Evolution, 55(7), 2001, pp. 1408–1418

RAPID EVOLUTION IN THE *NEBRIA GREGARIA* GROUP (COLEOPTERA: CARABIDAE) AND THE PALEOGEOGRAPHY OF THE QUEEN CHARLOTTE ISLANDS

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Abstract.—Morphological differentiation in the ground beetles of the *Nebria gregaria* group, found on the Queen Charlotte Islands, has been used as support for the glacial refugium proposed for the northwest coast of North America. Two members of this species group, *N. charlottae* and *N. louiseae,* are restricted to cobble beaches in this archipelago. A third, *N. haida,* is found only in alpine regions of the archipelago and the adjacent mainland. The remaining two species of the *gregaria* group, *N. lituyae* and *N. gregaria,* show highly restricted distributions in the mountains of the Alaska panhandle and on the beaches of the Aleutian Islands, respectively. To determine the relationships of the five species, we conducted phylogenetic analyses on nucleotide sequence data obtained from five regions of the mitochondrial DNA. In total, 1835 bp were analyzed. The results suggest that one species, *N. lituyae,* does not belong in the *gregaria* group, and that only seven mutations separated the two most divergent of the four remaining species. We also conducted random amplified polymorphic DNA fingerprinting analyses on genomic DNA extracted from the five species. Analyses of genetic diversity revealed a lack of molecular differentiation among the Queen Charlotte species, suggesting that these populations may be postglacial in origin and that together *N. gregaria, N. charlottae, N. louiseae,* and *N. haida* might represent local variations of a single species. These results are consistent with conclusions derived for the morphological and genetical differentiation among *Gasterosteus* populations in the archipelago.

Key words.—Carabidae, glacial refugium, mitochondrial DNA, molecular evolution, phylogenetic analysis, Queen Charlotte Islands, random amplified polymorphic DNA.

Received August 29, 2000. Accepted March 25, 2001.

The glacial cycles of the late Pleistocene have defined the current biogeography of North America, affecting both the composition and distribution of the animal and plant species. The regular glacial advances and retreats have resulted in the movement of species across vast distances and the reorganization of many ecological communities in their wake. Glacial advances have occurred with a period of approximately every 100,000 years with the most recent (Wisconsin) ice age beginning approximately 70,000 years ago, reaching a maximum extent during the Frasier glaciation between 25,000 and 15,000 years ago. (Liu 1992). At the height of the Frasier glaciation ice sheets occupied much of the northern half of the continent, leaving only a scattering of localities along the coast uncovered (Blaise et al. 1990). These ice-free refugia existing along the edge of the ice sheets are known for their role in maintaining species diversity through the glaciations by sheltering species and subspecies that would otherwise have risked extirpation. Refugia have also contributed to the biogeography of North America by allowing these species to rapidly colonize adjacent land in the wake of the glacial retreat (Pielou 1991). Whether coastal refugia have contributed to speciation, however, is still a topic of debate. In the South American and African tropics, the periodic fluctuations in global temperature that have characterized the late Pleistocene have promoted speciation through the creation of ecological refugia—isolating populations in fragmented forest and grassland ecosystems (Haffer 1977; Colinvaux 1998; Danin 1999) or on mountains and highlands during periods of global warming (Fjeldsa and Lovett 1997; Roy 1997; Smith et al. 1997). The physical refugia generated by the ice sheets along the coast of northern North America may have played a similar role in promoting speciation by maintaining populations in isolation from each other for periods of several tens of thousands of years.

On the northwest coast of North America, glacial refugia may have existed on Kodiak Island (Karlstrom and Ball 1969), the Alaskan panhandle (Kavanaugh 1988), and the Queen Charlotte Islands (Foster 1965), with the evidence based primarily on taxa that are either endemic to the refugia or display disjunct distributions. For the Queen Charlotte Islands, the largest of the hypothesized western refugia, the assemblage of endemic, dispersing, and disjunct taxa has included mammals (Foster 1965), birds (Cowan 1989), insects (Hamilton 1982; Ferguson 1987; Kavanaugh 1989), plants (Schofield 1989; Taylor 1989), and lichens (Brodo 1995). Although the glacial geology of the islands has suggested no refugia other than possible coastal beaches and mountaintop nunataks (Brown and Nasmith 1962), recent data suggest a possible refugial source area in the now submerged continental shelf separating the archipelago from the continent, as lowered sea levels may have exposed wide stretches of this terrain during the glacial period (Barrie et al. 1993; Josenhans et al. 1995).

One criterion used in evaluating evidence for glacial refugia is the extent of morphological differentiation or endemism in local biota. Similarity between the source populations on the continent and those on the adjacent islands suggests recent, presumably postglacial colonization, where-

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as marked dissimilarity suggests long-term separation. Yet the latter possibility pivots on the broad assumption that morphological change accrues slowly within isolated populations and that only the most minor of differences between populations were likely to have occurred in postglacial time (McCabe and Cowan 1945; Foster 1965). Under a more rigorous evaluation, however, the basis for this assumption would appear to be unfounded. Numerous examples of rapid postglacial morphological change have been documented, including the repeated evolution of benthic and limnetic morphs of fish (McPhail 1992) and size differences in insular rodent fauna (Angerbjorn 1986). On the Queen Charlotte Archipelago, studies on threespine stickleback demonstrate that the extreme population variation in body form and armor is an adaptive response to differences in the predator regimes among localities (Moodie and Reimchen 1976; Reimchen et al. 1985; Reimchen 1994), although there is evidence for deep phylogenetic branching among populations (O'Reilly et al. 1993; Deagle et al. 1996). Similarly, a molecular study of west coast black bears demonstrated that the morphologically distinct Queen Charlotte Islands subspecies (*Ursus americanus carlottae*) is very similar genetically to the coastal mainland bear population, indicating that the island and coastal subspecies have diverged very recently and that the morphological differences are likely an adaptation to differences in their respective habitats (Byun et al. 1997, 1999). The evidence that morphology can change rapidly, and has done so repeatedly through the postglacial period, necessitates a reexamination of the endemic Queen Charlotte Islands and western coastal taxa that have been used to support evidence for glacial refugia during the Fraser glaciation.

