Molecular reevaluation of the *Nebria gregaria* infragroup and the implications for the existence of an ice age refugium on the Queen Charlotte Islands

by

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ABSTRACT

Discussion on the origins of many of the endemic and disjunct organisms on the north-western coast of North America have centered on the role of the Queen Charlotte Islands as a refugium during the Fraser glaciation. Extended periods of isolation on the archipelago has been proposed as an explanation for the morphologically divergent island varieties of mammals, birds, insects, and plants. However, many of the endemic subspecies are differentiated from the mainland forms on the basis of small morphological changes that may instead be the result of rapid post-glacial evolution. One group of animals that has been used as evidence for the existence of a glacial refugium is the *Nebria gregaria* infragroup. Two members of this species group, *N. charlottae* and *N. louisea*, are restricted to cobble beaches on the Queen Charlotte Islands. A third, *N. haida*, is found only in alpine regions of the archipelago and the adjacent mainland. The remaining two species of the *gregaria* infragroup, *N. lituyea* and *N. gregaria*, show highly restricted distributions in the mountains of the Alaskan panhandle and on the beaches of the Aleutian Islands, respectively. Because of the morphological similarity among the five species, a morphometric study was performed on body length and pronotal shape measurements in order to determine if each species was distinct. The results of a discriminant analysis on the measurements indicate instead that the five species grade into each other, forming a morphological continuum. To determine the relationships of the five species, a phylogenetic analysis was conducted on

1856 bp from five regions of the mitochondrial DNA, comprising the NADH subunit 1 gene, the cytochrome oxidase subunit 1 gene, the cytochrome B gene, and two regions of the cytochrome oxidase II gene. The results of the phylogenetic analysis indicated that one species, *N. lituyea*, did not belong in the *gregaria* infragroup and that a very close relationship existed among the four remaining species. Only seven mutations separated the two most divergent species, *N. louisea* and *N. haida.* To further explore the relationships of the three Queen Charlotte Island members of the infragroup, a neighbour-joining tree was constructed from the DNA fingerprint patterns of *N. haida*, *N. charlottae*, and *N. louisea.* Genetic diversity was highest in the *N. haida* populations and lowest in *N. charlottae* and the eastern populations of *N. louisea.* In addition, the tree indicated that the latter two populations were sisters, derived from the western *N. louisea* populations, which were in turn derived from *N. haida.* The molecular data indicate that the morphological differences between the three Queen Charlotte species may be post-glacial in origin and that together with *N. gregaria*, these four should be considered as local variations of a single species. The implications for a glacial refugium on the Northwest coast are reevaluated in light of these new findings.

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CHAPTER 1: INTRODUCTION

Biogeography and Insular Populations

Explanations for the geographical distribution of species have generated a number of competing views in biogeographical theory. Traditionally, all higher taxa had a centre of origin from which the various extant species had dispersed and their dispersal was across a geologically stable planet that had persisted over the millennia with only minor changes in shape and structure. Centres of origin were identified on a range of often conflicting evidence, including abundance of primitive species, abundance of derived species, location of fossil beds, abundance of extant taxa, etc. (Darlington, 1957). Biogeographers focused on the distributions of a single taxon and only rarely were the distributions of unrelated taxa compared for overriding patterns (Humphries and Parenti, 1986).

The revolution in geology brought about by Wegner's theory of plate tectonics and the introduction of cladistic methods to the field of systematics radically changed the focus of biogeography. With the realization that the continents could break up, coalesce, and otherwise rearrange themselves, biogeographers began to look for patterns in the distribution of organisms that would indicate that some event had isolated the populations of an ancestral species and resulted in the radiation of an extant taxon. Where the same patterns of vicariance could be found across a range of unrelated taxa, biogeographers would conclude that a major geological event, e.g., continental break-up

or mountain building, had been the cause of the diversification. At its extreme, vicariance biogeography rejected entirely the idea that dispersal could have any meaningful role in the distribution of species beyond a few minor examples, and held that if a pattern existed, a geological or ecological reason must exist (Humphries and Parenti, 1986). A more moderate view is that of Hedges (1996), who indicated that some biogeographic patterns could be explained better through large-scale mechanisms of dispersal than by vicariance. In his example, he demonstrated that the biogeographic pattern linking the Caribbean fauna to those of South America could be better explained by ocean currents rafting animals on debris from the Amazon and Orinoco rivers northwards into the Caribbean than by a series of vicariance events splitting apart and rejoining the islands to each other and to South America.

The introduction of cladistics into systematics both provided less ambiguous phylogenies upon which to base biogeographic theories and altered the focus of biogeography from the narrative contributions of early authors to a methodological attempt to link together the phylogenies and distributions of various unrelated taxa in order to determine the chronological progression of vicariance events that led to their diversification. In practice, the cladistic biogeographer constructs a set of area cladograms for each of the taxa by replacing the names of the species in their phylogeny with each of their locations. A consensus cladogram for the region can then be constructed by merging the various area cladograms using their shared branches as references. Areas occupied by widespread species or by one set of taxa and not by another are incorporated in such a way as to minimize incongruities with the various area

cladograms. The consensus cladogram can then be used to predict the existence and timing of vicariance events in the region in question. (Humphries and Parenti, 1986; Morrone and Crisci, 1995.) The cladistic method of biogeography has been used to piece together complex patterns of vicariance such as the break-up of Gondwanaland (Humphries and Parenti, 1986) and the geographic and ecological subdivision of Central America (Rosen, 1976).

In the case of late Pleistocene biogeography, the geological events are fairly well known and most biogeographic efforts are attempts to track the recolonization of northern North America as well as to identify possible refugia based on the presence of endemic species with highly localized distributions. Three major refugia that sheltered the bulk of the species during the height of the ice age can be identified: Beringia, located in present day Alaska and the western Yukon; the north-western United States; and the northern United States east of the Rocky mountains. Their identification is based on a combination of fossil evidence linking northern fauna with regions south of the ice sheet (Schwert and Ashworth, 1988) and on comparisons of the distribution pattern of extant fauna and flora to identify common patterns of migration (Pielou, 1991).

In addition to the three major refugia, a host of other minor refugia have also been proposed along the eastern and western coasts of North America. On the east coast, offshore regions adjacent to Nova Scotia, Sable Island, and Newfoundland have been hypothesized to be glacial refugia (Pielou, 1991). On the west coast, potential refugia exist on Kodiak Island (Karlstrom and Ball, 1969), the Alaskan panhandle (Kavanaugh,

1988), and the Queen Charlotte Islands (Foster, 1965). The identification of these minor refugia is based on the presence of endemic species and subspecies, as well as on geographic zones that could have been exposed during the height of the ice age. However, the process of identifying these minor refugia, particularly those based on endemic forms, is complicated by the tendency for insular populations to develop morphological differences relative to their mainland sisters, a process that for some species has been shown to occur over a comparatively short span of time. In particular, for the insular mammal populations of northern North America and Europe the trend of small mammals to increase in size and larger species to have diminutive island forms has led to a great deal of biogeographic controversy (Foster, 1964; Berry, 1996). Explanations for these observations have ranged from considering most island populations to be relicts of Pleistocene events (e.g., Beirne, 1952), through postulating unique genetic events for colonizing populations, to a consideration that islands present novel selection regimes to colonizing species that lead to differentiation over time.

The theory that most differentiated insular populations are by default relict species has largely fallen out of favour because of a wider appreciation of genetics and the power of natural selection (Foster, 1964). In its place, two schools of thought have coalesced; the first, initiated by Mayr (1963) and expanded by Carson (1975) and Templeton (1980), is founded on the idea that the colonizing process initiates a ‘genetic revolution’ that results in rapid differentiation of the new population and quick attainment of reproductive isolation. As Mayr originally conceived it, the colonization event entailed the arrival of a very small number of individuals from the mainland onto an island, where large scale

inbreeding broke up co-adapted gene complexes and allowed selection to operate on recessive alleles that would otherwise be hidden in the population. Criticisms of Mayr's theory were that it entailed a massive loss in genetic variability and created circumstances that would not favour the survival of the colonizing population (Carson and Templeton, 1984). As a result, based on their research on the speciation of Hawaiian *Drosophila,* Carson and Templeton each proposed variations on Mayr's theory that allowed for the colonizing populations to retain genetic variability. Carson's 'founder flush' theory proposed a period of relaxed selection pressure during the initial few generations following the colonization event to allow the break-up of the co-adapted gene complexes and the evolution of both new adaptive forms and reproductive isolation. Templeton proposed a 'genetic transilience' model that relied on chance alterations in the major alleles of the gene complexes to change the genetic background for the various modifier genes, allowing evolution in a direction away from the mainland form. Both theories gave genetic drift the primary role in the differentiation of island populations from the mainland, with natural selection modifying the results once the population had become too large for genetic drift to produce significant effects and after reproductive isolation had been achieved.

All three theories have been attacked on the grounds that the founding effect is neither the best nor the only method by which island populations could rapidly become differentiated from their mainland sisters. Most vociferous have been Barton and Charlesworth (1984), who have used mathematical models to demonstrate an inverse relationship in the strength of reproductive isolation occurring between the colonizing

and the mainland populations as a result of a founding event and the rate at which reproductive isolation is built up by genetic drift. Instead, Barton and Charlesworth favour the alternative school of thought, a model of gradual change and the accumulation of reproductive isolation as a result of different selective regimes guiding the evolution of island and mainland fauna. Similarly, Orr (1995) has revived and defended a theory of speciation based on the accumulation by allopatric populations of normally beneficial genes that cause sterility or inviability when brought together in hybrids. This theory, while not specifying whether selection or drift plays the most important role in later differentiation, allows reproductive isolation to arise in large populations without the requirement of bottle-necks.

The primary arguments against natural selection as the mechanism behind the differentiation of insular populations have been that in large populations, morphological change is either attained slowly or is non-existent because of genetic inertia caused by both the population size and an underlying architecture of co-adapted gene complexes (Mayr, 1963). Speciation was conceived as a process that occurred only at the periphery of the range of widespread species, and which produced 'superior' forms that would expand from the periphery to eventually replace their sisters. Recent studies, however, have demonstrated numerous examples of natural selection producing significant morphological differences and reproductive isolation between populations over short periods of time, without evidence of the founding events and genetic bottlenecks required by the theories of Mayr, Carson, and Templeton to allow genetic drift to operate. Seven species of freshwater fish that have only occupied the rivers and lakes of northern North

America in the 15,000 years since the glacial retreat have evolved morphologically specialized and reproductively distinct limnetic and benthic populations (summarized in Schluter, 1996), while the sympatric evolution of strictly freshwater populations has occurred multiple times in the normally anadromous Atlantic and Sockeye salmon (Verspoor and Cole, 1989; Taylor et al. 1996) during the same time period. Differences in the predator composition faced by island and mainland populations of the wood mice of Northern Europe have resulted in a size increase in island populations (Angerbjorn, 1986). A similar effect has been observed in the stickleback fish of the QCIs, where differences in the levels of fish and bird predation within different lakes have led to variation among populations in body size (Reimchen, 1991), spine length (Reimchen, 1980), and armor plating (Reimchen, 1992). Predator regimes have also influenced body form among island populations of lizards (Thorpe and Malhotra, 1996), albeit over a longer time period. Colonization experiments using Caribbean *Anolis* lizards introduced to uninhabited islands have demonstrated morphological adaptation to the new habitats over a time interval of just under two decades despite small founding populations that would theoretically be most affected by drift (Losos et al., 1997).

Thus, the morphological differentiation observed in insular populations need not arise by chance genetic events in small founding populations, but may be the result of large semi-isolated or island populations undergoing a different selective regime than that experienced by their mainland sister populations. Genetic drift still plays a role in those changes associated with neutral or nearly neutral characteristics (Kimura, 1991). However most of the evidence indicates that natural selection is the predominant force

shaping the characteristics for which there is differences in fitness among morphs.

Queen Charlotte Controversy

The Queen Charlotte Islands (QCIs), with their many endemic species and subspecies, have been at the centre of a controversy over the origins of insular species. On the one hand, accumulated evidence for a glacial refugium on the QCIs serves to explain the endemic forms as the product of long periods of geographical separation during the most recent (Fraser) glaciation. On the other, rapid evolution in response to natural selection has been clearly demonstrated for certain members of the QCIs endemic fauna.

Morphological differences between animal populations living on the islands and on the mainland demonstrate that some of the Queen Charlotte fauna have undergone a period of isolation from their mainland sister populations. Despite consensus amongst biologists that the QCIs had escaped glaciation (summarised in Brown and Nasmith, 1962) it was not until Foster's (1965) studies of the endemic species and subspecies of mammals that the question arose on the length of time the populations would need to be isolated to allow the observed degree of differentiation. Foster did not feel that the post-glacial period provided sufficient time for the species and subspecies of the islands to evolve and concluded that some part of the archipelago had served as a refugium during the period between 30,000 before present (B.P.) and 14,000 B.P. when the mainland was under the weight of the Cordilleran ice sheet. From these beginnings, the theory of a glacial refugium located on the Queen Charlotte Islands expanded in an attempt to explain much of the diversity of endemic plants, animals and fungi that characterised the

archipelago.

Authors who have studied the mammals and birds of the QCIs have noted that some species display subtle characteristics that clearly separate the island populations from their mainland relatives, although the degree of differentiation between populations varies greatly among species. The greatest degree of divergence between mainland and island populations can be observed in the Dawson caribou, a now extinct island species that displayed small stature, poorly developed antlers, and a dull grey coat that contrasted with the normal colour and decoration of the southern forest caribou, its putative closest mainland relative (but see Osgood, 1901, for a discussion of the 'discovery' of the Dawson caribou). However, of the other endemic mammal populations of the Queen Charlottes (including weasels, martens, bears, shrews, and mice) most variations between island and mainland populations consist of differences in the average skull measurements and changes in the shape and dimensions of the animal's dentition (Foster, 1965). A similar situation exists for the avifauna of the QCIs. Of the 42 species of land birds that nest on the islands, eight have developed sufficient morphological differentiation to allow them to be distinguished from their mainland relatives. Four populations of the island land birds have been granted subspecific status. None, however, is sufficiently different to be considered separate species (Cowan, 1989). Despite the small degree of the differences observed in the island species and subspecies and their mainland relatives, most early naturalists believed the differences were too great to have arisen in the short interval since the ice age ended, and that some longer period of isolation must have occurred (McCabe and Cowan, 1945; Foster, 1965; Beebe, 1960).

The evidence for a glacial refugium on the QCIs was further supported by the discovery on the archipelago of unusual species of vascular plants and bryophytes, some of which are endemic and others of which show extremely disjunct distributions. Of the 459 species of bryophytes recorded from the archipelago, Schofield (1989) cited five species that are endemic to the islands and seven species that are known in the Western hemisphere as being only from the QCIs, but with representatives in Europe and Asia. All of the twelve species are characterized by poor powers of dispersal and by ecologies that allow their survival on rocky cliffs and in alpine conditions. The remainder of the bryophyte species show broader dispersal capabilities and ecological tolerances, as well as an overwhelmingly North American origin. Only thirty species of bryophytes found on the QCIs show a distribution indicative of an origin from the Beringia refugium in Alaska. Their ranges are limited to a thin band along the pacific coast of North America but they have a much wider European and Asian distribution. This was interpreted by Schofield as proof that the QCIs contained the remnants of populations that once spanned the northern Pacific coasts of both continents, but that had been extirpated from mainland North America during the last glaciation. He believed all other bryophytes had colonized the QCIs following the deglaciation of North America. The eleven species and subspecies of vascular plants mentioned by Taylor (1989) as being either endemic or showing an extremely restricted distribution extending to NW Vancouver Island or the immediate mainland, also show an ability to survive in alpine and sub-alpine environments. An ongoing survey of lichens and lichenicolous fungi of the QCIs has reported 18 species on the island that are new to North America, and promises more discoveries of botanical links between the islands and Asia as the study progresses (Brodo, 1995).

Only nine invertebrate species have been cited as endemic or exhibiting a restricted distribution adjacent to the QCIs. Bousfield (1958) described the amphipod *Paramoera carlottensis* from brackish water on the west coast of the islands, while Foster (1965) mentions an unnamed (and uncited) species of oligochaete worm (probably *Arctiostrotus perrieri*) supposedly also restricted to the archipelago. Both species are now known to have a wider distribution along the Pacific coast. Of the insects, all but two of the endemic and restricted species fall within the ground beetle family Carabidae. Lindroth (1961) described the species *Nebria charlottae* as being endemic to the QCIs, and Kavanaugh (1984) added *Nebria haida* and *Nebria louisea*. Kavanaugh (1989) also mentions the species *Bembidion viator* and *B. oblonguloides* as occupying restricted ranges centred on the QCIs. The only insect species endemic to the QCIs that are not members of the ground beetles are the spittle bug, *Aprophora regina*, described by Hamilton (1982) and the moth, *Xanthorhoe clarkeata,* discovered by Ferguson (1987). The dragonfly subspecies *Somatochlora albicincta massettensis*, is also restricted to the QCIs, but this large variant is not universally recognized as a distinct subspecies (Cannings and Stuart, 1977).

There have been few other studies that have explicitly examined the insects of the QCIs. Surveys of black flies (Currie and Adler, 1986), weevils (Anderson, 1988), and small dung flies (Marshall and Wheeler, 1991) have found no evidence of endemic species or forms within these groups on the QCIs. All three groups would be expected to have been represented in a refugium capable of supporting large mammalian predators such as bears and pine martens.

Molecular evidence for the presence of a Queen Charlotte Island refugium comes from the work of Byun et al. (1997) on the taxonomy of the black bear (*Ursus americanus*) subspecies found in western North America. Although, despite clear morphological differences between island and coastal populations, the QCI subspecies (ssp. *carlottae*) could not be differentiated genetically from subspecies found on Vancouver Island (ssp. *vancouveri*), coastal B.C. (ssp. *kermodei*), and the Olympic Peninsula (ssp. *altifrontalis*) coastal bears formed a distinct clade separated from continental bear populations by a sequence divergence of 3.6%. The existence of a coastal lineage of bear that has remained genetically distinct from continental populations for approximately 360,000 years indicates that this lineage survived the Fraser glaciation in a refugium isolated from the continental lineage and supports the existence of a refugium on or near the QCIs.

The alternative explanations for the differences observed between populations of mainland and island flora and fauna held that the morphological variations observed in the plants and animals of the QCIs could have occurred as a result of random drift or local selection pressures on small populations that colonized the islands after the ice receded. Evidence that such a process has occurred in at least one group of organisms came from the stickleback fish of the Queen Charlotte Islands. The stickleback showed a remarkable level of morphological diversity among the different lakes and river systems of the islands and also relative to the mainland populations. Moodie and Reimchen (1976a) and Reimchen (1992, 1994) demonstrated that variation in body size and lateral plate number among island populations could be accounted for by differences in the

selective pressures imposed by fish and bird predation among different bodies of water. In their discussion of the refugium hypothesis (1976b), they concluded that the degree of morphological variation found among QCI stickleback populations was greater than that of mainland populations because of an overall lack of predator diversity in the lakes and river systems of the islands. They hypothesized that the reduced predation created unique selection regimens that were not found on the mainland and that produced the variation found on the QCIs. The results of the morphological studies, combined with preliminary surveys of the mtDNA diversity among stickleback populations on the QCIs (Gach and Reimchen, 1989.), indicated that all of the divergent morphologies had arisen within the 10,000 year period since the islands had been deglaciated.

A number of populations of highly derived stickleback on Graham Island in the QCIs possess a mtDNA haplotype that is different from other freshwater and marine haplotypes. This suggested that the Graham Island stickleback have been separated since long before the last glacial advance and reopened the possibility that at least some of the fish populations may have inhabited glacial refugia (O'Reilly et al., 1993). However, the locations of the aberrant populations on a glacial outwash plain, the documenting of identical haplotypes amongst the marine stickleback of Japan (Orti et al. 1994), and the discovery of stickleback possessing the same haplotype at other sites in Alaska and Northern Vancouver Island (Deagle et al., 1996) make it less likely that the Graham Island stickleback survived the ice age in a QCI refugia, although populations may have inhabited other refugia in parts of coastal Alaska.

Glacial History of the Queen Charlotte Islands

The early theories for a glacial refugium on the QCIs that were proposed to explain the islands endemic species, required that the archipelago be almost entirely free of glacial ice for the entire duration of the Fraser glaciation. Brown and Nasmith (1962) demonstrated that while the Cordilleran ice sheet on the mainland is thought to have extended across Hecate Strait only as far as the north-east corner of Graham Island, the pattern of valley formation, rock erosion and till deposition indicate that almost all of the terrain currently above sea level was either submerged under locally produced glaciers or was covered over by huge amounts of glacial till as the ice retreated. Based on the present dimensions of the QCIs, Brown and Nasmith claimed that only nunataks and some coastal beaches between ice tongues would have remained free of these glaciers, leaving less than ten square kilometres of fairly inhospitable land for refugia. In their discussion of the glacial history of the Islands, Brown and Nasmith failed to take into account fluctuations in sea levels that would have exposed portions of Hecate Strait and Queen Charlotte Sound, leaving open the possibility that these areas may have remained free of overriding glaciers.

