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Comparative phylogeography of two North American 'glacial relict' crustaceans

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Abstract

The Pleistocene glaciations represent the most recent and dramatic series of habitat changes since the Cretaceous. The impact of these events was particularly acute for aquatic taxa with poor powers of dispersal, but few organisms have evolutionary histories more intimately entwined with the advance and retreat of ice than the 'glacial relicts'. In this study, we used a mitochondrial gene, cytochrome *c* oxidase subunit I (COI), to examine and compare the phylogeographical structure of two glacial relict crustaceans (*Limnocalanus macrurus* and members of the *Mysis relicta* species group) across North America. In both cases, we found a sharp phylogenetic division between populations from inland lakes formed during glacial retreat, and arctic lakes isolated from polar seas via isostatic rebound. However, the depth of this phylogenetic partition varied between taxa. In *L. macrurus*, nucleotide sequence divergence of 2.2% between these zones is consistent with its current status as a single morphologically variable species, but in *Mysis* the split occurred among recently described, morphologically conserved species, at a divergence of 8.2%. The disparity in the depth of divergence indicates a history of recurrent freshwater invasions from the arctic seas, in concordance with previous studies of Eurasian glacial relicts. However, we suggest further consideration of a largely overlooked explanation that could account for some of the discrepancies between molecular divergences and glaciation events. Many cladogenetic events could have occurred in arctic seas prior to the transition to inland waters, a possibility supported both by the complex physical and ionic history of arctic seas and by high marine and estuarine lineage diversity in the north.

Keywords: biogeography, freshwaters, habitat shifts, marine, refugia, speciation

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Introduction

Biogeographers have long sought to understand the impacts of geological features and events on the distributions and evolutionary histories of species. An eclectic assemblage of species has received much attention for an interesting, and apparently shared, history showing close association with the environment (see Segerstråle 1982). Generally referred to collectively as 'glacial relicts' (but see Holmquist 1959, 1970; Dadswell 1974), these species originated in arctic seas and subsequently invaded various inland waters in the Holarctic. They may be found in freshwater lakes in formerly glaciated regions (e.g. Ricker 1959; Martin & Chapman 1965; Dadswell 1974) and in habitats recently formed following isostatic rebound in the arctic (Johnson 1962, 1964). Many of these 'relicts' are also found in the Caspian, representing an unusual polar marine element in that inland sea (Högbom 1917). Members of the glacial relict group include various crustaceans, one fish, and a seal species (Segerstråle 1982), which all share the property of poor powers of dispersal among water bodies (Carter 1969; Roff 1972; Dadswell 1974). These features, combined with detailed comparisons between species occurrences and glacial extents, have led to suggestions that the distributions of these species are closely dependent upon Pleistocene glacial and limnological history (Segerstråle 1962, 1982; Ricker 1959; Martin & Chapman 1965; Dadswell 1974).

However, and despite the 'glacial relict' designation, there has been much controversy and discussion of the history of these species. In particular, the timing of inland invasion and the degree of congruence in their biogeographies have

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been debated. The Caspian has become one focal point for these discussions, with major divisions between Pleistocene and Tertiary hypotheses. The primary Pleistocene scenario, originally proposed with regards to the Caspian fauna (Högbom 1917), was the 'sluicing-up' hypothesis, whereby taxa were pushed inland recurrently in lakes at the margins of advancing ice sheets. This idea subsequently gained support for other Eurasian and North American taxa, with inland dispersal also apparently linked to proglacial lake extents and drainage routes during glacial melts (Ricker 1959; Segerstråle 1962, 1976, 1982; Martin & Chapman 1965; Carter *et al*. 1980). By contrast, Holmquist (1959, 1970) argued for the Caspian taxa having been left behind when marine connections to the arctic receded in the mid-Tertiary [25–30 million years ago (Ma)]. According to this scenario, 'glacial relict' species have instead had a long history of inland occupancy.

Recent genetic work has made substantial contributions to this dispute, with earlier studies employing allozyme loci and later studies focusing on mitochondrial DNA (mtDNA) divergences. In particular, broader phylogeographical surveys have been especially useful for elucidating clade distributions and helping to assess timings of inland movements. Interestingly, results to date seem to demonstrate a wide variety of inland invasion histories (Väinölä & Varvio 1989; Väinölä *et al*. 2001; Kontula & Väinölä 2003; Audzijonytë & Väinölä 2005; Audzijonytë *et al*. 2005). Moreover, in some cases neither of the traditional scenarios is supported. For example, Audzijonytë *et al*. (2005) found that the timing of inland invasion for members of the *Mysis relicta* species group may have, in fact, been Miocene or Pliocene, rather than either Pleistocene vs. Tertiary as previously supposed. However, our understanding of glacial relicts remains incomplete as the majority of genetic studies to date have focused on Eurasian (including Caspian) glacial relict taxa. Due to the dramatic nature of the glaciations in North America, we use a comparative approach to contribute to a better understanding of glacial relict history on that continent. We first briefly introduce the two crustacean taxa that our work examines.

Relationships and distribution of Mysis

The genus *Mysis* has a northern Holarctic distribution and currently includes 14 described (and at least 15 known biological) species (Audzijonytë & Väinölä 2005; Audzijonytë *et al*. 2005), seven of which occur in either strictly marine conditions and/or primarily in estuarine waters of varying salinity. However, it is the enigmatic origin of two groups of continental species [one group Caspian, the other coastal- and lake-dwelling (the *Mysis relicta* species group)] that has provoked great interest in the biogeography and evolutionary history of this genus. It was initially believed that the continental species were all recently derived from the marine *Mysis oculata* following the latest glacial retreat (reviews in Väinölä *et al*. 1994; Audzijonytë & Väinölä 2005). Indeed, *M. relicta* (s.l.) was viewed to be one of the 'classical' glacial relicts (see Segerstråle 1982). However, revisions by Holmquist (1959) pushed the appearance of continental species back to the mid-Tertiary, setting up a dichotomy in biogeographical hypotheses ripe for testing using modern methods.

Recent genetic investigations have helped to elucidate *Mysis* species diversity and distributions, with key contributions being the construction of phylogenies for the genus, and especially the division of *M. relicta* s.l. into four distinct species (*M. relicta* s.s., found in northern European lakes; *M. segerstralei*, distributed in coastal lakes circumarctically; *M. salemaii*, found in northern Eurasia; and *M. diluviana*, restricted to lakes in inland North America) (Väinölä 1986; Väinölä *et al*. 1994; Audzijonytë & Väinölä 2005; Audzijonytë *et al*. 2005). Interestingly, neither the Quarternary scenario nor the competing Tertiary explanation for the shift to continental waters has been supported by genetic evidence, which instead is consistent with both inland invasion and species divergence at an intermediate period. For example, allozyme studies (Väinölä 1986) suggested some 6–13 million years (Myr) of divergence between *M. oculata* and the *M. relicta* group, while mitochondrial DNA evidence indicates 3–7 Myr of independent evolutionary history among the *M. relicta* group, Caspian group, and related circumarctic clades of marine/estuarine species (Audzijonytë *et al*. 2005). Moreover, allozyme, mtDNA, and morphological evidence have indicated that the four *M. relicta*-group species are themselves well-separated, with most pairs having diverged at least 3 Ma (Väinölä 1986; Väinölä *et al*. 1994; Audzijonytë & Väinölä 2005). Thus, traditional scenarios have been reconsidered, with Miocene-Pliocene cooling and Pliocene glaciation being factors potentially coincident with the origin and diversification of the continental *Mysis* species (Audzijonytë *et al*. 2005).

However, there linger a number of key questions about the origins and distribution of the continental *Mysis* species. For example, although it appears that inland invasion predated the Pleistocene, occurrence information does suggest a strong glacial role in determining inland distributions (e.g. Martin & Chapman 1965; Dadswell 1974; Carter *et al*. 1980). To investigate further the relationship between glacial history and cladogenesis, we build upon previous work by sampling additional localities for several species of *Mysis* (and combine these data with those from Audzijonytë & Väinölä 2005 and Audzijonytë *et al*. 2005) to give a fuller account of phylogeographical patterns within North American continental waters.

Relationships and distribution of Limnocalanus

The taxonomy of the calanoid copepod genus *Limnocalanus* has also been controversial. *Limnocalanus johanseni* has

long been regarded as a distinct species with a distribution limited to the Mackenzie Delta region and northern coastal areas in Alaska. By contrast, *Limnocalanus macrurus* is regarded as having a Holarctic distribution (Dadswell 1974) although it was previously partitioned into two species, *L. macrurus* and *Limnocalanus grimaldii*, based on morphological variation between marine and freshwater populations (Sars 1897). In particular, variation in the shape of the cephalothorax was used to separate this taxon into 'extreme *macrurus*' and 'extreme *grimaldii*' forms (Sars 1897). The apparent relationship between morphology and habitat salinity was taken as evidence that *L. macrurus* was a freshwater species recently derived from the brackish and marine species *L. grimaldii* (see Ekman 1913). However, Lindquist (1961) recognized that intermediate forms exist between these taxa, and Holmquist (1970) subsequently concluded that both morphotypes should be considered as a single widespread and euryhaline species, *L. macrurus*. As a result, *L. johanseni* and *L. macrurus* are currently the only recognized members of this genus, with the latter species thought to display a glacial-relict type of distribution (Dadswell 1974). However, there has been no genetic work on this group aimed towards investigating its history. This study addresses this void through a broad phylogeographical survey of *Limnocalanus* in North America.