The ground beetles of the *Nebria gregaria* group, distributed intermittently along the northwestern coast of North America from the Queen Charlotte Islands to the Aleutians, are considered to be a taxon for which the repeated isolation of populations in glacial refugia has played an important role in the promotion of speciation (Kavanaugh 1992). Composed of five morphologically similar species, the members of the *Nebria gregaria* group display a remarkable degree of endemism to locations associated with hypothesized glacial refugia. The three Queen Charlotte Island species consist of *N. charlottae,* which is restricted to cobble beaches on Graham Island; *N. louiseae,* which is found in similar habitats on Moresby Island and many of the smaller islands on the southeast of the archipelago (Kavanaugh 1989); and *N. haida,* found only in alpine locations on Graham and Moresby Islands and on a mountaintop north of Prince Rupert, British Columbia (Kavanaugh 1992). Their closest relatives are *N. lituyae,* found in alpine areas in the Alaska panhandle, and *N. gregaria,* which occupies beaches on a number of the Aleutian Islands. Together, these five species are characterized by similar morphology, flightlessness, and a preference for low-temperature rocky habitats that contrasts with the riparian habitat and fully winged morphology of their wide-ranging sister taxa, *N. sahlbergii, N. acuta,* and *N. arkansana.* The five species of the *N. gregaria* group are believed to originated from a winged, riparian ancestor in the early Pleistocene and to have radiated in glacial refugia through the mid- to late-Pleistocene (Kavanaugh 1989). The earliest division within the group is thought to have been associated with habitat, grouping the alpine-dwelling species *N. lituyae* and *N. haida* into one lineage and the beach-dwelling species *N. gregaria, N. charlottae,* and *N. louiseae* into a second. Later periods of refugial isolation acted to produce the five species, with the most recent division between *N. gregaria* and *N. louiseae* believed to have occurred as a product of the Fraser glaciation (Fig. 1; Kavanaugh 1979a, 1989, 1992).

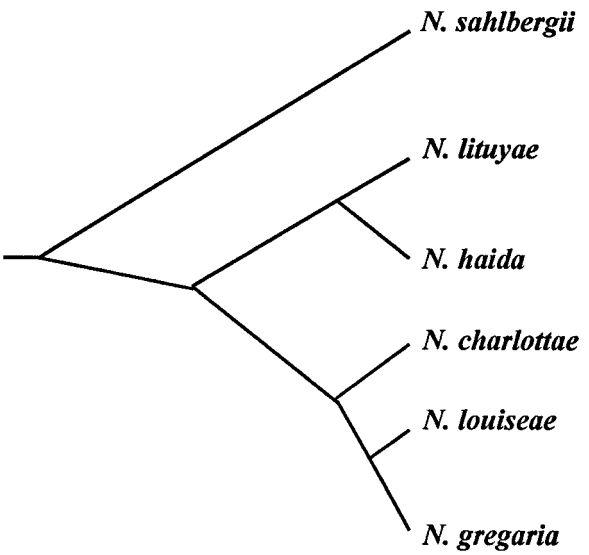

FIG. 1. Phylogeny of the *Nebria gregaria* infragroup (Kavanaugh 1989).

Evidence for morphological stasis and the extended periods of time required for reproductive isolation within the predacious ground beetles are based on data derived from paleoentomological studies of insect fossils from the high-arctic tundra, which show highly conserved morphology (Matthews 1980; Ashworth 1996). As such, the marginal morphological differentiation in the *gregaria* group is thought to represent multiple phases of speciation and stasis, beginning in the Early or Middle Pleistocene (Kavanaugh 1989). Yet, in view of the rapid morphological change observed in other Queen Charlotte taxa, we test here whether the diversification of the *gregaria* group is a recent event, either due to a rapid recolonization of northwest North America by a single *gregaria* species following the last glacial retreat, or the subdivision of a widespread ancestral species into Queen Charlotte, southwestern Alaskan, and Aleutian refugial populations during the most recent glacial advance. With no fossil evidence of long-term occupation of the Queen Charlotte Islands by the *gregaria* group and little probability that any such evidence could be found, given the extreme glacial conditions on the Pacific Northwest, we have used molecular markers to examine the phylogeny, age, and species status of these beetles to determine whether their biogeography indicates survival in a west coast refugium during the Fraser glaciation.

MATERIALS AND METHODS

Collections

Specimens of *Nebria* for use in DNA sequence analysis were collected into 80% ethanol from sites on the Queen