Core samples taken from Dogfish Bank to the east of the islands, from submerged banks in Queen Charlotte Sound, and from exposed cliff faces on eastern Graham Island, have indicated that low sea levels at the end of the glaciation exposed large areas of now submerged terrain to the east and south of the archipelago. Eustatically lowered sea levels across the globe, combined with isostatic uplift along the edge of the continental

shelf due to the crustal forebulge at the edge of the Cordilleran ice sheet, produced coastal sea levels ranging from 95m lower than present levels north of Vancouver Island (Luternauer et al. 1989a) to at least 170m lower than present levels to the east of Moresby Island (Josenhans et al. 1993). However such extremes were seen only near the end of the glacial period on the mainland (around 11,000 B.P. to 10,000 B.P) and were followed by a rapid marine inundation between 9000 B.P. and 7500 B.P. when sea levels rose to 15m higher than present (Clague et al., 1982). Carbon dated core samples taken from Dogfish Bank to the east of Graham Island indicate a more modest sea level drop of at least 37m, that exposed the terrain for a period of at least 3200 years beginning around 13,700 years B.P (Barrie et al. 1993). Middle Bank and Goose Island Bank in Queen Charlotte Sound are also believed to have been exposed during the height of the glaciation, with much of the Cordilleran ice being channelled into three deep troughs that separate the banks from each other and from the QCIs (Josenhans et al. 1995).

No continuous uninterrupted core that shows the habitation of the QCIs or the offshore banks through the entire period of the Fraser glaciation has been discovered, although an early carbon date taken from above glacial till on Cape Ball, Graham Island indicates the presence of diverse plant life as early as 16,000 +/- 570 years B.P. (Warner et al., 1982). Despite this early date for deglaciation of the QCIs, most samples taken from exposed cliff faces on eastern Graham Island indicate that early grass and sedge communities existed on top of thick layers of glacial till at different sites at dates ranging from 13,700 B.P to 12,000 B.P. (Barrie et al. 1993; Matthewes and Clague, 1982) Marine sediment cores taken from Queen Charlotte Sound date the end of till deposition

at around 12,900 B.P (Luternauer et al. 1989b). The earliest record for deglaciation of north-western Graham Island is that of a bog sample taken by Heusser (1955). The sample indicated the presence of lodgepole pine at its lowest stratigraphic level which was dated by Matthewes and Clague (1982) to 10,850 +/- 800 B.P. By comparison, deep sea cores in the Queen Charlotte Sound that measure ice rafted debris indicate the minimum date for the adjecent mainland glaciation as some time between 23,000 B.P. and 21,000 B.P., with the glacial maximum at 15,200, B.P., and rapid deglaciation beginning between 15,200 B.P. and 14,900 B.P. (Blaise et al. 1990).

Geological evidence accumulated over the past three decades has changed the theories of the number of possible sites for refugia on the QCIs from an initial belief that the entire archipelago escaped glaciation to the view that only some nunataks, coastal beaches, and now-submerged banks could have sheltered life through the Fraser glaciation. Despite evidence that the latter could have existed offshore from the glaciers and thus have sheltered a wide variety of life, the key piece of evidence, an uninterrupted core that reveals the existence of soil and vegetation through the entire duration of the ice age, has yet to be obtained.

The *Nebria*: Biology, Taxonomy, and Distribution

The genus *Nebria* is a predominantly Holarctic group, comprising over 500 species, of which 55 have been described from North America (Noonan, 1992). All species of *Nebria* demonstrate a requirement for cool or cold conditions and most are restricted to riparian habitats. Species diversity is highest in the mountain ranges of western North America. A few species occupy the eastern part of the continent and none are known from south of 30° latitude.

Seven species of *Nebria* are found on the QCIs (including one now extinct species represented by Holocene fossils); four are beach dwelling, one occupies river banks, and two (including the fossil species) are restricted to alpine conditions above the tree line. The three endemic species of *Nebria* found on the QCIs are *Nebria charlottae*, which is restricted to cobble beaches on Graham Island; *Nebria louisea*, found in similar habitats on Moresby Island and many of the smaller islands on the south-east of the archipelago; and *Nebria haida*, which was formerly believed to be found only in alpine locations on Graham and Moresby Islands, but has also been recorded on a mountain top north of Prince Rupert, B.C. (Kavanaugh, 1992). Their closest relatives are *Nebria lituyae* Kavanaugh, found in alpine areas in the Alaska panhandle, and *Nebria gregaria* Fischer, which occupies beaches on a number of the Aleutian Islands. Together, these five species comprise the *gregaria* infragroup, characterised by similar morphology, flightlessness, and a coastal distribution ranging from the Aleutian Islands to the QCIs,

with gaps in south-western coastal Alaska and mainland British Columbia. The remaining *Nebria* species on the QCIs are *N. sahlbergii sahlbergii* Fischer, *N. diversa* Leconte, and *N. mannerheimi* Fischer. Fossil specimens of *N. gyllenhalli* Schönherr have been recovered from alpine sites on the QCIs but it is no longer believed to exist on the islands (Kavanaugh, 1989).

The *gregaria* infragroup is placed within the *gregaria* group, which includes the species *N. lyelli* Van Dyke, *N. wallowae* Kavanaugh, and *N. acuta* Lindroth (*acuta* infragroup); *N. sahlbergii* (*sahlbergii* infragroup); *N. arkansana* Casey, *N. fragilis* Casey, and *N. zioni* Van Dyke (*arkansana* infragroup). The *acuta* infragroup extends along the Coastal Mountain range from northern California to southern Alaska but is not found on either Vancouver Island or the QCIs. *Nebria sahlbergii* is found in both the Coastal and Rocky Mountains, from Oregon to Southern Alaska and on to the proximal Aleutian Islands. The *arkansana* infragroup is restricted to the Rocky Mountains and in British Columbia is found only in the extreme north-east and south-east parts of the province.

The current distribution of most members of the *gregaria* group in north-western North America is a reflection of the preference of the species for cool climate habitats coupled with a northward range expansion from southern montane refugia following the path of the retreating glaciers. This pattern of northward dispersal following the glacial retreat is general for almost all of the Carabid fauna of north-western North America originating south of the ice sheets. In contrast, few of the arctic adapted species that sheltered either in the Beringia refugium, Kodiak Island, or the Aleutians have had any

success in extending their ranges beyond their glacial limits, and those that have are generally limited to the tundra north of the tree-line (Ball and Currie, 1997). Those few cold-adapted species with populations found in southern latitudes (e.g., *Amara alpina* and *Pterostichus* (*Cryobius*) *pinguedineus*) can be accounted for as being the last remnants of an arctic-tundra fauna that existed south of the ice sheet and that was largely extirpated due to a combination of rapid climatic warming coupled with a slow initial ice retreat. This resulted in the disappearance of habitat cool enough for the existence of many of these species (Schwert and Ashworth, 1988). The paucity of Carabid species in the eastern Arctic, despite an abundance of suitable habitat and an absence of physical barriers, implies that poor dispersal capabilities of the Beringian Carabid fauna were an additional factor in preventing their extension southwards, even marginally, into newly exposed terrain following glacial retreat (Danks, 1981).

Almost nothing is known about the life histories of the *Nebria* of the *gregaria* infragroup and, with two exceptions, very little has been published on the life histories of other *Nebria* species. Kavanaugh's (1978) revision of the Nearctic *Nebria* brought together a large amount of data on life zone and microhabitat preferences taken from field collecting and observations, but contains little in the way of direct experimental testing of *Nebria* biology. At the time of Kavanaugh's revision of the genus, only two of the five *gregaria* species were known, of which one (*N. charlottae*) was represented by a single museum specimen. The only extensively studied member of this genus is the atypical species *Nebria brevicollis* Fabricus, a forest dwelling and synanthropic beetle found in northern Europe and the United Kingdom that lacks the riparian and montane

requirements that characterize most other species in this genus. The degree to which the biology of *N. brevicollis* can be extrapolated to the members of the *gregaria* infragroup is unknown. However, it provides the largest window into the biological activity of this genus that exists at present.

In general, the narrow temperature requirements of members of this genus limit most species to montane habitats, where they are found occupying limited altitudinal ranges that are very often specific for a particular species in each mountain range. Only in northern latitudes are altitude requirements relaxed, allowing for a somewhat wider distribution of individuals (Kavanaugh, 1978; 1979a.). The narrow altitude ranges occupied by many *Nebria* species in the mountains of the southern United States, and the relaxation of these altitude requirements among northern and coastal representatives of the same species, are indicative of individuals tracking the movement of zones of suitable climate up or down in altitude until a level is found where conditions are acceptable. The other possible response to climate change, adaptation of the beetle's environmental tolerance limits to fit the new conditions, has yet to be demonstrated in any of the *Nebria* species that underwent shifts in distribution during the climatic upheavals of the late Pleistocene and Quaternary periods. The tendency of *Nebria* to respond to climate change by migration as opposed to adaptation is supported by the research of Butterfield (1996) into carabid communities at high altitudes in England. The two *Nebria* species in the study, *N. salinas* and *N. gyllenhali*, appeared to lack the plasticity in their life cycle to invade either a higher or a lower altitude range than that at which they occur naturally. Gereben (1995), in her study of *Nebria* communities colonizing recently exposed land in

the wake of a retreating glacier, and Kavanaugh (1978), in his survey of Nearctic *Nebria* collection localities, noted that a large number of microhabitat factors, including substrate size, temperature, humidity, and habitat stability, governed the selection of habitats for *Nebria*, allowing many species to co-occupy the same life zones and indicating that competitive exclusion was not a factor in limiting the beetles to specific altitudes.

Members of the genus *Nebria* have often been considered by naturalists to be specialist predators on springtails (for review, see Hengeveld, 1980a). Hengeveld (1980b), however, through dissection of the crops of adult beetles, demonstrated that the adults of *Nebria brevicollis*, in addition to springtails, consumed a wide variety of foods including plant matter, ants, spiders, caterpillars, flies and mites. Kavanaugh (1978) observed that adult *Nebria* of most species were fully capable of capturing and consuming prey items much larger than themselves, and speculated that crop content studies systematically underestimated the size range of potential prey items, as food fragments from large organisms would prove hard to identify. Kavanaugh's observation is supported by Hering and Plachter's (1997) survey of riparian beetles that documents the importance of emerging stoneflies as a diet component in the European species *N. picicornis*. Spence and Sutcliffe's (1982) examination of the mouthparts, food capture, and digestive processes of the larva of three North American *Nebria* species detailed a mandibular arrangement that functioned as a snap trap triggered by sensitive hairs extending into the mandibular space. This mechanism would allow the larva to capture prey that utilized rapid escape responses, such as that found in most springtails and some beach dwelling amphipods.

Nebria are primarily ambulatory insects, with long slender legs that give them the capability for rapid movement. Most observations on the ambulatory ability of *Nebria* have focused on their escape behaviour when disturbed, and very little has been done on their ability to migrate long distances, although *Nebria* have been captured on snowfields hundreds of meters from the nearest shelter (Kavanaugh, 1978). Despite a large proportion of the *Nebria* species possessing fully developed wings, flight has been recorded in only a few *Nebria* species, only one of which occurs in North America (Kavanaugh, 1978; Gereben, 1995). However, fully winged species occupy significantly larger ranges than short-winged or mixed-winged species, indicating that flight may hold an important role in colonizing new habitats (Kavanaugh, 1978). Examinations of the dispersal capabilities of *N. brevicollis* indicated that, although all adults possessed fully developed wings, almost all never developed their wing muscles to an extent that allowed the capability of flight (Nelemans, 1987). Flight muscle development was found to be linked to the amount of nutrition obtained by the larval beetles, with only those receiving an abnormally high amount of food as a larva showing any capability of flying. Despite the lack of flight in most adults, mark and recapture studies demonstrated that the adults were highly mobile and fully capable of dispersing over large distances and across unsuitable terrain (Nelemans et al., 1989).

One species of *Nebria* (*N. ingens* Horn) actively takes to underwater environments, and most species are capable of some swimming ability, enabling passive dispersal along streams (Kavanaugh, 1978). However, the aquatic capabilities and microhabitat requirements of *Nebria* would be insufficient to allow prolonged survival in

marine environments, either directly in the water or through rafting on logs. Dispersal by human activities is also unlikely because of life history factors, with the one exception of *N. brevicollis*, the synanthropic habits of which may have allowed its introduction twice into eastern North America (Kavanaugh, 1978).

Kavanaugh's (1989; 1992) proposed phylogeny of this group based on morphological features, holds that the five species of the *gregaria* infragroup represent the remnants of a widespread ancestral species whose population was subdivided and isolated during the last ice age for a period that was sufficient to allow the development of reproductive isolation. According to Kavanaugh early glacial events in the Pleistocene divided the ancestral population into two, one that occupied nunataks and a second that was confined to the rocky coastline. As further glaciations occurred, these two ancestral populations were subdivided into those beetles found in the Alaskan refugia and those that sheltered on the QCIs. The various populations were able to acquire sufficient genomic differences during their period of isolation from each other to prevent hybridization after the glaciers retreated. From the nunatak occupying population came the species *N. lituyae* and *N. haida,* while the shoreline population evolved into *N. gregaria, N. charlottae*, and *N. louisea.*

Alternative hypotheses to that of Kavanaugh's hold that the QCIs were unsuitable to the *Nebria* as a glacial refugium, and that the endemic species found there today are merely highly derived forms of existing mainland stocks that either colonized the archipelago after deglaciation when sea levels were lower and were then isolated from

their parent populations by the widening of the Hecate Strait, or that were introduced into the archipelago after sea levels had risen by rafting on logs or by human transport. Whichever ancestral population contributed to the extant Queen Charlotte *Nebria* may then have been eliminated or forced to migrate by the changing climatic conditions that marked the post glacial period. The effects of genetic drift and a selection regimen, different from that of the ancestral species, acting on a small population with a limited subset of the ancestral gene pool could then account for the observed morphological differences among the three Queen Charlotte species and their Alaskan sister species.

Geological evidence for deglaciation of the western coast of Alaska indicates that although ice began retreating from some sites as early as 14,700 B.P., marine inundation of coastal areas and the subdivision of ice-free areas of the Alaska panhandle by ice lobes would have proved a serious impediment to the migration of flightless beetles southward from refugia in Alaska and the Aleutian Islands (Mann and Hamilton, 1995). Alternatively, an ancestral population may have migrated northwards along the coast from a refugium in Washington State to Alaska, leaving behind a small cluster of populations in suitable habitat on the QCIs while the majority of the ancestral species settled on the Aleutians and in alpine areas of the panhandle. The presence of *Nebria gyllenhalli* (albeit as a now extinct population), a high altitude species on the QCIs, and the more lowland riparian species N. mannerheimi and N. sahlbergii, indicates that the Hecate Strait and the intervening terrain were not a significant barrier to dispersal from the mainland during the period of the glacial retreat.

Molecular Evolution

To evaluate the hypothesis that the radiation of the *gregaria* infragroup was dependent on refugia on the QCIs, it is necessary to determine whether the species of *Nebria* belonging to the infragroup show a pattern and level of divergence that would indicate a biological separation of species dating from before the Fraser glaciation or whether a non-refugial origin is indicated by the data. In order to determine the evolutionary pattern followed by the beetles of the *gregaria* infragroup, two lines of evidence are explored. The first involves discovering the order and timing of speciation events through comparisons of mitochondrial DNA sequences; the second seeks to evaluate the degree of overall genetic separation between the Queen Charlotte members of the infragroup by comparing nuclear markers generated through random amplified polymorphic DNA (RAPD) methods.

Because the primary focus of this research is on DNA sequences taken from the mitochondrial DNA (mtDNA) of the beetles, some background on insect mtDNA is required. No complete mtDNA genome exists for any member of the Coleoptera and the few mtDNA sequences from this order that have been published are of DNA fragments far too small to elucidate anything about the properties of the beetle mtDNA genome. However, complete mtDNA genomes exist for *Locusta migratoria, Drosophila yakuba, Drosophila melanogaster, Anopheles gambiae, Anopheles quadrimaculatus*, and *Apis mellifera*, as well as many other non-arthropod invertebrate species. Based on the properties of these mitochondrial genomes some inferences can be drawn about what can

be expected of beetle mtDNA.

The mitochondrial genome in almost all metazoa consists of circular molecules of DNA ranging in size from 14 thousand base pairs (kbp) to 39 kbp encoding 13 proteins involved in the oxidative phosphorylation process, 22 transfer RNAs, 2 ribosomal RNAs and a control region involved in replication. The order of genes in the mtDNA molecule varies considerably among phyla, but from the few complete mtDNA genomes known for the Insecta, the gene order appears to be uniform across the class, differing only in the location of the small tRNAs between protein coding and rRNA genes. Intergenic regions are small, averaging between 5 base pairs (bp) and 7 bp, and short overlapping regions of the sequences are not uncommon. The one exception is the common honey bee (*Apis mellifera*) which has a total of 811 bp of intergenic sequences that range in size from one nucleotide to 193 nucleotides long (Crozier & Crozier, 1993).

The control region of the mitochondrial genome is considered highly variable in insects and accounts for much of the mtDNA size difference observed among species. Within *Drosophila*, this control region is composed of two arrays of tandem repeats located on either side of the putative origin of replication. The Type I repeat unit is the most highly variable, both in size and nucleotide composition, while the Type II contains a conserved 300 bp region comprising two-thirds of the repeat units total length. (Lewis et al. 1994.) A similar situation is observed in *Locusta migratoria*, with two types of repeat arrays, one of which has a substantial conserved region. (Flook et al. 1995). No such obvious tandem repeats exist within the control region of *Apis mellifera*, and some

authors have hypothesized that the large intergenic region located between tRNA$_{leu}$ and the cytochrome oxidase II (COII) gene serves as the origin of replication (Corneut et al. 1991).

The mitochondrial genome of insects is characterized by a very high percentage of adenosine (A) and thymidine (T) nucleotides. Of the six complete insect mtDNA sequences, the total A+T content of the genome ranges from a low of 75% for *L. migratoria* to a high of 85% for *A. mellifera.* The four Dipteran species all have a fairly uniform A+T proportion of 78%, with protein coding genes possessing an A+T content of ~76%, ribosomal rRNAs having an A+T content of ~81%, and the control region having the highest A+T content at ~93% (Flook et al. 1995). Data taken from Howland and Hewitts (1995) survey of the cytochrome oxidase I (COI) gene across the Coleoptera places this order within the high end of the known A+T proportions, with values ranging from 80% to 85% for a 400 bp protein coding fragment. The effect of this bias within insects to preferentially incorporate adenosine or thymidine nucleotides during mutation events is to saturate those locations in the DNA that are not under selective pressure with the two nucleotides, thereby reducing the effective number of possible types of base substitutions from six to one. This bias is most pronounced in the two regions of the mitochondrial DNA that are the least conserved; the third position of each codon, and the control region of the mtDNA.

The mitochondrial genome is a favoured target for phylogenetic studies of insects as it lacks many of the complicating features that characterize nuclear DNA. There are

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no transposons, microsatellites, or introns within the mitochondrial genome, and the genome never undergoes recombination. The RNA copy for all mitochondrial protein coding genes, tRNAs, and rRNAs is transcribed as a single unit beginning at the origin of replication; thus there are no promoter regions or transcription factors except for those that might exist within the control region of the mtDNA molecule. Mitochondrial DNA in insects is inherited from the maternal parent, although some instances of small amounts of paternal mtDNA occurring in offspring have been recorded (Kondo et al. 1990; 1992). However, Meusel and Moritz (1993), experimenting with *Apis mellifera,* demonstrated that what little paternal mtDNA is transferred to the offspring diminishes in amount as the insect ages until it is undetectable in the adult insect. The possibility of paternal contributions of mtDNA to the next generation of insects can therefore be safely ignored. Most genetic changes that affect the mitochondrial DNA are base substitutions, with the occasional small addition or deletion of a few base pairs. Large changes in the size of the mitochondrial genome almost always occur in the control region due to the loss or gain of units from tandem repeat arrays.

One potential problem with the use of mtDNA is the possibility of accidentally amplifying, through the polymerase chain reaction, fragments of DNA that, while resembling mtDNA and having an evolutionary origin in the mitochondria, are located in the nuclear genome as they have undergone a separate evolutionary pathway from the mitochondrial genome (Zhang and Hewitt, 1996). Such transposition events have the potential to seriously confound mitochondrial based phylogenies, but are also thought to be easily detected. Nuclear copies of mitochondrial sequences would be revealed by the

presence of multiple PCR amplification bands, sequence heterogeneity within a single individual, and unexpected nucleotide sequences, frame shifts, and stop codons. The occurrence of transposed mtDNA genes, while widely observed in vertebrates, has only been observed in three species (within two orders) of insects. Sunnucks and Hale's (1996) research into the occurrence of transposed mitochondrial DNA fragments in the nuclear genome of *Sitobion* aphids noted that sequence differences were greater between mitochondrial fragments of different species than they were between nuclear and mitochondrial versions of the same gene within a single species (or individuals). They also noted that transposition events had only occurred within *Sitobion* and two other closely related genera, and that more distantly related genera of aphids did not show evidence of transposition events, indicating a species- or taxa-specific ability for the transposition of mitochondrial genes into the nuclear genome.

In order to determine the relationships among the members of the *gregaria* infragroup, sequences of mitochondrial DNA taken from the beetles are compared and a cladistic phylogeny is constructed using the mutations that had occurred between species as characters, with character polarity determined by the inclusion of outgroup species. The choice of mitochondrial regions to analyze was based on the known and inferred rate of evolution for Coleoptera mtDNA, given the limitations that a lack of any complete beetle mtDNA sequence presented.

