 Statistical parsimony network showing the relationships among 88 mitochondrial cytochrome *c* oxidase I haplotypes. Line connecting haplotypes indicates a single mutational step and the solid circles represent hypothetical intermediate haplotypes that were present in the data set. Loops indicate uncertainty in the relationships between haplotypes.

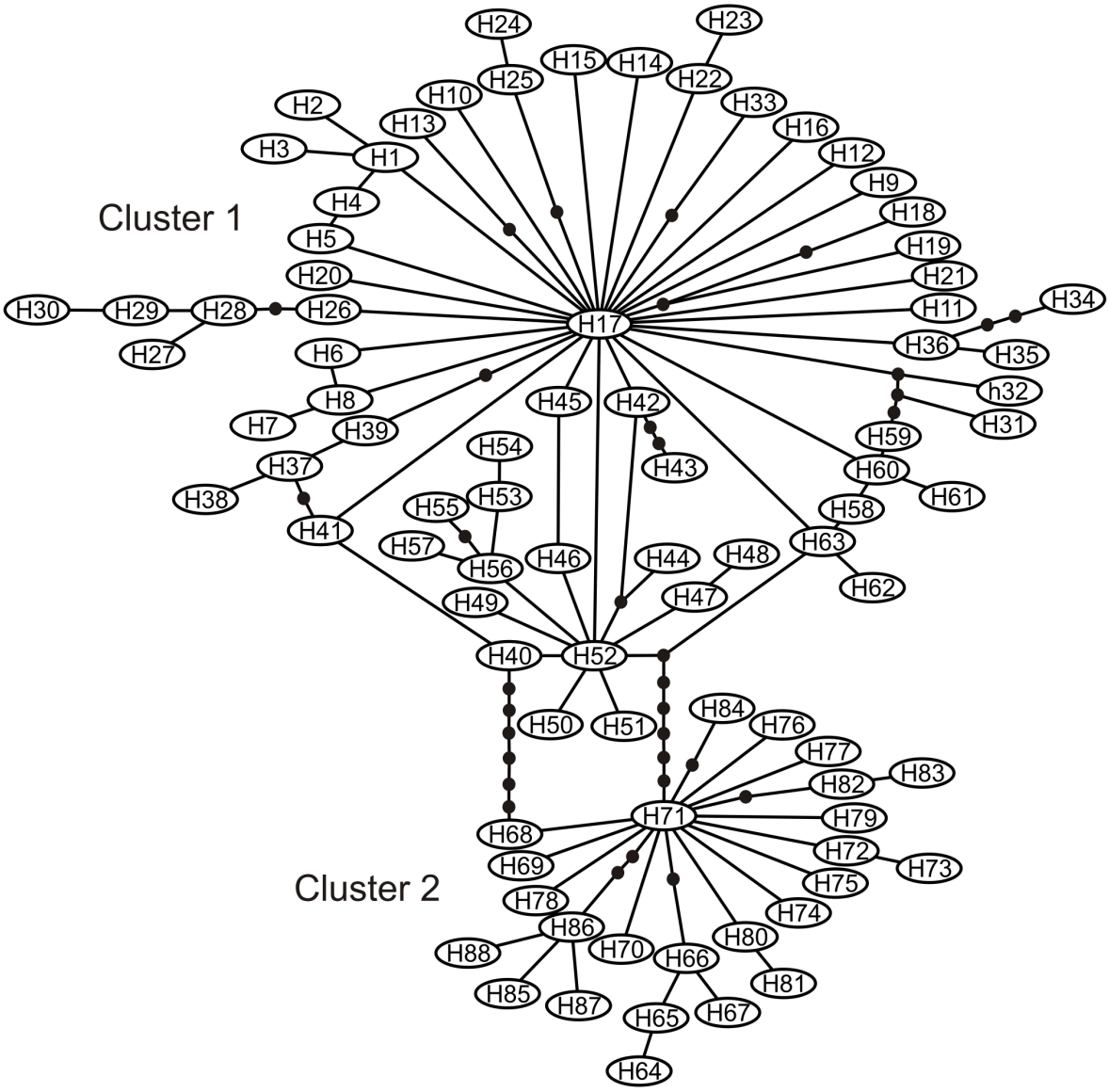


Table 1.1. Populations, sample size (n), haplotype diversity (h), nucleotide diversity (Π) and haplotypes presence (H) for the 260 *Diporeia* individuals sequenced from the Great Lakes. The haplotype designation follows Fig 1.2, and the number in parentheses beside the haplotype indicates the number of times it occurred in each population.

	Lake, Population	**n**	**h**	**Π**	H										
H1	Huron, Goderich	15	0.83810	0.00584	H17(5)	H24(1)	H26(2)	H42(1)	H52(3)	H71(3)					
H2	Huron, Hope Bay	15	0.83810	0.00381	H9(2)	H17(1)	H27(1)	H28(4)	H29(5)	H30(1)	H52(1)				
H3	Huron, North Channel	16	0.75000	0.00245	H25(2)	H40(2)	H44(1)	H49(1)	H50(1)	H51(1)	H52(8)				
M1	Michigan, 29m	15	0.92381	0.00411	H1(4)	H2(1)	H4(1)	H17(1)	H18(1)	H21(1)	H35(1)	H36(2)	H53(1)	H56(2)	
M2	Michigan, 45m	15	0.77143	0.00375	H1(1)	H3(1)	H14(1)	H17(3)	H34(1)	H54(1)	H56(7)				
M3	Michigan, 100m	15	0.77143	0.00297	H1(1)	H17(3)	H19(1)	H53(1)	H55(1)	H56(7)	H57(1)				
O1	Ontario, 50pt	15	0.83810	0.00222	H11(1)	H17(6)	H20(1)	H22(2)	H39(1)	H52(2)	H60(1)	H61(1)			
O2	Ontario, 85m	15	0.92381	0.00288	H6(1)	H15(1)	H16(1)	H17(4)	H22(2)	H32(1)	H39(1)	H45(1)	H47(1)	H60(2)	
O3	Ontario, Cobourg	15	0.94286	0.00492	H13(1)	H17(2)	H22(1)	H37(4)	H38(1)	H41(1)	H46(1)	H47(1)	H52(1)	H58(1)	H60(1)
O4	Ontario, Niagara on the Lake	15	0.92381	0.00411	H8(1)	H17(4)	H22(1)	H31(1)	H39(1)	H43(1)	H47(2)	H48(1)	H52(1)	H63(2)	
O5	Ontario, Port Credit	15	0.95238	0.00360	H5(1)	H10(1)	H17(2)	H22(1)	H23(1)	H39(1)	H47(3)	H52(1)	H59(1)	H60(2)	H63(1)
O6	Ontario, st41	15	0.84762	0.00282	H7(1)	H8(1)	H12(1)	H15(1)	H17(4)	H37(1)	H52(5)	H62(1)			
S1	Superior, Pancake pt	15	0.83810	0.00536	H33(1)	H65(2)	H66(5)	H71(4)	H78(1)	H82(1)	H83(1)				
S2	Superior, st2	15	0.76190	0.00291	H64(1)	H65(3)	H66(2)	H71(7)	H74(1)	H76(1)					

S3	Superior, st164	15	0.67619	0.00291	H69(1)	H71(8)	H80(3)	H87(3)								
S4	Superior, st221	15	0.96190	0.00474	H71(2)	H72(1)	H73(1)	H75(2)	H77(1)	H79(1)	H80(2)	H81(1)	H85(1)	H86(1)	H88(2)	
S5	Superior, Filicornis	10	0.53333	0.00207	H66(7)	H67(1)	H71(1)	H84(1)								
S5	Superior, Brevicornis	9	0.77778	0.00386	H65(4)	H68(2)	H70(2)	H71(1)								

Table 1.2. AMOVA partitions among *Diporeia* haplotypes identified in Lakes Huron, Michigan, Ontario and Superior. All sources of variation are significant ($p<0.05$, 1023 permutations).

Source of Variation	Covariance Component	Percentage of Variation
Among Lakes	1.91069	58.07
Among Populations Within Lakes	0.20340	6.18
Within Populations	1.17630	35.75
Total	3.29039	100

Table 1.3. Average F_{ST} values for all pairwise population comparisons of *Diporeia* between lakes. Standard errors are in parentheses.

Lake Comparison		FST
Superior	Huron	0.7253 (0.0273)
Superior	Michigan	0.7598 (0.0106)
Superior	Ontario	0.7652 (0.0084)
Huron	Michigan	0.2083 (0.0529)
Huron	Ontario	0.2488 (0.0371)
Michigan	Ontario	0.1721 (0.0177)

Chapter 2 - Phylogeographical analysis and evolutionary relationships of the North American glacial relict *Diporeia hoyi* (Order: Amphipoda)

2.1 Overview

The North American geographic distribution of glacial relict species has been strongly influenced by the postglacial lake system that formed during the last glacial retreat. In this study, mitochondrial cytochrome *c* oxidase I (COI) gene sequences are used to determine the phylogeographic distribution of the relict *Diporeia hoyi*, and to determine its evolutionary relationship with *Monoporeia affinis* and *Pontoporeia femorata*. Phylogenetic analyses and a statistical haplotype network revealed the existence of two genetically divergent *D. hoyi* lineages that exhibited a paraphyletic relationship and a nucleotide sequence divergence of 1.75%. The first lineage dispersed from a Missourian refugia and primarily occurs in the former lake Agàssiz basin, Northern Ontario (including Lake Superior) and Northern Quebec. Populations located in the Great Lakes basin (excluding Superior) and the St. Lawrence/Gatineau region alternatively dispersed from a Mississippian refuge. Phylogenetic analyses of the three genera identified *D. hoyi* and *M. affinis* as sister taxa and the average sequence divergence between the two is 21.56%. Two markedly distinct lineages of *P. femorata* were identified, indicating that it represents a cryptic species complex.

2.2 Introduction

The advance and retreat of the Pleistocene glaciers had a profound impact on the distributions of species across the Northern Hemisphere. There were four major glaciations

during the Pleistocene, the Nebraskan, Kansan, Illinoian and Wisconsinan, with multiple smaller advances and contractions between them (Ehlers and Gibbard, 2008). The Wisconsinan glaciation was the last major advance to impact North America, and reached its maximum approximately 18,000 years ago. Two ice sheets, the Laurentide and the Cordilleran, covered most of Canada as well as parts of Alaska, and extended as far Indiana and Ohio (Dyke and Prest, 1987). The vast quantities of water trapped in the glaciers caused a dramatic drop in sea level of up to 120 meters, exposing the continental shelves, and resulting in the recession of the marine littoral zone (Rohling *et al.,* 1998; Faure *et al.,* 2002). Species that escaped the ice were sundered into small pockets of unglaciated regions known as refugia, while others migrated south to warmer or more favorable environments. Populations that managed to survive were isolated and reduced to a fraction of their original size, causing population bottle necks that ultimately resulted in the loss of genetic variation (Pielou, 1991; Hewitt, 2004; Soltis *et al.,* 2006).

With the retreat of the glaciers came opportunities to re-colonize new habitats. The melting ice produced a large influx of freshwater that formed proglacial lakes, a series of large water bodies along the ice sheet margins that were interconnected at various times during the last 15,000-8,000 years. Aquatic species that gained access to the proglacial lake systems were subsequently able to disperse over large geographic areas, while the migrations of terrestrial species were hindered by these extensive water bodies (Pielou, 1991; Gagnon and Angers, 2006; Hailer *et al.,* 2007). As the melt waters drained into the oceans and the proglacial lakes receded, aquatic species that inhabited them became stranded and isolated in remnant water bodies.

Traditional biogeographic studies focused on morphologically described species and the palaeoecological record (through the use of pollen data) to determine the distributions and colonization histories of the North America flora and fauna. A reliance on morphological data alone can result in marked underestimates of biological diversity due to cryptic or convergent evolution, a problem which has been pervasive among aquatic invertebrate taxa (Witt and Hebert, 2000; Witt *et al.,* 2006; Finston *et al.,* 2007; Segers and Shiel, 2008; Belyaeva and Taylor, 2009). The development of molecular techniques to study the relationships among organisms led to the emergence of phylogeography, a subdiscipline of biogeography. Phylogeography is concerned with the geographical distribution of genetic lineages and the forces that influence those distributions. By examining the genetic structuring of species over their entire range and combining it with geological data, phylogeographical studies can more accurately describe the history of species and potentially trace lineages to the refugia from which they dispersed (Provan and Bennett, 2008).

Morphology based biogeographical studies have identified several key areas that served as refugia in North America during the last glacial maximum, and the existence of these refugia have been reaffirmed by recent phylogeographic studies. The Beringia refuge located in the North included Alaska, most of the Yukon, sections of Eastern Siberia and portions of the Bering and Chuckchi Sea that were exposed as a consequence of reduced sea level (Pielou, 1991; Bernatchez and Wilson, 1998; Hewitt, 2004). Numerous phylogeographical studies have confirmed the important roles that the Mississippian (Danzmann and Ihssen, 1995; Wilson and Hebert 1998; Van Houdt *et al.,* 2005; Gagnon and Angers, 2006) and Missourian basins (Ferguson and Duckworth, 1997; Stepien and Faber, 1998; Van Houdt *et al.,* 2005)

have played as refugia for an array of fish species. The reduction in sea level and exposure of the continental shelves created the coastal Pacific (Latch *et al.,* 2009) and Atlantic (Danzmann *et al.,* 1998; Colbeck *et al.,* 2008) refugia. A phylogenetic study on Lake Whitefish identified another refuge situated in Nahanni National Park in the Northwest Territories (Foote *et al.,* 1992; Stamford and Taylor, 2004)

As more phylogenetic studies on aquatic taxa were conducted, a paradox began to emerge concerning levels of genetic divergence within active and passively dispersed organisms. Passively dispersed species are capable of colonizing new habitats by the use of wind or animal vectors, and has been reported among many aquatic organisms (Bilton *et al.,* 2001; Figuerola *et al.,* 2002; Vanschoenwinkel *et al*., 2008a; Vanschoenwinkel *et al*., 2008b). Traditional biogeographers hypothesized that populations of these species would be genetically similar to each other as consequence of gene flow resulting from their strong dispersal capabilities. Unexpectedly, molecular studies revealed an opposite pattern, and many of these aquatic organisms exhibited deep intraspecific genetic divergences (Taylor *et al.,* 1998; Witt and Hebert, 2000; Cox and Hebert, 2001; Gomez *et al*., 2002; Adamowicz *et al.,* 2009). Given the strong potential for gene flow among such mobile organisms, the deep genetic divergences within these species are paradoxical.

Habitats are typically colonized by a few individuals, creating a strong founder effect in newly established populations (Boileau *et al.,* 1992). Founder effects can account for the initial genetic differences among populations, but cannot explain the failure to detect gene flow. The most prominent explanation for the apparent lack of gene flow is the monopolization hypothesis, which postulates that once a population becomes established, it

is difficult for new immigrants to survive and successfully reproduce (De Meester *et al.,* 2002). New immigrants must compete with locally adapted individuals for resources and potential mates, and even if reproduction is achieved, there is a high probability that their alleles will be lost within a few generations due to genetic drift (De Meester *et al.,* 2002; Bohonak and Jenkins, 2003; Weisse, 2008). The combination of founder events and the failure of migrants to contribute to established populations could account for the high level of intraspecific genetic divergence observed among passively dispersed species.