A comparative approach

This study investigates and compares the history of these two glacial relicts in North America. Specifically, cytochrome *c* oxidase subunit I (COI) phylogenies for a broad geographical sample of individuals are constructed to gain a perspective on the affinities of lacustrine and coastal marine populations in both groups. Phylogeographical patterns are subsequently compared between these groups and with previous studies on other glacial relicts, to gain an estimate of the relative lengths of inland tenancy and of the degree of congruence among groups. The implications of these results are discussed in terms of the diversity of evolutionary histories among glacial relicts. Finally, we suggest that pre-Pleistocene marine diversification could have played a larger role in the origins of glacial relict taxa than currently recognized.

Materials and methods

Specimen collections

Populations belonging to the *Mysis relicta* species group were collected from 15 lakes in North America, and an additional sample was contributed from Lough Neagh, Northern Ireland (Fig. 1; Appendix I). Several marine species of *Mysis* were collected as well for comparison: two populations of *M. oculata* from the Arctic Ocean, two individuals of *M. litoralis* from near Churchill, Manitoba, and one specimen of *M. stenolepis* collected offshore near St Andrews, New Brunswick (Appendix I). *Limnocalanus macrurus* was obtained from 85 lakes in North America, while an additional specimen was provided from the Baltic Sea (Fig. 2; Appendix II). Finally, three populations of *Limnocalanus johanseni* were collected from sites in the western Canadian Arctic (Appendix II). Specimens from inland lakes, which were collected in summer (July–September) over several years (1990–2000), were taken from below the thermocline using either a 50-cm diameter plankton net or a benthic drag with mesh sizes of 200-μm and 500-μm, respectively. Arctic samples were collected between August 1991 and August 2000. Collections were made from boat or helicopter in vertical hauls using nets with a mesh size of 200-μm. Samples were sorted in the field and either flash-frozen in liquid nitrogen or preserved in ethanol for genetic analysis.

Molecular protocols and sequence analysis

Sequence variation was examined in the mitochondrial gene COI from 37 individuals of *Mysis* and 119 individuals of *Limnocalanus*. Typically, one individual per location was sequenced for *Limnocalanus*. When possible, two individuals were sequenced per location for *Mysis*. In both cases, additional individuals were sequenced when novel haplotypes were detected from a locality. Template DNA was extracted from whole individuals of *Limnocalanus* and from the abdominal tissue of *Mysis* in 50-μL volumes of extraction buffer using the modified proteinase K methods described by Schwenk *et al*. (1998). Following incubation at 50 °C for 12–24 h, extractions were placed in a 96 °C water bath for 10 min to denature proteinase K. A 710-bp fragment of the COI gene was PCR-amplified (polymerase chain reaction) using the primer pair LCO1490 and HCO2198 (Folmer *et al*. 1994). Each 50-μL PCR consisted of 40-μL of 10× PCR buffer, 2.2 mM $MgCl_2$, 200-μM of each dNTP, 1 U of *Taq* polymerase, 0.3-μM of each primer, and 2–5-μL of DNA template. The thermal profile of the amplification protocol consisted of a 1 min cycle at 94 °C; 5 cycles of 1 min at 94 °C, 1.5 min at 45 °C, 1.5 min at 72 °C; 34 cycles of 1 min at 94 °C, 1.5 min at 51 °C, 1 min at 72 °C; and a final cycle of 5 min at 72 °C. Amplified products were stored at 5 °C prior to gel-based purification using the Qiaex II kit (QIAGEN). Purified COI gene fragments were sequenced with LCO1490 using an ABI PRISM 377 automated sequencer and the *Taq* FS dye rhodamine sequencing kit. All new sequences for both *Mysis* and *Limnocalanus* are available through the Barcode of Life Data (BOLD) Systems website (http://www.barcodinglife.org; see Appendices A and B for accession numbers, to be listed under 'published projects').

Sequence fragments were examined and aligned unambiguously by eye using the amino acid translation in SEQAPP 1.9a (Gilbert 1992). A 635-bp fragment of COI

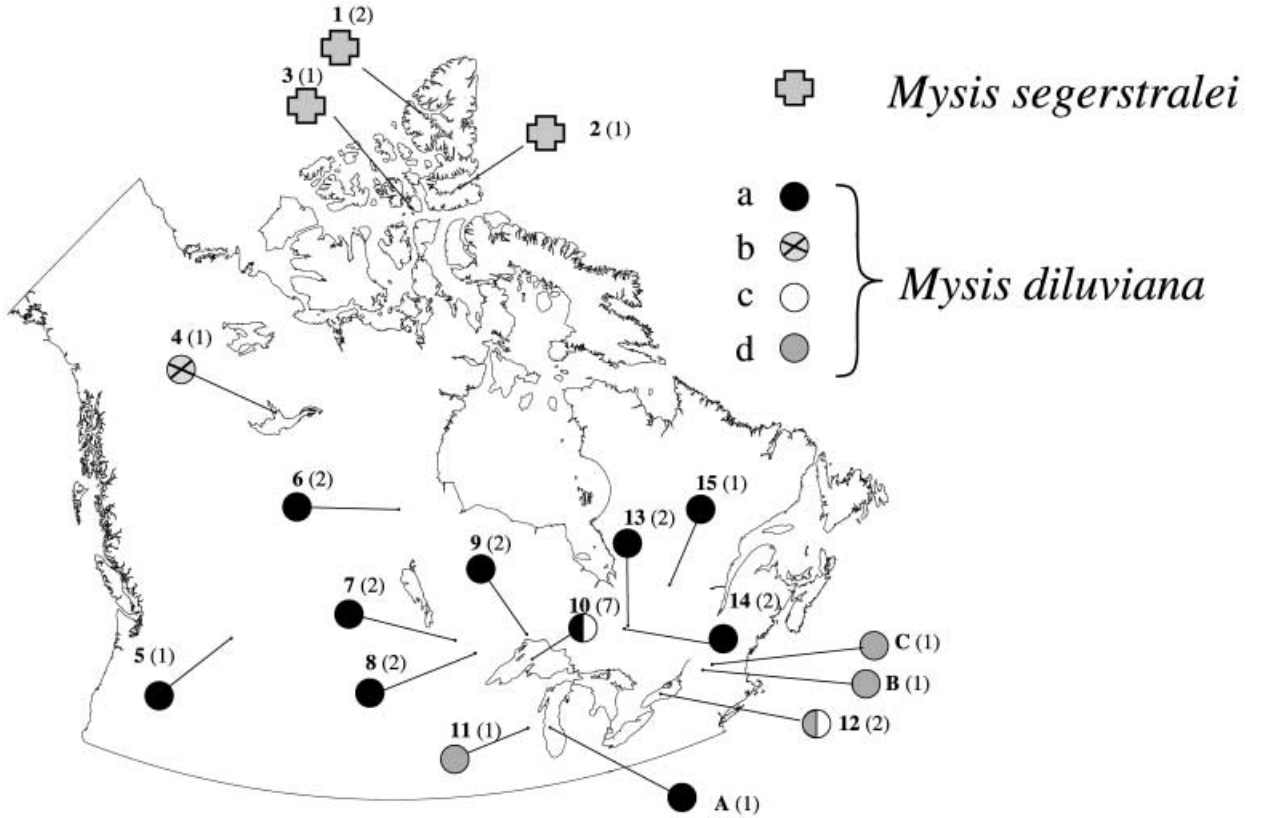

Fig. 1 Collection sites for two North American members of the *Mysis relicta* species group. Square cross symbols indicate *Mysis segerstralei*, while circles indicate *Mysis diluviana*. The distributions of COI haplotype clusters a–d (see Fig. 3) are also shown for *M. diluviana*. Labels for each site first show the site code (in bold), followed by the sample size of sequences obtained (in parentheses). Numeric site labels represent new collections (see Appendix I), while alphabetic labels represent *M. diluviana* data from Audzijonytė *et al*. (2005) (site A, GenBank sequence AY920494; B, DQ189154; C, DQ189155).

was aligned for *Mysis*, while a 601-bp fragment was examined for *Limnocalanus*. Unique haplotypes were used to calculate pairwise distance matrices according to Kimura's (1980) 2-parameter (K2P) model within *Mysis* and *Limnocalanus*, using the program MEGA2 version 2.1 (Kumar *et al*. 2001). This model was selected to facilitate genetic distance comparisons with other recent studies. Phenograms were then constructed using the neighbour-joining (NJ) method, with bootstrap support estimated using 10 000 replicates. Pairwise deletion of missing nucleotides was employed (analyses based upon complete deletion produced similar findings and are not reported further).

All COI sequences for *Mysis* that are available from GenBank (mostly from Audzijonytė *et al*. 2005; also from Audzijonytė & Väinölä 2005 and Cristescu & Hebert 2005) were included in a pairwise distance matrix, to clarify the identity of the North American populations in comparison with recently described species (Audzijonytė & Väinölä 2005). Including all *Mysis* species also allowed examination of patterns of intra- and interspecific genetic distance, as well as exploration of the depth of divergence among inland species compared with the marine species. The *Mysis* sequences included in the pairwise matrix along with our new data were: *M. mixta* (DQ189168), *M. stenolepis* (DQ189166, DQ189167), *M. gaspensis* (DQ189165), *M.* cf. *litoralis* (DQ189164), *M. litoralis* (DQ189162, DQ189163), *M. oculata* (DQ189160, DQ189161), *M. microphthalma* (DQ189158, DQ189159), *M. caspia* (DQ189157), *M. amblyops* (DQ189156), *M. diluviana* (AY529027, AY920494, DQ189153, DQ189154, DQ189155), *M. segerstralei* (AY920493, DQ189151, DQ189152), *M. salemaai* (AY920492, DQ189147, DQ189148, DQ189149, DQ189150), *M. relicta* s.s. (AY920491, DQ189144, DQ189145, DQ189146). Sequences are referred to by GenBank number when used in the phenogram figures.