The rate at which insect mitochondrial DNA evolves is unknown for most orders. It can, however, be inferred that those portions of the mtDNA that are highly conserved

are unlikely to have undergone many base substitutions in the timespan available to the *gregaria* infragroup. These conserved regions consist of the transfer RNAs, the ribosomal RNAs, and the first two base positions of the codon of protein coding genes. Changes to these regions that impair the efficiency of the resulting protein or RNA will result in the rapid elimination of the mtDNA haplotype from the population. Due to the fourfold degeneracy of the third base position of the codon for most amino acids, base substitutions at this position are not directly affected by selection pressures and theoretically any base change will be tolerated (Simon et al. 1994). In practice, the lack of conservation of the third base position makes it susceptible to the effects of the A+T substitution bias found in insects, resulting in genomes with 90% to 95% of the third codon positions being either adenosine or thymidine (Flook, 1995). The strong bias for inserting adensosine or thymidine during third base position mutation events can lead to underestimates of sequence divergence and unreliable phylogenies when incorporated into the analysis of older species. However, the analysis of recently diverged species that have undergone a much lower number of substitution events between taxa will not be adversely affected by the A+T substitution bias.

The remaining unconserved regions of the mitochondrial DNA, comprising the spacer regions between genes and the control region of the mtDNA, are unsuitable for phylogenetic analysis. From the known mtDNA sequences for insects, the untranscribed spacer regions between genes appear to be too small and too dispersed to be effectively targeted and sequenced. While large regions of untranscribed nucleotides similar to those found in *A. mellifera* may exist in the beetle mtDNA, their presence and location cannot

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be predicted beforehand. The control region of the mtDNA is often used as a target sequence for studies of vertebrates that involve a short time-span for speciation. However, the presence of tandem repeated units combined with over 90% of the nucleotides composed of adenosine and thymidine has produced a region of the mtDNA that in many species is hypervariable, with the potential to differ in size and sequence even within a single individual (Solignac et al. 1983). In addition, the presence of tandem repeated units makes amplification of the control region using the polymerase chain reaction difficult, often producing multiple bands containing different numbers of repeat units. All published sequences of insect mtDNA control regions have been obtained through restriction enzyme digestion of the control region followed by cloning and sequencing of the fragments (Lewis et al. 1994). The protein coding sequences, with particular reference to the third position of each codon, therefore remain the best possible targets for obtaining useful data.

The choice of which protein coding genes to sequence is less a question of mtDNA theory and more a problem of obtaining suitable sites for annealing of oligonucleotide primers for the polymerase chain reaction. As only a limited number of regions of the mtDNA for beetles have been sequenced to date, the identities of suitable primer binding sites have to be inferred from regions of the mitochondrial genome that are heavily conserved across many orders of insect. Such sites are generally located in transfer RNAs, ribosomal RNAs, and portions of protein encoding DNA crucial for enzymatic activity. The wide range of target sites that have been incorporated into this study reflect the ability of molecular researchers to discover and utilize such sites.

Sequence Analysis

The two methods used for determining the pattern of speciation from sequence data are phenetic (distance based) methods and parsimony analysis. The underriding assumption of distance based methods is that a single value for sequence similarity in a pairwise comparison can provide an accurate measure of the degree of divergence that has occurred between two taxa, and that this value is both objective and repeatable. These methods involve converting the sequence data into a measure of genetic distance between species and constructing the phylogenetic tree by progressively grouping the most similar taxa. In cluster analysis, its simplest form, taxa are progressively linked in a phylogenetic tree based on the raw genetic distances, with the underlying assumption that rates of evolution are equal across taxa and therefore the genetic distances are both additive and ultrametric. The more complex neighbour-joining method uses a rate-corrected distance matrix to link both terminal and internal nodes of the phylogenetic tree, under the assumption that there will be differences in the rates of evolution along different branches and that these differences must be adjusted for in the process of constructing the tree. The neighbour joining method, while still retaining the assumption of additivity in the data, no longer requires the data to be ultrametric (Swofford and Olson, 1990).

Cladistic methods group taxa together based on the presence of shared derived characters (synapomorphies) within the taxa. The product of a cladistic analysis is nested sets of monophyletic groupings (groupings of taxa sharing a common ancestor and

consisting of all of the extant descendents of that ancestor) that reveal the historical order in which taxa differentiated from each other. In DNA sequence data, the characters are the nucleotides of the raw DNA sequence, or the amino acid products of a translated DNA sequence.

Raw sequence data consist of long strings of letters representative of the four possible nucleotide bases in DNA: adenosine, thymidine, cytidine, and guanidine. Each nucleotide position in the sequence is considered a discrete and theoretically independent character, having one of four possible states representative of the identity of the nucleotide. The characters can be ranked as either ordered, partially ordered or unordered, depending upon what is known of the probabilities of the possible character state changes in a particular sequence of DNA. An unordered state can change into any other, while an ordered state must progress in a predefined sequence of state changes. Partially ordered characters are those that fall anywhere between these two extremes. The classification of characters as ordered or unordered can improve the resolution of the phylogenetic tree (Slowinski, 1993). However, the ordering of molecular characters requires a thorough understanding of the probabilities of each possible mutation if it is to be a reliable tool. For this reason, DNA sequence data characters are generally considered unordered, even though physiological processes behind mutation events, as well as compositional biases towards certain nucleotides in the DNA, makes certain types of mutations much more likely to occur.

In order for two or more sequences to be compared, the base positions in each

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sequence must be aligned with their homologous positions in the other sequences. Gaps are added to sequences where it is believed that a deletion event occurred, or where insertion events have occurred in the comparison sequence. There are elaborate programs for computerized alignment of sequences, that involve rating alignments based on assignment of gap and mismatch penalties, and the construction of distance trees to aid in multiple sequence alignment. Most recently diverged sequences, such as those used in this study, can be aligned visually without the aid of alignment algorithms and researchers recommend against the inclusion in the data of any region that cannot be easily aligned.(Swofford and Olson, 1990).

Once the homology of the characters has been determined by the alignment, the data can then be used to construct a dendrogram of species relationships. In theory, each character is evaluated in turn to determine which grouping of taxa its derived state supports and the results from all characters are compiled to form a branching arrangement of monophyletic groups. In practice, character polarity is often unknown at the outset of the analysis or, at best, is assumed from one or more outgroup taxa, while homoplasy (convergent or parallel evolution) produces conflicting results between different characters. Because DNA sequence data do not allow ontological examination of character origins, and because the number of possible character states is limited to four, the effects of unknown polarity and homoplasy present an even greater problem. Procedures for evaluating DNA sequence data generally involve examining every possible tree that could be produced with the data set to determine which dendrogram requires the least number of evolutionary changes with the assumption that the shortest

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tree best models the actual historical branching of the taxa. Where the number of taxa involved is large (>12) various 'short-cut' methods are employed to quickly eliminate long trees and to minimize the number of character state changes involved in reaching the optimal tree.

Two such 'short-cut' methods are the heuristic methods, and branch-and-bound methods (Swofford and Olson, 1990). The heuristic methods involve taking an initial tree of three taxa and sequentially adding taxa to the tree, each time evaluating various rearrangements of the positions of the added taxa in order to minimize the number of evolutionary steps required to produce the tree. While the heuristic method allows processing of data sets from large numbers of taxa (>25) within a reasonable time, it runs the risk of finding a 'locally' optimal tree as opposed to the most optimal tree for the data. Multiple heuristic examinations of the data have to be performed, using different starting taxa and methods of adding taxa to ensure that the true optimal tree has been found. The branch-and-bound methods begin with an initial upper bound limit to tree size, derived from a heuristically produced tree, and then proceed with an exhaustive evaluation of branching patterns, bypassing those branchings that would result in trees exceeding the upper bound limit. When a tree that has a length less then the current upper bound is produced, the tree length is taken as the new upper size limit and the process continues. In this way, a search of the different tree patterns can be performed in a manner similar to that of an exhaustive search, but allowing the rapid bypassing of unproductive dendrograms.

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Because of their extreme complexity, there is as yet no way to calculate confidence limits for trees. However, a number of methods and statistics can be used to infer how accurately the tree represents the actual history of the taxa. The first statistic considered is the tree length, using the assumption that the shortest possible tree is the best representation of the data. A second statistic, the Consistency Index (CI) evaluates the amount of homoplasy in the data by dividing the minimum amount of change needed for a character by the actual amount of character change, summed over all characters in the data. Because homoplasy will increase the amount of observed change over that of the minimum, a CI value of 1 would represent a situation of no homoplasy, with the CI declining as instances of parallel and convergent evolution occur. The lower boundary of the consistency index is defined by the distribution of character states and can only approach zero as the data increase in complexity. A second estimate of the degree of homoplasy, the Retention Index (RI) produces a value between the range of 1 and 0, with 1 representing a dataset without any homoplasy, and 0 a data set that is effectively random and unresolvable. The RI is calculated by dividing the value of the maximum amount of change possible for a character, minus the length required by the character on the tree evaluated, with the value of maximum change minus the minimum amount of change that the character may show in any tree, summed over all characters in the data.

A third statistic, the Skew, represents the degree to which the distribution of tree lengths from an exhaustive search resembles that of a normal distribution. A pattern of tree length distribution that is not normally distributed would indicate that the data have a strong phylogenetic signal. Normally distributed tree lengths would infer that the data

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have been randomized by convergent evolution and multiple character changes.

For evaluating the degree of support for specific branches in a dendrogram there are few options other than to use bootstrap resampling. The original data set is randomly sampled to produce a bootstrap data set of the same size, which is then analyzed to produce a tree. This procedure is repeated from 100 to 1000 times and the resulting trees are compared to determine the frequency with which particular nodes or branches reoccur. The frequency with which a node or branch is present in the data is then converted to a percentage measure of confidence that is superficially similar to that of statistical confidence levels.

In order to improve the resolution of the tree-building algorithms, weighting systems are often applied to the data set to enhance the importance of particular parts of the data in producing the final tree. In DNA sequence data, weighting systems are generally used to account for multiple nucleotide substitutions occurring at a single site, a common occurrence at loosely conserved sites in distantly related taxa. When base positions undergo multiple substitutions, the possibility exists that an informative mutation will be changed back to its original nucleotides, thus obscuring two character changes in the data. In addition, the number of character changes separating different species becomes underestimated to a degree relative to the level of divergence between species. Multiple substitutions occur most often in areas of the genome where a nucleotide change will not have an effect on the fitness of the organism, such as intergenic regions, introns, and the third base position of each codon. Various weighting

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systems can account for the possibility of multiple substitutions in the third base position, while introns and large intergenic regions are often analyzed separately from coding regions of the DNA.

A second cause of multiple substitutions are biochemical processes that favour certain types of mutations over others resulting in a greater probability that these mutations will fix in the population. The most common of these differentially occurring mutations is the observed difference in most sequence data between the number of transitions (ts) versus the number of transversions (tv). Transitions from purine to purine or pyrimidine to pyrimidine are far more likely to occur than transversions from pyrimidine to purine or vice-versa (Martin, 1995). In recently diverged lineages, the observed ts/tv ratio can be as great as 45:1, but, as the time since separation increases, multiple mutations force the observed ts/tv ratio towards 1:1 (Besansky et al. 1994). By using this ratio as a guide, weightings can be applied to the different classes of mutations to emphasize the rarer transversions over the more common transition mutations. Even finer grades of mutation weighting can be applied, using different weights for each of the six types of nucleotide changes (e.g., Knight and Mindell, 1993). The bias in insects towards the incorporation of adenosine and thymidine nucleotides at silent locations in the DNA reverses the biochemical bias, resulting in a preponderance of transversions being observed between DNA sequences (DeSalle 1992), and requiring that weighting systems based on mutation type for insects be calculated separately for each gene and not be based on the *a priori* assumption that transitions will predominate.

The Molecular Clock

The method used in this study of the *Nebria*, that of obtaining phylogenetic characters from sequences of the mitochondrial DNA, has the advantage of allowing an impartial determination of the time since two species diverged from their common ancestor from the number of base changes that have occurred between the two organisms. Although a calibration with fossil data or biogeographical events is required to allow the molecular clock to set an accurate date on the speciation events, an uncalibrated clock can still contribute a probability that a speciation event took place within the timespan under consideration. By combining phylogenetic and molecular clock data obtained from the mitochondrial DNA, and examining both in light of what is known about environmental conditions during the last glacial period, the hypothesis that the QCIs served as a refugium for one or more of its endemic *Nebria* species can be tested.

The concept of the molecular clock is based on Kimura's (1983) neutral theory of evolution, which holds that mutations accumulate at a steady rate within the genome over time, and that the rate of mutation accumulation is roughly equal for all taxa. According to theory, one need only determine the level of sequence divergence between two species and compare it to a pair of reference taxa with a known date of divergence to date the point of genetic separation for the two species. However, researchers have increasingly found examples of varying rates of evolution between different genes (Simon et al., 1994), and different taxa at both the level of families and orders (Li et al., 1987) and at the level of species (Ballard and Kreitman, 1994). The reasons for

variation in the rate of evolution between taxa are not yet fully understood, but researchers have noted that factors such as generation time (Li et al., 1987), metabolism (Martin and Palumbi, 1993), and DNA repair mechanisms (Britten, 1986) may contribute to variations in mutation accumulation within species. While Bromham et al.'s (1996) survey of variation in evolutionary rate across a wide range of mammals strongly implicated generation time as the source of synonymous mutations in mitochondrial DNA, no such survey has been performed on insect sequences. The choice of taxa to calibrate the molecular clock should therefore be taxonomically close to those species under consideration, to minimize lineage and life history differences in mutation rate.

While numerous studies involving sequence evolution have been performed with insects, very few have used taxa that had a dated divergence point that could be used to calibrate a molecular clock, and even fewer have involved the order Coleoptera. The most cited study involving sequence divergence rate calibrations is that of Brower (1994), in which seven arthropods from different habitats, continents and orders that all have their divergence occurring as the result of a dated geographic event, are used to estimate an 'overall' rate of insect mtDNA evolution of 2.3% sequence divergence per million years. However, Brower's estimate makes use of a single outlying species group (*Alpheus* spp., a decapod crustacean) with a sequence divergence of 2.35% sequence divergence per million years in combination with two insect divergence estimates of 1.7 % divergence / million years and a cluster of three estimates taken for durations of less then 100,000 years. The result is an estimate of sequence divergence for arthropods severely skewed in favour of that obtained for the single crustacean species group.

Of greater importance is Brower's phylogenetic tree of *Heliconius* butterfly morphotypes, that allows some idea of the degree of genetic variation to be expected from a single glaciation event. The various wing pattern morphs of the *Heliconius* butterfly are believed to be the result of morphological differentiation occurring in populations isolated by shrinkage and subdivision of the South American rainforest during glacial maxima. During the interstadial periods when *Heliconius* populations are reintroduced, the differences in wing pattern are maintained by Mullerian mimicry complexes that result in the rapid elimination of hybrid individuals by predation. The most recently diverged populations in Brower's phylogenetic tree would represent the result of the climate cooling and forest subdivision that would have occurred at the same time as the Fraser glaciation in North America. Mitochondrial sequence divergence between these sister populations of the *Heliconius* butterfly for the most recent glacial advance is in the order of 0 to 2 mutations over a 400 bp fragment.

Of the few molecular phylogenetic studies that have been performed on beetles, only two address questions of zoogeography, and of these only one attempts to calibrate the rate of molecular evolution based on a known geographic event. Vogler and DeSalle (1993) examined genetic variation between populations of the east coast tiger beetle *Cicindela dorsalis* and noted a divergence as great as 5% in mtDNA sequences between eastern and Gulf of Mexico populations. Vogler and DeSalle attributed their observations to the emergence of the Florida peninsula sometime in the Pliocene/Pleistocene era, but were unable to assign a discrete date to the moment of population divergence.

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Su et al. (1996a,) were able to estimate a divergence date for the splitting of the carabid genus *Damaster* on the Japanese islands from that of the genus *Acoptolabrus*, found on mainland Asia. Su et al. calculated that under Brower's estimate of 2.3% sequence divergence / million years, the timing of the *Damaster/ Acoptolabrus* split would have been 5.5 million years ago, and the radiation of *Damaster* and the related genus *Ohomopterus* would have taken place between four and five million years ago (Sue et al. 1996b). However, the estimate of the *Damaster / Acoptolabrus* split based on Brower's arthropod sequence divergence would have been five million years after the date placed on a fossil specimen of *Ohomopterus* and ten million years after the separation of Japan from the mainland, the hypothesized 'founding event' for the genus. Instead Su et al. based their estimations of the rate of sequence divergence on the pattern of vicariance between species and genera within these Japanese Carabids and the corresponding dates at which the various islands of the Japanese archipelago had become isolated from the mainland and each other. For this lineage of the Carabidae, Su et al. came to an estimated rate of molecular evolution of 1.2% sequence divergence / million years.

RAPD Analysis

The Random Amplified Polymorphic DNA (RAPD) technique is a method for assessing the degree of relatedness between taxa based on the presence or absence of markers located in their nuclear DNA. RAPDs belong to a class of techniques related to Restriction Fragment Length Polymorphisms (RFLP) and the Jeffrey's fingerprints in which DNA fragments from different taxa are used to form banding patterns on gels which can then be used in a phenetic analysis of taxon similarity. Unlike the RFLP and the Jeffrey's fingerprint, both of which depend on the presence or absence of restriction enzyme cut sites in the DNA, the RAPD band profile is generated by using arbitrary primers in a polymerase chain reaction to generate a pattern of amplified DNA bands that can then be separated on either agarose or acrylamide gels. The bands are produced in the polymerase chain reaction wherever two primers are able to bind to opposite strands of a section of genomic DNA within a sufficient distance of each other such that the polymerase enzyme can synthesize the intervening stretch of DNA. With the number of bases in the genomic DNA in the order of billions, the probability that two primer binding sites will occur in the correct orientation is exceedingly high. However, the non-random ordering of base pairs in the DNA, and the inability of most polymerases to synthesize bands greater then a few thousand kb, conspire to limit the number of bands produced to less then twenty or so per primer.

Primers have ranged in size from a low of 5 bp in length (Caetano-Anolles et al. 1991) to 36 bp long (Welsh and McClelland, 1990), and have included truly randomly

designed primers to microsatellite based primers consisting of tandem repeated di-, tri-, and quadramers with an anchoring nucleotide at the 3' end (Zietkiewicz et al. 1994). However, the standard primer in RAPDs is that of Williams et al. (1990) who found that a ten base primer with at least a 50% G-C content could be used to produce a range of banding patterns between 100 and 3000 bases in length.

One of the greatest practical difficulties with RAPDs is ensuring the reproducibility of results. Depending upon the author consulted (Hadrys et al, 1992; Bowditch et al. 1993), RAPD reactions have been cited to be sensitive to even minor changes in a wide range of factors including magnesium concentration, enzyme concentration, DNA template concentration, template purity, and ramping conditions. The factor that has appeared to have the greatest effect on reproducibility has been the magnesium concentration of the buffer and the enzyme concentration (Innis and Gelfand, 1990). RAPD reactions appear to be tolerant of wide variation in DNA concentration (Micheli et al. 1994) and only gross errors in pipetting of primers are likely to produce variation in band patterns. The use of standardized mixtures of enzyme and buffer ensure a high degree of reproducibility between reactions

Interpreting RAPD fingerprints involves scoring bands for the presence or absence across the individuals or taxa used in the study, and analyzing the data either through distance or parsimony methods. Shared bands between genomes are usually considered to be derived from homologous regions within the DNA and are treated as such in the data analysis. RAPD fragment band losses between genomes can be caused

either by a substitution at the primer binding site, a mutation causing changes in the secondary structure near the primer binding location, or a deletion or insertion event that alters the size of an amplified region between genomes. Limitations in the resolving ability of agarose gels and a lack of knowledge about the structure of the genomes being studied introduces the possibility that two bands occurring at the same level on the gel may be from entirely different regions in the genome, and may share no common evolutionary ancestor. Such events, known as co-migrations, are often accepted by researchers as inherent risks in RAPD analysis, with the hope that they are rare events that can be overridden by a large volume of accurate bands. However, band identity can be determined by probing gels with radioactively labelled DNA, allowing the identification of co-migrating non-homologous bands as well as band losses that are due to internal size changes and not the loss of primer sites.

Statistical tests of within and between population variance devised for RFLP data can be applied to the RAPD fingerprints (e.g., Excoffier et al. 1992), as can distance based methods of determining phylogenetic relatedness (e.g., Liu et al. 1994). Some researchers have also treated RAPD bands as discrete characters and have applied cladistic analysis to RAPD data sets (e.g., Landry et al. 1993). The use of cladistic methods on RAPD data introduces the requirement that the individual bands produced by the RAPD reaction be independent of each other. For this requirement to be satisfied, presence or absence of a particular band must be due entirely to the presence or absence of the corresponding primer sites and not to co-migration of different bands or internal size changes within a band. The competition for primers by binding sites on the DNA

that occurs during the RAPD reaction can also violate the assumption of independence by allowing strongly amplified bands to outcompete weakly amplified regions for primers, resulting in amplification bands for the latter regions that cannot be visualised on the gel (Backeljau, et al. 1995; Smith, et al. 1994).