Conversely, actively dispersing aquatic species are only capable of gaining access to new habitats by direct movement through the use of corridors (i.e. water ways, rivers streams etc.). If there are no channels present to allow migration, the populations become effectively isolated from one another, and allopatric divergence will occur. It is therefore expected that populations within these species would quickly diverge from one another as a result of isolation, and multiple divergent lineages would exist over large geographical ranges. In the Northern Hemisphere however, many actively dispersing species exhibit low levels of genetic divergence between lineages (Danzmann *et al.,* 1998; Van Houdt *et al*., 2003; Audzijonytė and Väinölä, 2006; Dooh *et al.,* 2006; Barrette *et al*., 2009; Kawamura *et al.,* 2009), contradicting traditional expectations.

The glacial marine relicts are a group of actively dispersing organisms confined to deep, cold freshwater lakes. In North America they consist of the crustaceans, *Mysis diluviana*, *Diporeia hoyi*, *Limnocalanus macrurus*, *Senecella calanoides* and one fish species, *Myoxocephalus thompsonii*. The distributions of all five relicts are restricted to lakes that occur within the former proglacial lakes basins (Dadswell, 1974).

The origin of the relicts has been somewhat enigmatic for the last century and until recently, little was known about their evolution. The "sluicing-up" hypothesis has been the most prominent explanation, which asserts that the relicts are remnant populations of arctic marine species that were trapped by the advancing ice sheets and forced inland (Hogbom, 1917). After gradually adapting to freshwater, the relicts eventually would have become established as independent freshwater species. The strong morphological resemblance between the relicts and their arctic marine relatives, combined with their co-distributions, led early researchers to suggest that the relicts evolved together during the Pleistocene (Ricker, 1959; Dadswell, 1974; Segersträle, 1977). Holmiquist (1959, 1970) however, argued that they were ancient and stable freshwater species, and suggested an origin as far back as the Oligocene.

Recent molecular studies have revealed that the glacial relicts did not invade the continent in unison, but rather have independently adapted to the freshwater environments. *Limnocalanus macrurus* and *M. thompsonii* appear to have diverged from their marine relatives during the Pleistocene as first hypothesized, though they likely invaded the continent independently during one of the earlier glaciations. Alternatively, *M. diluviana* displays a deeper level of genetic divergence with respect to its marine relative (*M. segerstralei*), suggesting a Miocene origin (Audzijonytė, 2005). Not only does the timing of the invasions vary, but the relicts also exhibit different phylogeographic patterns across North America. *Limnocalanus macrurus* and *M. thompsonii* appear to have re-colonized North America from a single refuge each (Kontula and Väinölä, 2003; Dooh *et al*., 2006; Sheldon *et al.,* 2008). The dispersal of *M. diluviana* was slightly more complicated and a

total of four refugial lineages have been identified, with overlapping ranges in the Great Lakes region, indicating the potential for secondary contact of different stocks (Dooh *et al.,* 2006). All lineages within these three species exhibit low levels of mitochondrial DNA sequence divergence despite their poor dispersal abilities.

The distribution of the amphipod *Diporeia*, like the other North American relicts, is restricted to the former proglacial lake basins, but a population also occurs in Lake Washington. A very low level of intraspecific genetic variation was detected among *Diporeia* populations using allozymes (Väinölä and Varvio, 1989), but the authors note that their study was geographically limited (restricted to the area surrounding Lake Ontario) and more of the species' range needs to be sampled for an accurate estimate of diversity. As discussed in the previous chapter, *Diporeia* populations in Lake Superior are genetically unique in comparison to those in the other Great Lakes, suggesting that Väinölä and Varvio's (1989) genetic diversity estimates are likely conservative. It has also been suggested that the morphotypes of *Diporeia* (see chapter one) may represent unique species (Bousfield, 1989). Väinölä and Varvio (1989) also provided insights into the relationships among the genera *Diporeia, Monoporeia* and *Pontoporeia*. Their allozyme study revealed the presence of strong genetic divergences between the three lineages, pushing back the estimated divergence of these genera from the Pleistocene into the Tertiary (Väinölä and Varvio, 1989).

This study investigates the history of *Diporeia* and the effect that the last glacial retreat had on its distribution across North America. I test the hypothesis that the two lineages of *Diporeia* identified within the Great lakes, one residing in Lake Superior, and the other in Lakes Huron, Michigan and Ontario, represent different refugial lineages. These two lineages

are expected to exhibit a wider geographic distribution across North America. The mitochondrial cytochrome *c* oxidase gene (COI), as well as the nuclear ribosomal internal transcribed spacer (ITS-1) gene will be employed to determine the number of glacial refugia *Diporeia* survived in, and subsequently dispersed from. The mitochondrial COI gene has been used in many phylogeographic studies (Dooh *et al.,* 2006; Browne *et al.,* 2007; Lefébure *et al.,* 2007; Audzijonytė *et al*., 2009; Carlini *et al.,* 2009) because it rapidly accumulates mutations allowing the detection of recent evolutionary events (Brown, 1979; Brown, 1982; Gissi *et al.,* 2008). A secondary goal of this study is to clarify the relationship among the three genera, *Diporeia*, *Monoporeia* and *Pontoporeia* in the family Pontoporeiidae by utilizing phylogenetic reconstruction techniques. The allozyme study by Väinölä and Varvio (1989) indicated that the three genera have been independently evolving for millions of years, and that *Diporeia* and *Monoporeia* are the most closely related. The problem with allozyme data is that they are based on allele frequencies and are not reliable for phylogenetic reconstruction (Likhnova and Lebedev, 1995). It is expected that *Diporeia, Monoporeia* and *Pontoporeia* will each form monophyletic lineages reflecting their independent evolutionary trajectories.

2.3 Materials and Methods

2.3.1 Collections

Diporeia specimens were collected from 42 locations across North America between 1994 and 2001 (Fig. 2.1). Specimens were taken at depths ranging between 29 and 100 meters

using a benthic drag and classified as *Diporeia hoyi* or *Diporeia hoyi erythrophthalma* (Waldron, 1953).

Samples of *Monoporeia affinis* were obtained from the Beaufort sea, off Tuktoyaktuk and *Pontoporeia femorata* was collected from Hudson Bay off Churchill Manitoba, Resolute Bay (Cornwallis Island, Nunavut, Canada) and Turton Bay (Igloolik Island, Nunavut, Canada). All specimens were stored in absolute ethanol for future analysis. A North American specimen of *Gammarus lacustris* was collected for use as an outgroup for the phylogenetic analyses of the genera *Diporeia*, *Monoporeia* and *Pontoporeia*.

2.3.2 DNA sequence analyses

Total DNA was extracted from 4-16 *Diporeia* individuals from each population by grinding a leg in 50 µL of proteinase K extraction buffer (Schwenk, 1996). A total of 3-9 specimens of *Monoporeia* and *Pontoporeia* from each location were also extracted. A 637 base pair fragment of the mitochondrial cytochrome *c* oxidase I (COI) gene was amplified using the primers LCO1490 and HCO2198 (Folmer *et al.,* 1994). The 50 µL PCR reactions contained 2.0-3.0 µL of DNA template, 5.0 µL 10x PCR buffer, 0.2 mM of each primer, 0.2 mM of each dNTP and 0.5 unit of Taq polymerase. The PCR conditions consisted of 1 min at 94°C followed by 5 cycles of 1 min at 94°C, 90 s at 45°C, 1 min at 72°C; followed by 35 cycles of 1 min at 94°C, 90 s at 51°C, 1 min at 72°C; followed by 5 min at 72°C. The PCR products were gel purified using the Qiaex kit (Qiagen Inc.) and sequenced using the ABI prism BigDye terminator sequencing kit (30 cycles, annealing at 55C). Products were sequenced in one direction using primer LCO1490 and electrophoresis was carried out on an ABI 3730

automated sequencer (Applied Biosystems). The sequences were aligned by eye in MEGA 4.1 (Tamura *et al.*, 2007).

The primers ITS1crF and ITS1crR were used to amplify a 505 base pair portion of the intertranscribed spacer (ITS1) region for 30 *Diporeia* specimens. The PCR reactions and sequencing protocol was identical to the COI methods. The sequences were aligned by eye in MEGA 4.1.

All haplotypes were identified by constructing a distance tree using the unweighted pair group with arithmetic averages (UPGMA) using a matrix of nucleotide differences between all pairwise combinations of sequences in MEGA 4.1. Nucleotide composition, nucleotide (p-distance) distance matrices, mean pairwise transition/transversion ratios and amino acid translations (invertebrate mitochondrial code) for all mitochondrial COI haplotypes were calculated in MEGA 4.1. Nucleotide sequence divergences between *Monoporeia*, *Diporeia* and *Pontoporeia* were estimated using the Tamura and Nei (Tamura and Nei, 1993) model of nucleotide substitution.

2.3.3 Phylogenetic analysis of *Diporeia*

A phylogenetic analysis using the Neighbour-Joining (NJ) (Saitou and Nei, 1987) distance method and the Jukes and Cantor (JC) (Jukes and Cantor, 1969) model of nucleotide substitutions was conducted using MEGA 4.1 on 138 *Diporeia* COI haplotypes. Confidence in the NJ analyses was assessed using the bootstrap method and interior branch test with 1000 replicates each.

As a result of computational constraints, the data set was reduced to 55 haplotypes for use in all subsequent phylogenetic analyses. A second NJ analysis of the 55 haplotypes was constructed using the same criterion as the first. The program MODELTEST version 3.0 (Posada and Crandall, 1998) was used to estimate the best fit model of 56 models of nucleotide substitution using the Akaike Information Criterion (Posada and Buckley, 2004).

A maximum likelihood analysis (ML) was conduced in PAUP 4.0b10 (Swofford, 2001) using the model and parameters estimated by MODELTEST. The ML analysis was executed using a heuristic search with the starting tree obtained using the NJ method, and the tree bisection reconnection (TBR) branch swapping algorithm. Confidence in the tree was assessed using the bootstrap method with 500 pseudoreplicates. A Bayesian analysis was executed in MrBayes, version 3.1.2 (Ronquist and Huelsenbeck, 2003) using the nucleotide substitution model estimated by MODELTEST. Four Markov chains were started from random trees and were run for six million generations, with trees sampled every 100 generations. A total of 31,210 trees sampled prior to convergence were discarded as burnin.

Phylogenetic methodologies assume a bifurcating branching pattern, and that ancestral haplotypes are not extant. An evolutionary network analysis better describes the history among closely related haplotypes, as it does not assume that ancestral haplotypes are extinct. A statistical parsimony network with 95% connection limits was constructed with all 138 *Diporeia* COI haplotypes using the program TCS version 1.21 (Clement *et al.,* 2000).

2.3.4 Phylogenetic analyses of Diporeia, Monoporeia and Pontoporeia

A NJ phylogeny of 9 *Diporeia*, 2 *Monoporeia affinis* and 6 *Pontoporeia femorata* COI haplotypes was constructed using the Tamura-Nei (Tamura and Nei, 1993) model of nucleotide substitution. A sequenced derived from *Gammarus lacustris* was used as an outgroup in the analysis. Confidence in the NJ analysis was estimated using the bootstrap method and interior branch test with 1000 replicates each.

Maximum likelihood and Bayesian analyses were conducted using the same methods previously described for the *Diporeia* dataset. The Bayesian analysis was run for one million generations and 3,210 trees were discarded as the burnin.

2.3.5 Molecular clock analysis

The determination of the time span that lineages have been evolving independently can be a difficult task. Even with a fossil record, establishing the point of divergence between morphologically similar species can be complex. The idea that proteins and nucleic acids evolve at a consistent rate (referred to as the molecular clock hypothesis) provided a new method for inferring the age of genetic lineages. The molecular clock hypothesis relies on the principles of the neutral theory, which predicts that portions of the DNA sequence that are not subject to selection pressures will undergo a constant rate of molecular evolution given a set mutation rate (Kimura, 1983). This has resulted in the application of molecular clocks to estimate the divergence times between organisms in many phylogenetic studies (Avise, 2004). However, caution must be taken when applying a molecular clock because many factors can influence DNA mutation rates, resulting in inconsistent 'clocks' for different

species, genera and even the same genes. Variations in population size, generation times, efficiency of DNA repair mechanisms and selection pressures are just a few potential causes of discrepancies in rates of sequence evolution (Bromham and Penny, 2003; Kumar, 2005; Pulquério and Nichols, 2006). To minimize the error introduced, if possible it is best to choose a molecular clock calibrated using closely related organisms that share many traits.

Examination of the genus *Alpheus* (subphylum: Crustacea) identified an average rate of 1.4% COI sequence divergence per million years (Knowlton and Weigt, 1998). This rate of nucleotide evolution was used to estimate how long *Diporeia*, *Monoporeia* and *Pontoporeia* lineages have been diverging.

2.4 Results

2.4.1 Collections

Samples collected at 41 locations yielded *Diporeia hoyi* individuals and specimens of *D.h. erythrophthalma* were only identified from Lake Washington.

2.4.2 DNA sequence analyses

A total of 401 *Diporeia* COI sequences were obtained and 138 haplotypes were identified among them. The final alignment of the sequences was 637 base pairs in length with 116 variable positions. Amino acid translations did not reveal any gaps, deletions or nonsense codons within the sequences. The average nucleotide composition among the 138 haplotypes was (T) 0.365, (C) 0.192, (A) 0.247, and (G) 0.196 and the mean pairwise transition /transversion ration (Ts/Tv) for all pairwise sequence comparisons was 2.936 (SE=1.6).

Two unique *Diporeia* COI haplotypes were sequenced from a population in Lake Cayuga that were significantly divergent from all other *Diporeia* haplotypes. When the sequences were translated into amino acids, multiple nonsense codons and rare amino acid substitutions were detected in the protein sequence. Considering the importance of the cytochrome *c* oxidase I gene in the mitochondria, it is not possible to introduce nonsense codons into a functioning copy of the gene and it is therefore probable that the sequences were psuedogenes and were excluded from all phylogenetic analyses. A single ITS haplotype was identified in all 30 *Diporeia* specimens sequenced.

Twenty-one *Pontoporeia femorata* and 3 *Monoporeia affinis* sequences were obtained, with 6 and 2 haplotypes identified among them respectively. The final length of the sequence alignment was 637 base pairs with 186 variable sites among them. Amino acid sequence translations were unambiguous, and no gaps or nonsense codons were detected among the 6 haplotypes.

2.4.3 Phylogenetic analyses of *Diporeia*

The initial NJ analysis of *Diporeia* revealed two well supported clusters of haplotypes that exhibited a paraphyletic relationship (Fig. 2.2). The first group of haplotypes (H1-H86) were identified in Southern Ontario, Eastward into New York state, and Southern Québec (Fig. 2.1). This group of haplotypes (South-Eastern region) formed a single monophyletic clade with an average nucleotide sequence divergence between haplotypes of 0.622% (SE=0.085%). The second group (H87-H138) is paraphyletic with respect to the South-Eastern group and these haplotypes were obtained from Northern Ontario, Northern Québec

and Western Canada (Fig. 2.1). The nucleotide divergence between all haplotypes in the western group was 0.573% (SE=0.092%), while the average sequence divergence between the two groups was 1.75% (SE=0.39%).