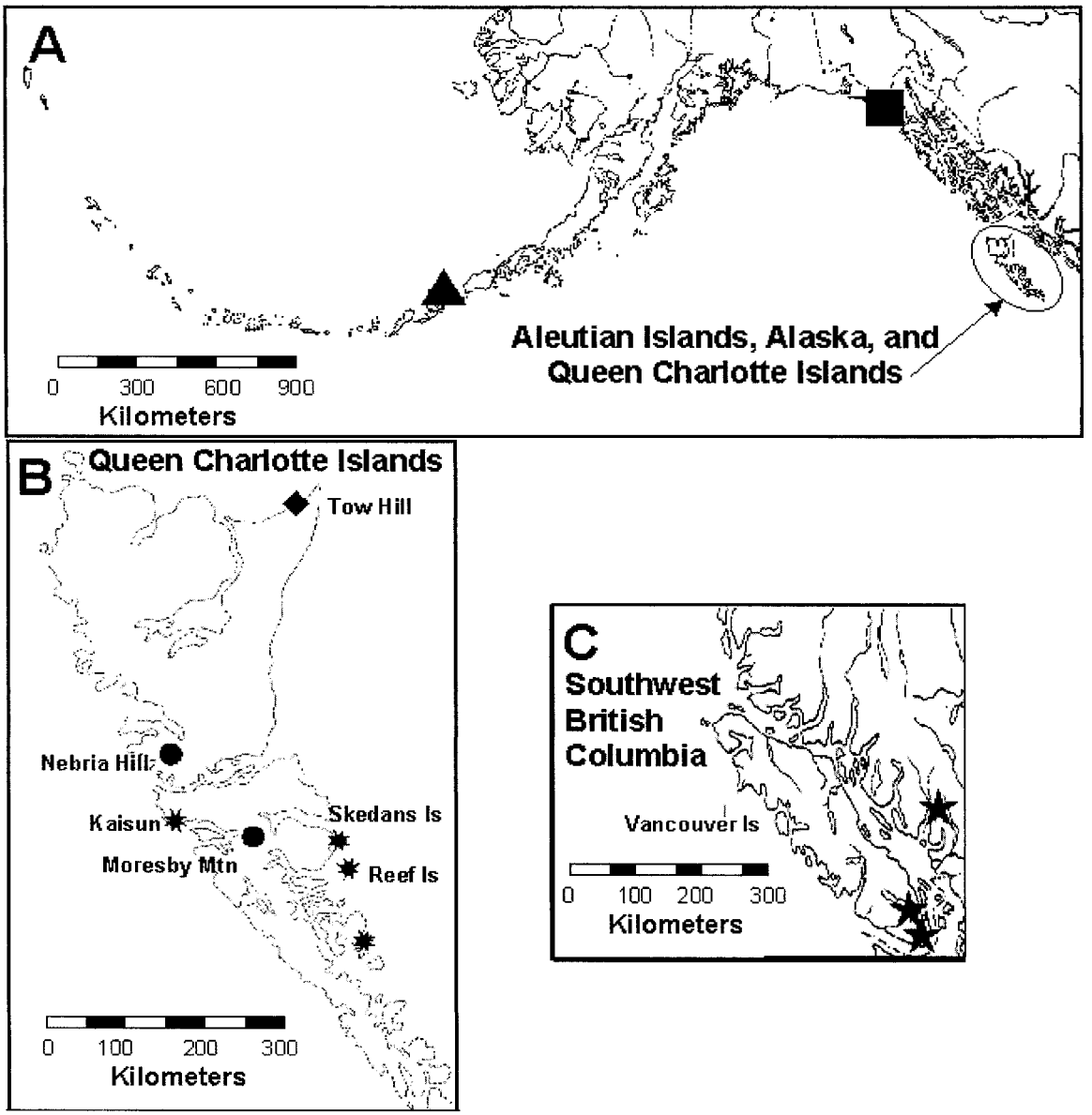

FIG. 2. Sites from which *Nebria* were obtained in western North America. (A) Map of collection sites of *Nebria gregaria* (▲) and *Nebria lituyae* (■) in the Aleutian Islands and Alaska, and the location of the Queen Charlotte Islands; (B) Collection sites of *Nebria charlottae* (♦), *Nebria haida* (●), and *Nebria louisea* (֍) on the Queen Charlotte Islands; (C) Collection sites of *Nebria sahlbergii sahlbergii* (★) in southwestern British Columbia, including Vancouver Island.

Charlotte Islands and the west coast of British Columbia (Fig. 2A). *Nebria charlottae* was obtained from cobble beaches at the base of Tow Hill and surrounding Estrado Lagoon, near Masset township, Graham Island (Fig. 2B). *Nebria louiseae* was collected from Kaisun village, Moresby Island, and from cobble beaches at Skedans and Reef Islands to the east of Moresby (Fig. 2B). *Nebria haida* was obtained from above the treeline on Nebria Hill, located south of Shields Bay, Graham Island, and from Mount Moresby, on north Moresby Island (Fig. 2B). *Nebria sahlbergii sahlbergii* and *N. gebleri* were both collected from stream banks in Manning Park, British Columbia (Fig. 2C). *Nebria diversa* was obtained from North Beach, Graham Island, and Cape Scott, Vancouver Island (Fig. 2B, C). One specimen each of *N. gregaria* collected from the Aleutian Islands and *N. lituyae* from the mountains near Juneau, Alaska (Fig. 2A) were obtained from the collection at the California Academy of Sciences. The *N. gregaria* specimen had been killed and stored in ethyl acetate before pinning, whereas the *N. lituyae* specimen was preserved in silica gel. Three specimens from each of the Tow Hill, Kaisun Village, Skedans Island, Reef Island, and Nebria Hill beetle populations as well as single specimens of *N. charlottae* from Masset, *N. louiseae* from Skedans Village site, *N. lituyae* and *N. gregaria* were selected for DNA extraction and nucleotide sequence analysis. Three specimens of *N. sahlbergii*, two of *N. gebleri*, and two each of the Queen

TABLE 1. Gene locus, direction of amplification, and nucleotide sequence of primers used for polymerase chain reaction amplification of *Nebria* mitochondrial DNA sequences.

NDI forward primer	5′-GCATCACAAAAGGCTGAGGA-3′
NDI reverse primer	5′-ACATGATCTGAGTTGAAACC-3′
COI forward primer (mtd-6)	5′-GGAGGATTTGGAAATTGATTAGTTCC-3′
COI reverse primer (mtd-9)	5′-CCCGGTAAAATTAAAATATAAACTTC-3′
COIIa forward primer (mtd-16)	5′-ATTGGACATCAATGATATTGA-3′
COIIa reverse primer (mtd-18)	5′-CCACAAATTTCTGAACATTGACCA-3′
COIIb forward primer (mtd-13)	5′-AATATGGCAGATTAGTGCA-3′
COIIb reverse primer (mtd-15)	5′-TCATAAGTTCARTATCATTG-3′
Cyt *b* forward primer (mtd-26)	5′-TATGTACTACCATGAGGACAAATATC-3′
Cyt *b* reverse primer (mtd-28)	5′-ATTACACCTCCTAATTTATTAGGAAT-3′

Charlotte and Cape Scott *N. diversa* were also processed for use as outgroups.

DNA Extraction

Beetle DNA used in polymerase chain reactions (PCRs) was extracted using either the nondestructive DNA extraction protocol of Phillips and Simon (1995) or the QIAamp tissue kit following the manufacturer's protocol (Qiagen, Mississauga, Ontario, Canada). The former method involves perforating dried beetles with a sterilized size 1 inset pin and extracting DNA with a solution of 8% dodecyltrimethyl-ammonium bromide (DTAB), 1.5 M NaCl, 100 mM Tris-HCl (pH 9.0), 50 mM EDTA at 68°C for 12 h, followed by chloroform extraction and DNA precipitation with 5% cetyltrimethylammonium bromide (CTAB) and 0.4 M NaCl. Pellets were resuspended in 1.2 M NaCl and reprecipitated with ethanol, followed by suspension in TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 7.5).

Sequence Analysis

Five regions of the mitochondrial DNA (mtDNA) were selected to be amplified, from total DNA extracted from the beetles, using the PCR. The regions of mtDNA amplifed were located inside the NADH subunit I gene (NDI region), within the cytochrome oxidase subunit I gene (COI), the cytochrome *b* gene (Cyt *b*), and within two sections of the cytochrome oxidase II gene (COIIa and COIIb; Table 1). PCR amplifications of fragments NDI and COI for beetles from Tow Hill, Kaisun Village, Nebria Hill, as well as all outgroup species were performed in 50-μl volumes containing 50–100 ng of beetle DNA, 83 ng of each primer, 200 μM of each deoxyribonucleotide triphosphate (dNTP), 15 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 60 mM KCl, and either 10 units of Taq DNA polymerase or 10 units of Pfu DNA polymerase, with 35 cycles of 94°C for 45 sec, 47°C for 60 sec, and 72°C for 60 sec. Fragments were ligated using T4 ligase (New England Biolabs, Beverly, CA) into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA). PCR amplifications of all other fragments were performed in 50-μl volumes containing Pfu polymerase (Stratagene, La Jolla, CA) and either $<$ 10 ng of beetle DNA (*N. gregaria*) or 50–100 ng of beetle DNA (all others), 83 ng of each primer, 200 μM of each dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.1% Triton X-100. Fragments were ligated into *Eco*RV (New England Biolabs) cut pBluescript (Stratagene) plasmid using T4 DNA ligase.