RAPD data sets are more often converted to measures of genetic distance between taxa and analyzed using the phenetic methods of cluster analysis and neighbour joining. A wide variety of formulae exist for producing values of genetic distance from the binary data of RAPD bands, of which the most basic are the Coefficient of Jaccard and the Simple Matching Coefficient (Sneath and Sokal, 1973). Both coefficients produce a value for genetic distance by dividing the number of shared characters by the total number of characters under consideration. They differ, however, in their treatment of those characters that are negative (or scored 0) between two taxa. The Coefficient of Jaccard assumes that shared absences in the data are phylogenetically meaningless, and removes such characters from consideration in both the numerator and the denominator of the equation. In contrast, the Simple Matching Coefficient holds that all matches are meaningful and retains shared negative characters in the equation.

The nature of RAPD bands lends itself to the use of the Coefficient of Jaccard and the exclusion of shared absences from the analysis. Without an extensive knowledge of the genome in question, it cannot be determined whether a shared absence is due to the loss of a band along a common branch of the taxonomic tree, or whether the shared absence merely signifies that the proper combination of nucleotides required to produce

the RAPD band never evolved and is a meaningless ancestral condition.

Most other coefficients used to determine genetic distance are based on simple monotonic functions of the Jaccard and Simple Matching Coefficients, designed to emphasise either the similarities or the mismatches between samples. As there is no set of general rules that can guide the researcher in the choice of appropriate coefficients, two modifications of the Jaccard coefficients have been selected for comparison in the analysis of the RAPD data, based on their popularity in the literature. The two coefficients are that of Nei and Li (1979), which doubles the value of shared presence in both the numerator and denominator of the distance equation, and that of Sokal and Sneath (1963), which doubles the value of mismatches.

The degree of difference between the neighbour-joining tree produced by using a particular coefficient of genetic distance and that of a random tree can be tested using the Permutation Tail Probability (PTP) analysis (Faith and Cranston, 1991). The PTP test generates a value based on the number of standard deviations difference in tree length between the tree produced from the data set and the mean tree length generated from a population of random trees. From the PTP test it can be determined if the data are highly structured and contains phylogenetic information, or whether the data are essentially randomized and the resulting tree is of little phylogenetic significance.

Objectives and Organization

The objectives of this study were to investigate through molecular means the possibility that the members of the *gregaria* infragroup of *Nebria* have an evolutionary radiation tied to the existence of a QCIs refugium during the last ice age. The background history of the QCI refugia question, *Nebria* taxonomy, and molecular methods are reviewed in Chapter 1. Three approaches were used in this study; the first (Chapter 2) evaluated the degree to which each species is morphologically distinct by using morphometric methods to re-examine the characters that delineate the species; the second (Chapter 3) involved sequencing regions of the mtDNA of various relevant *Nebria* species and using the data to construct a phylogeny of their radiation, using genetic distances to date speciation events; the third (Chapter 4) assessed the degree of genetic relatedness between the three Queen Charlotte *Nebria* species within the *gregaria* infragroup through random amplified polymorphic DNA fingerprints, in order to determine if the three species show a degree of nuclear DNA divergence in line with a period of isolation within a glacial refugium. In Chapter 5, the implications of the genetic evidence to both the taxonomy of the *Nebria* and the glacial history of the QCIs are discussed.

CHAPTER 2: Morphology and the Delineation of Species

Introduction

The species *Nebria gregaria*, the nominal species of the *gregaria* infragroup, was first described by Fischer in 1821 from two specimens collected on the Aleutian island of Unalaska (Lindroth, 1961). In 1961, Lindroth, in his monumental review of the Carabidae of Canada and Alaska, first described *N. charlottae* from a single male specimen collected at an unknown locality on the Queen Charlotte Islands. Lindroth described the beetle as being closely related to *N. gregaria*, agreeing in wing reduction and general form of the penis, but differing somewhat in size, colour, and prothoracic shape. The pronotal hind angles of *N. charlottae* were described as being produced backwards, and the front angles as triangularly protruding with respect to *N. gregaria*. Lindroth placed both species within his *gregaria* group, along with six other species including *N. sahlbergii.* The *gregaria* group, as first defined by Lindroth, included those *Nebria* without prothoracic marginal seta, with a well-defined reflexion of the sides of the prothorax, and with right or acute prothoracic hind-angles.

Kavanaugh's 1978 revision of the Nearctic *Nebria* subdivided Lindroth's *gregaria* group and created the *gregaria* subgroup (=infragroup) to hold *N. charlottae* and *N. gregaria*, but did not expand further on this species group because of a lack of specimens at the time of the revision. Collecting expeditions to Alaska and the Queen Charlotte Islands furnished the material to allow Kavanaugh to describe *N. lituyae*

(Kavanaugh, 1981), and *N. haida* and *N. louisea* (Kavanaugh, 1984). Published descriptions of the three newly described beetles were minimal, to fulfil the requirements of the International Code of Zoological Nomenclature, while awaiting a pending revision of the Nearctic members of the genus. As a consequence, descriptions of the male genitalia, generally considered the basis upon which beetle species are separated, were not included with the species descriptions. However, as noted by Lindroth (1961) the male genitalia of *Nebria* are very simple and lack chitinised structures, reducing their usefulness and implying that few characters could be derived from them. In Kavanaugh's original (1978) manuscript, he mentions that characters of the female genitalia are valuable for separating the members of the *gregaria* group. No females of *N. charlottae* were known at the time, and the usefulness of these characters for the *gregaria* infragroup cannot be evaluated from this statement.

Of the other characters used by Kavanaugh to delineate the species of the *gregaria* infragroup, the shape of the pronotum, elytral silhouette, and leg length are the most important (Kavanaugh, 1992). *N. charlottae* is described as having a short pronotum, with the basal sinuation of the lateral margin short but deep, an elytral silhouette that is relatively short and broad, and comparatively short legs. *N. haida* and *N. louisea* both differ in having a longer, slender pronotum, a basal sinuation of the lateral margin that is longer and only moderately deep, a longer slender elytral silhouette, and longer legs. *N. haida* is mentioned as having short apical angles and a narrower elytral silhouette, with a somewhat lighter colour to the legs than *N. louisea*. *N. gregaria* is described in Lindroth (1961) as having the hind-angles of the prothorax less produced

than *N. charlottae* and reddish brown tibia. The diagnostic combination of *N. lituyae* includes a broad lateral explanation of the pronotum, rectangular hind-angles, and a basal sinuation of moderate length and depth (Kavanaugh, 1981).

The original analysis by Kavanaugh (1978) of the genus *Nebria* used 171 characters, each consisting of two or more states, ranging from discrete presence/ absence to more ambiguous judgements of curvature or size (e.g., very slightly arcuate/ moderately arcuate/ markedly arcuate, etc.). Cladistic methods were then used to judge the relationship between beetles based on shared common character states. Few measurements were explicitly incorporated in the diagnoses of each species, and no apparent attempt was made to determine if character states from different but related species would grade into each other at some point in their range.

By applying a morphometric approach to the evaluation of those characters that delineate members of the *gregaria* infragroup, the importance of each character can be evaluated and the hypothesis that each species is morphologically distinct can be empirically tested. Discriminant analysis, performed on a set of measurements from members of the five species, should be able to assign each individual to its proper species if each species is discrete and there is sufficient morphological differentiation. The weight assigned to each of the functions of the discriminant analysis will then indicate critical characters for distinguishing species. An inability of the analysis to assign individuals to species could reflect a lack of discrete morphological boundaries among these species, calling into question the current species designation, or it could be an

indication of poor character choice. To minimize the possibility of the latter situation, characters for morphometric analysis are chosen from the keys and descriptions published by Kavanaugh, to include those that seem most useful in separating species. However, the lack of information on the beetles' genitalia and the sparse descriptions that exist in the literature for the two Alaska species prevent complete confidence that the characters chosen are the best possible.

Materials and Methods

The beetles used for the morphometric analysis were selected from samples collected into ethanol from the Queen Charlotte Islands, from specimens stored in the collection of the Royal British Columbia Museum (R.B.C.M.) lent by R. Cannings, and from specimens on loan from the California Academy of Sciences lent by Dr. D. Kavanaugh. Beetles preserved in ethanol were removed from the liquid, pinned, positioned, and dried before measurements were taken. The *gregaria* infragroup specimens consisted of 15 individuals (5M, 10F) from Toe Hill, 26 (16M, 10F) from Kaisun village site, five (2M, 3F) from Skedans Island, 24 (12M, 12F) from Reef Island, 20 (10M, 10F) from Lyell Island, one (1M) from Skedans village site, Louise Island, 26 (13M, 13F) from mountains on Graham Island, and 19 (9M, 10F) from mountains on Moresby Island. Twenty specimens (10M, 10F) each of *N. lituyae* and *N. gregaria* were also included in the analysis. The outgroup consisted of 15 (7M, 8F) specimens of *N. sahlbergii sahlbergii*, obtained from various sites around British Columbia. Maps indicating the geographic locations of the collection sites are given in Figure 1.

For each beetle, measurements were taken of the midline pronotal length, midline elytra length, pronotal shoulder length (apex of left shoulder to basal left corner), head width (measured from between the supra-orbital setae), right hind tibial length, and the length of the basal four antennal segments (Figure 2). Measurements were performed using an optical micrometer at 6X magnification for the elytra, 12X magnification for the tibia, and 25X magnification for all other dimensions, and multiplied by 0.134, 0.200,

and 0.0319 respectively to obtain the actual lengths in millimetres.

Shape analysis of the pronotum consisted of producing line drawings of the pronotal shape as viewed from above using the aid of a Camera Lucida. All drawings except for the six specimens of *N. sahlbergii sahlbergii* obtained from the R.B.C.M. were performed using a 10X ocular lens combined with a 25X magnification. The remaining drawings were done with 5X ocular lens and a 50X magnification. Measurements of the pronotal width were performed on the drawings using the Autocad program and a drawing tablet. Measurements across the pronotum were taken at intervals of one-tenth the maximum length of the pronotum (measured from the basal left corner to the apex of the left pronotal shoulder). Due to difficulties in accurately measuring the width of the pronotum between the apex of each shoulder, a further width measurement was taken at a distance of one-twentieth of the maximum length from the apex of the pronotal shoulder. Pronotal dimensions were converted to millimetres by multiplying by 0.0180 for the R.B.C.M. specimens, and by 0.0349 for all others. Measurements across the pronotum were further converted into percentages of the maximum pronotal width (taken as the value of the width measurement at four-tenths the maximum length from the apex). This conversion isolated the effects of changing pronotal shape from the differences in overall body size that existed between beetles, breaking up the high degree of correlation that existed between the unconverted measurements. For convenience, pronotal width ratios are referred to as M0, M1, M2, M3...M10. where M1 represents the width measurements taken at one tenth of the maximum pronotal length from the apex divided by the maximum width. M0.5 refers to the ratio calculated from the extra measurement taken

between M0 and M1.

Errors in measurements were assessed by performing replicate body measurements and pronotal drawings for twenty of the specimens and calculating both the real and the absolute difference for each measurement between the original and the replicate. Mean and standard deviations of the error were calculated from the absolute differences, from which a mean percentage error for each variable could be obtained. The skew towards over or under estimation of the measurements was assessed using the real difference between measurements. Variables with an excessive margin of error or a highly skewed distribution were dropped from further analysis (see Table 1).

Morphometric analysis of the data was performed using the SPSS version 6.0 statistical package. For the purpose of the analysis, Graham Island and Moresby Island specimens of *N. haida* were grouped into a single population Specimens of *N. louisea* were either grouped into a single population or divided into east Moresby and west Moresby populations. Principle components analysis were conducted using samples for each sex and a combined data set. An orthogonal rotation using the varimax rotational program was performed on those factors extracted from the principle components analysis having eigenvalues greater then one.

Separate sets of discriminant analysis were performed for each sex, and for each of two combinations of species (*N. sahlbergii* + *gregaria* infragroup and *gregaria* infragroup alone). Within each set, an analysis was conducted using: pronotal mid-line

length, unadjusted tibia, elytra, and pronotal shoulder lengths, and pronotal width ratios; size-standardized tibia, elytra, and pronotal shoulder lengths and pronotal width ratios; and pronotal width ratios alone. Size standardization was performed using the pronotal mid-line as the measure of body-size and the formula. Two further discriminant analyses were performed on male and female data sets within which *N. louisea* had been partitioned into east and west Moresby populations using body and pronotal width ratios.

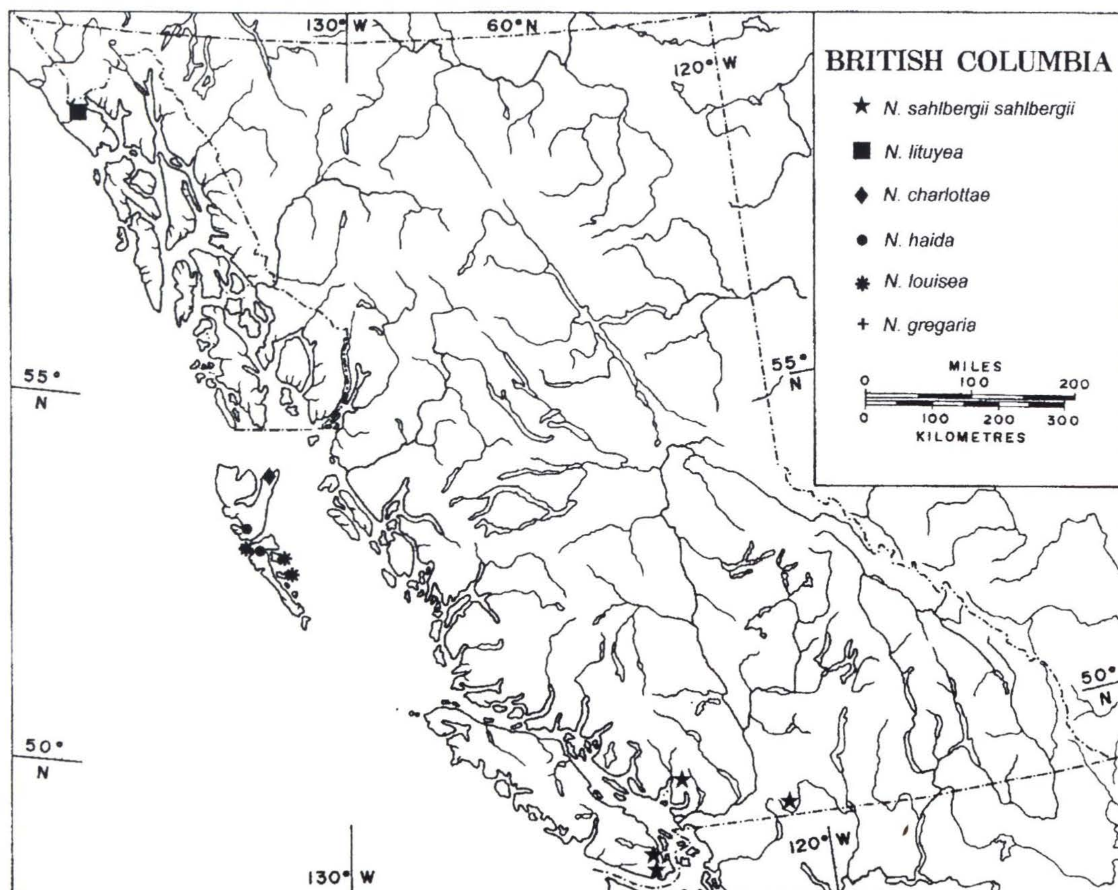


Figure 1a: *Nebria* collection localities for British Columbia

Figure 1b: *Nebria* collection localities for Alaska and the Aleutian Islands.

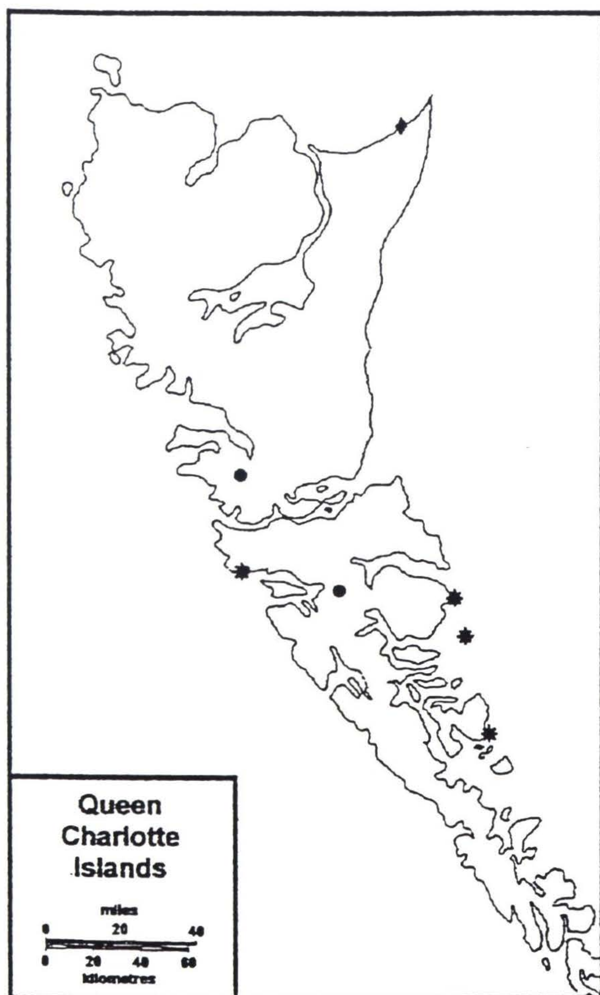
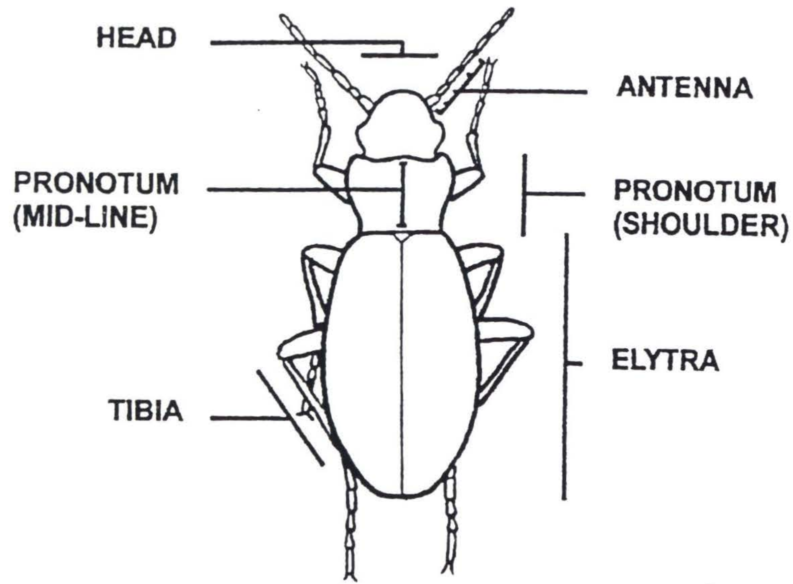


Figure 1c: *Nebria* collection localities for the Queen Charlotte Islands



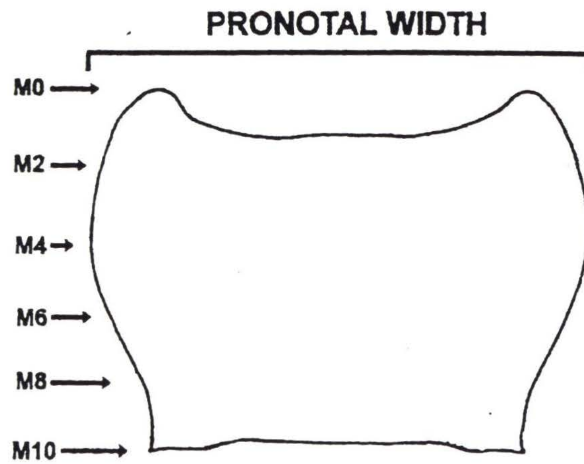


Figure 2: Locations of morphometric measurements.

Table 1: Percentage measurement error. Variables in italics were dropped from the analysis due to a high error rate.

Variable	Percent mean error	Standard Deviation	Skew
Pronotum mid-line	0.47	0.74	0.95
M4	0.99	0.76	0.80
M2	0.99	0.72	0.57
M1	1.02	0.78	0.73
M3	1.11	0.75	0.38
M5	1.17	0.90	1.40
M9	1.18	1.14	1.15
M0	1.21	1.06	1.04
Tibia	1.33	1.60	1.47
M6	1.34	1.01	1.58
M0.5	1.45	1.12	0.37
Elytra	1.54	1.20	0.23
Pronotal shoulder	1.58	1.18	0.78
M10	1.60	1.06	0.26
M7	1.60	1.17	0.54
M8	1.82	1.31	0.83
Antenna 3	2.07	2.09	0.20
Antenna 1	2.30	2.97	0.59
Antenna 4	2.56	3.81	1.55
Head	2.57	4.31	3.55
Antenna 2	4.09	5.38	1.06

Results

The initial survey of the ranges of measurements for each species indicated a large variance within and a high degree of overlap between the populations (Figure 3). No single measurement is capable of discriminating between the members of the *gregaria* infragroup and only one species, *N. sahlbergii sahlbergii*, was distinguished.