For *Limnocalanus* haplotypes, the K2P distance estimates within and between both species were examined. To test for saturation, the relationship between transition/transversion ratios (Ts/Tv) and nucleotide p-distances was explored at three levels: within species, between *L. macrurus* and *L. johanseni*, and between these two species and other calanoid copepods. Thus, a 566-bp fragment of COI was aligned for all *Limnocalanus* haplotypes and for 10 other species of calanoid copepods belonging to three families. Two of these species (*Osphranticum labronectum*

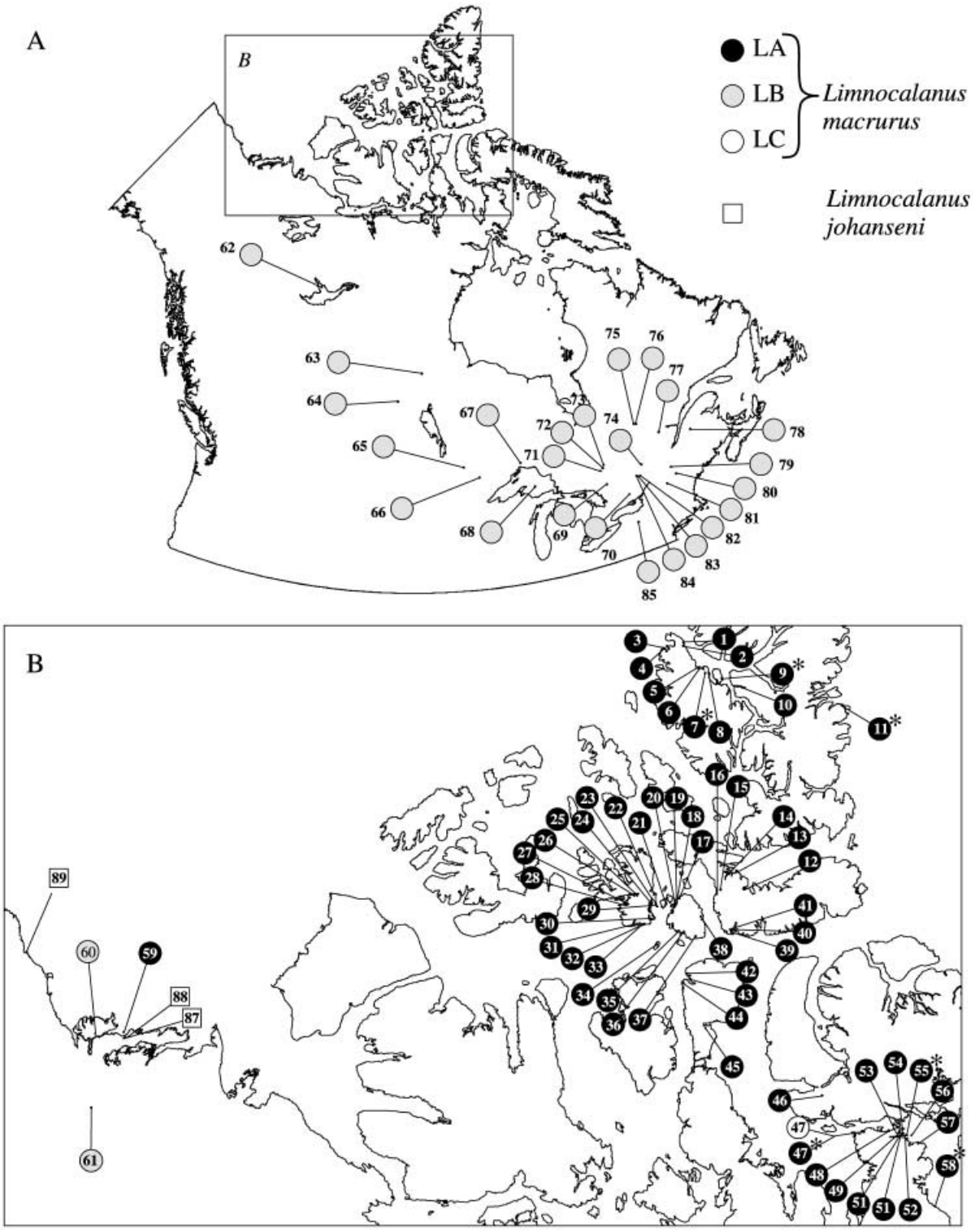

Fig. 2 Collection sites for *Limnocalanus macrurus* (also see Appendix II). All samples from inland regions (part A) were collected from freshwater lakes. Most *L. macrurus* from the Canadian Arctic (part B) were collected from lakes which have been recently isolated from the ocean by isostatic rebound. However, one sample was collected from the Beaufort Sea, and two samples were collected from lakes in the MacKenzie Delta region. The geographical distributions of the three major COI haplotype groups (see Fig. 4) detected in North American populations are shown. Black circles indicate lineage LA, and grey circles indicate lineage LB. The white circle indicates the sole location containing lineage LC, which coexists with several LA haplotypes. Six populations harbour haplotypes of the same LA sublineage that contains the sequence from the Baltic Sea (see Fig. 4); they are identified by asterisks.

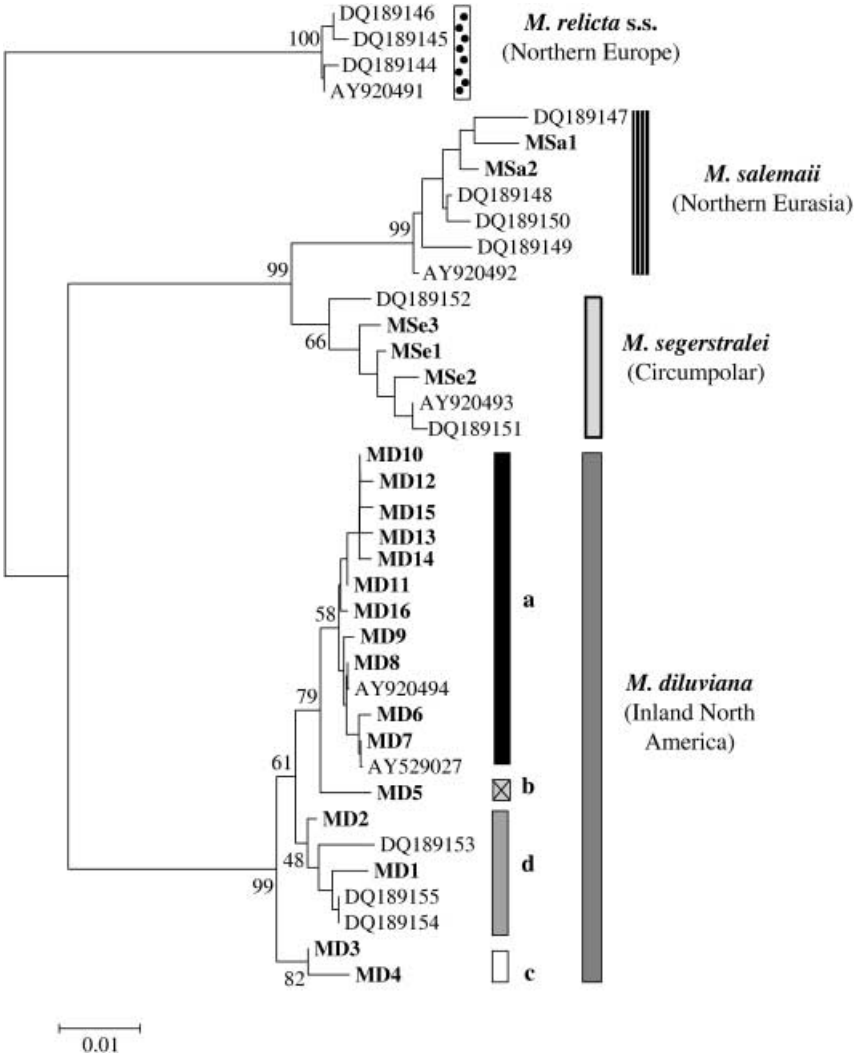

Fig. 3 NJ phenogram of all unique haplotypes for four species belonging to the *Mysis relicta* species group, based upon COI mtDNA sequences. All sequences new to this study are indicated by code (see Appendix I) and are in bold, while other sequences are refereed to by GenBank number (mainly from Audzijonytë *et al.* 2005). Four haplotype clusters (lettered a–d) are designated for the North American species *Mysis diluviana*. Bootstrap values (based upon 10 000 replicates) are given for select nodes. The scale bar indicates nucleotide sequence distances based upon the K2P model.

and *Centropages furcatus*) belong to the same family (Centropagidae) as *Limnocalanus* (SJA, unpublished data). Eight additional COI sequences from more distantly related species from two families were obtained from GenBank: *Pseudocalanus newmani* (AF332796) and *P. moultoni* (AF332795) (Clausocalanidae); and *Calanus pacificus oceanicus* (AF332764), *C. marshallae* (AF332768), *C. hyperboreus* (AF332770), *C. helgolandicus* (AF332760), *C. glacialis* (AF333039), and *Calanoides acutus* (AF332791) (Calanidae) (all sequences from Hill *et al.* 2001 and Bucklin *et al.* 2003). Upon detecting saturation between *L. macrurus* and *L. johanseni*, an NJ tree was subsequently built using haplotypes for *L. macrurus* alone.