Within the reduced data set of 55 *Diporeia* COI sequences, 80 sites were variable. The mean base frequencies were T:0.364, C:0.192, A:0.248 and G:0.196 and there was no indication of heterogeneous nucleotide composition among the sequences including the outgroup (homogeneity χ^2=7.204, d.f.=162, *P*=1). The Akaike information criterion indicated that out of the 56 DNA sequence substitution models the HKY+G model best explains the data, with Ts/Tv = 4.6259 and α =0.2720. The ML and Bayesian analyses completed on the reduced data set recovered the two haplotypic groups and the paraphyletic relationship identified in the initial analysis of all 138 *Diporeia* COI haplotypes (Fig. 2.3).

The statistical parsimony network of all 138 *Diporeia* COI haplotypes indicated that the two clusters are separated by 5 mutational steps between H85 and H87 (Fig. 2.5). Most haplotypes in the South-Eastern group were only one or two mutational steps away from H47, which was the most broadly distributed haplotype in the group accounting for 12.7% of all *Diporeia* sequences analyzed. The network identified multiple loops, which represent uncertainty in the South-Eastern group. In the North-Western group, H107 was the most broadly distributed (8% of all sequences obtained) and the network suggested that the majority of haplotypes in this group were derived from this haplotype (Fig. 2.5). A single loop was identified in the North-Western group.

2.4.4 Phylogenetic analyses of *Diporeia*, *Monoporeia* and *Pontoporeia*

The generic data set consisted of 9 *Diporeia*, 2 *Monoporeia* and 6 *Pontoporeia* COI haplotypes as well as the outgroup *Gammarus lacustris*. Among the 17 ingroup sequences there were 229 variable positions, with 218 of those sites being informative using the parsimony criterion. The average nucleotide frequencies among all haplotypes was T:0.358, C: 0.198, A: 0.235 and G: 0.209, and the mean Ts/Tv was 5.363 (SE=2.082). The heterogeneity test revealed no disparities in nucleotide frequencies among sequences (homogeneity χ^2=28.817, d.f.=51, P>0.99). The program MODELTEST indicated that the data was best explained by the HKY+G model of sequence substitution with α = 0.2484 and Ts/Tv = 4.4564.

The NJ analysis identified all three genera as well supported monophyletic clades and placed the *Diporeia* and *Monoporeia* lineages closer to each other (Fig. 2.4). Two distinct lineages of *Pontoporeia femorata* were identified with an average nucleotide sequence divergence between haplotypes in the two groups of 17.64% (SE = 1.77%) (Table 2.2). Identical tree topologies were recovered by the ML and Bayesian analyses and all clades were well supported (Fig. 2.4).

2.4.5 Molecular clock analysis

The average sequence divergence between *Diporeia* and *Monoporeia* is 21.56% (SE = 1.95%) (Table 2.2) and the application of a molecular clock rate of 1.4% nucleotide sequence divergence per Myr (Knowlton and Weigt, 1998) suggests that they have been independently evolving for approximately 15.4 Myr. *Diporeia* and *Monoporeia* appear to have been

diverging from the *Pontoporeia* lineage for about 21.6 Myr. (average divergence of 30.26% (SE = 2.23%)).

2.5 Discussion

This study is the first genetic survey of the glacial relict *Diporeia hoyi* across of its range in North America. The phylogenetic analyses identified two well supported mitochondrial clusters or lineages, with an average nucleotide sequence divergence of 1.75% between haplotypes within the two groups (Fig. 2.2). The statistical parsimony analysis also resolved these two haplotype clusters, which were separated by 5 mutational steps (Fig. 2.5). Strong phylogeographical patterning of the two lineages was identified, and the only overlap between their distributions occurred where Lakes Huron and Superior connect (Table 2.1). A single ITS-1 haplotype was identified in *Diporeia* individuals sequenced from both mitochondrial clusters.

The generic level phylogenetic analyses of *Diporeia*, *Monoporeia* and *Pontoporeia* indicated that they all form well supported monophyletic lineages, and are strongly differentiated from one another (Fig. 2.4). *Diporeia* was revealed to be the sister taxa to the genus *Monoporeia*. The mitochondrial molecular clock calibration of 1.4% nucleotide sequence divergence per million years (Knowlton and Weigt, 1998) suggests that *Diporeia* and *Monoporeia* have been independently evolving for approximately 15.4 Myr, and that the two diverged from *Pontoporeia* 21.6 Myr. ago. Surprisingly, the phylogenetic analysis also revealed two markedly divergent *Pontoporeia femorata* lineages (Fig. 2.4), with an average nucleotide sequence divergence 17.64% between haplotypes within the two groups. Using

the same molecular clock calibration it is estimated that the two lineages have experienced 12.6 Myr of independent evolutionary history.

2.5.1 North American phylogeography

The average nucleotide sequence divergence between the two *Diporeia* lineages is 1.75%, suggesting that the two began to evolve independently prior to the last glacial maximum, 18,000 years ago. Haplotypes from the South-Eastern cluster (Fig. 2.2) were identified in the Great Lakes basin (excluding Lake Superior), the St. Lawrence/Gatineau region and the Finger Lakes (Fig. 2.1). This pattern is indicative of colonization from a Mississippian or Atlantic refuge.

Diporeia may have gained access to Lake Chicago or Lake Maumee from smaller lakes in the Mississippi refuge approximately 14,000 years B.P, and as the glaciers continued to retreat, dispersed into Lake Algonquin providing access to the upper Great Lakes basin (Dadswell, 1974). Lake Superior would have been prevented by being colonized from this refugia as the Superior Lobe of the Laurentide ice sheet was still covering the basin (Farrand and Drexler, 1985). *Diporeia* could have dispersed into Lake Iroquois (present day Lake Ontario) directly from Lake Erie, but individuals would have to get past Niagara Falls. The alternative rout is that *Diporeia* dispersed from Lake Algonquin, through the Fossmill outlet (part of the North Bay outlets) and into the Champlain sea (Dadswell, 1974). Once in the Champlain Sea, *Diporeia* could then disperse into the St. Lawrence/Gatineau region, Lake Vermont, Lake Iroquois and the Finger Lakes (Dadswell, 1974; Muller and Prest, 1985).

Diporeia's apparent intolerance to salt waters lead Bousfield (1989) to reject the idea that brackish waters such as the Champlain Sea could have acted as a dispersal mechanism, but a study showed that *Diporeia* can tolerate up to 20 grams of salt per liter of water for 28 days in cold temperatures before adverse effects began to occur (Gossiaux *et al.* 1992). This level of tolerance for brackish waters could have allowed *Diporeia* to disperse through the upper zones of estuaries where the salt concentration was relatively low.

Alternatively, lakes from the Atlantic region have been suggested as potential refugia for *Diporeia*, including Lake Albany and the Salamanca reentrant (Dadswell 1974). *Diporeia* could have entered Lake Vermont and the Champlain Sea from an Atlantic refugia and dispersed westward. However, *Diporeia* is only capable of active dispersion, and can not swim against strong currents. The fast moving waters of the North Bay outlets and the physical barrier presented by Niagara falls would have prevented *Diporeia* from dispersing into the upper Great Lakes from an Atlantic refuge.

The range of the North-Western group of haplotypes spans across the proglacial lake systems in Saskatchewan, Manitoba, Northwest Territories and Northern Ontario and Northern Québec. Lake Waterton, which is in the extreme south west of Alberta and Lake Washington along the West coast, also contains *Diporeia* populations belonging to the North-Western group. The North-Western group most likely dispersed from a Missourian refugia because populations of *Diporeia* have not been identified in the Beringian refugium. The presence of this lineage in Waterton Lake supports a Missourian source because the population is located near the southwestern glacial margin, which is located at a higher altitude that was only accessible from a southern dispersal route (Kontula and Väinölä,

2003). A significant portion of the lineages distribution correlates with the known extent of Lake Agàssiz, which *Diporeia* could have entered early on and dispersed northward into Lake McConnell, gaining access to Great Bear and Great Slave Lakes. *Diporeia* would have dispersed eastward into Northern Ontario and Northern Québec through a connection between Lake Agàssiz and Lakes Ojibway and Barlow. The corridor that connected Lakes Agàssiz and Ojibway also periodically flooded into Lake Nipigon and down to the Superior basin (Leverington and Teller, 2003). The identification of the *Diporeia* population in Green Lake Wisconsin belonging to the North-Western lineage was surprising because it is located in the Mississippi basin, east from Lake Michigan. *Diporeia* may have dispersed to this location through the channeling route of flood waters from Lake Agàssiz or Lake Superior (Teller *et al.*, 2005).

The most puzzling of all populations is the presence of the North-Western lineage in Lake Washington. The lake is situated outside the former proglacial lakes system and is the only known location west of the continental divide to contain *Diporeia*. It was initially thought that *Diporeia* gained access to Lake Washington by a marine route (Ricker, 1959). Segersträle (1971b) rejected this idea and contended that populations from the Missouri refuge could have gained access to the Fraser River system and then dispersed southward in pooling water along the margins of the Cordilleran ice sheet. This scenario can be rejected because there are no *Diporeia* populations along the proposed route. The alternative hypothesis was that they were transported by draining water from the proglacial lakes down the continental slope (Segersträle, 1971b).

Glacial Lake Missoula was an ice dammed lake situated to the south-west of present day Waterton Lake. The breaking of the ice dams resulted in multiple flash floods draining westward into Washington though the channeled Scablands (Shaw *et al*., 1999; Benito and O'Connor, 2003; Smith 2006). This could have provided a potential path for *Diporeia* to reach Lake Washington and establish a population.

The South-Eastern and North-Western lineages exhibited low levels of genetic divergence both within and among groups (average haplotype divergence between groups is 1.75%). These results are comparable to the low levels of genetic divergence detected in *Diporeia* using allozymes (Väinölä and Varvio, 1989). The low levels of intraspecific genetic divergence is congruent with pattern observed in many active dispersers, including the North American Glacial relicts *Limnocalanus macrurus* (Dooh *et al.,* 2006), *Mysis diluviana* (Väinölä, 1994; Audzijonytė, 2005; Dooh *et al.*, 2006), and *Myoxocephalus* thompsonii (Kontula and Väinölä, 2003).

2.5.2 *Diporeia* species

Eight morphotypes of *Diporeia* have been identified in North America and it has been suggested that they each represent distinct species (Bousfield, 1989). It is important to note that all the morphological distinguishing features of these "species" have only been identified in sexually mature males. The morphotypes *D. hoyi* (*D. h. filicornis*), *D.h. brevicornis* and *D h. erythrophthalma* were included in the phylogenetic analysis, which did not provide any evidence to support the existence of more than one *Diporeia* species.

In the previous chapter *D.h. filicornis* and *D.h. brevicornis* were analyzed and discussed in detail. The two morphotypes failed to meet the requirement for recognition as distinct phylogenetic species. The morphotype *D.h intermedia* was identified from single specimen collected in Great Bear Lake and Lake Cayuga (Segersträle, 1971a). Several of their secondary sexual characteristics morphologically resemble a mix between *brevicornis* and *filicornis* types. The most prominent feature is their antennae, which contain a similar number of segments as *filicornis*, but the segments are shorter, reducing the overall antennal length (Segersträle, 1971a). As previously suggested with *brevicornis* individuals, it is far more probable that these males failed to fully develop their secondary sexual traits, rather than represent a distinct species.

A single specimen was used to diagnose *D.h. kendalli* from Chamberlain Lake, Maine, USA (Norton 1909). However upon further investigation Segersträle (1937) concluded that the specimen in question was identical to *D.h. filicornis* and that he could not locate any of the features Norton (1909) claimed were present. Later sampling in Chamberlain Lake did not yield *Diporeia* leading to the conclusion that "*D.h. kendalli*" was mislabeled and was likely sampled from Lake Champlain instead (Segersträle 1971a; Dadswell 1974).

Lake Washington is the only known location of *Diporeia* west of the continental divide. This population was classified as a separate sub species by Waldron (1953) on the basis of morphological characteristics and was designated *Pontoporeia affinis erythrophthalma* (currently *Diporeia hoyi erythrophthalma*). These specimens possess a unique red colouring of the eyes and bifurcate spines on the basal lobe of the first maxilla. Caution is advised when taking this morphological data as evidence of a separate species because samples from

Lake Washington were compared to Finnish *Monoporeia affinis*, rather than North American specimens of *Diporeia* (Segersträle, 1977). Four unique haplotypes (H90-H93) were sequenced from this population that formed a monophyletic cluster in the phylogenetic analysis, but the clusters was poorly supported (Fig. 2.5). These haplotypes were distinguished by at least two mutational steps (Fig. 2.5) from H89. Since the colonization of Lake Washington was likely accompanied by profound population bottlenecks the effects of genetic drift could have been accelerated allowing faster fixation of mutations relative to other *Diporeia* populations.

2.5.3 Evolutionary divergence among genera

The results of this study indicate that *Diporeia*, *Monoporeia* and *Pontoporeia* are ancient lineages that began to diverge during the Miocene epoch, and all speculation of a Pleistocene origin of *Diporeia* should be rejected. The identification of the three genera as ancient genetic lineages reaffirms similar conclusions on their evolutionary history based on morphological and allozyme data (Bousfield, 1989; Väinölä and Varvio, 1989).

The relationships among the three genera have been debated over the last century and the current classification was established within the last two decades. *Monoporeia* was originally identified as *P. affinis* (Lindstrom 1855) and is distributed throughout the Holarctic, mainly occupying brackish lakes, estuaries and European marine-glacial lakes (Bousfield, 1989). *Diporeia*, first identified in Lake Superior (Smith, 1871), was classified as a North American version of the European *P. affinis*, but after more rigorous morphological comparisons it was designated to *P. hoyi* (Smith, 1874), although the name did not enter common use until over

a century later. The "species" *P. affinis* and *P. hoyi* were both considered to be close relatives of *P. femorata*, although there was considerable disagreement regarding there relationship.

It was initially hypothesized that the ancestor of *Diporeia* and *Monoporeia* diverged from *Pontoporeia* in arctic brackish-waters, and that the two genera invaded and adapted to continental waters independently (Lomakina 1952; Ricker 1959; Dadswell 1974). Segersträle (1977) initially agreed with this scenario, but later morphological comparisons lead him to suggest that both genera were directly derived from *P. femorata*. The phylogenetic analyses support the traditional hypothesis of a monophyletic relationship between *Diporeia* and *Monoporeia*, and that their common ancestor diverged from *Pontoporeia*.

Diporeia specimens were originally designated as *Pontoporeia* "*affinis*" because they were believed to be conspecific populations of European *P. affinis* (currently classified as *Monoporeia affinis*). When the North American freshwater specimens were reclassified as *Diporeia*, distinct populations that inhabit the coastal waters in Eastern Canada maintained the name *P. "affinis"*. These populations morphologically resemble European *M. affinis* (Bousfield, 1958; Bousfield 1989) and a molecular analyses of the two revealed that they are closely related (Väinölä and Varvio, 1989). However, the genetic divergence among them is strong and the authors concluded that they warranted recognition as independent species. North American populations of *P. "affinis"* should be formally renamed to reflect its phylogeny and relationship with European *M. affinis*.