Principle components analysis using female and combined data sets extracted four factors with eigenvalues greater then one, accounting for 78.3% and 77.7% of the variance respectively (Table 2). The principle components analysis of the male specimens extracted three factors accounting for 71.9% of the variance. In all three analyses the first factor loaded on the pronotal width ratios above the middle (M0 to M3). In the analysis of the female and combined data sets, the second factor loaded on the body measurements, the third factor loaded on the pronotal width ratios just below the middle (M5 to M7) while the last factor loaded on the pronotal base (M8 to M10). In contrast, the second factor in the analysis of the male data set loaded heavily on all of the lower pronotal width ratios (M5 to M10) while the third factor loaded heavily on the body measurements with a small negative contribution from the pronotal base (M9 and M10).

Tables 5 through 28 indicate the discriminant functions, variation accounted by each function, mean and standard deviations of each population for each function, and classification success for each of the twelve discriminant analyses. Overall,

classification success was high for analyses using body length measurements and only the analyses that used pronotal width ratios alone achieved a consistently low level of success (Table 30). The species *N. sahlbergii* was the most clearly defined of the six species, having a consistent 100% classification rate for those analyses that used body measurements and having a mean and standard deviation for the discriminant functions that clearly placed this species as separate from the *gregaria* infragroup (Tables 5,7,9,11; Fig 4a, 4b). High rates of successful classification were also obtained for *N. lituyae*, *N. charlottae*, and *N. gregaria*, although the success rate for *N. gregaria* was somewhat lower in the presence of *N. sahlbergii* than when only *gregaria* infragroup species were considered. The species *N. louisea* and *N. haida* had an overall lower success rate for the classification of their component specimens. For males, misclassification of individuals of these species were generally exchanges between the two taxa, while misclassified females of these species were often assigned to the species *N. lituyae* or *N. gregaria*.

An examination of the pattern of variable loadings on the discriminant functions indicates sex differences in the important variables used to differentiate the species (Table 31). For the females, the pronotal width ratios M0, M2, M6, and M10 consistently appear with discriminant loading values greater then 0.700 in the first or second discriminant function. Of the body-size measurements, only the elytral length demonstrates any significant importance, occurring in the first or second function for three of the four relevant analyses. In contrast, for the males strong emphasis is placed on the pronotal shoulder length, elytral length and M6 in those analyses using body-size measurements, and the variables M0, M1, M9, and M10 in those analyses that used only

pronotal width ratios. The presence of *N. sahlbergii* in the analyses placed greater emphasis on pronotal mid-line length for females but had little effect on the pattern of discriminant loadings for males. Partitioning *N. louisea* into eastern and western populations did not significantly change the results of the discriminant analysis for the females (Tables 32, 33; Fig 5a) but resulted in high levels of misclassification between the eastern *N. louisea* population and the species *N. haida* (Tables 34, 35; Fig 5b).

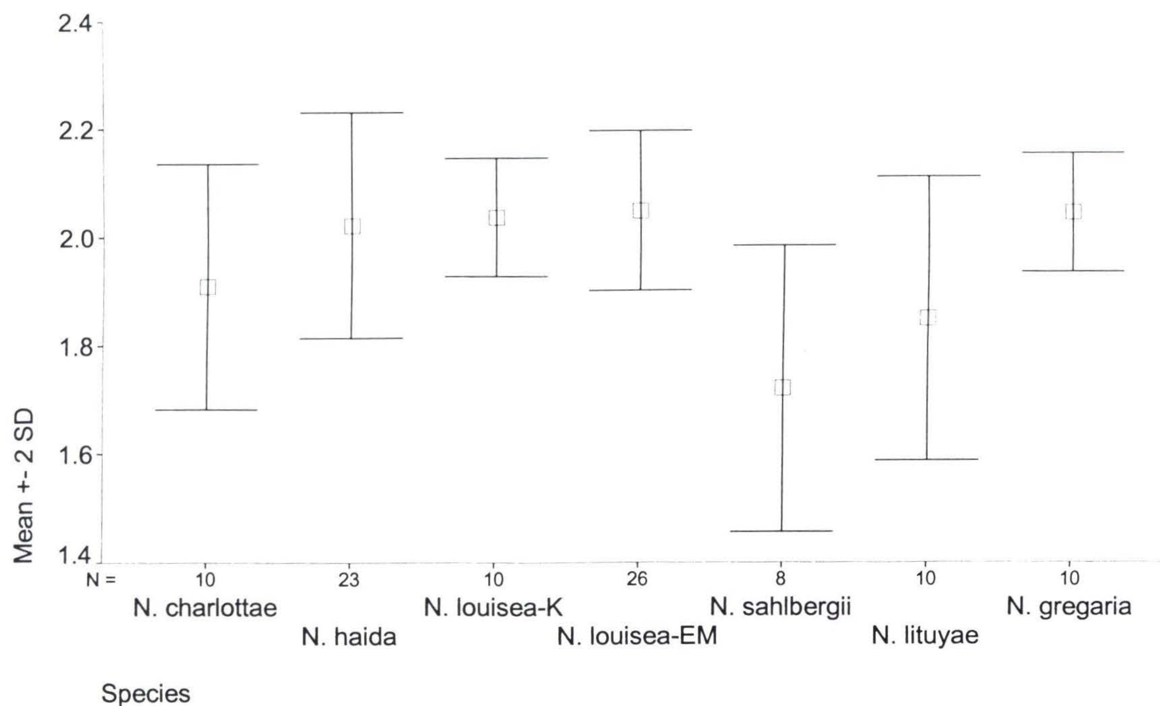


Figure 3a: Pronotal mid-line length, mean and two standard deviations, females.

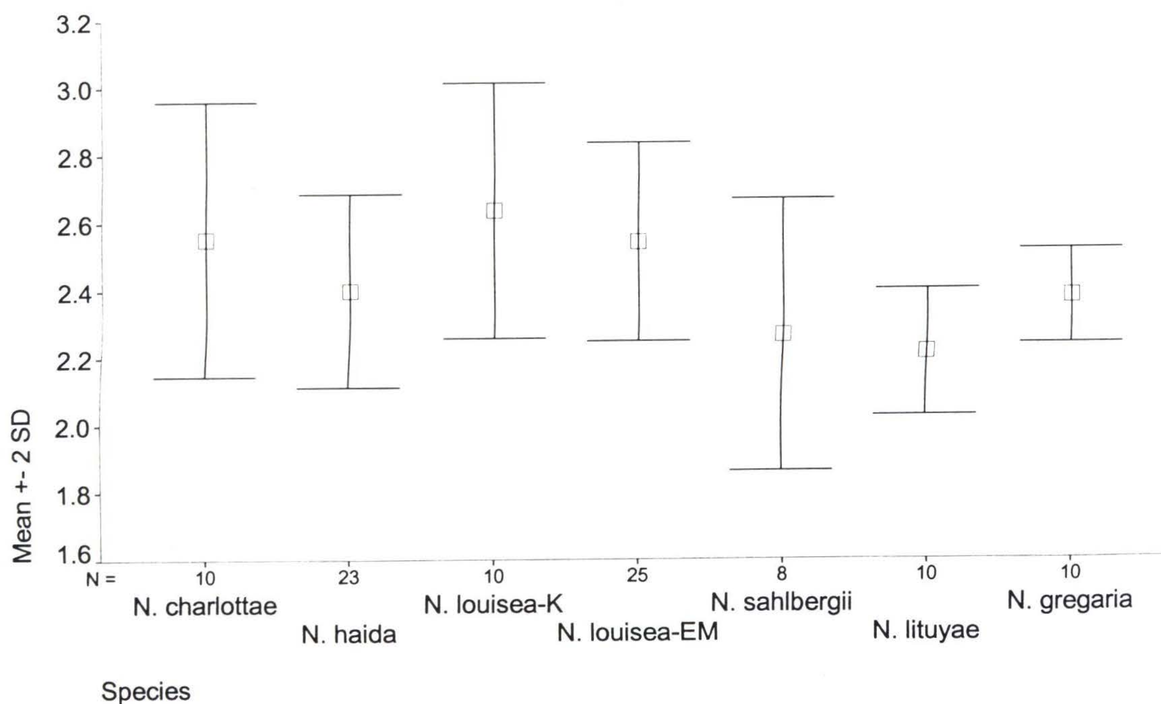


Figure 3b: Pronotal shoulder length, mean and two standard deviations, females.

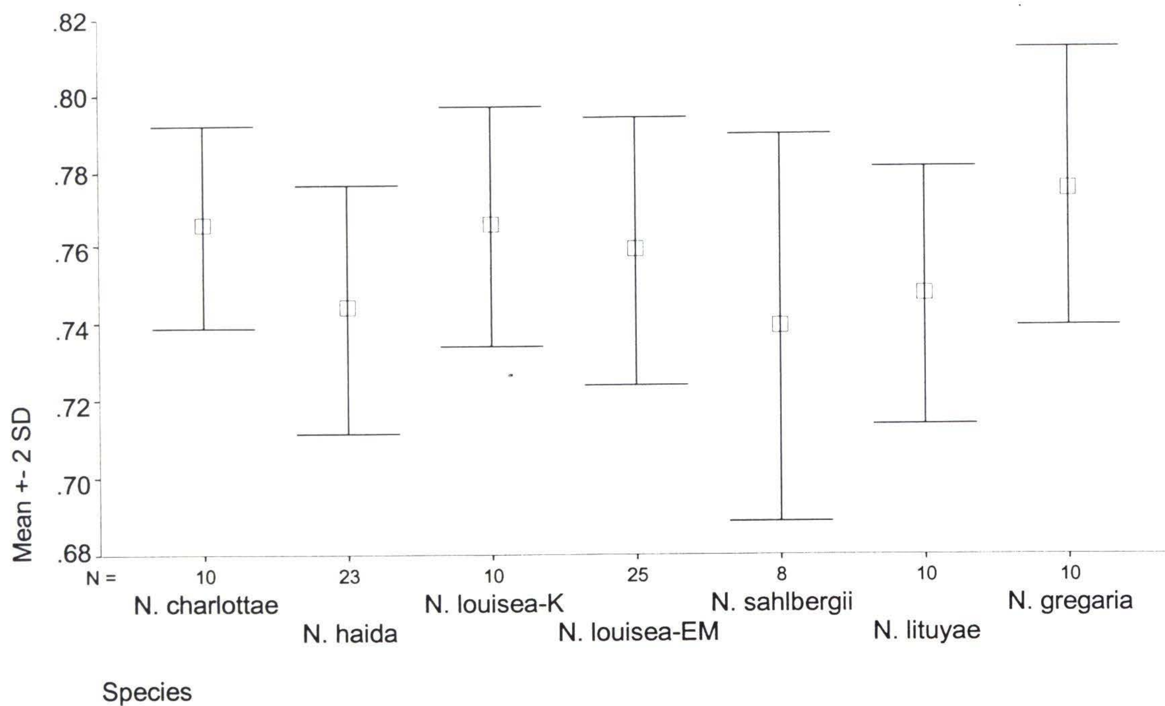


Figure 3c: Pronotal width ratio M0, mean and two standard deviations, females.

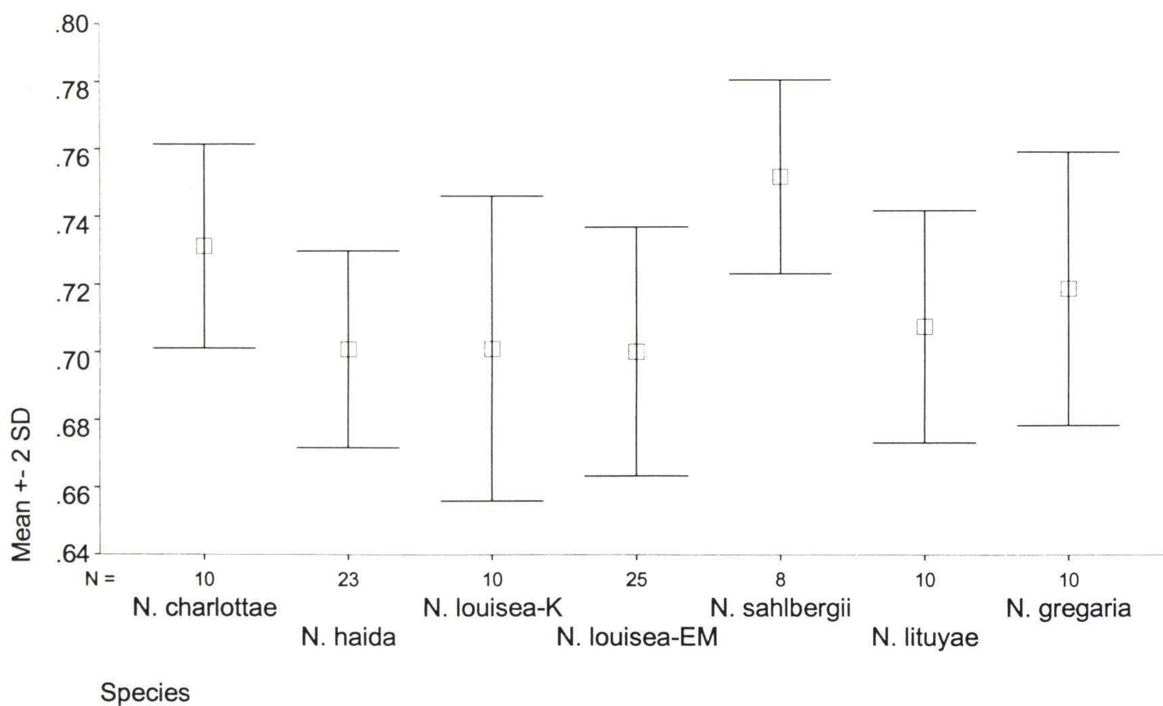


Figure 3d: Pronotal width ratio M10, mean and two standard deviations, females.

Table 2a: Rotated principle components factor matrix of male specimens.

Variable	Factor 1	Factor 2	Factor 3
Pronotum (mid)	-0.068	0.001	0.906
Elytra	0.016	-0.046	0.870
Tibia	-0.052	0.049	0.818
Pronotum (shoulder)	0.122	-0.119	0.700
M0	0.759	-0.029	0.235
M0.5	0.891	-0.164	-0.044
M1	0.924	-0.118	-0.107
M2	0.880	-0.073	-0.033
M3	0.696	-0.066	-0.046
M5	-0.478	0.591	0.026
M6	-0.386	0.792	0.026
M7	-0.193	0.911	0.057
M8	0.032	0.861	-0.089
M9	0.322	0.677	-0.450
M10	0.489	0.444	-0.479

Table 2b: Percentage of variation explained by each function

Factor	Eigenvalue	Pct. of Variation	Cumulative Percentage
1	4.646	31.0%	31.0%
2	3.799	25.3%	56.3%
3	2.347	15.6%	71.9%

Table 3a: Rotated principle components factor matrix of female specimens.

Variable	Factor 1	Factor 2	Factor 3	Factor 4
Pronotum (mid)	0.049	0.868	-0.014	-0.239
Elytra	0.123	0.896	-0.024	-0.133
Tibia	-0.005	0.810	-0.324	0.099
Pronotum (shoulder)	0.040	0.615	0.236	-0.368
M0	0.842	0.052	0.034	-0.201
M0.5	0.931	0.012	-0.133	0.016
M1	0.940	-0.025	-0.155	0.121
M2	0.833	0.071	-0.247	0.272
M3	0.743	0.170	-0.297	0.201
M5	-0.234	-0.050	0.817	0.008
M6	-0.158	-0.064	0.905	0.212
M7	-0.174	-0.008	0.729	0.519
M8	-0.108	-0.080	0.376	0.796
M9	0.186	-0.198	0.099	0.867
M10	0.314	-0.269	0.045	0.625

Table 3b: Percentage of variation explained by each function

Factor	Eigenvalue	Pct. of Variation	Cumulative Percentage
1	4.724	31.5%	31.5%
2	3.690	24.6%	56.1%
3	2.129	14.2%	70.3%
4	1.197	8.0%	78.3%

Table 4a: Rotated principle components factor matrix for combined data set.

Variable	Factor 1	Factor 2	Factor 3	Factor 4
Pronotum (mid)	0.016	0.893	-0.021	-0.197
Elytra	0.054	0.907	-0.081	-0.096
Tibia	-0.004	0.822	-0.083	-0.034
Pronotum (shoulder)	0.019	0.741	-0.012	-0.151
M0	0.735	0.144	-0.162	0.137
M0.5	0.875	0.016	-0.255	0.146
M1	0.911	-0.029	-0.209	0.160
M2	0.843	0.053	-0.223	0.123
M3	0.615	-0.088	0.126	-0.234
M5	-0.236	-0.101	0.821	-0.054
M6	-0.190	-0.079	0.909	0.122
M7	-0.148	0.010	0.832	0.379
M8	-0.057	-0.073	0.567	0.643
M9	0.112	-0.248	0.216	0.882
M10	0.234	-0.243	0.025	0.811

Table 4b: Percentage of variation explained by each function

Factor	Eigenvalue	Pct. of Variation	Cumulative Percentage
1	4.467	29.8%	29.8%
2	3.701	24.7%	54.4%
3	2.229	14.9%	69.3%
4	1.032	6.9%	76.2%

Table 5a: Standardized discriminant function coefficients for females of all six species using pronotal ratios and unadjusted body measurements

Variable	Function 1	Function 2	Function 3	Function 4
Pronotum (mid)	**-0.849**	-0.005	-0.554	0.683
Pronotum (shoulder)	0.436	0.378	**-0.860**	-0.474
Elytra	-0.587	0.398	**1.171**	0.134
Tibia	0.431	-0.665	0.251	-0.559
M0	**-1.260**	0.183	-0.179	-0.292
M0.5	0.664	0.250	-0.098	-0.133
M1	0.515	-0.250	0.698	-0.014
M2	-0.138	**0.833**	-0.316	**0.737**
M3	0.025	-0.387	0.084	-0.162
M5	-0.498	0.076	0.288	-0.484
M6	**0.827**	0.271	-0.386	**1.242**
M7	-0.054	-0.415	0.293	-0.045
M8	-0.174	-0.579	-0.209	-0.470
M9	-0.101	-0.468	-0.066	0.496
M10	**0.881**	**0.805**	0.115	-0.277

Table 5b: Percentage of variation explained by each function

Function	Eigenvalue	Pct. of variation	Cumulative Pct.
1	4.7892	48.99	48.99
2	2.6055	26.65	75.64
3	1.4457	14.79	90.43
4	0.6796	6.95	97.39

Table 5c: Discriminant functions evaluated at group means.

Species	Function 1		Function 2		Function 3		Function 4	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
N. charlottae	2.040	1.031	3.044	1.235	-1.613	1.265	0.9206	0.724
N. haida	-0.539	1.017	-1.729	1.037	-0.188	0.868	1.022	1.024
N. louisea	-1.552	0.841	0.317	0.976	-0.508	1.067	-0.729	0.933
N. sahlbergii	5.872	1.394	-0.701	0.824	0.587	0.806	-0.786	1.549
N. lituyae	0.881	0.976	-1.515	0.923	0.559	1.026	-0.502	0.905
N. gregaria	-1.056	1.145	2.110	0.923	3.107	0.810	0.458	0.965

Table 6: Classification results

Actual group	No. of cases	*Nebria charlottae*	*Nebria haida*	*Nebria louisea*	*Nebria sahlbergii*	*Nebria lituyae*	*Nebria gregaria*
N. charlottae	10	9	0	0	0	1	0
		90.0%	0%	0%	0%	10.0%	0%
N. haida	23	0	20	1	0	2	0
		0%	87.0%	4.3%	0%	8.7%	0%
N. louisea	35	0	0	33	0	1	1
		0%	0%	94.3%	0%	2.9%	2.9%
N. sahlbergii	8	0	0	0	8	0	0
		0%	0%	0%	100.0%	0%	0%
N. lituyae	10	0	1	0	0	9	0
		0%	10.0%	0%	0%	90.0%	0%
N. gregaria	9	0	0	0	0	0	9
		0%	0%	0%	0%	0%	100.0%

Percent of cases correctly classified: 92.63%

Table 7a: Standardized discriminant function coefficients for males of all six species using pronotal ratios and unadjusted body measurements

Variable	Function 1	Function 2	Function 3	Function 4
Pronotum (mid)	0.651	-0.468	-0.159	0.623
Pronotum (shoulder)	-0.108	**-1.067**	0.186	-0.204
Elytra	0.394	**1.021**	0.551	-0.081
Tibia	-0.144	0.399	-0.372	-0.678
M0	0.374	-0.207	0.083	-0.429
M0.5	0.316	0.150	0.023	-0.124
M1	-0.431	-0.198	0.142	0.070
M2	-0.086	0.040	0.512	0.231
M3	0.146	0.144	-0.202	0.083
M5	0.132	0.278	0.359	-0.116
M6	-0.367	**-0.849**	-0.060	0.439
M7	0.227	0.669	-0.094	0.481
M8	0.137	-0.043	-0.133	-0.032
M9	-0.270	-0.157	**-0.799**	**-1.138**
M10	-0.460	0.339	**1.038**	**0.901**

Table 7b: Percentage of variation explained by each function

Function	Eigenvalue	Pct. of variation	Cumulative Pct.
1	3.2946	43.33	43.33
2	2.6237	34.51	77.84
3	1.0665	14.03	91.87
4	0.5158	6.78	98.65

Table 7c: Discriminant functions evaluated at group means.