Results

Genetic patterns in Mysis

Twenty-one COI haplotypes were detected among the new sequences obtained for the *Mysis relicta* species group, while three, two, and one additional haplotypes were observed within the marine *Mysis* species *M. oculata*, *M.* cf *litoralis*, and *M. stenolepis*, respectively (Fig. 3; see also Appendix I). For the populations belonging to the *M. relicta* species group, NJ analysis (Fig. 3) revealed that all new haplotypes grouped closely with sequences from three species recently described by Audzijonytë & Väinölä (2005): *Mysis diluviana* (haplotypes coded MD) and *Mysis segerstralei* (coded MSe) for the North American populations, and *Mysis salemaii* (coded MSa) for the Irish samples (Fig. 3). Indeed, all of the new and GenBank *Mysis* sequences formed tight, monophyletic clusters supported by high bootstrap values, supporting recent designations of four species in this group (Audzijonytë & Väinölä 2005).

Genetic divergences tended to be limited within species, with mean K2P distances among haplotypes within species ranging from 0.4 to 1.1% (but up to an average of 2.9% in the multispecies Caspian group) (see Table 1). The maximal extent of intraspecific divergence was also limited, with

Table 1 Genetic distances for the COI gene within and among *Mysis* species, based upon new (see Appendix I) and GenBank sequence data (mainly from Audzijonytë *et al.* 2005). The lower half of each section gives average between-group genetic distances (using the K2P model and pairwise deletion of missing sites). Haplotype groupings for *Mysis diluviana* are as in Fig. 3. Along the diagonal (delineated in bold), mean within-group nucleotide divergences are presented, with the maximum within-group values indicated in parentheses

	M. relicta group (inland and coastal)				Caspian	Marine and estuarine environments				
Mysis:	*diluviana* (*n* = 21)	*segerstralei* (*n* = 6)	*salemaii* (*n* = 7)	*relicta* s.s. (*n* = 4)	Caspian group (*n* = 4)*	*oculata/litoralis* (*n* = 7)*	cf *litoralis* (*n* = 3)	*gaspensis* (*n* = 1)	*stenolepis* (*n* = 3)	*mixta* (*n* = 1)
M. diluviana	**1.0 (2.3)**									
M. segerstralei	7.7	**0.8 (1.4)**								
M. salemaii	8.7	2.9	**1.1 (1.7)**							
M. relicta s.s.	8.4	9.2	9.3	**0.3 (0.5)**						
Caspian group	10.5	10.0	10.0	9.6	**2.9 (3.7)**					
M. oculata/litoralis	9.6	10.4	10.6	8.6	10.8	**0.7 (1.6)**				
M. of litoralis	9.2	11.2	10.6	8.8	10.5	9.6	**0.4 (0.5)**			
M. gaspensis	13.8	13.4	12.8	12.8	14.1	12.7	11.9	**N/A**		
M. stenolepis	14.3	14.8	15.8	13.0	14.7	16.4	13.5	16.7	**0.8 (1.2)**	
M. mixta	12.9	13.7	13.8	11.5	14.2	12.8	11.8	15.3	15.7	**N/A**

M. diluviana haplotype groups	Group a (*n* = 13)	Group b (*n* = 1)	Group c (*n* = 2)	Group d (*n* = 5)	**Key summary statistics**
Group a	**0.4 (0.8)**				Mean K2P distance between *M. diluviana* and *M. segerstralei* + *M. salemaai*: 8.2%
Group b	1.1	**N/A**			Range of mean divergences among members of the *M. relicta* group: 2.9%–9.3%
Group c	1.6	1.5	**0.3 (0.3)**		Range of mean divergences among marine *Mysis* species: 9.6%–16.7%
Group d	1.4	1.6	1.7	**0.6 (1.4)**	

*These two monophyletic groups were pooled, as deeper divergences were primarily of interest.

maxima ranging from 0.5 to 2.3% (and up to 3.7% for the monophyletic Caspian group). Within *M. diluviana*, four haplotype clusters showing 1.1–1.7% divergence from one another were apparent (Table 1; Fig. 3), but sampling was too sparse in the other species to draw conclusions about intraspecific patterning. Interspecific divergences were much higher, with a low of 2.9% between *M. segerstralei* and *M. salemaii* but ranging between 7.7% and 16.7% for all other pairs of species (Table 1). The inland North American species (*M. diluviana*) was an average of 8.2% divergent from its sister group (*M. segerstralei* + *M. salemaii*). The maximum extent of divergence among members of the *M. relicta* complex (9.3%) was similar to the lower end of the range of divergences among marine species (9.6–16.7%).

Phylogeographical patterns in Mysis *spp.*

The additional intraspecific geographical mtDNA sampling presented here confirmed that the two recently described North American species of the *M. relicta* group displayed a pronounced distributional disjunction (Fig. 1) that coincided with habitat occupation. *M. diluviana* occupied all inland lakes derived from the proglacial waterways formed during the last glacial retreat, while *M. segerstralei* occupied lakes that have been recently isolated (< 10 000 years ago) from the Arctic Ocean through isostatic rebound.

Within *M. diluviana*, the pairwise divergences of up to 2.3% are indicative of deep historical subdivisions within this inland species. Moreover, at least four distinct clusters of COI sequences may be identified (see Figs 1 and 3). Although bootstrap support is limited for two of them in the NJ phenogram, these clusters were also supported using other phylogenetic methods (maximum parsimony, results not shown). The geographical distribution of these haplotype groups, although not indicative of complete allopatry, indicates a spatial pattern of haplotype diversity. Among these four clusters, one was found in Great Slave Lake in arctic Canada alone (group b), while another cluster was widely distributed across the remaining inland regions sampled (group a). The Great Lakes region was the only area to harbour greater haplotype diversity, with three of the four haplotype clusters detected in that area (a, c, d), although we note that sampling was greater in that region (Fig. 1). Group c was only found in two Great Lakes, while group

d was only detected in southern populations, in Wisconsin, Lake Ontario, in southern Quebec, and in New York.

Lineage diversity in Limnocalanus

Forty-two COI haplotypes were detected within *Limnocalanus macrurus*, while five haplotypes were identified in *Limnocalanus johanseni* (Appendix II). Deep sequence division was present between these two species (average K2P distance of 27.0%, s.e. ± 2.5%), which was more than an order of magnitude greater than typical genetic divergences within *L. macrurus* (average K2P distance of 1.5%; s.e. ± 0.3%; maximum of 4.3%). Almost all third-position nucleotides showed substitutions between the two species. Ts/Tv vs. distance estimates between all *Limnocalanus* haplotypes and 10 other copepod species indicated a clear difference between intra- and interspecific comparisons. The p-distance between the two *Limnocalanus* species (22.1%, s.e. ± 1.6%) was comparable to those observed between copepods of different genera and families (*c.* 25%–35%). Ts/Tv values for pairwise comparisons within *L. macrurus* ranged between 0.33 and 13, but calculations were not possible for some within-species pairs due to a lack of transversions. The Ts/Tv ratios between *L. johanseni* and *L. macrurus* were within the range of values observed for comparisons between other species (0.82–2.93), as well as among members of different genera and families (0.82–2.14), indicating that COI nucleotide substitutions between *L. johanseni* and *L. macrurus* are saturated. Based on these results, *L. johanseni* haplotypes were removed from a subsequent analysis of *L. macrurus*, in order to explore better the shallower patterns within the latter species.

The topology of the *L. macrurus*-only NJ phenogram indicated three primary distinct haplotype lineages (LA, LB, LC), which were well supported by bootstrap analysis (Fig. 4) and by maximum parsimony analyses as well (Dooh 2003). However, there was evidence of further phylogenetic partitions. LA was the most diverse lineage examined, with a mean pairwise K2P distance of 1.1% (s.e. ± 0.2%; max. 2.5%) among its component haplotypes. By contrast, members of LB and LC displayed mean within-group distances of only 0.4% (s.e. ± 0.1%; max. 1.0%) and 0.7% (between the two haplotypes), respectively. These three lineages showed averages of 2.2–3.6% K2P divergence from one another. Inspection of the NJ tree and sequence data revealed that much of the observed haplotype diversity within individual lineages was related to single nucleotide changes. A significant departure from this pattern was observed within lineage LA, whose 23 component haplotypes appear to form two sublineages, showing an average of 1.5% (s.e. ± 0.3%) divergence from one another. The single haplotype from the Baltic Sea grouped with one of the LA clusters (containing haplotypes LA14-LA23) with strong bootstrap support (82%).