The most surprising result of this study was the identification of two highly divergent genetic lineages within *P. femorata* (COI sequence divergence = 17.64%). The lineages

formed well supported monophyletic clusters and meet the criteria for classification as independent species under the phylogenetic species concept. In addition, the genetic divergence detected between the lineages is comparable to the level of variation observed among the mitochondrial DNA of established crustacean species (Witt and Hebert 2000; Lefébure *et al.,* 2006) and approaches the observed degree of divergence among *Diporeia* and *Monoporeia* (Table 2.2). Implementing the molecular clock calibrations, the two lineages have been independently evolving for an estimated 12.6 Myr. Evolutionary processes of this time can maintain or select similar phenotypes, resulting in morphologically cryptic species (Bickford *et al*., 2006).The identification of cryptic species within the order Amphipoda is common (Witt and Hebert, 2000; Gervasio *et al*., 2004; Hogg *et al.*, 2006 Witt *et al.*, 2006; Murphy *et al.*, 2009) and the high prevalence of morphological similarities among these taxa supports the use of phylogenetic tools for species identification. North American *Pontoporeia femorata* represent a species complex and I recommend further molecular investigation of the genus *Pontoporeia* throughout its Holarctic distribution to adequately describe the diversity within the genus.

The notion that the North American glacial marine relicts diverged from their arctic marine relatives, invaded and dispersed across the continent in unison during the Pleistocene glaciations must be disregarded (Ricker, 1959; Dadswell, 1974; Segersträle, 1977). The relicts are a diverse group of organisms, each with a unique evolutionary history. *Diporeia* is an ancient lineage that has likely inhabited freshwater environments for millions of years, similar to the pattern observed in *M. diluviana* (Audzijonytė and Väinölä, 2005; Dooh *et al.*, 2006). These results are more congruent with Holmiquist's views of the relicts being ancient,

and stable freshwater species (Holmiquist, 1959, 1970). This is in stark contrast to the recent Pleistocene origin revealed in *L. macrurus* (Dooh *et al.,* 2006) and *M. thompsonii* (Kontula and Väinölä, 2003), which have recently diverged from their marine counterparts.

2.6 Conclusions

This genetic investigation contributes to our knowledge on the influence of the Pleistocene glaciations on actively dispersed species. The proglacial lakes systems have played a substantial role in the dispersal and genetic distribution of the North American glacial marine relicts. Colonization of the proglacial lakes has resulted in their co-distribution over a large geographical range and low levels of genetic divergence within each species. This study underscores the important roles that the refugia have played, as isolation and dispersal from different refugia have resulted in the incongruent phylogeographical distributions of the relicts. Molecular analyses also indicate that the relicts possess an array of divergence times from their marine relatives, ranging from the Miocene up until the Pleistocene. Despite their similar geographic distributions, the relics do not share evolutionary and phylogeographic histories. The identification of *Pontoporeia femorata* as a cryptic species complex reveals the underestimated level genetic diversity present within arctic marine populations. Future investigations on the speciation events that resulted in the formation of the marine cryptic lineages may provide more insight into the factors that resulted in the various phylogenetic ages of the relicts.

Figure 2.1. Map of North America indicating all sample locations for *Diporeia*, *Monoporeia affinis* and *Pontoporeia femorata*. Names of *Diporeia* sample locations follow Table 1.

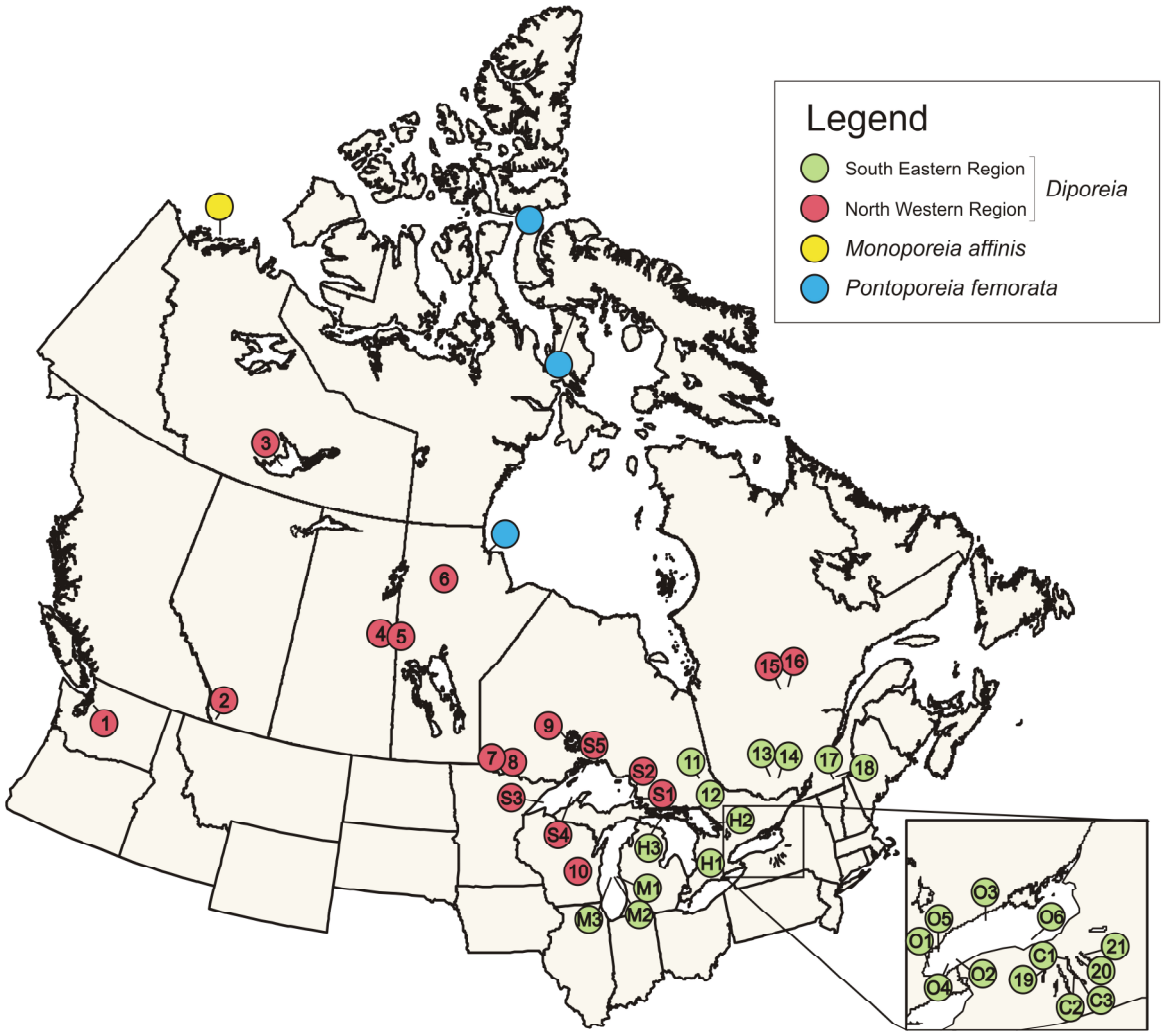
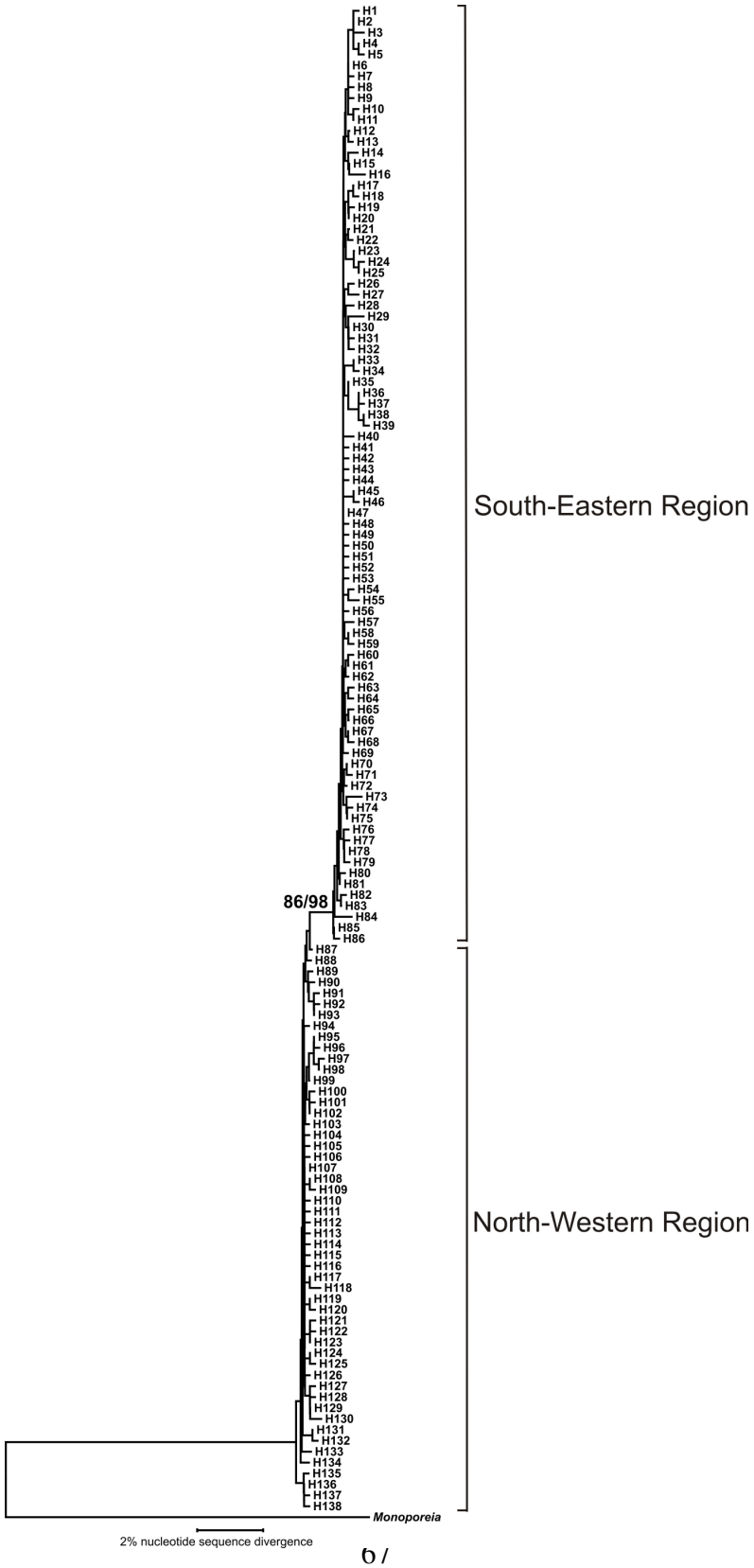


Figure 2.2. NJ phenogram showing the relationship of all 138 cytochrome *c* oxidase I (COI) haplotypes. Numbers above the node indicate NJ bootstrap and interior branch test percentages respectively.


H1
H2
H3
H4
H5
H6
H7
H8
H9
H10
H11
H12
H13
H14
H15
H16
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86/98
H83
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H123
H124
H125
H126
H127
H128
H129
H130
H131
H132
H133
H134
H135
H136
H137
H138
Monoporeia
2% nucleotide sequence divergence
South-Eastern Region
North-Western Region

Figure 2.3. NJ phenogram showing the relationships among 55 cytochrome *c* oxidase I (COI) haplotypes. Numbers above the nodes represent NJ bootstrap percentages, interior branch test percentages, ML bootstrap percentages and Bayesian consensus values.

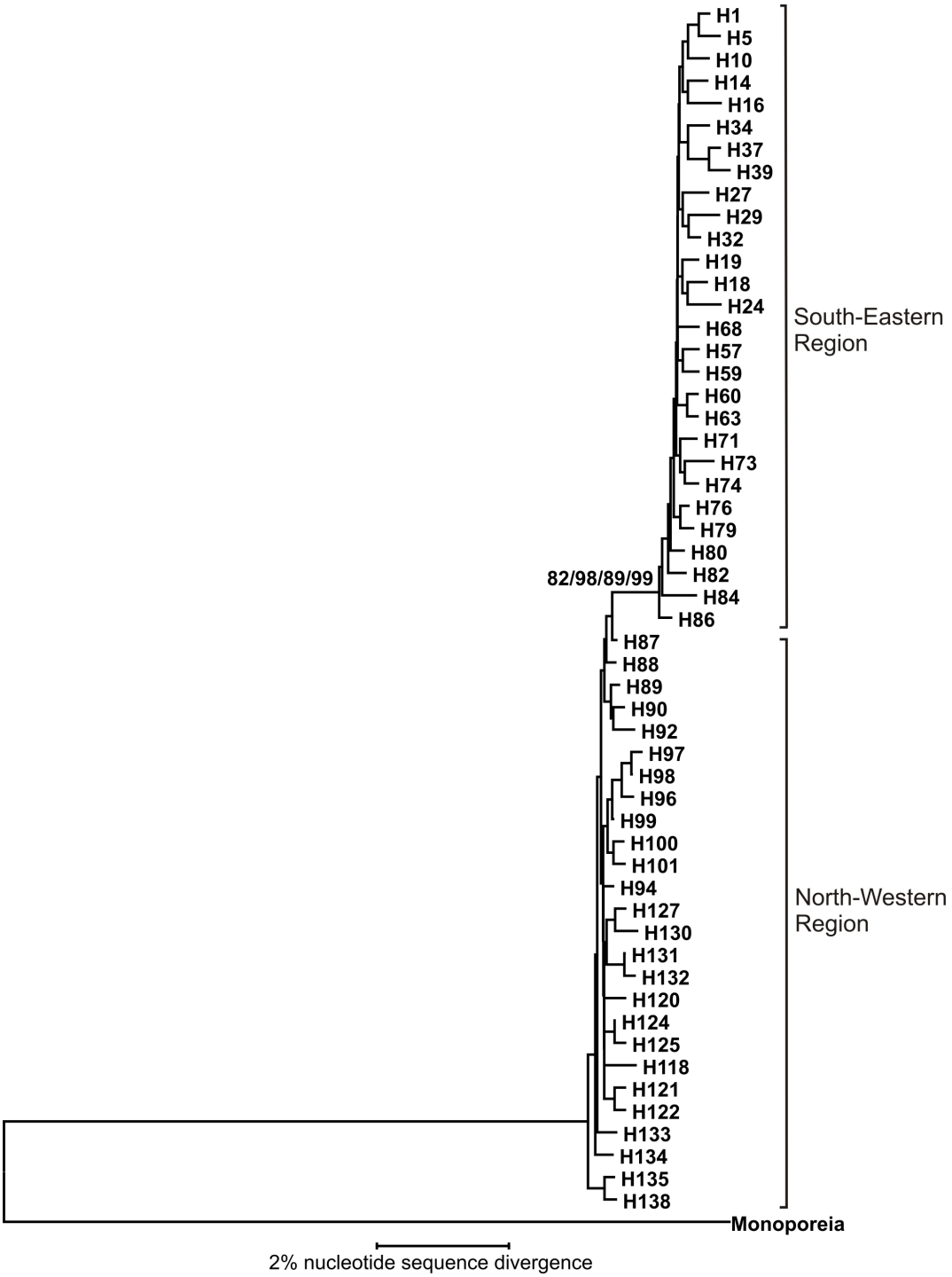

Figure 2.4. NJ phenogram showing the relationships among *Diporeia*, *Monoporeia* and *Pontoporeia* COI haplotypes. Values above the nodes represent NJ bootstrap percentages, interior branch test percentages, ML bootstrap percentages and Bayesian consensus values.