Species	Function 1		Function 2		Function 3		Function 4	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
N. charlottae	-1.388	0.965	-3.574	0.816	2.190	0.402	1.712	1.030
N. haida	0.612	1.111	0.027	0.913	-1.340	1.120	0.587	1.266
N. louisea	0.785	1.021	-0.870	1.085	0.113	1.093	-0.592	0.982
N. sahlbergii	-5.709	1.072	0.543	1.161	-0.014	1.032	-0.844	0.767
N. lituyae	-1.411	0.798	1.570	0.737	-0.452	0.880	0.591	0.810
N. gregaria	1.322	0.803	3.142	1.020	1.615	0.477	0.133	0.650

Table 8: Classification results

Actual group	No. of cases	*Nebria charlottae*	*Nebria haida*	*Nebria louisea*	*Nebria sahlbergii*	*Nebria lituyae*	*Nebria gregaria*
N. charlottae	5	5	0	0	0	0	0
		100.0%	0%	0%	0%	0%	0%
N. haida	20	0	15	4	0	1	0
		0%	75.0%	20.0%	0%	5.0%	0%
N. louisea	38	0	5	31	0	1	1
		0	13.2%	81.6%	0%	2.6%	2.6%
N. sahlbergii	6	0	0	0	6	0	0
		0%	0%	0%	100.0%	0%	0%
N. lituyae	10	0	0	0	0	10	0
		0%	0%	0%	0%	100.0%	0%
N. gregaria	10	0	0	0	0	0	10
		0%	0%	0%	0%	0%	100.0%

Percent of cases correctly classified: 86.52%

Table 9a: Standardized discriminant function coefficients for females of all six species using pronotal ratios and size-adjusted body measurements

Variable	Function 1	Function 2	Function 3	Function 4
Pronotum (shoulder)	0.486	0.421	-0.610	0.416
Elytra	**-0.935**	0.145	0.593	-0.171
Tibia	0.547	-0.503	0.419	0.572
M0	**-1.215**	0.056	-0.403	0.216
M0.5	0.628	0.321	0.028	0.186
M1	0.375	-0.263	**0.726**	0.054
M2	0.006	**0.888**	-0.025	-0.647
M3	-0.138	-0.431	-0.175	0.044
M5	-0.590	-0.015	0.116	0.453
M6	**1.027**	0.446	0.047	**-1.166**
M7	-0.170	-0.486	0.143	0.049
M8	0.017	-0.555	-0.220	0.454
M9	-0.098	-0.465	-0.148	-0.558
M10	**0.826**	**0.891**	0.395	0.397

Table 9b: Percentage of variation explained by each function

Function	Eigenvalue	Pct. of variation	Cumulative Pct.
1	3.8523	45.49	45.49
2	2.6061	30.78	76.27
3	1.2086	14.27	90.54
4	0.6689	7.90	98.44

Table 9c: Discriminant functions evaluated at group means.

Species	Function 1		Function 2		Function 3		Function 4	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
N. charlottae	1.603	0.790	3.394	1.314	-1.202	0.931	-0.962	0.720
N. haida	-0.017	0.949	-1.671	1.065	-0.125	0.856	-1.010	1.040
N. louisea	-1.301	0.964	0.205	0.962	-0.570	1.176	0.713	0.928
N. sahlbergii	5.283	1.123	-0.213	0.863	1.018	0.992	0.956	1.573
N. lituyae	0.501	0.909	-1.588	0.864	0.073	0.907	0.310	0.874
N. gregaria	-1.931	1.401	1.656	0.798	2.885	0.685	-0.317	0.943

Table 10: Classification results

Actual group	No. of cases	*Nebria charlottae*	*Nebria haida*	*Nebria louisea*	*Nebria sahlbergii*	*Nebria lituyae*	*Nebria gregaria*
N. charlottae	10	9	0	0	0	1	0
		90.0%	0%	0%	0%	10.0%	0%
N. haida	23	0	20	1	0	2	0
		0%	87.0%	4.3%	0%	8.7%	0%
N. louisea	35	1	0	29	0	3	2
		2.9%	0%	82.9%	0%	8.6%	5.7%
N. sahlbergii	8	0	0	0	8	0	0
		0%	0%	0%	100.0%	0%	0%
N. lituyae	10	0	2	0	0	8	0
		0%	20.0%	0%	0%	80.0%	0%
N. gregaria	9	0	0	0	0	0	9
		0%	0%	0%	0%	0%	100.0%

Percent of cases correctly classified: 87.37%

Table 11a: Standardized discriminant function coefficients for males of all six species using pronotal ratios and size-adjusted body measurements

Variable	Function 1	Function 2	Function 3	Function 4
Pronotum (shoulder)	**-0.772**	0.573	0.091	0.175
Elytra	**0.959**	0.110	0.370	0.012
Tibia	0.093	-0.557	-0.220	0.647
M0	0.185	**0.790**	-0.112	0.377
M0.5	0.315	0.021	0.018	-0.166
M1	-0.540	-0.543	0.305	-0.360
M2	0.012	0.119	0.489	-0.035
M3	0.230	0.101	-0.241	0.151
M5	0.323	0.013	0.341	-0.029
M6	**-0.923**	0.144	-0.029	0.546
M7	0.690	-0.199	-0.087	**-0.932**
M8	0.067	0.222	-0.183	**1.207**
M9	-0.329	-0.309	**-0.766**	**-1.641**
M10	-0.058	-0.481	**1.162**	**1.155**

Table 11b: Percentage of variation explained by each function

Function	Eigenvalue	Pct. of variation	Cumulative Pct.
1	2.7900	44.16	44.16
2	1.8418	29.15	73.31
3	1.0428	16.50	89.81
4	0.5356	8.46	98.27

Table 11c: Discriminant functions evaluated at group means.

Species	Function 1		Function 2		Function 3		Function 4	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
N. charlottae	-3.577	0.759	1.544	0.922	2.136	0.474	-1.690	1.107
N. haida	0.335	1.017	0.045	1.054	-1.323	1.156	-0.614	1.275
N. louisea	-0.174	1.059	0.961	0.843	-0.018	1.059	0.582	0.960
N. sahlbergii	-3.077	0.929	-3.408	1.764	0.340	1.066	0.955	0.838
N. lituyae	0.367	0.921	-1.769	0.754	-0.219	0.929	-0.643	0.794
N. gregaria	3.256	0.920	-0.699	1.130	1.660	0.442	-0.070	0.660

Table 12: Classification results

Actual group	No. of cases	*Nebria charlottae*	*Nebria haida*	*Nebria louisea*	*Nebria sahlbergii*	*Nebria lituyae*	*Nebria gregaria*
N. charlottae	5	5	0	0	0	0	0
		100.0%	0%	0%	0%	0%	0%
N. haida	20	0	14	4	0	2	0
		0%	70.0%	20.0%	0%	10.0%	0%
N. louisea	38	0	4	31	0	2	1
		0%	10.5%	81.6%	0%	5.3%	2.6%
N. sahlbergii	6	0	0	0	6	0	0
		0%	0%	0%	100.0%	0%	0%
N. lituyae	10	0	1	0	0	9	0
		0%	10.0%	0%	0%	90.0%	0%
N. gregaria	10	0	0	0	0	0	10
		0%	0%	0%	0%	0%	100.0%

Percent of cases correctly classified: 84.27%

Table 13a: Standardized discriminant function coefficients for females of all six species using pronotal ratios only.

Variable	Function 1	Function 2
M0	**-0.819**	**-0.974**
M0.5	0.519	-0.021
M1	0.212	0.644
M2	**0.767**	-0.512
M3	-0.469	0.176
M5	-0.268	-0.282
M6	**1.027**	0.220
M7	-0.460	0.236
M8	-0.389	0.346
M9	-0.429	0.633
M10	**1.236**	-0.293

Table 13b: Percentage of variation explained by each function

Function	Eigenvalue	Pct. of variation	Cumulative Pct.
1	2.3565	53.85	53.85
2	1.5228	34.80	88.65

Table 13c: Discriminant functions evaluated at group means.

Species	Function 1		Function 2	
	Mean	SD	Mean	SD
N. charlottae	2.505	0.814	-0.847	0.979
N. haida	-0.911	0.945	1.347	0.957
N. louisea	-0.989	1.115	-0.914	0.890
N. sahlbergii	2.813	1.079	2.014	1.300
N. lituyae	-0.710	0.871	-0.685	1.094
N. gregaria	1.679	0.878	-1.499	1.156

Table 14: Classification results

Actual group	No. of cases	*Nebria charlottae*	*Nebria haida*	*Nebria louisea*	*Nebria sahlbergii*	*Nebria lituyae*	*Nebria gregaria*
N. charlottae	10	8	0	0	0	0	2
		80.0%	0%	0%	0%	0%	20.0%
N. haida	23	0	17	1	0	4	1
		0%	73.9%	4.3%	0%	17.4%	4.3%
N. louisea	35	0	1	27	0	4	3
		0%	2.9%	77.1%	0%	11.4%	8.6%
N. sahlbergii	8	1	0	0	7	0	0
		12.5%	0%	0%	87.5%	0%	0%
N. lituyae	10	0	2	1	0	7	0
		0%	20.0%	10.0%	0%	70.0%	0%
N. gregaria	9	2	0	1	0	0	6
		22.2%	0%	11.1%	0%	0%	66.7%

Percent of cases correctly classified: 75.79%

Table 15a: Standardized discriminant function coefficients for males of all six species using pronotal ratios only.

Variable	Function 1	Function 2
M0	**-0.755**	0.600
M0.5	-0.366	0.212
M1	**0.813**	-0.200
M2	0.301	0.467
M3	-0.276	-0.204
M5	0.165	0.225
M6	0.259	-0.090
M7	-0.336	-0.031
M8	-0.247	-0.164
M9	0.092	**-0.749**
M10	**1.013**	0.503

Table 15b: Percentage of variation explained by each function

Function	Eigenvalue	Pct. of variation	Cumulative Pct.
1	1.5744	56.20	56.20
2	0.7597	27.12	83.32

Table 15c: Discriminant functions evaluated at group means.

Species	Function 1		Function 2	
	Mean	SD	Mean	SD
N. charlottae	1.549	1.153	1.665	1.228
N. haida	-0.927	1.170	-1.032	1.015
N. louisea	-0.653	0.841	0.449	0.997
N. sahlbergii	3.300	1.170	-0.644	1.256
N. lituyae	1.065	1.099	-0.941	0.817
N. gregaria	0.343	0.903	0.975	0.823

Table 16: Classification results

Actual group	No. of cases	*Nebria charlottae*	*Nebria haida*	*Nebria louisea*	*Nebria sahlbergii*	*Nebria lituyae*	*Nebria gregaria*
N. charlottae	5	5 100.0%	0 0%	0 0%	0 0%	0 0%	0 0%
N. haida	21	0 0%	13 61.9%	4 19.0%	0 0%	4 19.0%	0 0%
N. louisea	39	1 2.6%	7 17.9%	24 61.5%	1 2.6%	2 5.1%	4 10.3%
N. sahlbergii	7	0 0%	0 0%	0 0%	6 85.7%	1 14.3%	0 0%
N. lituyae	10	0 0%	1 10.0%	0 0%	1 10.0%	6 60.0%	2 20.0%
N. gregaria	10	1 10.0%	0 0%	1 10.0%	0 0%	1 10.0%	7 70.0%

Percent of cases correctly classified: 66.30%

Table 17a: Standardized discriminant function coefficients for females of the *gregaria* infragroup using pronotal ratios and unadjusted body measurements

Variable	Function 1	Function 2	Function 3	Function 4
Pronotum (mid)	-0.263	0.115	-0.458	**0.822**
Pronotum (shoulder)	0.489	-0.083	**-0.884**	-0.058
Elytra	-0.124	**0.854**	**0.905**	0.029
Tibia	-0.537	-0.086	0.022	-0.170
M0	-0.662	**0.969**	-0.646	-0.042
M0.5	**0.764**	-0.444	0.111	-0.432
M1	-0.178	-0.132	0.688	-0.068
M2	**0.747**	0.448	-0.216	**1.122**
M3	-0.192	-0.399	0.176	-0.601
M5	-0.310	0.575	-0.005	-0.669
M6	**0.871**	**-0.770**	0.147	**0.981**
M7	-0.214	-0.135	0.310	0.325
M8	-0.655	-0.153	-0.330	-0.491
M9	-0.568	-0.287	0.022	0.192
M10	**1.220**	-0.014	0.223	-0.139

Table 17b: Percentage of variation explained by each function

Function	Eigenvalue	Pct. of variation	Cumulative Pct.
1	3.0573	41.57	41.57
2	2.2602	30.73	72.31
3	1.3753	18.70	91.01
4	0.6613	8.99	100.00

Table 17c: Discriminant functions evaluated at group means.

Species	Function 1		Function 2		Function 3		Function 4	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
N. charlottae	4.443	1.113	-1.011	0.954	-0.592	1.472	0.247	1.068
N. haida	-1.197	1.075	-1.363	1.036	0.352	0.787	0.906	1.030
N. louisea	-0.581	1.031	1.025	0.890	-1.020	1.052	-0.212	0.927
N. lituyae	-0.432	0.847	-1.816	0.856	1.051	1.019	-1.806	1.059
N. gregaria	0.859	0.589	2.637	1.443	2.559	0.484	0.240	1.065

Table 18: Classification results

Actual group	No. of cases	*Nebria charlottae*	*Nebria haida*	*Nebria louisea*	*Nebria lituyae*	*Nebria gregaria*
N. charlottae	10	9 90.0%	0 0%	0 0%	1 10.0%	0 0%
N. haida	23	0 0%	21 91.3%	1 4.3%	1 4.3%	0 0%
N. louisea	35	0 0%	0 0%	33 94.3%	1 2.9%	1 2.9%
N. lituyae	10	0 0%	1 10.0%	0 0%	9 90.0%	0 0%
N. gregaria	9	0 0%	0 0%	0 0%	0 0%	9 100.0%

Percent of cases correctly classified: 93.10%

Table 19a: Standardized discriminant function coefficients for males of the *gregaria* infragroup using pronotal ratios and unadjusted body measurements

Variable	Function 1	Function 2	Function 3
Pronotum (mid)	-0.263	0.037	-0.237
Pronotum (shoulder)	**-1.083**	0.231	-0.049
Elytra	**0.986**	0.549	0.109
Tibia	0.476	-0.091	-0.428
M0	-0.203	0.421	-0.429
M0.5	-0.086	0.108	-0.203
M1	-0.017	-0.068	0.397
M2	-.088	0.319	0.422
M3	0.145	-0.129	-0.172
M5	0.375	0.487	0.011
M6	**-0.909**	-0.297	0.354
M7	0.550	-0.261	0.175
M8	0.067	0.046	-0.323
M9	-0.233	-0.680	-0.560
M10	0.416	0.574	**1.140**

Table 19b: Percentage of variation explained by each function

Function	Eigenvalue	Pct. of variation	Cumulative Pct.
1	2.9654	53.78	53.78
2	1.1893	21.57	75.34
3	1.0690	19.39	94.73

Table 19c: Discriminant functions evaluated at group means.

Species	Function 1		Function 2		Function 3	
	Mean	SD	Mean	SD	Mean	SD
N. charlottae	-3.815	0.845	0.783	0.889	2.810	1.038
N. haida	0.119	0.927	-1.150	1.074	-0.637	1.133
N. louisea	-0.810	1.100	0.503	1.067	-0.558	0.976
N. lituyae	1.358	0.729	-1.639	0.840	1.451	1.132
N. gregaria	3.389	1.012	1.635	0.704	0.538	0.525

Table 20: Classification results

Actual group	No. of cases	*Nebria charlottae*	*Nebria haida*	*Nebria louisea*	*Nebria lituyae*	*Nebria gregaria*
N. charlottae	5	5 100.0%	0 0%	0 0%	0 0%	0 0%
N. haida	20	0 0%	13 65.0%	5 25.0%	2 10.0%	0 0%
N. louisea	38	0 0%	5 13.2%	31 81.6%	1 2.6%	1 2.6%
N. lituyae	10	0 0%	0 0%	0 0%	10 100.0%	0 0%
N. gregaria	10	0 0%	0 0%	0 0%	0 0%	10 100.0%

Percent of cases correctly classified: 83.13%

Table 21a: Standardized discriminant function coefficients for females of the *gregaria* infragroup using pronotal ratios and size-adjusted body measurements

Variable	Function 1	Function 2	Function 3
Pronotum (shoulder)	0.495	-0.305	-0.687
Elytra	-0.123	**0.954**	0.430
Tibia	-0.447	-0.015	0.002
M0	-0.296	**0.873**	**-0.737**
M0.5	0.535	-0.437	0.011
M1	-0.328	0.066	0.555
M2	**1.016**	0.067	0.390
M3	-0.394	-0.196	-0.128
M5	-0.228	0.677	-0.366
M6	**0.794**	**-1.035**	**0.825**
M7	-0.325	0.020	0.263
M8	-0.615	-0.091	-0.468
M9	-0.614	-0.177	0.018
M10	**1.134**	-0.177	0.334

Table 21b: Percentage of variation explained by each function

Function	Eigenvalue	Pct. of variation	Cumulative Pct.
1	2.8798	44.77	44.77
2	2.1282	33.08	77.85
3	1.1419	17.75	95.60

Table 21c: Discriminant functions evaluated at group means.

Species	Function 1		Function 2		Function 3	
	Mean	SD	Mean	SD	Mean	SD
N. charlottae	4.066	1.370	-1.796	0.671	0.024	0.967
N. haida	-1.358	1.030	-1.168	1.024	0.787	0.896
N. louisea	-0.152	1.002	0.828	0.905	-1.083	1.119
N. lituyae	-1.342	0.737	-1.035	0.968	0.101	0.998
N. gregaria	1.034	0.582	2.909	1.524	2.062	0.741

Table 22: Classification results

Actual group	No. of cases	*Nebria charlottae*	*Nebria haida*	*Nebria louisea*	*Nebria lituyae*	*Nebria gregaria*
N. charlottae	10	9	0	0	1	0
		90.0%	0%	0%	10.0%	0%
N. haida	23	0	18	1	4	0
		0%	78.3%	4.3%	17.4%	0%
N. louisea	35	0	0	30	3	2
		0%	0%	85.7%	8.6%	5.7%
N. lituyae	10	0	2	0	8	0
		0%	20.0%	0%	80.0%	0%
N. gregaria	9	0	0	0	0	9
		0%	0%	0%	0%	100.0%

Percent of cases correctly classified: 85.06%

Table 23a: Standardized discriminant function coefficients for males of the *gregaria* infragroup using pronotal ratios and size-adjusted body measurements

Variable	Function 1	Function 2	Function 3
Pronotum (shoulder)	**-0.980**	0.198	0.195
Elytra	**0.819**	0.520	0.197
Tibia	0.446	-0.202	0.297
M0	-0.227	0.306	**0.726**
M0.5	-0.079	0.046	0.271
M1	0.025	0.014	-0.614
M2	0.069	0.433	-0.307
M3	0.135	-0.165	0.162
M5	0.389	0.465	0.100
M6	**-0.916**	-0.189	-0.464
M7	0.536	-0.186	-0.199
M8	0.065	-0.052	0.281
M9	-0.228	**-0.799**	0.498
M10	0.400	**0.868**	**-1.090**

Table 23b: Percentage of variation explained by each function

Function	Eigenvalue	Pct. of variation	Cumulative Pct.
1	2.9470	55.79	55.79
2	1.1740	22.22	78.01
3	0.9214	17.44	95.45

Table 23c: Discriminant functions evaluated at group means.

Species	Function 1		Function 2		Function 3	
	Mean	SD	Mean	SD	Mean	SD
N. charlottae	-3.940	0.861	1.598	0.601	-2.354	1.009
N. haida	0.149	0.934	-1.312	1.090	0.112	1.152
N. louisea	-0.774	1.104	0.310	1.088	0.711	0.921
N. lituyae	1.243	0.716	-1.066	0.864	-1.560	1.200
N. gregaria	3.371	0.987	1.713	0.625	-0.190	0.695

Table 24: Classification results

Actual group	No. of cases	*Nebria charlottae*	*Nebria haida*	*Nebria louisea*	*Nebria lituyae*	*Nebria gregaria*
N. charlottae	5	5 100.0%	0 0%	0 0%	0 0%	0 0%
N. haida	20	0 0%	13 65.0%	5 25.0%	2 10.0%	0 0%
N. louisea	38	0 0%	4 10.5%	31 81.6%	2 5.3%	0 0%
N. lituyae	10	0 0%	1 10.0%	0 0%	9 90.0%	0 0%
N. gregaria	10	0 0%	0 0%	0 0%	0 0%	10 100.0%

Percent of cases correctly classified: 81.93%

Table 25a: Standardized discriminant function coefficients for females of the *gregaria* infragroup using pronotal ratios only

Variable	Function 1	Function 2
M0	-0.243	**-1.139**
M0.5	0.486	0.063
M1	-0.211	**0.726**
M2	**1.033**	0.076
M3	-0.475	-0.069
M5	-0.186	**-0.872**
M6	**0.908**	**1.223**
M7	-0.337	0.345
M8	-0.645	-0.332
M9	**-0.742**	0.321
M10	**1.231**	0.189

Table 25b: Percentage of variation explained by each function

Function	Eigenvalue	Pct. of variation	Cumulative Pct.
1	2.1141	59.46	59.46
2	1.1823	33.25	92.71

Table 25c: Discriminant functions evaluated at group means.