Sequencing was performed using the Sanger dideoxy sequencing method (Sanger et al. 1977) on Automated Laser Fluorescence sequencing machines (Pharmacia, Uppsala, Sweden) with fluorescene-labeled dideoxy nucleotides. To avoid sequence errors introduced by the PCR process, between three to five different clones were sequenced from fragments amplified with Taq polymerase. Because Pfu polymerase exhibits a much higher degree of fidelity then Taq polymerase, only two to three different clones were sequenced from fragments amplified with Pfu polymerase.

Individual sequences were assembled using the Seqman program in the Lasergene package (DNASTAR, Madison, WI). Homologous sequences from each beetle were aligned to each other using the Clustal V alignment option of the Megalign program (also from the Lasergene package). Genetic distance relationships between beetles were obtained from the output of the Megalign program, and cladistic analysis of the sequence data was performed using the PAUP 3.1 program (Swofford 1993). The species *N. gebleri* was used to root the trees (Kavanaugh 1978). Sequences are stored in Genbank under accession codes AF093761–AF093770, AF095196–AF09514, AF095468–AF095477, and AF096699–AF096708.

Random Amplified Polymorphic DNA Fingerprinting Analyses

The beetles used in the random amplified polymorphic DNA (RAPD) analysis were all selected from ethanol-collected material from the Queen Charlotte Islands, with the exception of the silica-gel-preserved *N. lituyae* specimen supplied by D. H. Kavanaugh (Table 2).

DNA for RAPD analysis was extracted from the beetles using the QIAamp tissue kit following the manufacturer's protocol (Qiagen). The concentration and purity of the DNA was tested using a spectrometer and by running samples on agarose gels. Aliquots of the extracted DNA were diluted with ddH_2O to a concentration of 6 ng/μl for use in the RAPD reactions. Ready-to-go RAPD analysis beads (Amersham Pharmacia Biotech, Piscataway, NJ) were used to perform the RAPD reactions. Each reaction contained 6 ng of genomic DNA, 25 pmol of a 10-mer oligonucleotide primer, and a RAPD bead containing of AmpiTAQ and Stoffel fragment thermostable DNA polymerases, 400 μM of each dNTP 0.1 g/ml BSA, 3 mM $MgCl_2$, 30 mM KCl, and 10 mM Tris pH 8.3 in a total reaction volume of 25 μl. The nucleotide sequences of primers used for the RAPD reactions were: 5′-

GGTGCGGGAA-3′, 5′-GTTTCGCTCC-3′, 5′-GTAGACCCGT-3′, and 5′-AAGAGCCCGT-3′. Reactions were overlaid with mineral oil to prevent evaporation. The thermocycler program used for the RAPD reactions was as follows: one cycle at 95°C for 5 min, followed by 45 cycles of 95°C for 1 min, 36°C for 1 min, and 72°C for 2 min. Bands were separated using 2% agarose gels, stained with ethidium bromide, and scored by hand from photographs and computer images taken of the gels. Each beetle sample was tested using each of four different primers in separate reactions. Because banding pattern produced by the RAPD reaction has been demonstrated to be sensitive to the reaction conditions (Innis and Gelfand 1990), RAPD reactions were standardized through the use of a manufactured kit containing preformulated amounts of enzyme and buffers and by using only high-purity extractions of DNA. Replicate RAPD reactions and blank control reactions were used to eliminate artifactual banding.

Rooted neighbor joining trees were constructed for the 47 samples using the Jaccard coefficient option of the RAPDistance package (ver. 1.04; Armstrong et al. 1994) to calculate genetic distance. The Jaccard coefficient calculates distance based on the number of shared bands divided by the sum of shared bands and mismatches. Alternative methods of determining genetic distance were calculated using two variants of the Jaccard coefficient, the Nei and Li coefficient, which doubles the value of shared bands to emphasize genetic similarities among taxa, and the Sokal and Sneath coefficient, which doubles the value of mismatches to emphasize differences between taxa. The average heterozygosity of each population was calculated using Nei's (1978) formula for estimating heterozygosity from small sample sizes. An analysis of molecular variance using the WINAMOVA (ver. 1.04; Excoffier 1992; Excoffier et al. 1992) was used to compare variation between the six different populations as well as among *N. sahlbergii sahlbergii* and the other five populations together.

Results

Mitochondrial DNA Sequence Analyses

Five fragments were amplified and sequenced from the mitochondrial DNA of the *Nebria* beetles for a total of 1835 bp, of which 214 bp were variable and 47 bp were phylogenetically informative. No differences in sequence could be found between beetles taken from the same population, with the exception of *N. louiseae,* for which three different haplotypes were discovered (designated KV, SKA, and SKB). KV and SKB haplotypes were restricted to populations from Kaisun Village and the islands east of Moresby Island, respectively, whereas the SKA haplotype was found at both sites.

Twenty-four base-pair substitutions occurred among the species of the *gregaria* species group, 135 between the *gregaria* species group and the species *N. diversa,* and 106 between the *gregaria* species group and *N. gebleri* (of 1363 bp sequenced for *N. gebleri*). Within the *gregaria* species group seven of the protein coding base-pair substitutions occurred at the first codon position and the remaining 15 occurred at the third codon position, with two mutations taking place in intergenic regions. The transition:transversion ratio (Ti:Tv) within the *gregaria* species group was 20:4. In contrast, 22 first codon position, nine second codon position, and 96 third codon position mutations occurred between *N. diversa* and the *gregaria* species group, with eight substitutions taking place in nonprotein coding regions of the DNA. Eighteen first, 13 second, and 64 third codon position substitutions occurred between *N. gebleri* and the *gregaria* species group. The Ti:Tv ratio was 78:57 between *N. diversa* and the *gregaria* species group and 53:51 between *N. gebleri* and the *gregaria* species.