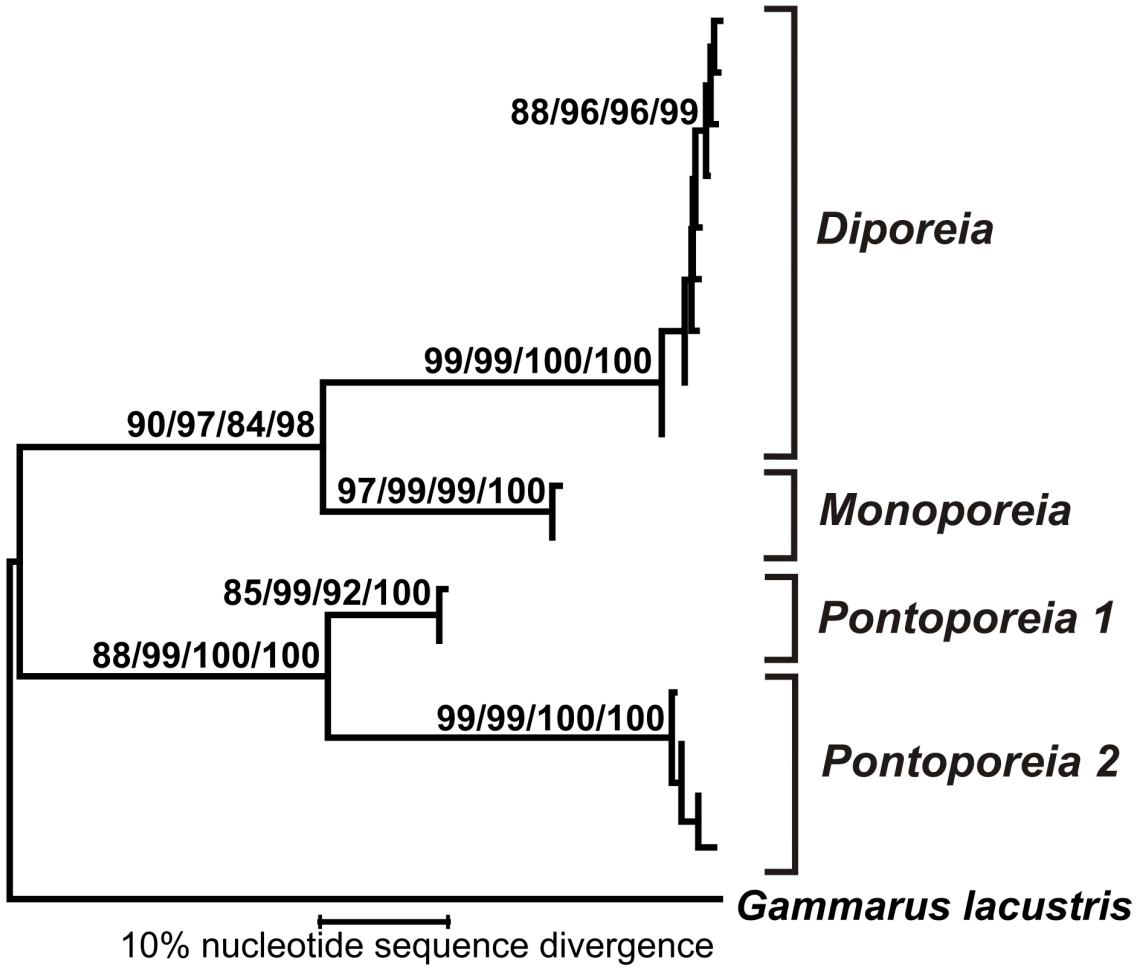

Figure 2.5. Statistical parsimony network showing the relationships among the 138 COI haplotypes. Solid circles represent a single mutational step.

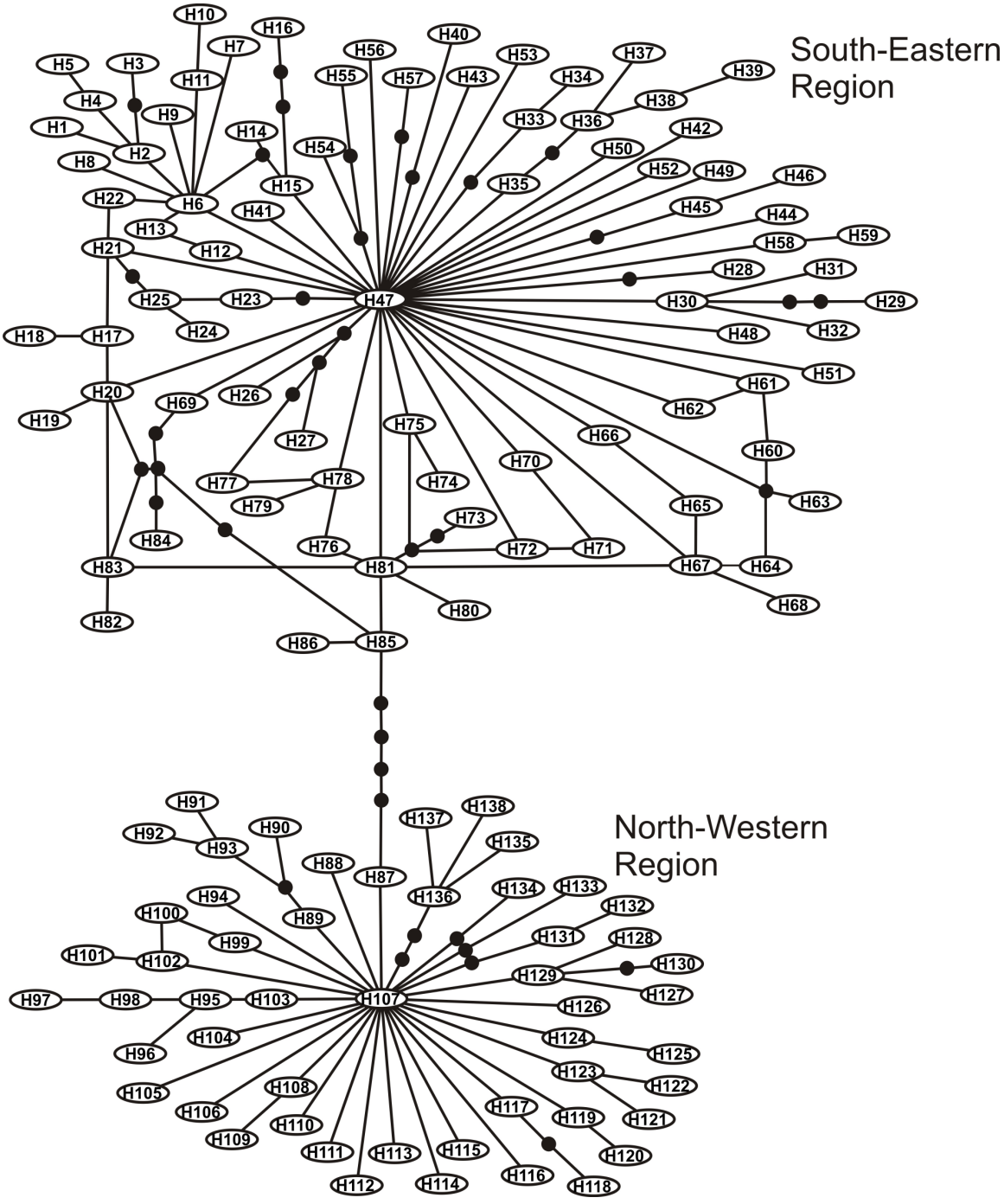

Table 2.1. Population, sample size (n) and haplotype presence (H) for the 401 sequenced *Diporeia* mitochondrial cytochrome *c* oxidase I gene. Haplotype designations follow that of Fig. 2.2, and the number in parentheses beside the haplotype indicates the frequency of its occurrence.

	Sample Location	N	H									
1	Lake Washington	7	H90(1)	H91(1)	H92(2)	H93(3)						
2	Waterton Lake	6	H117(2)	H118(4)								
3	Great Slave Lake	5	H103(3)	H104(1)	H107(1)							
4	Amisk Lake	9	H100(1)	H101(2)	H102(6)							
5	Lake Athapapuskow	8	H107(1)	H127(1)	H129(6)							
6	Southern Indian Lake	5	H99(4)	H107(1)								
7	Lake of the Woods	7	H89(1)	H124(5)	H125(1)							
8	Rainy Lake	7	H107(1)	H128(1)	H129(2)	H130(1)	H134(2)					
9	Lake Nipigon	4	H107(1)	H112(2)	H122(1)							
10	Green Lake	7	H88(2)	H111(5)								
11	Lake Temagami	5	H41(1)	H47(4)								
12	Lake Joseph	7	H45(5)	H46(1)	H57(1)							
13	Blue Sea Lake	6	H47(1)	H52(1)	H82(1)	H83(3)						
14	Lake Pemichangan	4	H85(1)	H86(3)								
15	Lake Gilman	6	H87(5)	H107(1)								
16	Lake Chibougamau	2	H121(1)	H123(1)								
17	Lake Memphremagog	7	H31(2)	H47(4)	H63(1)							
18	Lake Massiwippi	5	H17(1)	H18(1)	H19(1)	H20(2)						
19	Lake Canandaigua	6	H71(3)	H72(2)	H73(1)							
20	Owasco Lake	5	H47(3)	H50(1)	H53(1)							
21	Skeneateles Lake	5	H59(4)	H67(1)								
C1	Cayuga, site 1	2	H47(1)	H56(1)								
C2	Cayuga, Long pt.	4	H47(3)	H69(1)								
C3	Cayuga, Myers pt.	3	H47(1)	H59(1)	H84(1)							
H1	Huron, Goderich	15	H6(3)	H15(1)	H34(1)	H35(2)	H47(5)	H107(3)				
H2	Huron, Hope Bay	15	H6(1)	H36(4)	H37(1)	H38(5)	H39(1)	H47(1)	H49(2)			
H3	Huron, North Channel	16	H6(8)	H7(1)	H8(1)	H9(1)	H14(1)	H22(2)	H33(2)			
M1	Michigan, site 1	15	H2(2)	H4(1)	H30(2)	H32(1)	H47(1)	H55(1)	H65(1)	H67(4)	H68(1)	H70(1)

M2	Michigan, site 2	15	H2(7)	H5(1)	H29(1)	H47(3)	H48(1)	H64(1)	H67(1)						
M3	Michigan, site 3	15	H1(1)	H2(7)	H3(1)	H4(1)	H47(3)	H54(1)	H67(1)						
O1	Ontario, 50pt	15	H6(2)	H23(1)	H47(6)	H51(1)	H58(1)	H75(2)	H78(1)	H79(1)					
O2	Ontario, site 2	15	H11(1)	H12(1)	H23(1)	H26(1)	H43(1)	H44(1)	H47(4)	H62(1)	H75(1)	H78(2)			
O3	Ontario, Cobourg	15	H6(1)	H11(1)	H13(1)	H21(1)	H24(1)	H25(4)	H40(1)	H41(1)	H47(1)	H75(1)	H76(1)	H78(1)	
O4	Ontario, Niagara on the	15	H6(1)	H10(1)	H11(2)	H16(1)	H23(1)	H27(1)	H47(4)	H61(1)	H70(1)	H81(2)			
O5	Ontario, Port Credit	15	H6(1)	H11(3)	H23(1)	H41(1)	H47(2)	H66(1)	H74(1)	H75(1)	H77(1)	H78(2)	H81(1)		
O6	Ontario, station 41	15	H6(5)	H25(1)	H42(1)	H44(1)	H47(4)	H60(1)	H61(1)	H80(1)					
S1	Superior, Pancake pt.	15	H28(1)	H95(5)	H98(2)	H107(4)	H115(1)	H131(1)	H132(1)						
S2	Superior, station 2	15	H95(2)	H97(1)	H98(3)	H107(7)	H110(1)	H116(1)							
S3	Superior, station 164	15	H105(1)	H107(8)	H119(3)	H137(3)									
S4	Superior, station 221	15	H108(1)	H109(1)	H107(2)	H113(2)	H114(1)	H119(2)	H120(1)	H126(1)	H135(1)	H136(1)	H138(2)		
S5	Superior, *D.h filicornis*	10	H95(7)	H96(1)	H107(1)	H133(1)									
S5	Superior, *D.h brevicornis*	9	H98(4)	H94(2)	H106(2)	H107(1)									

Table 2.2. Mean genetic distances for the COI gene between the genera *Diporeia*, *Monoporeia* and *Pontoporeia*. Groups correspond with lineages identified in Fig. 4 and sample size is beside the group name in parentheses. Distances are presented in percent values and standard errors are in parentheses.

	Diporeia	*Monoporeia*	*Pontoporeia* 1	*Pontoporeia* 2
Diporeia (9)	_			
Monoporeia (2)	21.56 (1.95)	_		
Pontoporeia 1 (2)	28.99 (2.55)	26.12 (2.37)	_	
Pontoporeia 2 (4)	31.72 (2.71)	28.57 (2.48)	17.61 (2.00)	_

Chapter 3 – Phylogeography of *Gammarus lacustris* in North America: investigating the effects of dispersal abilities

3.1 Overview

Previous phylogeographic studies have highlighted the impacts of active and passive dispersal strategies on the genetic distribution of aquatic organisms. The discovery of marked genetic divergences within passively dispersed species, despite their strong potential for gene flow between populations, came as a surprise to phylogeographers. In this study a phylogeographic analysis of the passively dispersed amphipod species *Gammarus lacustris* was conducted over a portion of its North American range using the mitochondrial cytochrome *c* oxidase I (COI) gene. The results of this study show that North American *G. lacustris* contain six identifiable haplotype groups with an average nucleotide sequence divergence of 1.52%. A similar level of genetic divergence was detected between haplotypes in two refugial groups of actively dispersed *Diporeia hoyi* (average 1.8% mtDNA divergence). The low sequence divergences detected within North American *G. lacustris* are not congruent with the pattern observed in other passively dispersed species and is likely the result of the amphipods recent invasion into North America from Asia populations.

3.2 Introduction

After the retreat of the Pleistocene glaciers, new aquatic habitats were available for colonization across the northern hemisphere. Migrants that initially gained access to these aquatic environments did not have to compete with locally adapted individuals and could easily establish new populations. The distribution of genetic lineages within species

occupying formally glaciated regions will reflect their postglacial dispersal patterns. Comparisons between the genetic structuring within a species and its dispersal capabilities provides an opportunity to examine the impacts of different dispersal strategies on phylogeographical patterns. There are two modes of dispersal for aquatic organisms, active and passive. Active dispersers can only colonize new habitats via connected waterways, while passive dispersers are capable of long distance colonization through wind, animal or avian vectors. It was initially hypothesized that populations within actively dispersing species would exhibit higher levels of genetic divergence in comparison to passive dispersers because of the lack of geneflow between distant populations. Phylogeographical studies however, have revealed that active dispersers in previously glaciated regions of the northern hemisphere generally exhibit the opposite pattern, with low levels of intraspecific divergence (Kontula and Väinölä 2003; Audzijonytė and Väinölä, 2006; Dooh *et al.,* 2006; Barrette *et al*., 2009). This was surprising as the lack of gene flow between isolated populations would be conducive to allopatric divergence. The wide distributions and low genetic variation within many of these organisms have been attributed to their dispersal via glacial melt waters from a single or a few isolated refugial populations.

In the previous chapter it was revealed that the actively dispersing amphipod *Diporeia hoyi* colonized North America from two major refugia, and exhibits low levels of genetic variation (average 1.8% mtDNA divergence) between phylogroups despite weak dispersal abilities. This is attributed to *D. hoyi's* strict reliance on the proglacial lakes system as dispersal routes across North America (Dadswell, 1974).