Phylogeographical patterns in L. macrurus

The two dominant lineages of *L. macrurus* detected in this study exhibited strong phylogeographical structure (Fig. 2). Lineage LA was found in lakes throughout the Canadian Arctic islands, and also occurred in the Beaufort and Baltic Seas. By contrast, the second dominant lineage (LB) occupied freshwater lakes, from the Mackenzie Delta to the St Lawrence River valley. These two lineages occurred in close proximity within the Mackenzie Delta region, but did not occur sympatrically as LA was found in the Beaufort Sea, while LB occurred in adjacent lakes. Thus, while both lineages have extensive ranges, they are nonoverlapping.

The remaining lineage (LC) included just two haplotypes, which were detected at a single locale (site 47) on the Melville Peninsula (Fig. 2; Appendix II). This lake also contained four haplotypes belonging to lineage LA (Appendix II), including the relatively divergent haplotypes LA22 and LA23. This site occurred at one of the highest altitudes (50 m above sea level) within this study, making it one of the oldest arctic lake populations investigated.

Within each lineage, most sites possessed one of a few widely distributed haplotypes. For example, LB1 occurred in 10 of 26 inland lakes, from the westernmost site (60), to the easternmost site (78). LB8 and LB16 were the only other haplotypes of lineage LB found in more than one lake, with LB16 occurring from the Mackenzie Delta (site 60) to Quebec (site 78), while LB8 was limited to three lakes in eastern Ontario and western Quebec (Appendix II). A similar pattern was observed in the Arctic, where three haplotypes (LA1, LA2, LA3) were dominant, collectively occurring in 45 of the 59 sites. By contrast, the other LA sublineage of 10 haplotypes was restricted to six locations in the eastern Arctic, and occurred at the one locality sampled in the Baltic Sea.

Discussion

This study provides the first extensive survey on phylogeographical structure for North American populations of the crustacean glacial relicts *Limnocalanus macrurus* and the *Mysis relicta* species group. Our work reveals strong concordance in their phylogeographical patterns, as both taxa are partitioned into two major groups: one restricted to mainland lakes, and a second to arctic lakes recently isolated from the sea through isostatic rebound. In addition, the polar lineage within each group (i.e. *Limnocalanus* lineage LA and *Mysis segerstralei*) shows close affinities with specimen(s) from Europe, while inland lineages (i.e. lineage LB and *Mysis diluviana*) appear to be North American endemics. However, there is also a key difference: sequence divergences between arctic and inland species of *Mysis* are much deeper, indeed fourfold greater, than between similarly distributed phylogroups within the species

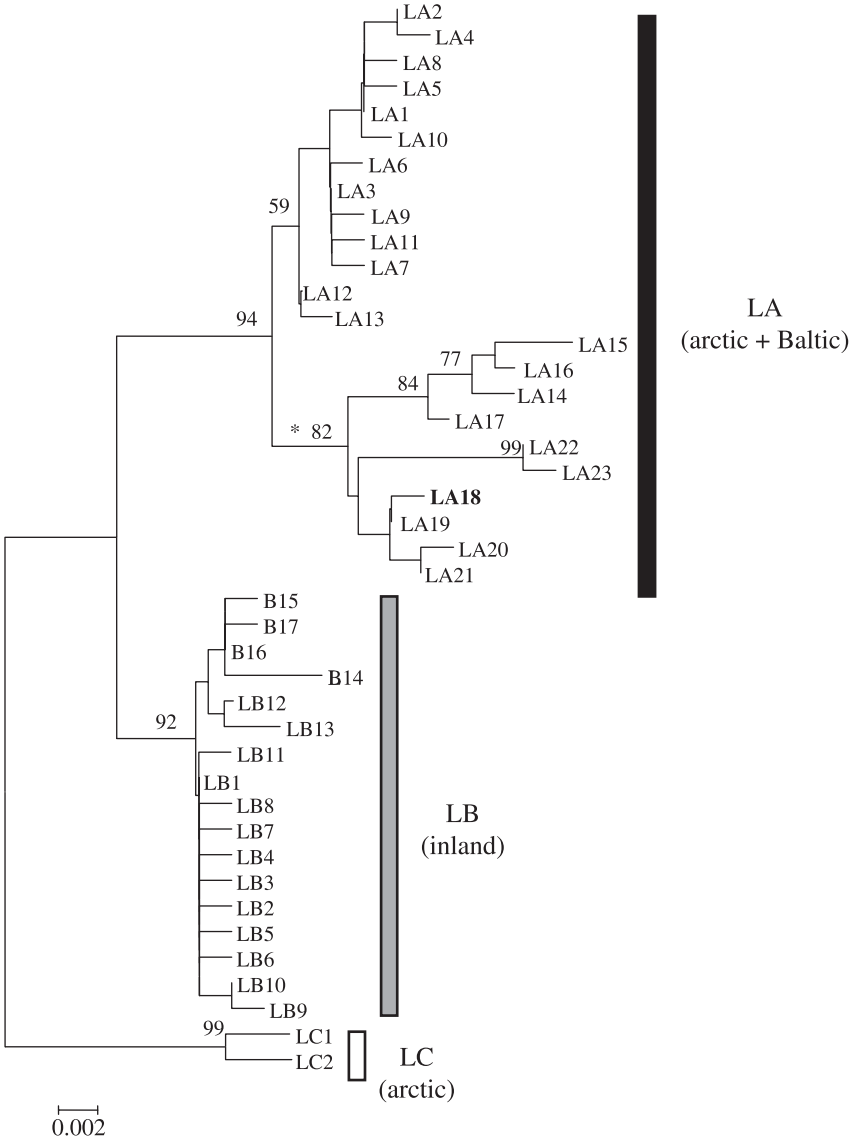

Fig. 4 NJ phenogram for *Limnocalanus macrurus* COI sequences, based upon K2P genetic distances. Three main haplotype clusters (LA, LB, and LC) are designated. An asterisk indicates the subgroup within lineage LA which contains the sole haplotype (LA18, highlighted in bold) from the Baltic Sea. Bootstrap values are presented for deep nodes, and for those having support greater than 70%. We note that *Limnocalanus johanseni* haplotypes showed 27% divergence from *L. macrurus*; these sequences were omitted from this phenogram in order to demonstrate better the shallower phylogenetic structure within *L. macrurus*.

L. macrurus. This discrepancy, combined with evidence from previous studies, suggests that the immigration history of glacial relicts is complex, involving multiple inland invasions over a long timeframe. The balance of this discussion considers the histories of both taxa in greater detail, and we propose a largely overlooked explanation that may account for the frequent discrepancy between molecular vs. geological estimates for the timing of inland invasion in glacial relicts.

Before the ice: the ages of glacial relict taxa

This study contributes to a growing body of evidence that many glacial relict taxa are more ancient than the Pleistocene origin that was previously supposed. Indeed, for three of the four glacial relict crustaceans so far examined from a molecular perspective, the levels of sequence or allozyme divergence between arctic and inland populations are suggestive of a more ancient divergence. Moreover, the lack of congruence of estimates among the different taxa is puzzling for this suite of species once thought to share a largely similar biogeographical history. We briefly review the results for the different glacial relict taxa investigated to date.

For *Mysis*, an average divergence of 8.2% in COI separates the inland lacustrine North American species *M. diluviana* from its circumarctic relatives. Cautiously applying several mitochondrial DNA molecular clocks (1.4% divergence per Myr, Knowlton & Weigt 1998; 2% per Myr, Brown *et al*. 1979; 2.3% Myr, Brower 1994) is suggestive of about 3.6–5.9 Myr of independent evolutionary history. Thus, this level of divergence does suggest an origin predating the onset of

the Pleistocene 2.4 Ma. Similarly, levels of divergence between arctic marine and inland Eurasian *Mysis* (i.e. *Mysis relicta* s.s and the Caspian group) are indicative of independent Eurasian origins of inland taxa prior to the Pleistocene (Audzijonytë *et al*. 2005).

Genetic results for two other glacial relict crustaceans, the amphipods *Gammaracanthus* and *Diporeia*, are similarly indicative of a pre-Pleistocene origin. COI results for the northern European lacustrine species *Gammaracanthus lacustris* indicate a divergence from marine species *c*. 5–8.5 Ma (based upon a COI divergence of 12% from its closest estuarine relative; Väinölä *et al*. 2001). However, the age of the Caspian *Gammaracanthus* species at about 2–3.5 Myr (5% COI divergence from its northern estuarine sister species) is consistent with an independent invasion of inland waters possibly occurring in the early Pleistocene. Allozyme results for the North American genus *Diporeia* are suggestive of an even older age, showing perhaps tens of millions of years of independent evolutionary history compared with marine and brackish relatives (Väinölä & Varvio 1989). Future work involving DNA sequencing may help to clarify the age of this taxon. Similarly, genetic data would be desirable for the final North American crustacean member of the glacial relict assemblage of species (Dadswell 1974), *Senecella calanoides*, as well as amphipod *Pallasea quadrispinosa* and isopod *Mesidotea entomon* from Europe (see Segerstråle 1982).

In contrast to these previous results for other glacial relict crustaceans, the present study revealed that *L. macrurus* alone displays genetic patterning that closely mirrors traditional expectations (e.g. Ricker 1959) for a taxon with a glacial relict distribution. It shows only shallow divergence between a haplotype cluster found in coastal arctic lake populations (recently captured from the sea by isostatic rebound) and one exclusively inhabiting inland lacustrine environments. The mean COI divergence of 2.2% is suggestive of independent evolution between these groups of just 0.9–1.6 Myr, well within a Pleistocene scenario for inland invasion. Indeed, evidence of rapid sequence divergence early in the history of lineage isolation (Howell *et al*. 1996; Ho *et al*. 2005) suggests that these lineages could have been separated even more recently. Interestingly, in showing such shallow divergences between arctic and inland lineages, *Limnocalanus* more closely resembles the glacial relict fish *Myoxocephalus quadricornis* (see Kontula & Väinölä 2003) than the other glacial relict crustaceans studied to date.