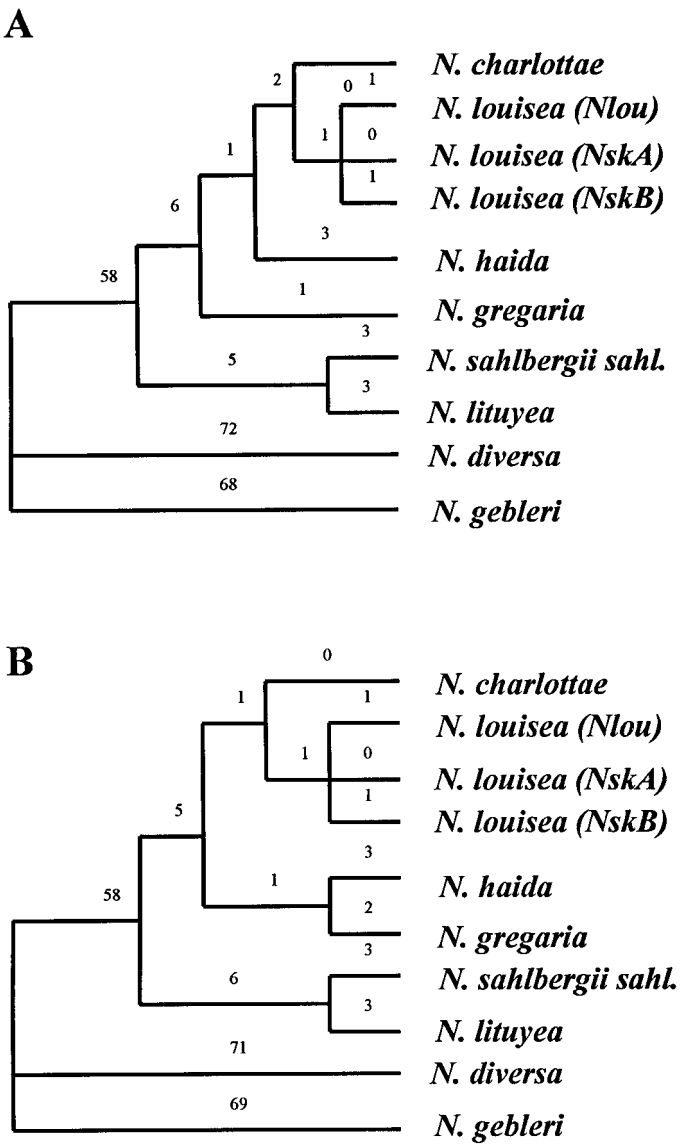

FIG. 3. The two most parsimonious trees resulting from phylogenetic analyses of *Nebria* species using mitochondrial DNA sequences. Numbers above the branches indicate the number of synapomorphies supporting each clade.

Cladistic analyses using the branch-and-bound algorithm of the total dataset produced two most parsimonious trees, each of 225 steps (Fig. 3). Both trees produced a trichotomy at the basal node, consisting of *N. gebleri, N. diversa,* and a clade containing all other species (the *N. gregaria* species

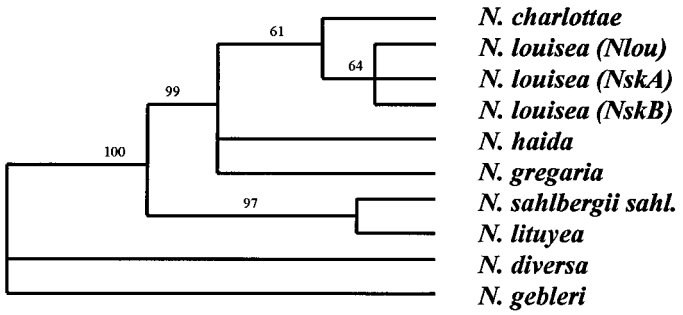

FIG. 4. Bootstrap tree of the total *Nebria* mitochondrial DNA sequence dataset using 1000 replicates. Numbers above the branches indicate the number of synapomorphies supporting each clade.

group, sensu lato). Within the *gregaria* group, both trees aligned the species *N. lituyae* as sister to *N. sahlbergii,* thus removing it from the *gregaria* group. The two trees differed from each other only in the placement of *N. gregaria,* either as a sister taxon to all of the Queen Charlotte Islands *Nebria* or as the sister taxon to *N. haida.* Very little difference existed between the members of the *gregaria* group (exclusive of *N. lituyae*). In both trees, no more than two character changes separated any of the *N. louiseae* haplotypes from *N. charlottae,* whereas the greatest distance between any two of the *gregaria* group taxa (excluding *N. lituyae*) involved seven mutations. Both trees had a consistency index of 0.978 and a retention index of 0.921.

Homoplastic base changes were concentrated between *N. gebleri* and the *N. gregaria* species group in tree A and between *N. gebleri* + *N. diversa* and the *gregaria* species group in tree B. The degree of sequence divergence between members within the *gregaria* group, exclusive of *N. lituyae,* ranged from 0.05% to 0.38%, whereas sequence divergence between the two represented lineages of the *gregaria* species group reached a maximum of 0.98%, between either of *N. sahlbergii* or *N. lituyae* and *N. haida.*

A bootstrap analysis of the data, using 1000 replicates and the branch-and-bound algorithm, supported the clade formed by *N. sahlbergii sahlbergii* and *N. lituyae,* the clade formed by the remaining four members of the *gregaria* group, and the sister relationship between the two groups of taxa (Fig. 4). Within the latter clade, the group formed by *N. charlottae* and *N. louiseae* was maintained as a distinct clade from *N. haida* and *N. gregaria* in 61% of the replicates.

Random Amplified Polymorphic DNA Analyses

In total, 77 bands were produced using RAPDs (Table 2). Across the 47 samples, only one band was uniformly present, although 12 other bands each occurred in 45 or more of the samples. Eleven bands were unique, each occurring in only a single individual. The populations with the greatest degree of fixation for bands were the eastern *N. louiseae* and *N. charlottae* populations, each with fixation rates exceeding 70% and average heterozygosity of less then 0.090. The Kaisun Village *N. louiseae* showed a greater degree of heterozygosity, with only 60.0% of the bands present in all individuals and an average heterozygosity of 0.132. *Nebria haida,* when considered as a single population, contained the greatest heterozygosity, with only 30% of the bands fixed across all 12 individuals and an average heterozygosity of 0.210, comparable to the outgroup species *N. sahlbergii sahlbergii.*

The neighbor-joining tree calculated using the Jaccard coefficient of genetic distance groups the five specimens of *N. sahlbergii sahlbergii* together to form an outgroup cluster with a genetic distance of 0.166 (Fig. 5) from the cluster containing the members of the *gregaria* group. Members of the Kaisun Village population of *N. louiseae, N. charlottae,* and the eastern *N. louiseae* population each formed distinct clusters, whereas *N. haida* formed a single cluster with *N. lituyae* as sister group. Moresby Island and Graham Island populations of *N. haida* could not be differentiated. The four populations of the *gregaria* group from the Queen Charlotte Islands were linked by a simple hierarchy of descent with *N. haida* and *N. lituyae* forming the basal group from which the Kaisun Village population was derived. The Kaisun Village population, in turn, represented the ancestor from which the sister clusters of *N. charlottae* and the eastern *N. louiseae* were derived.