Passively dispersing species were originally hypothesized to exhibit low levels of genetic divergence between distant populations. The migration of individuals was expected to slow or counteract evolutionary forces, such as selection and genetic drift that would cause populations to genetically diverge. Again, biogeographers were surprised to discover that these populations show marked genetic structuring and divergence (De Meester *et al.,* 2002; Gomez *et al*., 2002; Figuerola *et al.,* 2005; Ketmaier *et al.,* 2008; Adamowicz *et al.,* 2009). The inconsistency between the results of the phylogeographical studies and the expected genetic pattern exhibited by passive and active dispersers indicated a lack in understanding regarding the impact of dispersal capabilities on population divergence. This resulted in the re-evaluation of the mechanisms influencing the distribution and colonization patterns of species (Bilton *et al.,* 2001; De Meester *et al.,* 2002; Gomez, 2002; Bohonak and Jenkins, 2003).

Gammarus lacustris is a passively dispersed amphipod species that inhabits the shallow vegetated areas of fresh water lakes, rivers and streams, and possesses strong long distance dispersal capabilities, including avian mediated migration (Segerstråle, 1954; Vainio and Väinölä, 2003). It has been recorded in part of northern Asia, including China and Siberia, North America, extending as far south as New Mexico and California, and has been identified in discontinuous populations across Europe. Populations of *G. lacustris* on all three continents have been subject to multiple displacement and re-colonization events due to the Pleistocene glaciations.

Like all other amphipods, *G. lacustris* releases live young and they do not possess a diapause stage that is observed in many crustaceans, which would allow them to withstand

desiccation and transport (Fryer, 1996; Hairston and Caceres, 1996; Caceres, 1998; Butorina, 2008). It is via this resting stage that the majority of passively dispersed crustaceans migrate to new habitats by latching onto birds, animals or other aquatic vertebrates (Bilton *et al.,* 2001; Slusarczyk and Pietrzak, 2008). Aquatic amphipods are directly exposed to fluctuating temperatures and dry environments when they utilize out of water dispersal mechanisms, and as a result many are restricted to active dispersal. *Gammarus lacustris* has been observed to latch onto the feathers of waterfowl, and it has been estimated that they may be able to tolerate travel distances of up to 120-140km per flight (Segerstråle, 1954). Avian mediated passive dispersal provides *G. lacustris* with the ability to colonize new habitats quickly, which likely contributed to its extensive distribution. It also provides an opportunity for geneflow between distant populations.

Currently, there are two published phylogenetic studies (Meyran *et al.,* 1997; Hou *et al.,* 2007) and one phylogeographical paper (Vainio and Väinölä, 2003) concerning *G. lacustris*. The phylogeographic study was conducted in Northern Europe and identified marked allozyme allele frequency differences between Eastern and Western populations of *G. lacustris* (Vainio and Väinölä, 2003). These two lineages were revealed to have an average genetic distance (Nei's distance) of 0.3, which is attributed to isolation and dispersal from separate refugia during the last Pleistocene glaciation. Populations within each of these lineages also exhibited strong genetic divergences, with genetic distances between them estimated as high as 0.12 (Vainio and Väinölä, 2003).

The high genetic distances estimated between the European populations and refugial lineages indicate that little or no gene flow is occurring. Often new habitats are colonized by

only a few individuals creating a strong founder effect within newly formed populations (Boileau *et al*., 1992). The monopolization hypothesis postulates that once a population is established, new immigrants have a reduced prospect of survival as they are competing with locally adapted individuals and there is a high probability that their alleles will be lost in a few generations due to drift (De Meester *et al.,* 2002). This could account for the apparent lack of gene flow observed in *G. lacustris* and other passively dispersing species.

Two sub species of *G. lacustris* have been identified in North America. The first, *Gammarus. l. lacustris,* spans the majority of North America from Baffin Island, to Alaska, the Rocky Mountains, and south to New Mexico and California. Eastern Canada contains *G.l. limnaeus*, which is confined to the St. Lawrence drainage basin and parts of western Ontario to eastern Québec (Bousfield, 1958; Holsinger, 1972). Breeding experiments on these two sub-species demonstrated that hybridization between the two is possible, though there was a reduction in offspring vitality (Hynes and Harper, 1972). The two sub species were distinguished on the basis of morphological differences, but it has been shown that morphology is unreliable for taxonomic classification in the genus *Gammarus* (Meyran *et al.*, 1997; Müller, 2000; Hou *et al.,* 2007).

This study investigates the phylogeographical distribution of *G. lacustris* in North America using the mitochondrial cytochrome *c* oxidase (COI) gene. The rapid evolution of the mitochondrial genome makes it an excellent tool for the detection of recent evolutionary events (Brown, 1979; Brown, 1982; Gissi *et al.*, 2008). In this study I test the hypothesis that the passively dispersing amphipod *G. lacustris* will possess a greater level of genetic divergence between lineages in comparison to that observed in the amphipod *Diporeia hoyi*,

which is restricted to active dispersal. Unlike *D. hoyi*, which is only distributed in the former proglacial lakes basins (Dadswell, 1974), *G. lacustris* has been identified across formerly glaciated regions and its range extends into the Southern United States which were unglaciated. These unglaciated regions are expected to contain greater levels of genetic variation than populations of *G. lacustris* located within formally glaciated regions. Haplotypes within glaciated regions are also expected to show a greater levels of divergence than that which was observed in *D. hoyi*.

3.3 Materials and Methods

3.3.1 Collections

Gammarus lacustris specimens were collected between 2005 and 2008 from a total of 52 ponds, lakes and streams within Canada and the United States (Fig. 3.1). Samples were collected using a dip net and stored in 95% ethanol for later analyses. A single specimen of *Gammarus pseudolimneaus* was obtained for use as an outgroup in the phylogenetic analysis.

3.3.2 DNA sequence analyses

Total DNA was extracted from 3-5 *G. lacustris* individuals from each population by grinding a leg in 50 µL of proteinase K extraction buffer (Schwenk, 1996). A 654 base pair fragment of the mitochondrial cytochrome *c* oxidase I (COI) gene was amplified using the primers COIcrustDF1 and HCO2198 (Folmer *et al.,* 1994). The 50 µL PCR reactions contained 2.0-3.0 µL of DNA template, 5.0 µL 10x PCR buffer, 0.2 mM of each primer, 0.2 mM of each dNTP and 0.5 unit of Taq polymerase. The PCR conditions consisted of 1 min at 94°C followed by 5 cycles of 1 min at 94°C, 90 s at 45°C, 1 min at 72°C; followed by 35 cycles of

1 min at 94°C, 90 s at 51°C, 1 min at 72°C; followed by 5 min at 72°C. PCR products were gel purified using the Qiaex kit (Qiagen Inc.) and sequenced using the ABI prism BigDye terminator sequencing kit (30 cycles, annealing at 55C). Products were sequenced in one direction using primer COIcrustDF1 and electrophoresis was carried out on an ABI 3730 automated sequencer (Applied Biosystems). The sequences were aligned by eye in MEGA 4.1 (Tamura *et al.*, 2007).

All haplotypes were identified by constructing a distance tree using the unweighted pair group method with arithmetic averages (UPGMA) using a matrix of nucleotide differences between all pairwise combinations of sequences in MEGA 4.1. Nucleotide composition, nucleotide (p-distance) distance matrices, mean pairwise transition/transversion ratios and amino acid translations (invertebrate mitochondrial code) for all mitochondrial COI haplotypes were also calculated in MEGA 4.1.

3.3.3 Phylogenetic analyses

A phylogenetic analysis was conducted on all *G. lacustris* COI haplotypes identified using the Neighbour-Joining (NJ) (Saitou and Nei, 1987) distance method and the Jukes and Cantor (JC) (Jukes and Cantor, 1969) model of nucleotide substitutions in MEGA 4.1. Confidence in the NJ analyses was assessed using the bootstrap method and interior branch test with 1000 replicates each. A North American specimen of *Gammarus pseudolimnaeus* was used in the analysis as an outgroup.

Since the relationships among haplotypes in phylogenetic trees are assumed to have a bifurcating branching pattern, most phylogenetic methods do not accommodate the presence

of ancestral haplotypes in the data set. As a result, the evolutionary relationships among closely related sequences are better resolved using evolutionary networks as opposed to phylogenetic trees (Posada and Crandall, 2001; Morrison, 2005). A statistical parsimony network with 95% connection limits was constructed with all 57 *G. lacustris* COI haplotypes using the program TCS version 1.21 (Clement *et al.* 2000).

3.4 Results

3.4.1 Collections

Gammarus lacustris specimens were obtained from shallow, vegetated portions of the shoreline at all 52 locations.

3.4.2 DNA sequence analyses

A total of 111 *G. lacustris* COI sequences were obtained and 54 haplotypes were identified among them. The final alignment of the sequences was 654 base pairs in length and there were 67 variable positions among them, 44 of these positions being phylogenetically informative using the parsimony criterion. The average nucleotide frequencies among all 54 haplotypes is T: 0.34, C: 0.22, A: 0.23 and G: 0.21, and the mean pairwise haplotype sequence divergence is 1.41 % (SE=0.23%). The overall mean pairwise transition/transversion ratio was 5.894 (SE =1.471). Amino acid sequence translations were unambiguous, and no gaps or nonsense codons were detected in the data set.

3.4.3 Phylogenetic analyses

The NJ analysis of all 57 haplotypes revealed low levels of genetic variation among the sequences within *G. lacustris* in North America. The average mitochondrial nucleotide sequence divergence between all *G. lacustris* haplotypes is 1.41% (SE=0.23%) with values ranging from 0.15% to 2.65%. The haplotypes in the NJ analysis can be divided into 6 groups based on their clustering and the geographical distribution of the haplotypes. All haplotype groups formed monophyletic clusters with the exception of group F_1, which is paraphyletic with respect to clusters A to E. Only haplotype groups A, B_1, C_1 and D have high bootstrap support, but all groups were revealed to have strong interior branch test values (Fig. 3.2). The average nucleotide sequence divergence within and between each group is estimated as 0.58% (SE = 0.17%) and 1.52% (SE = 0.37%) respectively.

Group A was only identified in the Southern USA, including California, Nevada and Oregon. Haplotypes in Group E were also restricted to the USA and only identified in Idaho, which also contained H15, the only sequence belonging to group B_2 (Table 3.1). Two haplotypes from cluster C_2 were sequenced from California and Nevada, while the remaining haplotypes were identified in Inuvik, the Northwest Territories. British Columbia contained both B_1 and C_1 haplotypes. The largest group of haplotypes, group F_1, is distributed across western North America. Arizona, Utah and Idaho all contained F_1 haplotypes, and their range extended into Alberta and Saskatchewan. The distribution of F_2 haplotypes is concentrated in the northern Canadian prairie provinces, and had some range overlap with the F_1 group in Saskatchewan and Alberta (Table 3.1). Haplotypes belonging to groups F_1 and F_2 accounted

for 56% of all sequences obtained. Samples collected in Northern Québec only contained haplotypes belonging to group D and were not identified at any other location.

All 6 haplotype groups identified in the NJ analysis (Fig. 3.2) were resolved in the network analysis (Fig. 3.3). The haplotype network revealed that groups A, B, C_1 and C_2 diverged from a singe ancestral haplotype and would be better represented with a polytomous relationship rather than the bifurcating pattern imposed by the NJ analysis. A polytomous relationship was also identified for the divergence between groups A-C, D, E and F. The multifurcating relationships account for the low bootstrap support observed for these groups in the NJ analysis, which assumes each divergent event is bifurcating in nature. The network indicates that haplotypes within each group are closely related, often separated by one or two mutational steps. A single loop, indicating uncertainty, was identified in the network between haplotypes 8, 9 and 11.

3.5 Discussion

This study revealed low levels of genetic diversity within North American populations of *Gammarus lacustris*, with an average mitochondrial haplotype sequence divergence of 1.41%. Six groups of haplotypes were identified in the phylogenetic analysis (Fig. 3.2), but not all of the groups showed strong support. The haplotype groups were also observed in the statistical parsimony network and several of them exhibit polytomous relationships (Fig. 3.3). The average nucleotide sequence divergence between haplotypes in each of the six groups is estimated at 1.52%.

The geographical distributions of the haplotype groups overlap at several locations, with the exception of group D identified in Québec (Fig. 3.1). The south-western United States was revealed to contain the greatest level of genetic diversity, with populations containing haplotypes belonging to five different groups (Table 3.1).

3.5.1 Phylogeography of *Gammarus lacustris*

The distribution of the F_1 haplotypes extends into the southern USA, expanding up into the Canadian prairies where it overlaps with the range of the F_2 group in Alberta and Saskatchewan. Populations containing F_1 haplotypes in Arizona, Idaho and Utah may have acted as a refuge during the Pleistocene, dispersing northward from one or more of these locations into the Prairie Provinces during the last glacial retreat. However, there were 5 haplotype groups identified in the USA, but the F_2 group was the only one that appeared to have dispersed northward into previously glaciated regions.

Group F_1 was only identified in populations in Northern Manitoba, Saskatchewan, Alberta and British Columbia (Fig. 3.1). The absence of F_1 haplotypes from southern populations suggests that these individuals likely colonized the area from unglaciated regions in the north. There currently are insufficient data to determine the origin of the F_1 group and additional samples from the North West Territories, Yukon and Alaska are required to identify where they have dispersed from.

Groups B and C were located in a few isolated locations in formally glaciated regions. Haplotypes belonging to B_1 were only identified in southern British Columbia (H12 and H14) and in central Manitoba. This group is most closely related to B_2, which was only

identified in Idaho. However, the two exhibited a strong level of genetic divergence between them (Fig. 3.3), indicating that B_2 is not a likely source for the recently established B_1 populations and the region from which the B_1 group dispersed can not be currently determined.

Haplotypes from group C_2 were located in both Canada and the USA. However, the network analysis indicates that the two C_2 haplotypes identified in California and Nevada (H19 and H22) are derived from H20 and H21 (Fig. 3.3), which was located in Inuvik, Northwest Territories. This suggests that the group may have first diverged in the north and later dispersed south into the USA. Populations containing H20 and H21 are located near the north-western boundary of the Laurentide ice sheet and were potentially colonized by *G. lacustris* from a northern refuge.

Populations in Québec contained four unique haplotypes (group D) that were not identified in any other location. These haplotypes however, showed only limited divergence from the remaining *G. lacustris* sampled in North America, and they were separated from each other by single mutational steps (Fig. 3.3). Québec *G. lacustris* likely dispersed Northward during the last glacial retreat from a southern region, perhaps from the Mississippian or Atlantic refugia, rather than from the west, which did not possess group D haplotypes. To understand how this group dispersed into Québec will require additional sampling in Eastern Canada and the United States.

Although the ranges of haplotype groups did overlap, populations that were sampled more than once contained haplotypes from a single group, with the exception of three populations

in the former Lake Agàssiz basin in Alberta. If *G. lacustris* dispersed from more than one refugia, or if multiple haplotype groups gained access to the proglacial lakes, then populations could contain a mix of lineages, which may be the case with populations in the Lake Agàssiz basin.