Species	Function 1		Function 2	
	Mean	SD	Mean	SD
N. charlottae	2.815	0.909	1.019	0.802
N. haida	-1.203	0.984	1.313	0.965
N. louisea	-0.346	1.129	-1.148	0.904
N. lituyae	-0.920	0.879	0.179	1.101
N. gregaria	2.314	0.613	-0.222	1.460

Table 26: Classification results

Actual group	No. of cases	*Nebria charlottae*	*Nebria haida*	*Nebria louisea*	*Nebria lituyae*	*Nebria gregaria*
N. charlottae	10	8 80.0%	0 0%	0 0%	0 0%	2 20.0%
N. haida	23	0 0%	17 73.9%	1 4.3%	4 17.4%	1 4.3%
N. louisea	35	0 0%	1 2.9%	27 77.1%	3 8.6%	4 11.4%
N. lituyae	10	0 0%	2 20.0%	1 10.0%	7 70.0%	0 0%
N. gregaria	9	2 22.2%	0 0%	0 0%	0 0%	7 77.8%

Percent of cases correctly classified: 75.86%

Table 27a: Standardized discriminant function coefficients for males of the *gregaria* infragroup using pronotal ratios only

Variable	Function 1	Function 2
M0	-0.056	**-0.998**
M0.5	-0.186	-0.567
M1	0.471	**0.801**
M2	0.531	-0.017
M3	-0.287	0.038
M5	0.313	0.053
M6	0.053	0.012
M7	-0.072	0.298
M8	-0.266	0.044
M9	**-0.811**	-0.164
M10	**1.242**	**0.807**

Table 27b: Percentage of variation explained by each function

Function	Eigenvalue	Pct. of variation	Cumulative Pct.
1	.9584	49.72	49.72
2	.6523	33.84	83.56

Table 27c: Discriminant functions evaluated at group means.

Species	Function 1		Function 2	
	Mean	SD	Mean	SD
N. charlottae	2.560	0.599	0.466	1.636
N. haida	-1.177	1.164	0.403	1.074
N. louisea	-0.032	1.005	-0.726	0.919
N. lituyae	0.108	1.081	1.643	0.741
N. gregaria	1.210	0.536	0.108	1.008

Table 28: Classification results

Actual group	No. of cases	*Nebria charlottae*	*Nebria haida*	*Nebria louisea*	*Nebria lituyae*	*Nebria gregaria*
N. charlottae	5	5 100.0%	0 0%	0 0%	0 0%	0 0%
N. haida	21	0 0%	13 61.9%	4 19.0%	4 19.0%	0 0%
N. louisea	39	2 5.1%	5 12.8%	26 66.7%	2 5.1%	4 10.3%
N. lituyae	10	0 0%	1 10.0%	0 0%	8 80.0%	1 10.0%
N. gregaria	10	1 10.0%	0 0%	1 10.0%	1 10.0%	7 70.0%

Percent of cases correctly classified: 69.41%

Table 29: Summary of classification success for each species over all twelve discriminant analyses. Numbers within grid indicate percentage of successfully classified individuals. Code above columns indicates analysis as follows: F=female; M=male; 5= *gregaria* infragroup; 6= *gregaria* infragroup + *N. sahlbergii*; LBP=pronotal width ratios + unadjusted body measurements; BP=pronotal width ratios + size standardized body measurements; P=pronotal width ratios only.

	F6LBP	F6BP	F6P	F5LBP	F5BP	F5P	M6LBP	M6BP	M6P	M5LBP	M5BP	M5P
N. cha	90	90	80	90	90	80	100	100	100	100	100	100
N. hai	87	87	73.9	91.3	78.3	73.9	75	70	61.9	65	65	61.9
N. lou	94.3	82.9	77.1	94.3	85.7	77.1	81.6	81.6	61.5	81.6	81.6	66.7
N. lit	100	100	87.5	90	80	70	100	100	85.7	100	90	80
N. greg	90	80	70	100	100	77.8	100	90	60	100	100	70
N. sahl	100	100	66.7	n/a	n/a	n/a	100	100	70	n/a	n/a	n/a

Table 30: Summary of variables loading greater then 0.700 for each analysis. Numbers within grid indicate function within analysis. Code above columns indicates analysis as follows: F=female; M=male; 5= *gregaria* infragroup; 6= *gregaria* infragroup + *N. sahlbergii*; LBP=pronotal width ratios + unadjusted body measurements; BP=pronotal width ratios + size standardized body measurements; P=pronotal width ratios only.

	F6LBP	F6BP	F6P	F5LBP	F5BP	F5P	M6LBP	M6BP	M6P	M5LBP	M5BP	M5P
Pro(M)	1	n/a	n/a	4	n/a	n/a		n/a	n/a		n/a	n/a
Pro(S)	3		n/a	3		n/a	2	1	n/a	1	1	n/a
Elytra	3	1	n/a	2,3	2	n/a	2	1	n/a	1	1	n/a
Tibia			n/a			n/a			n/a			n/a
M0	1	1	1,2	2	2,3	2		2	1		3	2
M0.5				1								
M1		3				2			1			2
M2	2,4	2	1	1,4	1	1						
M3												
M5						2						
M6	1,4	1,4	1	1,2,4	1,2,3	1,2	2	1		1	1	
M7								4				
M8								4				
M9						1	3,4	3,4	2		2	1
M10	1,2	1,2	1	1	1	1	3,4	3,4	1	3	2,3	1,2

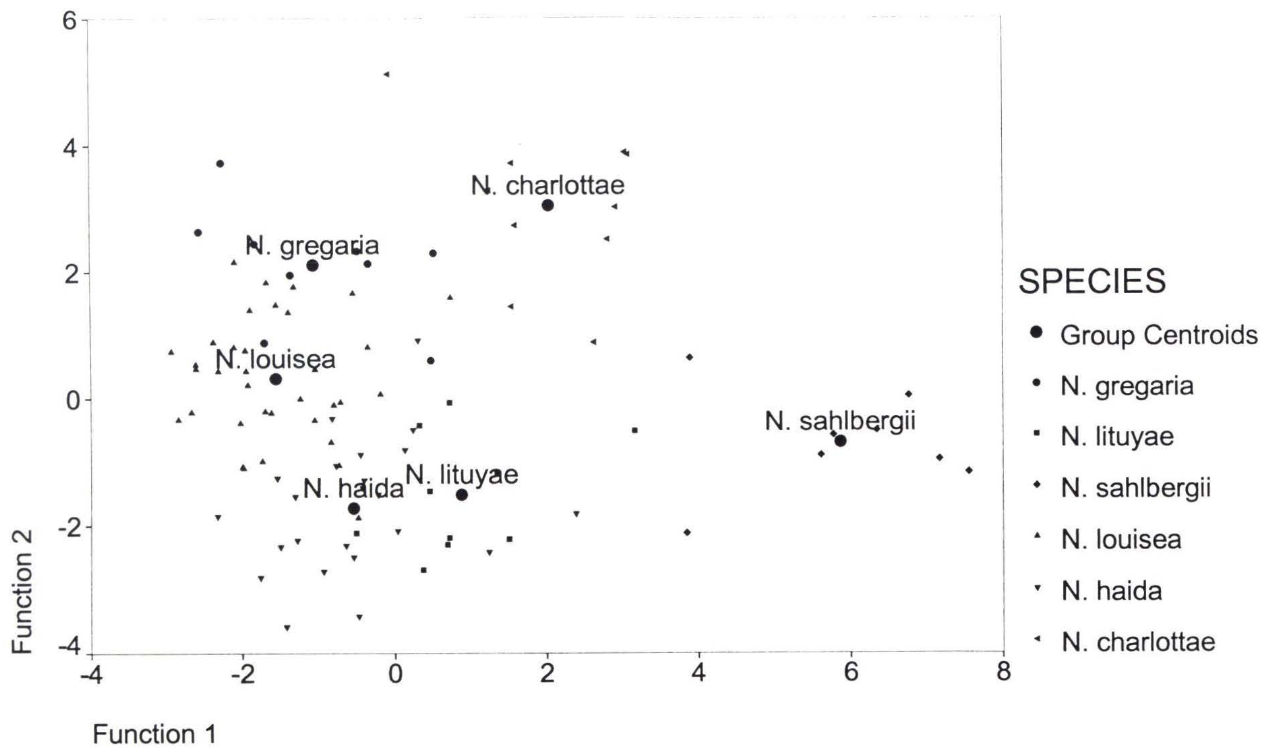


Figure 4a: Plot of discriminant function values and population centroids from analysis of females, using all six species and both pronotal and body measurements.

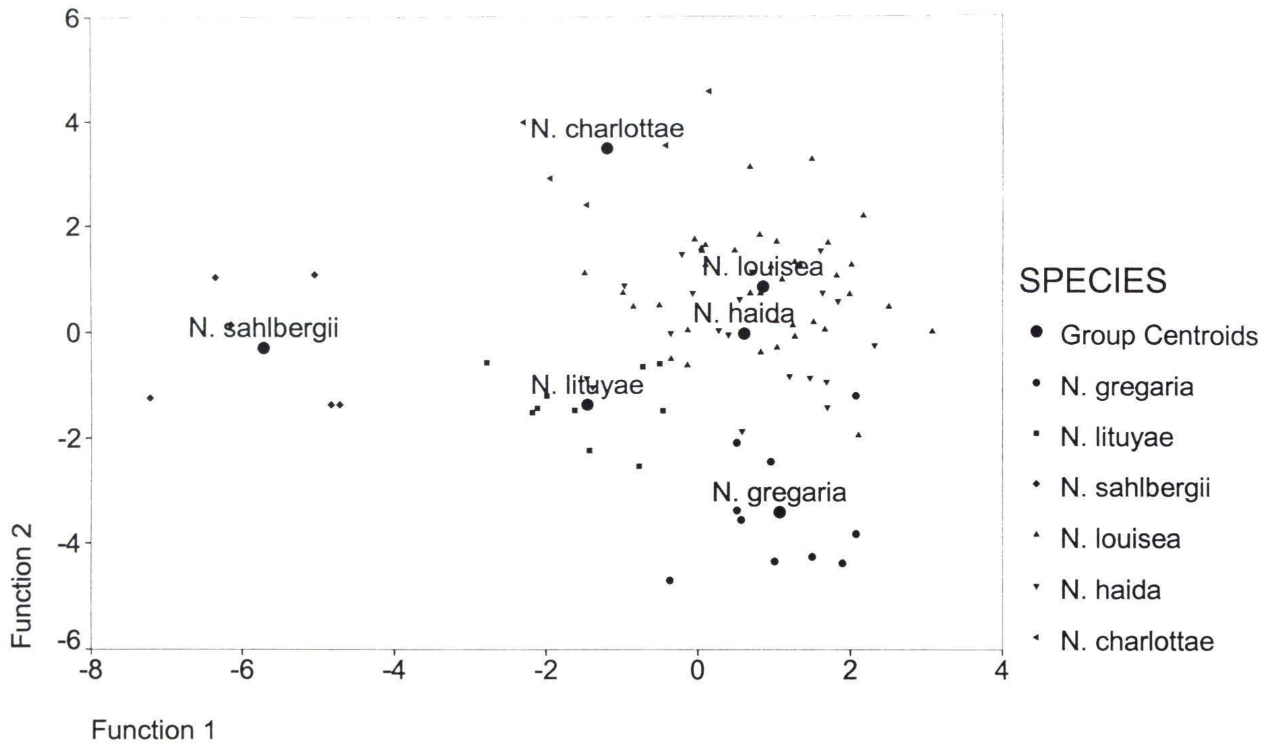


Figure 4b: Plot of discriminant function values and population centroids from analysis of males, using all six species and both pronotal and body measurements.

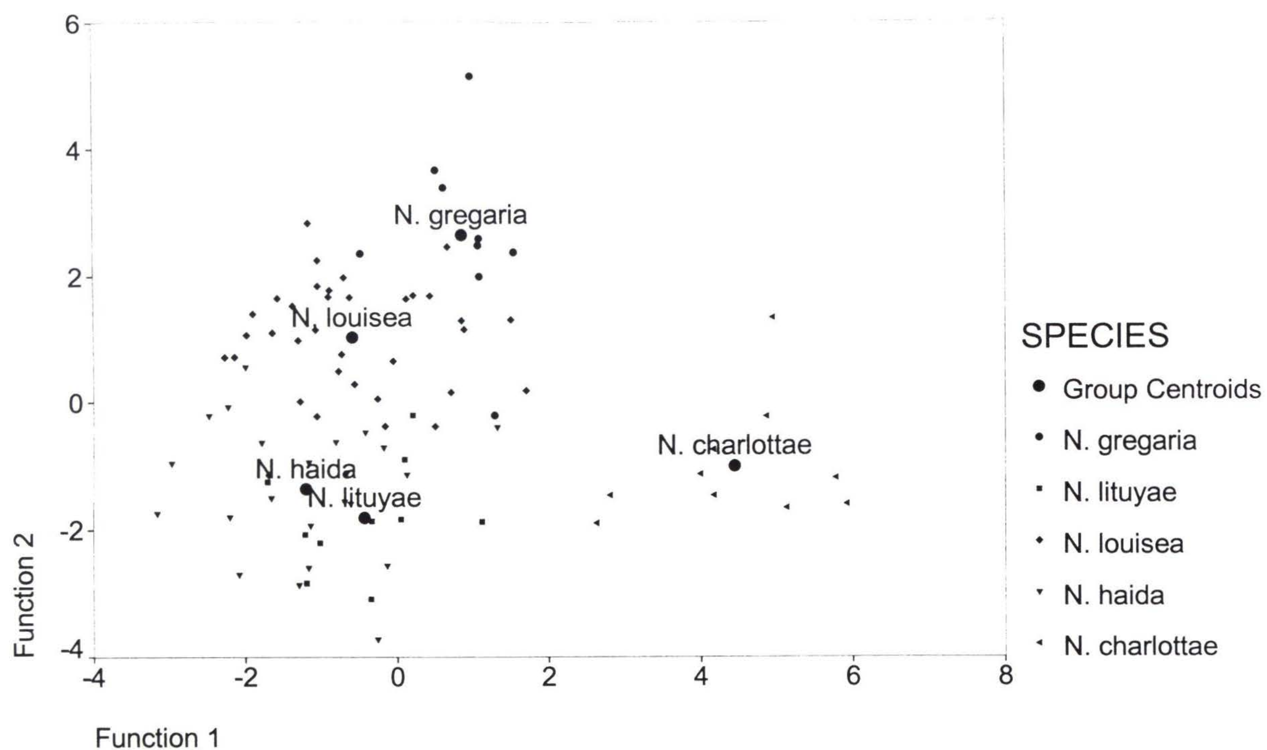


Figure 4c: Plot of discriminant function values and population centroids from analysis of females, using the *gregaria* infragroup and both pronotal and body measurements.

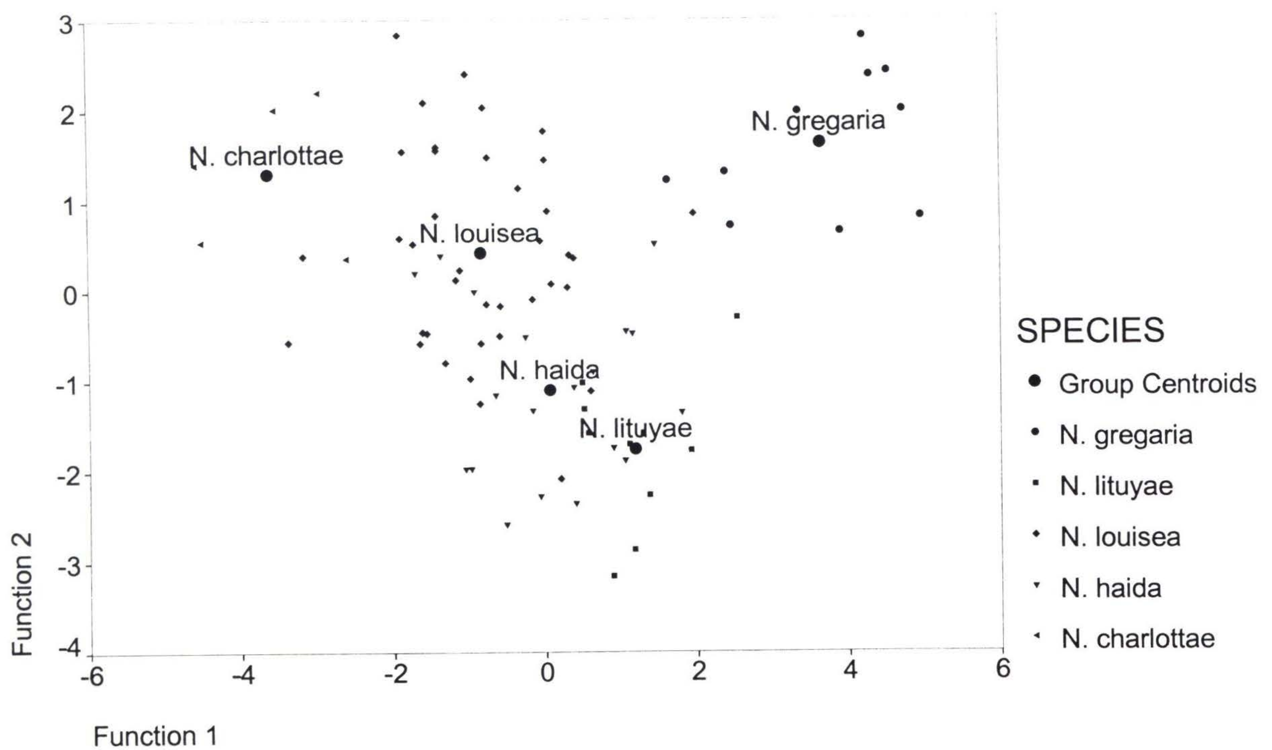


Figure 4d: Plot of discriminant function values and population centroids from analysis of males, using the *gregaria* infragroup and both pronotal and body measurements.

Table 31a: Standardized discriminant function coefficients for females of the *gregaria* infragroup using pronotal ratios and unadjusted body measurements. *N. louisea* partitioned into east and west Moresby populations.

Variable	Function 1	Function 2	Function 3	Function 4
Pronotum (mid)	-0.295	0.022	0.449	**0.791**
Pronotum (shoulder)	0.369	-0.385	**0.890**	-0.088
Elytra	-0.017	**0.962**	-0.465	0.120
Tibia	-0.444	0.128	-0.324	-0.217
M0	-0.682	**0.736**	**0.973**	-0.034
M0.5	**0.739**	-0.462	-0.198	-0.428
M1	-0.074	0.159	**-0.849**	-0.066
M2	**0.774**	0.329	0.330	**1.096**
M3	-0.253	-0.406	-0.144	-0.559
M5	-0.228	0.638	0.036	-0.674
M6	**0.826**	**-0.773**	-0.312	**0.969**
M7	-0.169	0.016	-0.410	0.317
M8	-0.709	-0.245	0.358	-0.453
M9	-0.653	-0.357	0.110	0.233
M10	**1.306**	0.059	-0.362	-0.169

Table 31b: Percentage of variation explained by each function

Function	Eigenvalue	Pct. of variation	Cumulative Pct.
1	3.103	38.08	38.08
2	2.376	29.16	67.24
3	1.719	21.10	88.34
4	0.664	8.16	96.50

Table 31c: Discriminant functions evaluated at group means.

Species	Function 1		Function 2		Function 3		Function 4	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
N. charlottae	4.241	1.143	-1.583	0.736	0.609	1.695	0.227	1.027
N. haida	-1.278	1.061	-1.120	1.018	-0.722	0.796	0.901	1.037
N. louisea-KV	-0.993	0.915	0.052	0.883	2.267	0.846	-0.107	1.081
N. louisea-EM	-0.438	1.057	1.041	0.999	0.811	1.054	-0.300	0.880
N. lituyae	-0.456	0.904	-1.378	0.896	-1.588	0.741	-1.765	1.083
N. gregaria	1.381	0.592	3.205	1.375	-1.841	0.628	0.359	1.013

Table 32: Classification results

Actual group	No. of cases	*Nebria charlottae*	*Nebria haida*	*Nebria louisea*-KV	*Nebria louisea*-EM	*Nebria lituyae*	*Nebria gregaria*
N. charlottae	10	9	0	0	0	1	0
		90.0%	0%	0%	0%	10.0%	0%
N. haida	23	0	21	0	1	1	0
		0%	91.3%	0%	4.3%	4.3%	0%
N. louisea-KV	10	0	0	8	2	0	0
		0%	0%	80.0%	20.0%	0%	0%
N. louisea-EM	25	0	0	3	20	1	1
		0%	0%	12.0%	80.0%	4.0%	4.0%
N. lituyae	10	0	1	0	0	9	0
		0%	10.0%	0%	0%	90.0%	0%
N. gregaria	9	0	0	0	0	0	9
		0%	0%	0%	0%	0%	100.0%

Percent of cases correctly classified: 87.36%

Table 33a: Standardized discriminant function coefficients for males of the *gregaria* infragroup using pronotal ratios and unadjusted body measurements. *N. louisea* partitioned into east and west Moresby populations.