The statistical significance of the neighbor-joining tree was assessed using a permutation tail probability test. Twenty random trees were generated from the RAPD data of the *Nebria* populations collected on the Queen Charlotte Islands,

TABLE 2. Summary of random amplified polymorphic DNA statistics for *Nebria* specimens from Queen Charlotte Island and mainland localities. *N,* population size; FB, number of fixed bands; VB, number of variable bands; SB, number of single bands; AB, number of absent bands; %UFB, percentage of unfixed bands; AH, average heterozygosity.

Population	*N*	FB	VB(2+)	SB	AB	%UFB	AH
Eastern *N. louiseae* (A + B)	10	33	10	3	31	28.3%	0.082
Reef Island (A)	5	36	6	4	31	21.7%	0.089
Skedans Isle (B)	5	38	4	2	33	13.6%	0.053
Tow Hill	10	35	8	1	33	20.5%	0.089
Kaisun Village	9	29	13	6	28	40.0%	0.132
N. haida (total)	12	17	31	8	21	69.6%	0.210
N. haida (Graham Island)	8	19	26	8	24	64.2%	0.217
N. haida (Moresby Island)	4	29	11	6	31	37.0%	0.210
N. lituyae	1	37	n/a	n/a	40	n/a	n/a
N. sahlbergi sahlbergi	5	20	9	8	40	46.0%	0.192
All *Nebria*	47	1	65	11	n/a	98.7%	0.200

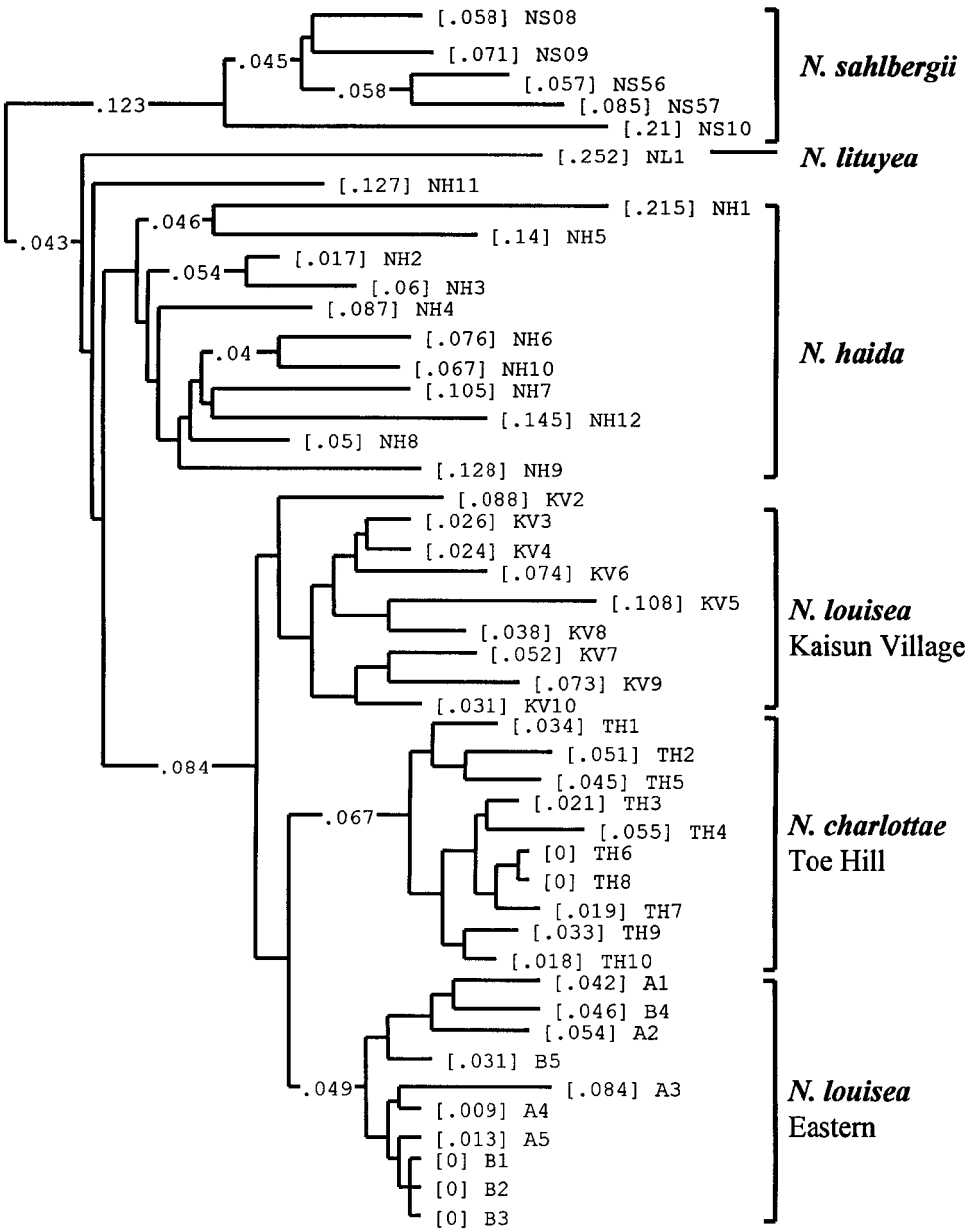

FIG. 5. Neighbor-joining tree from RAPD analysis data using the Jacard coefficients of relatedness.

having a mean length of 6.60 and a PTP value of 0.06 (equivalent to a standard deviation), compared to a length of 4.05 for the original tree. The mean length of the neighbor-joining tree exceeding the mean length of randomly generated trees by 41.94 standard deviations.

An analysis of molecular variance (AMOVA) using the genetic distances calculated from the three coefficients divided the variation fairly evenly, with between-population variance accounting for 53.4% and within-population variance comprising 46.6% of the total. The PHI-statistic represents the correlation of random haplotypes within populations relative to that of pairs of haplotypes drawn randomly from the whole species and is essentially equal to the variance among populations. PHI equals 0.534 for the Jaccard distance matrix, with a 99% level of significance.