The low level of intraspecific genetic variation identified in *G. lacustris* is surprising and contradicts the observed pattern of marked genetic structuring and divergent lineages in other passively dispersed species (Taylor *et al.,* 1998; Witt and Hebert, 2000; Cox and Hebert, 2001; Gomez *et al*., 2002; Adamowicz *et al.,* 2009). Investigations into the passively dispersed amphipod *Hyalella azteca* revealed the presence of seven distinct lineages within glaciated North America, representing a morphologically cryptic species (Witt and Hebert, 2000). The mitochondrial sequence divergence between these lineages ranged from 8.5% to 27.5%. The phylogeographic distribution of each *H. azteca* species within glaciated North America varied, suggesting each lineage was independently isolated and dispersed from different refugia during the last glacial retreat (Witt and Hebert, 2000). A substantial portion of the diversity within the *Hyalella* genus is restricted to the southern Great Basin, which contains an extraordinary level of cryptic diversity and endemism (Witt *et al*., 2003; Witt *et al*., 2006).

Gammarus lacustris has strong dispersal capabilities which have resulted in its holarctic distribution. A phylogenetic analysis of the genus *Gammarus* has identified Asia as the most probable origin for *G. lacustris* based on mitochondrial and nuclear DNA (Hou *et al.*, 2007). The shallow level of sequence divergence identified in North American *G. lacustris* is likely a result of its recent evolutionary and dispersal history. The phylogeny of the genus

Gammarus revealed that North American *G. lacustris* formed a monophyletic cluster nestled within the Asian specimens. Examination of the *G. lacustris* sequences presented by Hou *et al.* (2007) revealed an average of 1.8% nucleotide sequence divergence between all haplotypes and 2.5% between the North American and Asian haplotypes. The formation of a monophyletic cluster and low molecular divergence observed in North American *G. lacustris* suggests a single, recent invasion. *Gammarus lacustris* likely invaded North America by traveling through Siberia, across the Beringian straight and into present day Alaska, a rout which has been established numerous times (Rohling *et al*., 1998; Hewitt, 2004; Lo *et al.*, 2009). This study did not contain any *G. lacustris* sample from unglaciated regions of Alaska, but if North America was invaded through the Beringian straight, this region will likely possess greater levels of variation.

In contrast with the low genetic divergence detected in North America, a study of *G. lacustris* in Northern Europe using allozyme data revealed high levels of genetic variation between populations (genetic distance as high as 0.12) (Vainio and Väinölä, 2003). Two distinct races were identified and the level of molecular divergences between them suggested that they may have been independently evolving for up to 3 million years (Vainio and Väinölä, 2003). These authors also refer to unpublished mitochondrial sequences showing 6% nucleotide sequence divergence between the two lineages, and suggested that the Eastern race was descended from Asian populations (Vainio and Väinölä, 2003). There appears to be a greater level of genetic divergence between European and Asian populations, indicating that *G. lacustris* invaded Europe before it invaded North America. If *G. lacustris* invaded Europe first, European populations would have had more time to diverge then North

American populations, and would explain the discrepancies in the level of molecular variation observed within the two continents. When the entire range of *G. lacustris* is taken into consideration, it is apparent that the passively dispersed amphipod does possess some markedly divergent lineages.

3.5.2 Comparative phylogeography

The low level of genetic variation observed between haplotypes in *G. lacustris* was initially surprising (average nucleotide sequence divergence of 1.41%) and is not significantly higher than that identified in the actively dispersing species *Diporeia hoyi* (1.15%, see chapter 2). The two amphipods appear to have similar levels of genetic divergence, but upon closer examination it emerges that they differ in their distributions.

Diporeia, unlike *G. lacustris*, is not capable of passive dispersal and can only colonize new habitats through connecting waterways. This resulted in the geographic distribution of *Diporeia* being restricted to the former proglacial lakes basins (Dadswell, 1974), with the exception of Lakes Washington and Waterton to the west. This is in sharp contrast with the distribution of *G. lacustris*, which is located both inside and outside the proglacial lakes system. The ability of *G. lacustris* to colonize new aquatic habitats through passive transport accounts for its presence in the proglacial lakes basins, as well as the Mountains of British Columbia and the western portion of the United States.

Two distinct and well supported genetic lineages of *Diporeia* have been identified with virtually non overlapping geographic ranges. The first lineage was primarily distributed throughout the former proglacial Lake Agàssiz basin, across northern Ontario (including

Lake Superior) and into northern Québec. The second lineage was identified in the Great Lakes basin (excluding Lake Superior), the St. Lawrence basin and surrounding regions (see chapter 2). The average nucleotide sequence divergence between haplotypes of the two lineages is 1.75%, just slightly higher than that observed between the haplotype groups identified in *G. lacustris*.

The geographical ranges of the *G. lacustris* haplotype groups overlapped unlike *Diporeia*, particularly in unglaciated regions in the United States. New populations established in the southern United States would have likely been colonized by a few individuals and would have experienced founder events and genetic bottlenecks, resulting in the rapid divergence of their haplotypes (Boileau *et al*., 1992; De Meester *et al.*, 2002). Passive dispersal of *G. lacustris* is a random event and colonization of local habitats is dependent upon the transporting vector. The stochastic nature of this type of dispersal resulted in geographically close populations containing different haplotype groups. The dispersal of *Diporeia* on the other hand was solely determined by the proglacial lakes system and newly colonized habitats would have had larger population sizes (McDonald *et al.,* 1990; Hondorp *et al.,* 2005), resulting in a slower rates of sequence evolution.

The low level of molecular divergence observed in North American *G. lacustris* only represents a fraction of the total genetic variation present in the species (Vainio and Väinölä, 2003; Hou *et al.*, 2007). The ability to passively disperse provides *G. lacustris* the opportunity to colonize new habitats that *Diporeia* can not, and is responsible for is holarctic distribution. The genetic divergence observed in *Diporeia* is significantly lower than the

molecular divergence present among Asian, European and North American lineages of *G. lacustris*.

3.6 Conclusions

This study revealed low sequence divergence within North American *G. lacustris,* similar to the level detected in actively dispersed species, contradicting the pattern of marked lineage divergence observed within other passively dispersed organisms. The recent evolution and dispersal of *G. lacustris* into North America from Asian populations accounts for the detection of low variation. The phylogeography of passively dispersed species is greatly influenced by local geological processes. Unglaciated regions are subject to less large scale extinction events resulting in a greater level of diversity, such as the south western USA. Populations in these areas can then diverge and undergo allopatric speciation, resulting in high levels of species endemism (Witt *et al.*, 2006), making conservation practices more difficult in these regions. The geographic range of passively dispersed species, although not restricted to, were still substantially influenced by the proglacial lakes systems, which facilitated the distribution of individual refugial populations over large areas in the same way that they influenced active dispersers. Use of the proglacial lakes network has resulted in the widespread distribution of a few genetic lineages in both active and passive dispersers across portions of previously glaciated regions. The phylogeographic distribution of passively dispersed species is complex and is heavily influenced by geological processes that ensue periodically.

Figure 3.1. Map showing all sample locations for *Gammarus lacustris* in North America. Location codes indicating sample sites follow Table 3.1 and haplotypes groups correspond to Fig. 3.1.

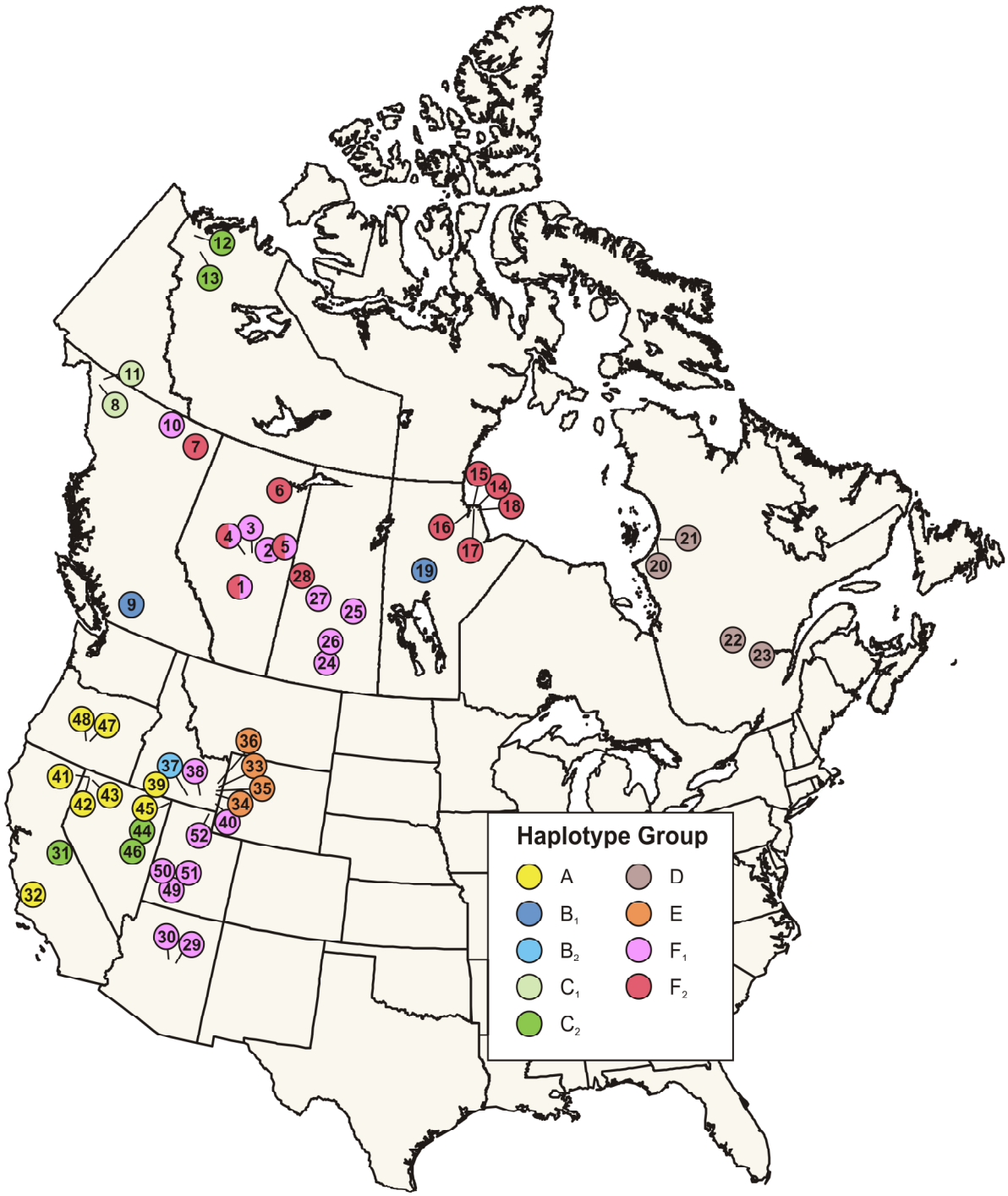
Haplotype Group
A
B1
B2
C1
C2
D
E
F1
F2

Figure 3.2. NJ phenogram showing the relationships among 57 *Gammarus lacustris* cytochrome *c* oxidase I (COI) haplotypes. Letter designations along the right indicate haplotype group and correlate with the haplotype groups in Fig. 3.3. Numbers above the nodes indicate the NJ bootstrap and interior branch test percentages respectively.

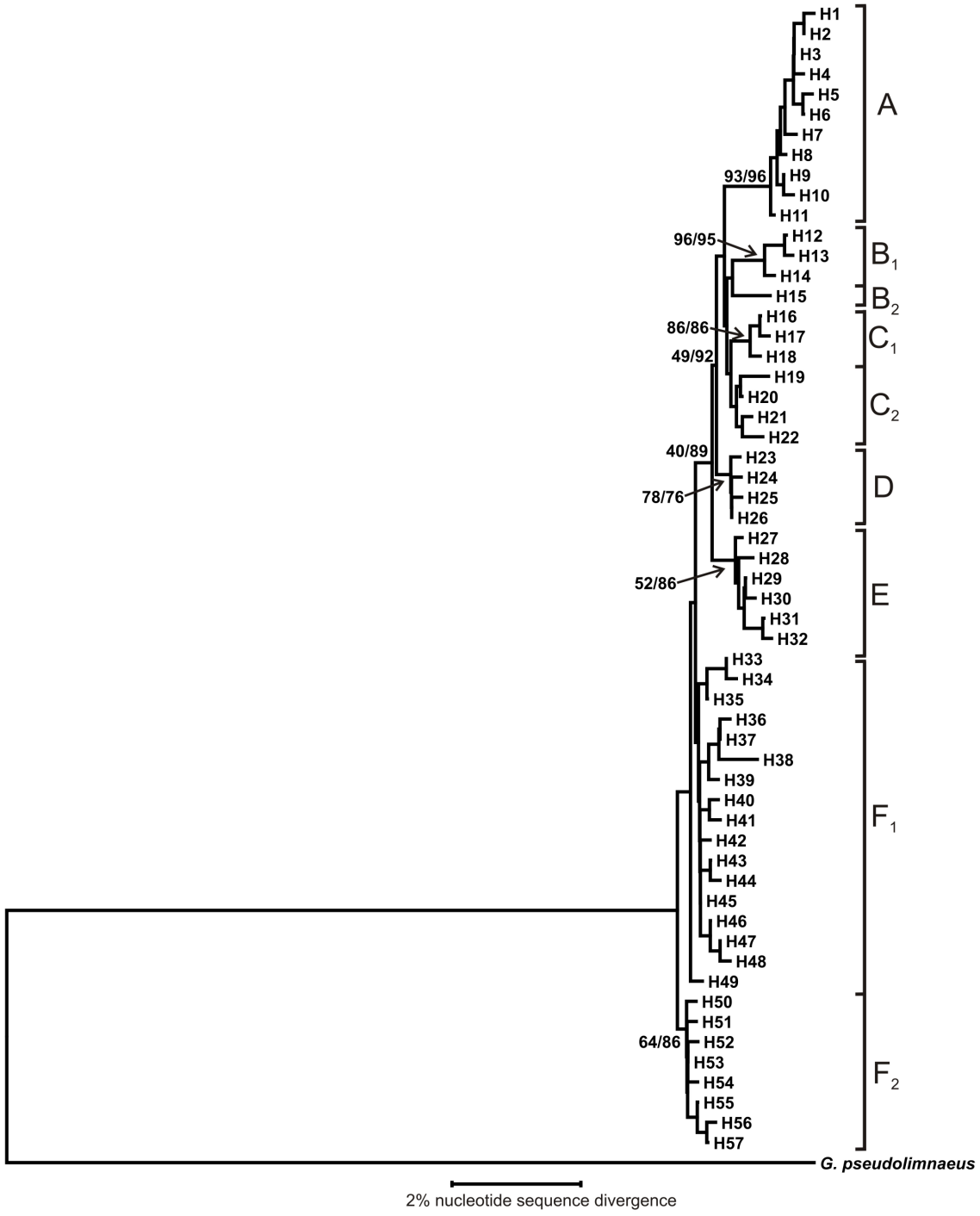

Figure 3.3. Statistical parsimony network representing the relationships among 57 *Gammarus lacustris* cytochrome *c* oxidase I haplotypes**.** Solid circles represent a single mutational step. Haplotype names and groupings correspond to the names and groups identified in the NJ analysis (Fig. 3.2).