The varied depths of sequence divergence among these taxa are suggestive of a complex history for the glacial relict fauna, reflecting multiple origins over a long time period from ancestral arctic marine and estuarine lineages. Moreover, most of these cladogenetic events appear to predate the Pleistocene. Thus, these results could lead one towards the conclusion that traditional scenarios of inland invasion, i.e. being pushed inland in lakes at the edges of advancing glacial sheets, may not apply to most of these taxa. Indeed, other mechanisms of inland invasion merit consideration (such as estuarine species moving inland through river systems, or euryhaline species being transported inland by other vectors such as birds). However, the close association between inland distributions and glacial extents, combined with the absence of these species in suitable habitats outside of proglacial drainage areas, suggests that the glacial-advance hypothesis should not be abandoned lightly. Moreover, the glacial relicts' lineages may have originated in an alternative setting.

Marine divergence?

Although most of the glacial relict lineages are older than previously supposed, as evidenced by deep divergences from their marine counterparts, it is possible that cladogenesis occurred in arctic waters prior to inland invasion. If so, this pattern could account for some of the discrepancies between molecular divergences and the geological events that seem most likely to have provoked inland movement. Thus, we suggest that there may be a propensity for lineage proliferation in the Arctic, producing a decoupling between cladogenesis and inland invasion.

Evidence of lineage diversification in arctic marine and estuarine settings suggests that a marine origin for current inland lineages is plausible. In the genus *Mysis*, the Caspian group of four species is exclusively inland, as is *M. relicta* s.s. in Europe and *M. diluviana* in North America. All of the remaining species occur in marine, estuarine and/or young coastal marine lakes (Audzijonytë & Väinölä 2005), as do all of the deep phylogenetic divergences in the genus (Audzijonytë *et al*. 2005), suggesting that marine settings have been the cradle of *Mysis* diversification, fostering most speciation events. Moreover, the timeframe for these marine speciation events is very similar to some of the divergences within the *M. relicta* species group. Within *L. macrurus*, lineage diversification is also apparent in the Arctic, as the diversity of haplotypes is greater in polar coastal than inland regions. Moreover, the two divergent haplotype lineages that are restricted to northern settings show a deeper split with one another (3.5%) than either does to the third, inland group (2.1–2.6%), indicating northern lineage genesis. Finally, allozyme evidence also reveals significant genetic structure among coastal lake populations of *L. macrurus* recently captured from the sea (Dooh 2003), indicating that modern marine lineages are not panmictic.

The geological and hydrological history of the Arctic Ocean reveals many opportunities for the sundering of populations. Many large rivers drain into the Arctic Ocean, producing large variability in salinity concentrations between estuaries and intervening waters, which may provide a setting promoting divergence. Indeed, estuaries have been

associated with genetic divergence in other taxa, such as the copepod *Acartia tonsa* along the Atlantic coast (Caudill & Bucklin 2004). Moreover, arctic seas receive large and variable amounts of freshwater from major north-flowing river systems, from glacial melts (Fisher & Smith 1994), and from sea ice (Aagaard & Carmack 1989). Physiological adaptation to freshwater conditions is a key feature of the glacial relict group, and differential salinity tolerance among conspecific populations may promote diversification (Lee 1999). Moreover, shifting ice-sheets and ice-free corridors throughout at least the late Pliocene (Williams 1993) and Pleistocene would have resulted in physical disjunctions forming and dissolving, facilitating allopatric population divergence. Therefore, aspects of both the ionic and physical structures of arctic waters may have set the stage for pre-Pleistocene, as well as Pleistocene, diversification.

Thus, if we accept that marine diversification is a *plausible* explanation for lineage origin, and that glaciation is the most *likely* explanation for the inland distributions, these premises jointly suggest a history of glacial relicts far more compatible with traditional scenarios. While most other studies have interpreted marine-inland genetic divergences to equate to the single best estimate for the timing of inland invasion, we suggest an alternative approach that considers a *range* of possibilities. We suggest treating the divergences of marine-inland sister taxa as maximum ages for the timing of inland invasion, while interpreting sequence variation among inland populations as providing a minimum timeframe for continental occupancy. For example, within *M. diluviana* in North America, average divergences among haplotype clusters were up to 1.7%, with maximum pairwise sequence divergence at 2.3%. These maximal within-species divergences are suggestive of a minimum occupancy of *M. diluviana* in inland waters of 0.7–1.6 Myr, figures that are fully compatible with Pleistocene scenarios for inland invasion.

As with *M. diluviana* in North America, *M. relicta* s.s. in Europe displays limited intraspecific sequence divergence (up to 0.5%). Likewise, divergences among members of the Caspian group of *Mysis* are also limited (1.4–3.7%). Shallow intraspecifc divergences are also known for *G. lacustris* (0.7%), although the geographical extent of mitochondrial DNA sampling is limited for this taxon (Väinölä *et al*. 2001). All of these values produce *minimum* estimates that place the movement inland within the realm of Pleistocene events.

If divergences among inland populations are considered for *L. macrurus*, these suggest an even more recent history of inland occupancy for this taxon. The very shallow divergences among its inland lineages (maximum of 1%) suggest continental invasion about 0.5 Ma or even more recently (Howell *et al*. 1996; Denver *et al*. 2000; Ho *et al*. 2005). Moreover, its distribution is easily harmonized with recent glacial events. However, within the genus *Limnocalanus*, *L. johanseni* presents somewhat of an enigma, occurring only in the Mackenzie delta and being highly divergent (27%) from *L. macrurus*. However, despite this great divergence, it could have been pushed inland relatively recently from arctic waters. Deciphering the history of its origins is problematic, due to a lack of close relatives in arctic waters. However, other types of data (such as physiological or palaeolimnological) may in the future help to elucidate its history. Thus, while further sequence data would be desirable (especially for *Diporeia* and *Senecella*), current evidence regarding continental sequence divergence suggests that inland invasion could have been more recent than would be indicated when considering marine-inland divergences alone.

While the above account outlines a plausible explanation for discrepancies between molecular observations and expectations, it is important to consider a number of caveats. First of all, this marine-divergence scenario requires many mitochondrial lineage extinctions in order to be compatible with the deep arctic-inland divergences observed. However, the deep divisions among arctic marine and estuarine species of *Mysis* appear to be compatible with such rates. Second, it may be the case that inland invasion did occur well prior to the Pleistocene, in accordance with the face-value genetic divergences, but that glaciation had such a dominant role in causing mtDNA lineage pruning that inland phylogeography does not reflect this more ancient move inland. Although this problem with our hypothesis cannot be dismissed, we note that the depths of divergence among inland phylogroups (at least in *Mysis*) are compatible with their survival through several glacial cycles. Third, although our explanation does not depend upon precise molecular clock-based divergence times, gross deviations from standard rates would profoundly influence even broad (e.g. Pleistocene vs. pre-Pleistocene) discussions about timeframes. While preliminary analyses showed molecular rates to be fairly consistent both within the *M. relicta* group and within *Limnocalanus*, a lack of absolute calibration points for both groups substantially limits conclusions about timing. Any analysis attempting to construct taxon-specific molecular clocks for these (and other) groups would be important contributions to our understanding of glacial relict evolution. Thus, the present marine-divergence proposal may be tested more definitively in the future.

Comparative continental phylogeography

Above, we reconsidered the potential timing of inland invasion of glacial relict taxa. Although we have argued that cladogenesis resulting in speciation may have occurred in arctic seas, with just one element pushed inland with the ice sheets, phylogeographical evidence suggests a major role for glaciation in structuring genetic patterns and distributional extents once taxa were inland. We briefly

consider the similarities and differences between the continental patterns of *L. macrurus* and *M. diluviana* and two comparison groups.

Our data set for *L. macrurus* represents the most comprehensive sampling to date of intraspecific variation in a glacial relict taxon across a large geographical area. The broad distribution of a single shallow cluster of mitochondrial haplotypes suggests recent colonization perhaps from a single — probably Mississipian — refugial source. Its distribution largely falls within the region influenced by the various outlets of glacial Lake Agassiz, a massive water body that has been recognized as an important dispersal corridor for freshwater fishes (e.g. Bernatchez & Dodson 1991; Rempel & Smith 1998; Wilson & Hebert 1998). Having reached its maximum size of some 440 000 km^2 at 9900 BP, it largely drained northwards (Fisher & Smith 1994). If *L. macrurus* was present in the Lake Agassiz region before then, it could have easily assumed much of its current inland distribution.

Similarly, *M. diluviana* also appears to bear a substantial phylogeographical signature from Lake Agassiz, with one main haplotype group displaying an extensive distribution across the proglacial lake chain in the Canadian Shield and in the Great Lakes Basin. However, inland haplotypes displayed deeper genetic divides than observed in *L. macrurus*, suggestive of a longer period of inland occupancy and divergence. Our results suggest four main haplotype groupings, but the overall patterning of haplotype diversity is not dependent upon this specific conclusion. In addition to the widespread haplotype group, two of these were present in the Great Lakes basin, suggesting admixture of different refugial stocks, such as Atlantic and Mississipian, similar to patterns seen in some fishes (Wilson & Hebert 1998; Turgeon & Bernatchez 2001). By contrast, a single distinct haplotype was found in Great Slave Lake, a location suggestive of a Beringian source. Väinölä *et al*. (1994) favoured a separate western refugium to account for allozyme affinities among populations from Lake Huron, Manitoba, and the Mackenzie Delta. Further COI sampling in the far north and western Canadian shield would be desirable to test this scenario, and to check for possible admixture of Agassiz and Beringian sources.