Nebria charlottae and the eastern *N. louiseae* populations displayed high degrees of genetic uniformity, with mean values of genetic distance to a common ancestor of 0.028 for eastern *N. louiseae* and 0.027 for *N. charlottae.* The Kaisun Village population displayed a mean genetic distance value of 0.057, whereas mean values of 0.101 and 0.096 were obtained for *N. haida* and *N. sahlbergii sahlbergii*, respectively. *Nebria lituyae* was represented by a single long branch with a length of 0.252.

Neighbor-joining trees calculated using the Nei and Li or Sokal and Sneath coefficients and their associated statistics (data not shown) did not differ significantly from those of the Jaccard neighbor-joining tree, with the one exception of the Sokal and Sneath neighbor-joining tree placing the specimen of *N. lituyae* within the species *N. haida.*

Discussion

The importance of geological complexity in the promotion of speciation has long been recognized in surveys of biological diversity (Cracraft 1985). The presence of mountainous or otherwise subdivided terrain coupled with the movement of climatic zones produced by glacial cycles provides a mechanism for the fragmentation of populations that can lead to allopatric speciation (Haffer 1969, 1977; Prance 1982). Pleistocene species radiations have been documented in biodiversity studies of African birds and plants (Fjeldsa and Lovett 1997), the biogeographical analysis of montane butterfly subspecies (Hammond 1990), and molecular studies of birds (Roy 1997) and butterflies (Britten and Brussard 1992; Brower 1996).

In contrast, the reigning paradigm of evolution and speciation within the predaceous ground beetles is that of long-term stasis. The age of most extant species is believed to extend through the Pleistocene into the late Tertiary, and very few examples of either speciation or significant phyletic evolution have been documented (Coope 1970; Matthews 1980; Elias 1994; Ashworth 1996). Although early hypotheses of beetle evolution postulated that the climatic upheavals of the Middle to Late Pleistocene had the effect of promoting speciation within the Carabidae, particularly within the forest- and tundra-dwelling genera of the northern Holarctic (e.g., Ball 1966), numerous studies of fossil coleopterans from northern temperate and arctic locations in Europe and North America were able to conclusively show that almost all of the beetle fauna of the Early Pleistocene and Late Pliocene that had been examined could be matched to extant species (e.g., Coope 1961, 1962; Matthews 1974). Rather than promoting speciation and phyletic evolution, the Pleistocene glacial period appeared to have resulted in massive range shifts for much of the ground beetle fauna of the Northern Hemisphere, with species tracking their optimal latitudes in response to climate change. The only phyletic evolution acknowledged for most ground beetle species was the adaptation to changes in day-length as populations migrated into new latitudes (Ashworth 1996).

However, the fossil surveys upon which theories of ground beetle evolution were based were not without bias. Most surveys of fossils had been performed in relatively stable lowland areas with little geological complexity and the majority of the insects studied typically occupied wide ranges during both glacial maxima and interglacials (Ashworth 1996). Despite the emphasis of paleoentomology on this narrow subset of possible habitats, the conclusions of these studies had an effect on phylogenetic hypotheses for ground beetles that occupied very different conditions, with assumptions on the rate of morphological change for other lineages modified to fit the evidence of slow, gradual evolution.

The strongest influence of the static ground beetle theory is seen in those phylogenetic hypotheses dealing with the speciation of alpine and subalpine beetles. The importance of montane habitat and climatic change to the process of speciation in Carabid beetles has been recognized in the two most comprehensive zoogeographical analyses of montane Nearctic ground beetle genera, *Harpalus* (Noonan 1990, 1992) and *Nebria* (Kavanaugh 1978, 1979b, 1988). However, most examples of speciation within these lineages have been taken to be old events predating the Pleistocene, based on the degree of morphological and ecological change between sister taxa. The few Pleistocene speciation events (in terms of both morphology and habitat) that have been proposed for the *Nebria* are thought to have occurred between only the most similar species (Kavanaugh 1979a), whereas no speciation events are thought to have occurred within the last 1.6 million years in the genus *Harpalus* (Noonan 1990).

The original phylogeny of the *gregaria* group proposed that the five species of the group survived the Fraser glaciation in a series of refugia centred on the Queen Charlotte Islands, the Alexander Archipelago, and the Aleutian Islands (Kavanaugh 1989). Evidence for the refugial survival of these species comes from their restricted distributions, loss of wings, adaptation to a low-temperature environment, and a lack of potential source populations in either the Beringia refugium or south of the ice sheets. The molecular sequence data presented in this study, however, suggest that four of the five members of the *gregaria* group are incipient species that have radiated from a single common ancestor, and one species (*N. lituyae*) does not belong within the *gregaria* group. However, the position of *N. lituyae* is inferred from a single specimen.

The low level of sequence divergence among the members of this species group indicates that the radiation of these species and populations has occurred relatively recently, however, dating the actual speciation events is complicated by a lack of a reliable molecular clock. Estimates of the rate of sequence divergence between species for arthropod mitochondrial DNA varies from 1.2% to 2.3% per million years (Brower 1994; Su et al. 1996a,b; Knowlton and Weigt 1998) based on a variety of techniques not limited to sequence data and using taxa that have diverged between 3 million and 20 million years ago. Among the few studies that have examined more recently derived insects (within the last 1 million to 3 million years), rates of sequence divergence between taxa have been higher, approaching 5% per million years (Vogler and DeSalle 1993; Juan et al. 1996). Calibrations of the molecular clock for vertebrates has demonstrated that recently derived taxa contain predominantly noncoding mutations (e.g., the third ''wobble'' base of each codon; Li et al. 1987), and that among these unconserved regions of the DNA the rate of sequence divergence can approach 10% per million years (Irwin et al. 1991). Given the wide range of possible estimates of the molecular clock, speciation dates for the *gregaria* infragroup have been estimated using the division between the infragroup and *N. sahlbergii* as a calibration.

If this divergence is assumed to have occurred during the glacial period preceding the Wisconsin, approximately 150,000 years ago (Liu 1992), this would give the *gregaria* species group a rate of sequence divergence of 5.7% per million years and indicate that the component lineages of this species group branched from each other between 20,000 and 50,000 years ago. Extending the age of separation between *N. sahlbergii* and the *gregaria* infragroup to the beginning of the Late Pleistocene, approximately 400,000 years ago, would still place the radiation of the *gregaria* infragroup within the Wisconsin glacial period. To allow the radiation of the *gregaria* infragroup to have occurred before the Wis-

consin, the division between the *gregaria* infragroup and *N. sahlbergii* would need to have occurred approximately 900,000 years ago and would require a molecular clock of 1% per million years, which is far slower then any other insect taxa and not in keeping with the low level of sequence divergence.