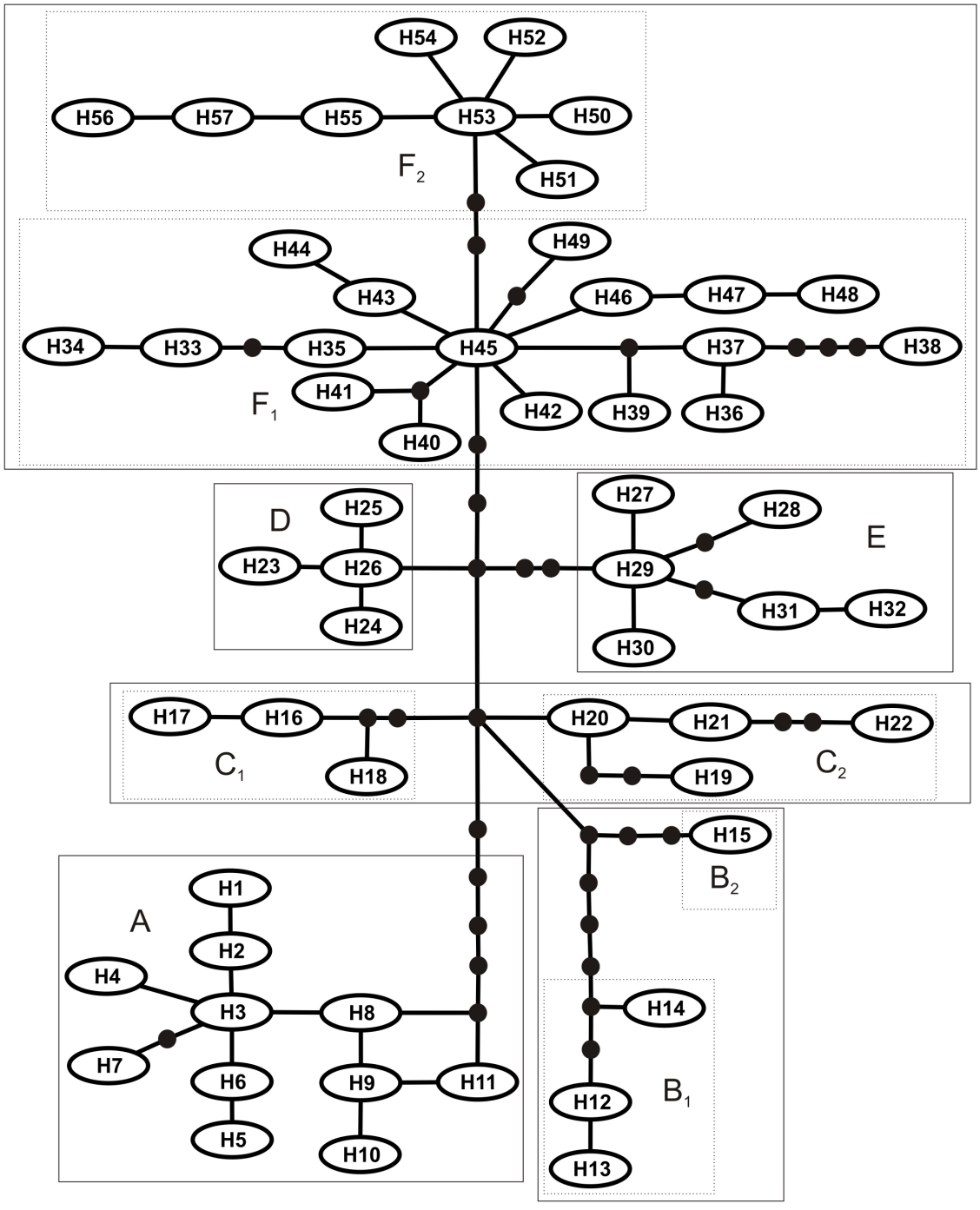

Table 3.1. Population location, sample size (n) and haplotype breakdown for all 57 *Gammarus lacustris* COI haplotypes. Numbers beside population name correspond with Fig. 3.1. Haplotype groups and designation follow Fig. 3.2 and Fig. 3.3

Population name		Location		n	Hap Group	Haplotype Breakdown		
1	Buffalo Lake	CA	Alberta	3	F1, F2	H43(1)	H51(2)	
2	Fork Lake	CA	Alberta	3	F1	H45(1)	H47(1)	H48(1)
3	Hanmore Lake	CA	Alberta	2	F1	H47(1)	H48(1)	
4	HWY 28 Creek	CA	Alberta	3	F1, F2	H45(2)	H53(1)	
5	Marie Creek	CA	Alberta	3	F1, F2	H42(1)	H45(1)	H50(1)
6	Pad 3	CA	Alberta	1	F2	H53(1)		
7	Andy-Baily Lake	CA	British Columbia	3	F2	H52(1)	H53(1)	H54(1)
8	Atlin Lake	CA	British Columbia	2	C1	H17(1)	H16(1)	
9	Kidd creek	CA	British Columbia	2	B1	H12(1)	H14(1)	
10	Liard River, Marsh	CA	British Columbia	2	F1	H44(2)		
11	McDonald Lake	CA	British Columbia	1	C1	H18(1)		
12	Boot Lake	CA	Inuvik, NWT	2	C2	H20(2)		
13	Travaillant Lake	CA	Inuvik, NWT	1	C2	H21(1)		
14	Eastern Creek	CA	Manitoba	3	F2	H55(2)	H56(1)	
15	Farnsworth Lake	CA	Manitoba	3	F2	H55(3)		
16	Goose Creek	CA	Manitoba	2	F2	H55(2)		
17	Landing Lake	CA	Manitoba	3	F2	H55(3)		
18	Paint Lake	CA	Manitoba	2	B1	H13(2)		
19	Twin Lakes	CA	Manitoba	3	F2	H55(1)	H57(2)	
20	KJQ-06	CA	Québec	2	D	H23(2)		
21	Kuujjuarapik	CA	Québec	2	D	H23(2)		
22	Lac Cummings	CA	Québec	1	D	H25(1)		
23	Lac Vert	CA	Québec	2	D	H24(1)	H26(1)	
24	Arm River	CA	Saskatchewan	3	F1	H45(3)		
25	Barrier River	CA	Saskatchewan	3	F1	H47(2)	H48(1)	
26	Last Mountain Lake	CA	Saskatchewan	3	F1	H46(1)	H49(2)	
27	Makwa River	CA	Saskatchewan	1	F1	H45(1)		
28	Shell River	CA	Saskatchewan	3	F2	H53(2)	H54(1)	
29	Dairy springs	USA	Arizona	2	F1	H39(2)		

30	West fork, Wall spring	USA	Arizona	2	F1	H38(1)	H42(1)	
31	Mono 3	USA	California	2	C2	H22(2)		
32	Thorn spr. #2	USA	California	1	A	H3(1)		
33	Big Flat Creek	USA	Idaho	3	A	H5(1)	H6(2)	
34	Chubb Spring	USA	Idaho	1	E	H29(1)		
35	Hooper Spr. Area	USA	Idaho	1	E	H30(1)		
36	Little Blackfoot River	USA	Idaho	3	E	H31(2)	H32(1)	
37	Outlet Creek	USA	Idaho	2	E	H27(1)	H28(1)	
38	Rock Creek	USA	Idaho	3	B2	H15(3)		
39	Sheep creek spr.	USA	Idaho	2	F1	H36(1)	H37(1)	
40	Zenda spr.	USA	Idaho	1	F1	H41(1)		
41	Catnip Creek	USA	Nevada	3	A	H9(1)	H10(1)	H11(1)
42	DWS05-41	USA	Nevada	3	A	H2(3)		
43	DWS05-45	USA	Nevada	3	A	H1(1)	H2(2)	
44	DWS05-109	USA	Nevada	1	C2	H19(1)		
45	DWS05-117	USA	Nevada	1	A	H7(1)		
46	Hot Creek	USA	Nevada	1	C2	H22(1)		
47	Ana River	USA	Oregon	3	A	H3(2)	H8(1)	
48	Summer Lake, Creek	USA	Oregon	1	A	H4(1)		
49	Big Swamp spr.	USA	Utah	3	F1	H33(1)	H34(1)	H35(1)
50	Antelope spr.	USA	Utah	1	F1	H45(1)		
51	Heppler spr.	USA	Utah	1	F1	H45(1)		
52	Logan River spr.	USA	Utah	3	F1	H40(2)	H46(1)	

Discussion and Conclusions

This thesis investigated the phylogeographical distribution of the two North American amphipods, *Diporeia hoyi* and *Gammarus lacustris*. The phylogenetic analyses of *Diporeia* populations did not indicate that it represented a cryptic species complex and there was no evidence to suggest that the eight plus morphotypes described by Bousfield (1989) are independent species. A close examination of the morphotypes *D.h. filicornis*, *D.h. brevicornis* and *D.h. erythropthalma* indicated that they do not warrant recognition as separate phylogenetic species. When considering the weaknesses with the morphological species concept (MSC), recent studies have focused on unrecognized genetic diversity (Avise, 2000; Bickford *et al*., 2006; Goodman *et al.*, 2009; Moussalli *et al.*, 2009). However, this study revealed that not all morphological variations observed indicate significant levels of genetic divergence and taxonomists should take equal care to prevent the over splitting of taxa on the basis of phenotypic variation.

Molecular comparisons between *Diporeia* and its closest relatives revealed that it has been independently evolving from *Monoporeia affinis* for approximately 15.4 Myr, and the two lineages diverged from arctic *Pontoporeia femorata* around 21.6 Myr. These lineages have been independently evolving for considerably longer time than previously assumed. The assumption that they diverged during the Pleistocene was in part based on similar morphological characteristics (Hogbom, 1917; Ricker, 1959). Phylogenetic analyses revealed substantial genetic divergences between the three genera, suggesting that they evolved prior to the Quaternary Period (last 2.4 Myr), ruling out a Pleistocene origin. Currently, there are

no publications suggesting that *P. femorata* represents a species complex and only a single species has been recognized using the MSC. Nonetheless, two genetic lineages *P. femorata* that show a level of divergence similar to that identified between *Diporeia* and *Monoporeia* were revealed. The morphological characteristics present within all three genera do not necessarily reflect their relationships and their underlying genetic diversity. This further supports the use of an integrative approach for species identification and taxonomists should attempt to utilize both morphological and molecular techniques whenever possible (Gibbs, 2009; Padial *et al.*, 2009).

Two genetically distinct lineages of *Diporeia* were identified in North America with an average sequence divergence of 1.75% between them and they should be treated as independent management units for conservational purposes (Moritz, 1994). After the Pleistocene glaciations, contemporary populations of *Diporeia* dispersed through the proglacial lakes system from two locations, the Mississippi and Missouri refugia. The low level of genetic divergence within *Diporeia* is consistent with the pattern observed in other active dispersing taxa (Kontula and Väinölä 2003; Audzijonytė and Väinölä, 2006; Dooh *et al.,* 2006; Barrette *et al*., 2009). The wide spread geographic distribution among these species is a direct result of their ability to enter proglacial lake systems that formed during the glacial retreat and utilize them to disperse across the continent. This process would recur each time the glaciers retreated, resulting in their repeated dispersal and subsequent low genetic divergence levels across glaciated regions. Major glacial events occur periodically every 21, 41 and 100 thousand years (Hewitt, 2000; Ruddiman, 2006), suggesting that dispersal

opportunities for active dispersers plays a substantial role in the phylogeographic distribution of these species, potentially influencing genetic variation more so than allopatric divergence.

Similar levels of genetic divergence observed in *Diporeia* were identified in the passively dispersed amphipod *G. lacustris* and there was no evidence indicating that it represents a species complex. Six haplotype groups were identified in North America, exhibiting an average mitochondrial sequence divergence of 1.52% between them. This is counterintuitive to the emerging trend of greater levels of intraspecific genetic divergences detected in passively dispersed species (Witt and Hebert, 2000; Gomez *et al.,* 2002; Adamowicz *et al.*, 2009). The unexpected low level of divergence observed is possibly because *G. lacustris* recently invaded North America from Asia (Hou *et al.*, 2007) and populations have not had sufficient time to diverge. *Gammarus lacustris* populations from these two continents do not show the level divergence observed in other passively dispersed species (Taylor *et al.,* 1998; Witt and Hebert, 2000; Cox and Hebert, 2001; Gomez *et al*., 2002; Adamowicz *et al.,* 2009). However, North American *G. lacustris* possess lower levels of genetic divergence than both Asian (Hou *et al.*, 2007) and European (Vainio and Väinölä, 2003) populations, which were observed to have mitochondrial sequence divergences as high as 6%.

The colonization of glaciated North American by *G. lacustris* was not dependent on the proglacial lake systems. The geographical ranges of the different groups of *G. lacustris* overlap, but with the exception of a few locations in the former Lake Agàssiz basin, populations only contained haplotypes from a single group. This could reflect local populations excluding new immigrants from becoming established through competition for

resources and mates (De Meester *et al.,* 2002), but it is important to note that the samples sizes were small.

Within North America, the unglaciated South-western United States contained the greatest level of *G. lacustris* diversity, with 5 haplotype groups indentified in the region. In general, unglaciated regions contain greater levels of genetic diversity among aquatic organisms (Bernatchez and Wilson, 1998; Cox and Hebert, 2001; Witt *et al.*, 2006; Adamowicz *et al.*, 2009). Populations persisted in these regions during the glaciations, allowing allopatric divergence to occur, resulting in the formation of more deeply diverged lineages. After the glaciers retreat, passively dispersed species have the opportunity to colonize formerly glaciated regions resulting in the establishment of new populations, which will subsequently diverge. However, any unique groups that emerge in glaciated regions are prone to extinction once the next glacial cycle begins. The ineffectiveness of gene flow in passively dispersed species (De Meester *et al.*, 2002; Bohonak and Jenkins, 2003; Weisse, 2008) will likely prevent the establishment of these lineages in unglaciated regions, resulting in little or no contribution of these populations to the overall genetic variation. Dispersal events initially play a significant role in the genetic divergence of passively dispersed species, but once populations are established their genetic variation is substantially influenced by allopatric divergence (Witt *et al.*, 2006; Adamowicz *et al.*, 2009).

The glaciation of the Northern Hemisphere has profoundly impacted both active and passively dispersed species, although the modes in which it has influenced their genetic divergences differ. This study highlights the importance of understanding the interaction of climatic processes, along with the ecology of a species to adequately understand its

geographic genetic distribution. Effective conservation practices can only be implemented when species diversity is accurately described.

Future Research

The recognition of only a single phylogenetic species in *Diporeia* does not exclude the possibility of recently diverged biological species. If the two morphotypes *D.h. filicornis* and *D.h. brevicornis* recently became reproductively isolated, sufficient time may not have passed to discriminate them using phylogenetic techniques. Further investigation of the two morphotypes would benefit by examining highly variable molecular markers such as microsatellites to determine if they are recently derived biological species.

Pontoporeia femorata specimens included in this thesis were collected from 3 populations in North America, but they are distributed in marine waters throughout the Holarctic. The specimens were revealed to represent a cryptic species complex and further phylogenetic investigation should be conducted across the species entire range.

Finally, the phylogeographic analysis of *G. lacustris* in this study only represents a fraction of the species holarctic range and probably does not accurately reflect its entire genetic diversity. *Diporeia* is only located in North America, a product of its limited dispersal abilities and the majority of its range was included in the analyses. The relationship of North American *G. lacustris* to Asian populations has already been established, but their relationship to European populations is not known. To adequately characterize the effects of active and passive dispersal abilities on the distribution and genetic divergences of amphipods their entire range should be incorporated into the analyses.

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