Because all glacial relict crustaceans release their eggs directly into deep water lakes (e.g. Roff 1972), they possess poor ability for dispersal among water bodies. Thus, it is not surprising that their continental phylogeographies largely mimic those of fishes found in previously glaciated regions (see Bernatchez & Wilson 1998; Wilson & Hebert 1998). In both groups, phylogenetic divergences are shallow (almost nonexistent within the recent continental invader *L. macrurus*) and seem primarily to reflect recent glacial history, including proglacial lake localities and drainage routes (although some differences result from fish being able to swim upstream; see Cox & Hebert 2001). By contrast, another group of crustaceans that has received continent-wide attention often displays a different pattern. The cladocerans differ in being (generally cyclic) parthenogens, which enables population establishment from a single individual, and in having resting eggs that can withstand desiccation and transport via animal dispersal vectors (see De Meester *et al*. 2002 for a review). Their phylogeographical patterns within formerly glaciated regions are sometimes characterized by deeper genetic divergence and crisper delineation among allopatric phylogroups than those of fish or glacial relict crustaceans (Cox & Hebert 2001). This pattern is perhaps attributable to their greater resistance to extinction in refugia, due to resting egg banks, through numerous glacial cycles (Cox & Hebert 2001) and to the lower potential for admixture of lineages due to large population sizes and resource monopolization (De Meester *et al*. 2002). Cladoceran phylogeography also reflects their potential for dispersal beyond particular glacial drainage routes via ice flows or bird-mediated dispersal (Weider *et al*. 1999; Cox & Hebert 2001; Figuerola *et al*. 2005). Thus, the glacial relict vs. cladoceran crustaceans exemplify virtually opposite dispersal syndromes, and highlight the diverse ways that the environment can impact biogeographical history depending upon organismal biology.

Broadening the scope of analysis beyond North America revealed a second interesting phylogeographical pattern in *Limnocalanus* and *Mysis*. In both *L. macrurus* (lineage LA) and in *M. segerstralei*, lineages in arctic North America and Europe were closely similar, while temperate lineages were apparently endemic (although more European sampling is desirable for *Limnocalanus*). In contrast to the continental results, this pattern mimics that observed in phylogeographical surveys of cladocerans. For example, in *Holopedium gibberum* and several species of *Daphnia*, close genetic associations exist between arctic lineages in Europe and North America, while temperate zones are generally characterized by endemism (Rowe 2000; Schwenk *et al*. 2000). The mechanism(s) underlying this biogeographical similarity between glacial relicts and cladocerans is not yet clear. While cladocerans may be dispersed via ice flows or circumarctic birds, the euryhaline glacial relicts may utilize marine and brackish dispersal corridors that bridge continents. Thus, this interesting pattern should be the focus of future study.

Concluding remarks

The glacial relict crustaceans exemplify the phenomenon of phylogeographical 'pseudocongruence' (Taylor *et al*. 1998), and, notably, they do so doubly. First, incongruence is observed between phylogeny and geology, in terms of the depth of sequence divergence compared with the timing of geological events that supposedly caused cladogenesis. Second, discordance in divergence is prevalent among taxa

previously thought to share a biogeographical history. These results have forced revisions of previous scenarios regarding the ages and phylogenetic relationships of inland invaders (e.g. Väinölä *et al*. 2001; Audzijonytė *et al*. 2005), and we extend this trend here. We have suggested that cladogenetic events in arctic marine waters may explain the discrepancy between deep phylogenetic ages on the one hand and inland distributions closely mirroring glacial extents on the other. While further DNA sequencing (e.g. on *Diporeia* and *Senecella*) will contribute to our understanding of the varied histories of the glacial relict fauna, and of our marine divergence hypothesis, the evidence for incongruence is now solid. Despite the apparent lack of a clear and homogenous explanation, the new perspectives presented here suggest that glacial relicts provide a rich body of natural experiments for future study. Further investigations of the interplay between the complex history of arctic waters, varied physiological regimes, and patterns of molecular evolution may provide valuable new insights into mechanisms for speciation.

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Robert Dooh's research on glacial relict crustaceans was completed as part of his MSc degree at the University of Guelph. He is currently Information Officer for the Canadian Barcode of Life Network. Sarah Adamowicz investigates comparative patterns of diversification and evolutionary trends in aquatic invertebrates; she recently completed her PhD at Imperial College London. Paul Hebert holds a Canada Research Chair in Molecular Biodiversity at the University of Guelph where his research spans a broad range of questions regarding the diversity and history of species. Most current work is directed towards the Barcode of Life Initiative (http://www.barcodeoflife.org/).

Appendix I

Collection localities of *Mysis* populations in North America and Europe (also see Fig. 1). Approximate coordinates for these sites are given in decimal degrees. 'N' designates the numbers of *Mysis* specimens sequenced from each site, and the codes for all sequences detected are provided

Site	Location	Latitude	Longitude	*N*	Haplotypes present	BOLD Accession no.
	M. segerstralei					
1	Axel Heiberg 9, Nunavut	80.405	−89.514	2	MSe1, MSe2	RDMYS001-06, RDMYS002-06
2	Char Lake, Nunavut	74.700	−94.833	1	MSe3	RDMYS003-06
3	Immerk Lake, Nunavut	75.683	−84.617	1	MSe3	RDMYS004-06
	M. diluviana					
4	Great Slave Lake, NWT	62.338	−114.318	1	MD5	RDMYS005-06
5	Waterton Lakes, Alberta	49.031	−113.905	1	MD16	RDMYS006-06
6	Southern Indian lake, Manitoba	56.812	−98.876	2	MD14, MD10	RDMYS007-06, RDMYS008-06
7	Lake of the Woods, Ontario	49.722	−94.462	2	2xMD10	RDMYS009-06, RDMYS010-06
8	Rainy Lake, Ontario	48.685	−92.979	2	MD6, MD7	RDMYS011-06, RDMYS012-06
9	Lake Nipigon, Ontario	49.436	−88.193	2	2xMD10	RDMYS013-06, RDMYS014-06
10	Lake Superior, Ontario	48.142	−87.697	7	MD4, MD8, MD12, MD13, 3xMD10	RDMYS015-06, RDMYS016-06, RDMYS017-06, RDMYS018-06, RDMYS019-06, RDMYS020-06, RDMYS021-06
11	Green Lake, Wisconsin	43.797	−89.003	1	MD1	RDMYS022-06
12	Lake Ontario, Ontario	43.747	−78.000	2	MD2, MD3	RDMYS023-06, RDMYS024-06
13	Lac des Quinzes, Quebec	47.569	−79.192	2	MD9, MD10	RDMYS025-06, RDMYS026-06
14	Lake Temiskaming, Ontario	47.460	−79.565	2	MD11, MD10	RDMYS027-06, RDMYS028-06
15	Lac Chibougamau, Quebec	49.785	−74.355	1	MD15	RDMYS029-06
	M. salemaai					
16	Lough Neagh, Northern Ireland	54.704	−6.266	2	MSa1, MSa2	RDMYS030-06, RDMYS031-06
	M. oculata					
17	Alexandra Fiord, Nunavut	78.673	−74.717	2	MO1, MO2	RDMYS032-06, RDMYS033-06
18	Resolute Bay, Nunavut	74.683	−94.883	1	MO3	RDMYS034-06
	M. c.f. litoralis					
19	Bird Cove, Churchill, Manitoba	*c.* 58.7	*c.* −94.25	2	ML1, ML2	RDMYS035-06, RDMYS036-06
	M. stenolepis					
20	Atlantic, near St Andrews, New Brunswick	*c.* 45.1	*c.* −67.1	1	MS1	RDMYS037-06

Appendix II

Collection locations for *Limnocalanus macrurus* in North America (1–85) (also see Fig. 2) and for one site in Europe (86). *Limnocalanus johanseni* (*) was collected from three freshwater lakes (87–89) in the western Arctic. Approximate site coordinates are given in decimal degrees. The number of specimens sequenced (N) and the haplotypes detected are provided for each site

Site	Location	Latitude	Longitude	*N*	Haplotypes	BOLD Accession no.
1	Nansen Sound 17, Nunavut	81.107	−91.901	1	LA1	RDCNA001-06
2	Nansen Sound 16, Nunavut	81.063	−92.009	1	LA9	RDCNA002-06
3	Nansen Sound 31, Nunavut	81.043	−94.881	1	LA11	RDCNA003-06
4	Nansen Sound 29, Nunavut	81.033	−94.623	1	LA3	RDCNA004-06
5	Nansen Sound 2, Nunavut	80.538	−90.417	1	LA3	RDCNA005-06
6	Nansen Sound 1, Nunavut	80.537	−90.295	1	LA9	RDCNA006-06
7	Axel Heiberg 9, Nunavut	80.405	−89.514	2	LA12, LA19	RDCNA007-06, RDCNA008-06
8	Axel Heiberg 10, Nunavut	80.383	−89.196	1	LA14	RDCNA009-06
9	Axel Heiberg 1, Nunavut	80.170	−87.725	1	LA12	RDCNA010-06
10	Eureka 5, Nunavut	79.867	−85.083	4	2xLA6, 2xLA3	RDCNA011-06, RDCNA012-06, RDCNA013-06, RDCNA014-06