RAPD analyses provide a measure of the variation between populations at the genomic level (Williams et al. 1990). There is a nested hierarchy of populations within the species of the Queen Charlotte Islands with both Moresby and Graham Island populations of *N. haida* clustering as a single genetically diverse unit, sister to a paraphyletic assemblage consisting of *N. charlottae* and distinct western and eastern populations of *N. louiseae.* The pattern of genetic diversity in the Queen Charlotte Island species indicates that the genetic variation is not strongly subdivided among the populations, and that much of the existing variation is associated with the species *N. haida,* the expected outcome of a recent radiation of the species group. The single specimen of *N. lituyae* displayed a DNA banding pattern that was similar to several individuals within the *N. haida* populations.

Scenarios that postulate long periods of isolation for each of the populations (e.g., a preglacial differentiation of the four populations) probably would result in much greater partitioning of variation among populations and some form of genetic break between Moresby and Graham Island populations of *N. haida.* In contrast, recent colonization of the Queen Charlotte Islands would reduce both mitochondrial and nuclear diversity through the process of the leading-edge effect during northward migration and concentrate uch of the remaining diversity in the beach populations (Hewitt 1993; Soltis et al. 1997). Given that most other *Nebria* species have responded in the past to warming climates by migrating into the mountains, following the altitude of optimal temperature (Kavanaugh 1979b), the most probable scenario to explain the molecular data would be that the ancestral *gregaria* species ascended into the alpine regions of the Queen Charlotte Islands following deglaciation, leaving behind small scattered populations in the lowland regions that would become the modern species *N. louiseae* and *N. charlottae.*

Where did the *Nebria* of the Queen Charlotte Islands come from? New evidence supports the existence of exposed terrain in the Hecate Strait during the height of the Fraser glaciation. Geologically, both the offshore banks of the Queen Charlotte Islands and coastal regions in the vicinity of Lituya Bay are believed to have had ice-free areas during the course of the Wisconsin glacial period (Barrie et al. 1993; Josenhans et al. 1995; Mann and Hamilton 1995), the product of changing ocean levels and crustal uplift in response to the weight of the mainland ice sheets. Remnants of vegetation discovered in an exposed cliff face on eastern Graham Island indicate that the deglaciation of the Queen Charlotte Islands began at least 1000 years earlier than the adjacent mainland and point toward a nearby reservoir of herbaceous and arboreal plants that would have supplied colonists for the Graham Island site (Warner et al. 1982). Molecular evidence for distinct west coast lineages of black bear (Byun et al. 1997), pine marten and weasel (Byun et al. 1999), as well as genetically disjunct populations of west coast plants (Soltis et al. 1997) are consistent with proposals of a biologically complex refugium located in the now submerged region between the Queen Charlotte Islands and Vancouver Island. The presence of *N. haida* on the mainland uplands would suggest that the common ancestor of the Queen Charlotte Island *Nebria* was also present in the putative refugia located on the inner coast.

This observation of rapid radiation within the *gregaria* infragroup has implications for the understanding of the rate and process of speciation and diversification among predacious ground beetles. The conservative nature of carabid systematics has reflected an understanding of ground beetle evolution based on insects that had extensive ecologically stable habitats with few physical boundaries and little in the way of opportunities for allopatric speciation (Ashworth 1996). Our study, however, has focused on a small group of beetles for which isolation, combined with strong selection pressure for environmental adaptation, appears to have promoted rapid speciation and diversification. The morphology and ecological preferences of the sister taxa to the *gregaria* infragroup would indicate that the common ancestor to the *gregaria* infragroup was macropterous and occupied riparian habitats without a particular altitude preference (Kavanaugh 1978). Based on the rate of molecular evolution proposed here, the species of the *gregaria* infragroup would therefore have acquired brachypterous wing morphology and cold-tolerance within a span of only 20,000 to 50,000 years. While this example of rapid change (in morphology, behavior, and physiology) in response to environmental pressures may represent an extreme brought about by the unique conditions of the west coast refugium, many genera within the predacious ground beetles occupy habitats that would have undergone repeated fragmentation during the cyclical climate changes of the Pleistocene period. The molecular reexamination of these more typical representatives of the family Carabidae will provide invaluable insight into the role played by the Pleistocene climate change in the diversification of one of the largest families of insects.

The results of this study also suggest that a revision of the taxonomy of *N. gregaria* group may be required. MtDNA analyses indicated that *N. haida, N. louiseae,* and *N. charlottae* are closely related. RAPD analyses also revealed close association among these three species. These two genetic targets combined with high morphological similarity among the three species raises questions concerning their taxonomic designations. Based on mtDNA sequence data, *N. lituyae* may be more closely related to *N. sahlbergii* than to other species of the *gregaria* group and, if so, should be excluded from the *gregaria* group. However, this suggestion is based on data derived from a single specimen of *N. lituyae,* and, clearly, more data must be gathered. *Nebria louiseae* and *N. charlottae* may not represent distinct taxonomic units but rather variants of a single beach-dwelling species, *N. charlottae.* Whether even *N. haida* can be considered a distinct species appears debatable, given the close genetic relationship and slight morphological differences between the alpine and beach-dwelling populations. Because only minor differences in morphology and biology and the physical separation of alpine from cobble beach habitat serves to differentiate *N. haida* as a distinct taxonomic unit, we feel that this species may be only at an incipient stage of speciation, and might more appropriately be accorded subspecific status within the

single species, *N. charlottae*. However, we must obtain additional molecular data for other genes, including nuclear genes, and from additional taxa to test this initial evidence further before formally proposing the taxonomic changes suggested by our results.

Acknowledgments

The authors thank L. Millar, J.-L. Wolfe, J. Huang, A. Byun, M. Voodouw, D. Walsh, and H. Ertle for assistance in the laboratory and D. Burles and S. Douglas for assistance in the field. We thank R. Cannings (Royal British Columbia Museum) for specimens. TER would also like to thank the Canadian Coast Guard and volunteers for assistance to TEC. This research was supported by an NSERC operating grant (A2354) to TER, funds from Canadian Parks Service to TER, an NSERC equipment grant to DBL and TER, an NSERC Industrial Partnership Grant to DBL, a University of Victoria Fellowship to TEC, and travel support from S. Cannings (Ministry of Environment, Government of British Columbia).

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