Appendix II *Continued*

Site	Location	Latitude	Longitude	*N*	Haplotypes	BOLD Accession no.
11	Alexandra Fiord 20, Nunavut	78.804	−74.845	2	2xLA15	RDCNA015-06, RDCNA016-06
12	Thomas Lee 1, Nunavut	75.576	−89.311	1	LA2	RDCNA017-06
13	Thomas Lee 2, Nunavut	75.961	−89.946	1	LA8	RDCNA018-06
14	Thomas Lee 3, Nunavut	75.954	−90.756	2	2xLA3	RDCNA019-06, RDCNA020-06
15	Beechy Region 2, Nunavut	75.642	−91.697	1	LA1	RDCNA021-06
16	Beechy Region 7, Nunavut	75.558	−92.078	1	LA1	RDCNA022-06
17	Disappointment Bay 8, Nunavut	75.541	−95.483	1	LA3	RDCNA023-06
18	Disappointment Bay 10, Nunavut	75.540	−95.366	1	LA3	RDCNA024-06
19	Disappointment Bay 2, Nunavut	75.465	−95.643	1	LA3	RDCNA025-06
20	Disappointment Bay 4, Nunavut	75.431	−95.654	1	LA2	RDCNA026-06
21	Little Cornwallis 10, Nunavut	75.470	−96.557	1	LA2	RDCNA027-06
22	Freemans Cove 14, Nunavut	75.681	−97.565	1	LA2	RDCNA028-06
23	Freemans Cove 11, Nunavut	75.564	−97.840	1	LA2	RDCNA029-06
24	Freemans Cove 7, Nunavut	75.466	−97.780	1	LA2	RDCNA030-06
25	Bracebridge 3, Nunavut	75.657	−99.078	1	LA4	RDCNA031-06
26	Bracebridge 4, Nunavut	75.643	−99.310	1	LA2	RDCNA032-06
27	Bracebridge 9, Nunavut	75.460	−99.911	1	LA2	RDCNA033-06
28	Bracebridge 10, Nunavut	75.433	−99.963	1	LA2	RDCNA034-06
29	Freemans Cove 8, Nunavut	75.453	−97.458	1	LA2	RDCNA035-06
30	Freemans Cove 20, Nunavut	75.139	−97.772	1	LA2	RDCNA036-06
31	Freemans Cove 26, Nunavut	75.034	−99.122	1	LA2	RDCNA037-06
32	Freemans Cove 23, Nunavut	75.027	−98.492	1	LA2	RDCNA038-06
33	Freemans Cove 24, Nunavut	75.015	−98.721	1	LA2	RDCNA039-06
34	Lowther Island 1, Nunavut	74.567	−97.439	1	LA2	RDCNA040-06
35	Resolute 2, Nunavut	74.827	−95.597	1	LA3	RDCNA041-06
36	Resolute 13, Nunavut	74.810	−95.200	1	LA1	RDCNA042-06
37	Resolute 16, Nunavut	74.678	−94.272	1	LA1	RDCNA043-06
38	Sophia Lake 1, Nunavut	75.098	−93.614	1	LA2	RDCNA044-06
39	Beechy Region 13, Nunavut	74.696	−91.247	1	LA1	RDCNA045-06
40	Beechy Region 14, Nunavut	74.698	−91.232	1	LA1	RDCNA046-06
41	Beechy Region 11, Nunavut	74.728	−91.337	1	LA1	RDCNA047-06
42	Aston Bay 23, Nunavut	73.879	−95.216	1	LA5	RDCNA048-06
43	Aston Bay 18, Nunavut	73.770	−94.996	1	LA1	RDCNA049-06
44	Aston Bay 17, Nunavut	73.678	−95.546	1	LA1	RDCNA050-06
45	Creswell Bay 9, Nunavut	72.653	−94.199	1	LA1	RDCNA051-06
46	Berlinguet Inlet 4, Nunavut	70.784	−86.864	1	LA1	RDCNA052-06
47	Encampment Bay 7, Nunavut	69.792	−84.808	7	LA1, 2xLA13, LA22, LA23, LC1, LC2	RDCNA053-06, RDCNA054-06, RDCNA055-06, RDCNA056-06, RDCNA057-06, RDCNA058-06, RDCNA059-06
48	Grinell 7, Nunavut	69.669	−83.250	2	2xLA1	RDCNA060-06, RDCNA061-06
49	Richards Bay 66, Nunavut	69.537	−82.489	1	LA1	RDCNA062-06
50	Richards Bay 32, Nunavut	69.477	−82.377	1	LA1	RDCNA063-06
51	Richards Bay 12, Nunavut	69.611	−82.694	1	LA1	RDCNA064-06
52	Richards Bay 58, Nunavut	69.480	−82.275	1	LA10	RDCNA065-06
53	Richards Bay 4, Nunavut	69.556	−82.766	1	LA1	RDCNA066-06
54	Richards Bay 20, Nunavut	69.545	−82.424	1	LA1	RDCNA067-06
55	Richards Bay 21, Nunavut	69.531	−82.487	2	2xLA17	RDCNA068-06, RDCNA069-06
56	Richards Bay 36, Nunavut	69.440	−81.997	1	LA1	RDCNA070-06

Appendix II *Continued*

Site	Location	Latitude	Longitude	*N*	Haplotypes	BOLD Accession no.
57	Foxe Plains 1, Nunavut	69.217	−81.717	2	2x LA1	RDCNA071-06, RDCNA072-06
58	Parry Bay 9, Nunavut	67.799	−81.844	3	LA16, LA20, LA21	RDCNA073-06, RDCNA074-06, RDCNA075-06
59	Beaufort Sea, NWT	69.438	−133.063	1	LA7	RDCNA076-06
60	MacKenzie Delta 19, NWT	69.141	−134.683	2	LB1, LB16	RDCNA077-06, RDCNA078-06
61	Arctic Red River 4, NWT	67.707	−132.574	3	LB9, 2xLB10	RDCNA079-06, RDCNA080-06, RDCNA081-06
62	Great Slave Lake, NWT	63.338	−114.318	2	2xLB1	RDCNA082-06, RDCNA083-06
63	Southern Indian Lake, Manitoba	56.812	−98.876	1	LB3	RDCNA084-06
64	Amisk Lake, Saskatchewan	54.685	−102.079	1	LB15	RDCNA085-06
65	Lake of the Woods, Ontario	49.722	−94.462	1	LB1	RDCNA086-06
66	Rainy Lake, Ontario	48.685	−92.979	1	LB5	RDCNA087-06
67	Lake Nipigon, Ontario	49.436	−88.193	1	LB1	RDCNA088-06
68	Lake Superior, Ontario	47.581	−86.917	2	LB13, LB16	RDCNA089-06, RDCNA090-06
69	Talon Lake, Ontario	46.316	−79.354	1	LB1	RDCNA091-06
70	Weslemkoon, Ontario	45.049	−77.405	2	2xLB8	RDCNA092-06, RDCNA093-06
71	Lake Temiskaming, Ontario	47.460	−79.565	1	LB14	RDCNA094-06
72	Lac des Quinzes, Quebec	47.569	−79.192	1	LB1	RDCNA095-06
73	Lac Remingy, Quebec	47.773	−79.233	1	LB1	RDCNA096-06
74	Lac Tapani, Quebec	46.882	−75.324	1	LB2	RDCNA097-06
75	Lac Gilman, Quebec	54.501	−74.341	2	2xLB1	RDCNA098-06, RDCNA099-06
76	Lac Chibougamau, Quebec	49.785	−74.355	1	LB16	RDCNA100-06
77	Saint Jean, Quebec	48.536	−72.204	1	LB17	RDCNA101-06
78	Lac Temiscouata, Quebec	47.707	−68.902	3	2xLB12, LB1	RDCNA102-06, RDCNA103-06, RDCNA104-06
79	St Francois, Quebec	45.829	−72.356	1	LB4	RDCNA105-06
80	Lac Massawippi, Quebec	45.218	−72.007	1	LB6	RDCNA106-06
81	Lake Champlain, Vermont	44.768	−73.290	2	2xLB1	RDCNA107-06, RDCNA108-06
82	Lac Pemichangan, Quebec	46.078	−75.856	2	2xLB8	RDCNA109-06, RDCNA110-06
83	Lac Cameron, Quebec	46.172	−75.921	1	LB11	RDCNA111-06
84	Blue Sea Lake, Quebec	46.211	−76.046	1	LB8	RDCNA112-06
85	Lake Canadaigua, New York	42.850	−77.269	1	LB7	RDCNA113-06
86	Baltic Sea	61.200	18.500	1	LA18	RDCNA114-06
87*	Tuktoyaktuk 3, NWT	69.404	−133.025	1	LJ5	RDCNA115-06
88*	Tuktoyaktuk 6, NWT	69.425	−133.001	2	LJ1, LJ3	RDCNA116-06, RDCNA117-06
89*	Demarcation Point 21, Alaska	69.669	−141.161	2	LJ2, LJ4	RDCNA118-06, RDCNA119-06