Variable	*Function 1*	*Function 2*	*Function 3*	*Function 4*
Pronotum (mid)	0.158	-0.378	0.208	-0.497
Pronotum (shoulder)	1.137	0.147	0.184	0.305
Elytra	-0.892	0.610	0.343	-0.015
Tibia	-0.534	-0.258	0.183	0.356
M0	0.169	-0.106	0.581	0.004
M0.5	0.033	-0.171	0.214	-0.012
M1	0.070	0.247	-0.287	-0.002
M2	0.054	0.597	-0.029	-0.047
M3	-0.118	-0.023	-0.019	0.245
M5	-0.346	0.290	0.375	-0.174
M6	0.771	-0.454	-0.380	-0.935
M7	-0.374	0.448	-0.370	0.659
M8	0.004	0.075	0.187	0.237
M9	0.036	-0.901	-0.164	0.929
M10	-0.114	1.302	-0.264	-0.699

Table 34b: Percentage of variation explained by each function

Function	Eigenvalue	Pct. of variation	Cumulative Pct.
1	3.113	47.54	47.54
2	1.664	25.41	72.96
3	1.148	17.53	90.49
4	0.367	5.61	96.10

Table 33c: Discriminant functions evaluated at group means.

Species	Function 1		Function 2		Function 3		Function 4	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
N. charlottae	4.351	0.589	2.103	0.895	-1.103	1.240	-1.297	0.739
N. haida	-0.394	0.960	-1.301	1.170	-0.477	1.098	-0.118	0.835
N. louisea-KV	1.300	1.010	0.853	1.092	0.604	0.800	0.950	1.163
N. louisea-EM	0.402	1.207	-0.976	0.960	0.840	1.072	-0.250	1.110
N. lituyae	-1.262	0.770	0.382	0.842	-2.201	0.965	0.390	1.011
N. gregaria	-3.089	0.874	1.952	0.681	0.891	0.803	-0.475	0.828

Table 34: Classification results

Actual group	No. of cases	*Nebria charlottae*	*Nebria haida*	*Nebria louisea*-KV	*Nebria louisea*-EM	*Nebria lituyae*	*Nebria gregaria*
N. charlottae	5	5	0	0	0	0	0
		100.0%	0%	0%	0%	0%	0%
N. haida	20	0	13	0	5	2	0
		0%	65.0%	0%	25.0%	10.0%	0%
N. louisea-KV	16	0	0	14	1	1	0
		0	0%	87.5%	6.3%	6.3%	0%
N. louisea-EM	22	0	3	4	15	1	0
		0%	13.6%	18.2%	68.2%	6.3%	0%
N. lituyae	10	0	1	0	0	9	0
		0%	10.0%	0%	0%	90.0%	0%
N. gregaria	10	0	0	0	0	0	10
		0%	0%	0%	0%	0%	100.0%

Percent of cases correctly classified: 79.52%

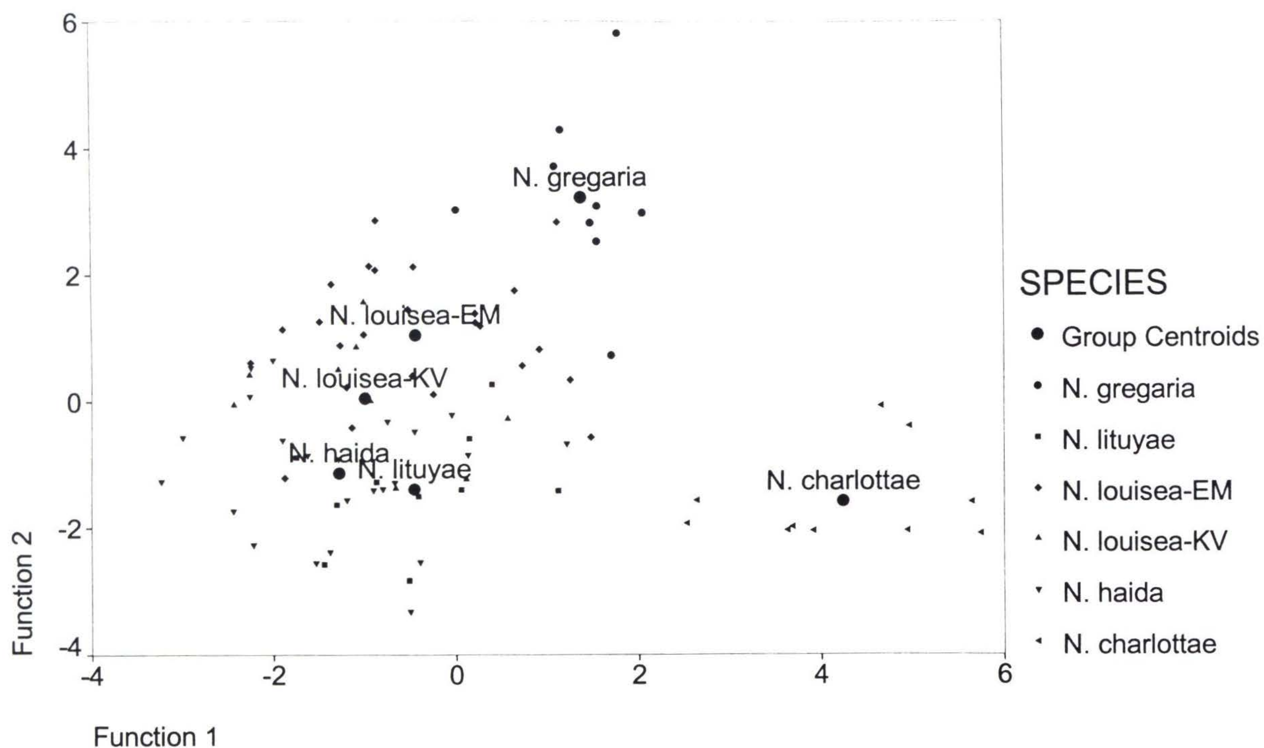


Figure 5a: Plot of discriminant function values and population centroids from analysis of females, using the *gregaria* infragroup and both pronotal and body measurements. *N. louisea* partitioned into east Moresby (EM) and Kaisun Village (KV) populations.

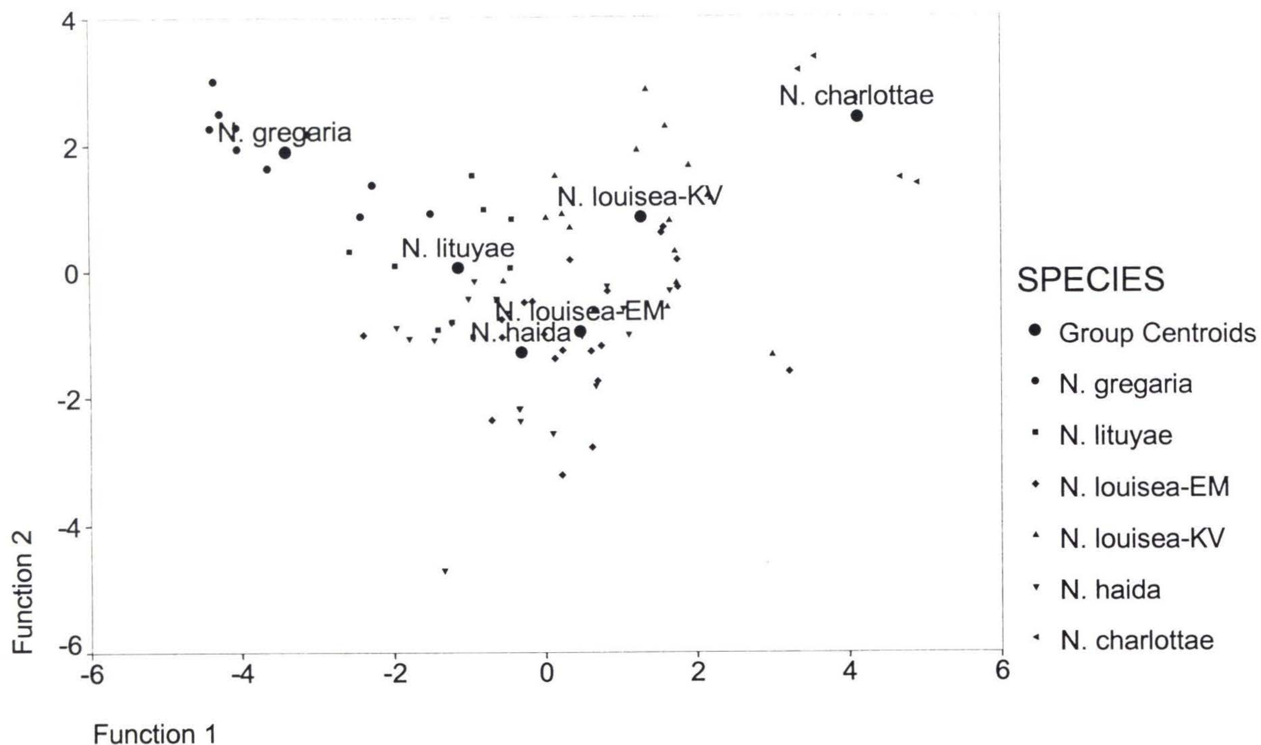


Figure 5b: Plot of discriminant function values and population centroids from analysis of males, using the *gregaria* infragroup and both pronotal and body measurements. *N. louisea* partitioned into east Moresby (EM) and Kaisun Village (KV) populations.

Discussion

An examination of the functions, population means, and classification results of the discriminant analyses would indicate some support for Kavanaugh's morphological separation of the five *gregaria* infragroup species. The species *N. charlottae and N. gregaria* are distinct in most analyses, and the remaining three species, although displaying some overlap, manage to occupy fairly discrete regions of the multi-dimensional space. However, only *N. charlottae* approaches the level of separation observed between *N. sahlbergii* and the *gregaria* infragroup, while most species appear to occupy regions of a morphological continuum across the *gregaria* infragroup. The species *N. lituyae* and *N. haida* are particularly closely associated, with most separation between these species occurring on the third and fourth discriminant function. Although the latter relationship mirrors the phylogeny proposed by Kavanaugh (1989), other species are not as clearly related. In particular, N. gregaria and N. charlottae, believed to be part of the same lineage in Kavanaugh's phylogeny, often occupy the opposite extremes of a discriminant function.

How the overlap and adjoining variation present in the different populations is dealt with becomes the important criterion for determining which of the populations deserve species status. Because the original phylogeny of the *gregaria* infragroup was based on cladistics, the morphological variation present in the beetles had to be condensed into a set of discrete character states, representing the mean values observed in each population, or more typically, predesignated values that attempt to reduce the

different mean values from different populations into a small number of manageable states (e.g., 'small', 'medium', and 'large') (Kavanaugh, 1978). Cladistic methods alone are thus not well adapted to dealing with characters that have a high degree of variation that can produce overlap between populations. Various methods have been described for incorporating variation into taxonomic decisions, ranging from calculating Coefficients of Difference (the difference in population means divided by the sum of standard deviations; Mayr, 1969) to discriminant analysis. However, there are no agreed upon criteria for separating species using either method.. While in theory, the category of species is only to be applied to groups of interbreeding natural populations which are reproductively isolated from other such groups, in practice the final decision on species status falls upon the judgement of the investigator and his or her preference for the lumping or splitting of taxa (Mayr, 1969; Sneath and Sokal, 1973). The most conservative judgement would be that, with the exception of *N. charlottae*, the five members of the *gregaria* infragroup do not represent distinct taxonomic units, but rather different populations of the same species occupying different positions on a morphological gradient. However, the low levels of overlap occurring in the morphometric analysis would still argue for some form of intraspecific subdivision of the component.

The question of whether morphological differences between populations actually represents the genetic history of the populations has particular importance for the Queen Charlotte Islands. The hypothesis of a QCI refugium began as a creation of biologists to explain the unusual differences in morphology between island populations of mammals

and their mainland conspecifics. Originally, this hypothesis had been fuelled, not by the discovery of supporting geological evidence, but by the belief that morphological change is, and has always been, a gradual process, and that the differences observed between mainland and island mammals could not have arisen in the 10 to 15 thousand years since deglaciation allowed recolonization of the archipelago. However, the status of many of the island's morphologically distinct subspecies and populations has been challenged by genetic evidence that indicates a recent origin to taxa considered to be the product of long periods of genetic isolation. The Queen Charlotte Black Bear (*Ursus americanus carlottae*) was first described as a distinct species by Osgood (1901) based on a larger body size, an elongate skull, larger teeth, and a last molar with a pronounced posterior heel. This last character was emphasized by Foster (1965) in his hypothesis that the QCI Black Bear was a glacial relict, as it showed a markedly greater degree of development then could be found in the other island dwelling subspecies of Vancouver Island and the Alexander archipelago. However, the molecular survey conducted by Byun et al. (1997) could not distinguish this subspecies from the three other coastal subspecies used in Foster's comparison. Instead they lumped all four coastal subspecies into a single lineage distinct from the continental subspecies *americanus* and *cinnamonum.* A similar situation exists for the Pine Marten *(Martes americana nesophila*), described by Osgood as a distinctly larger form with a shorter, heavier rostrum and larger premolars and proposed by Hagmeier (1955) as a glacial relic on the basis of this size difference between island and mainland forms. As with the bears, molecular evidence (Byun, pers. com.) groups together the Queen Charlotte populations of Pine Marten with a less derived form on Vancouver Island, that together form a genetic lineage distinct from mainland

populations. In both species, the differences between the QCI and the less derived mainland and Vancouver Island populations appear to be the result of rapid post-glacial adaptation to their different environments.

In comparison to the relatively conserved differences between island and mainland subpopulations of the QCI mammals, the stickleback fish (*Gasterosteus aculeatus*) of the QCIs show a wide variety of morphologies in different lakes and streams, despite all or most of the populations having a clear post-glacial origin from marine stickleback (Moodie and Reimchen, 1976b). Different populations vary in the number of spines, gill rakers, lateral plates, and development of the pectoral girdle as a result of adaptation to the different selective regimes that exist on the islands. Both spine reduction, plate loss, and body size (Reimchen, 1980, 1992a, 1994) were associated with differences in the numbers and types of vertebrate predators at each of the water bodies and reflected directional selection on each of these traits (Moodie and Reimchen, 1976a, Reimchen, 1995). The wide variety of selection regimes present on the QCIs relative to the mainland is itself a product of the depauperate fauna of the islands that has resulted in a reduced diversity of predators, particularly fish, in many of the stickleback habitats, with possibly an increased emphasis on those invertebrate predators that can ignore or take advantage of the stickleback's armour (Reimchen, 1980). The presence of two genetically distinct lineages of stickleback on the QCIs showing similar morphological derivations only further emphasizes that morphological change in response to selection pressure can occur relatively quickly, and that long periods of isolation are not required to explain changes in those traits on which selection can act (Deagle et al. 1996).

The lack of correlation between morphological change and degree of genetic relatedness observed for the bears, pine martens, and stickleback of the QCIs calls into question the genetic distinctiveness of the other QCI endemics identified as such on morphological grounds. While the degree of morphological difference observed between the populations of the *gregaria* infragroup is neither as great as that observed in the mammals and stickleback, nor as clearly associated with selective benefits, the *gregaria* infragroup as a whole is distinct from its near relative *N. sahlbergii sahlbergii*. The combination of wing loss, habitat shift, and morphometric difference between the *gregaria* and *sahlbergii* species infragroups supports the contention that some period of isolation has existed between the members of each infragroup. However, morphology alone is a poor mechanism with which to determine the length of phyletic divergence while certain characters, such as habitat preference and flightlessness, can be very plastic in the face of selection pressure (Roff, 1994). The impetus for re-examining the phylogeny of the *gregaria* infragroup of *Nebria* from a molecular viewpoint comes from a need for an unambiguous understanding of the relationships between the five beetle species, both in terms of their phylogenetic relationships to one another and the timing of the component speciation events, in order to accurately deduce the Quaternary biogeography of this beetle group and its relationship to the QCIs.

CHAPTER 3: Sequence Analysis

Introduction

In order to determine the phylogeny of the *Nebria gregaria* infragroup and to correlate the history of these five species of beetles with the glacial history of the QCIs, a suite of characters are needed that are large, unambiguous, allow an estimation of the rate of evolution, and that are not subject to excessive homoplasy. It has been demonstrated in the previous chapter that many of the key morphological characters available for these beetles overlap extensively and do not provide a clear breakdown of species relationships. In contrast, DNA sequence comparison has the potential to provide a large number of characters to bear on a phylogenetic question and can estimate the timing of speciation events. The degree of ambiguity and homoplasy that exists in the data can be limited by the researcher by the judicial choice of DNA regions to compare and can be estimated by various statistics.

By comparing regions of the mtDNA among the five species of the *gregaria* infragroup, a closely related species *N. sahlbergii sahlbergii*, and two more distantly related *Nebria* species, the phylogeny and age of the *gregaria* infragroup and its component species may be estimated. This phylogeny can then be compared with the known glacial history of the north-west coast of North America to determine what role, if any, a hypothetical glacial refugium centred on the QCIs and Hecate Strait played in the history of this species group.

Materials and Methods

Collection:

Specimens of *Nebria* for use in DNA sequence analysis were collected into 80% ethanol from sites on the Queen Charlotte Islands and the west coast of British Columbia (Figure 1). *Nebria charlottae* were obtained from cobble beaches at the base of Toe Hill and surrounding Estrado Lagoon, near Masset township, Graham Island. *Nebria louisea* were collected from Kaisun village, Moresby Island, and from cobble beaches at Skedans and Reef Islands to the east of Moresby. *Nebria haida* were obtained from above the tree-line on 'Nebria Hill', located south of Shields Bay, Graham Island. *Nebria sahlbergii sahlbergii* and *Nebria gebleri* were both collected from stream banks in Manning Park, B.C. *Nebria diversa* were obtained from North Beach, Graham Island, and Cape Scott, Vancouver Island. One specimen each of *Nebria gregaria* collected from the Aleutian Islands and *Nebria lituyae* from the mountains near Juneau were supplied by Dr. D. Kavanaugh from the California Academy of Sciences collection. The *Nebria gregaria* specimen had been killed and stored in ethyl acetate before pinning, while the *Nebria lituyae* specimen was preserved in silica gel.

The beetles selected for DNA extraction and sequencing were three specimens from each of the Toe Hill, Kaisun village, Skedans Island, Reef Island, and Nebria Hill beetle populations as well as single specimens of *N. charlottae* from Masset, *N. louisea* from Skedans village site, *N. lituyae* and *N. gregaria*. Three specimens of *N. sahlbergii*, two of *N. gebleri*, and two each of the Queen Charlotte and Cape Scott *N. diversa* were

also processed for use as outgroups. A total of 1856 bases from the mitochondrial sequence of *Drosophilia melanogaster* (Gene bank accession numbers J01404 and M37275) was used to provide an additional outgroup sequence for the purpose of rooting the *Nebria* phylogenetic tree.

DNA extraction:

Beetle DNA for polymerase chain reactions was extracted using either the nondestructive DNA extraction protocol of Phillips and Simon (1995) or the QIAamp tissue kit using the manufacturers protocol (Qiagen, 1996). For the former method, dried beetles were perforated using a sterilized size 1 insect pin on the underside of their abdomen and thorax and allowed to soak in a solution containing 8% dodecyltrimethyl-ammonium bromide (DTAB), 1.5 M NaCl, 100 mM Tris-HCl (pH 9.0), 50 mM EDTA at 68° C in a waterbath, overnight. Specimens were then removed and washed in chloroform before being transferred to alcohol or pinned.

Two chloroform extractions were performed on the DTAB extract solution before the DNA was precipitated with a solution containing 5% cetyltrimethylammonium bromide (CTAB) and 0.4 M NaCl. The DNA was pelleted by centrifugation at 15,000g for 30 minutes at 4° C. After the aqueous solution was discarded, the pellet was resuspended in 1.2 M NaCl to exchange the CTAB and precipitated again by the addition of 100% ethanol, followed by a second round of centrifugation. A final wash with 70% ethanol was performed before the pellet was dried under vacuum (Sorval Speed-vac) and resuspended in TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 7.5).

PCR amplification:

Five regions of the mitochondrial DNA were selected to be amplified from the beetle mitochondrial (mt) DNA using the polymerase chain reaction (PCR). The regions were located inside the NADH subunit I gene (ND1 region), within the cytochrome oxidase subunit 1 gene (CO1), the cytochrome B gene (CytB), and within two sections of the cytochrome oxidase II gene (COIIa and COIIb) (Table 35).

PCR amplifications of fragments ND1 and CO1 for beetles from Toe Hill, Kaisun village, Nebria Hill, as well as all outgroup species were performed in 50 μL volumes containing 50 ng to 100 ng of beetle DNA, 83 ng of each primer, 200 μM of each deoxyribonucleic acid (dNTP), 15 mM of Tris (pH 8.0), 2.5 mM $MgCl_2$, 60 mM KCl, and Taq polymerase, with 35 cycles of 94° C for 45 seconds, 47° C for 60 seconds, and 72° C for 60 seconds. Fragments were ligated using T4 ligase into the TA cloning vector pCR2.1 (Invitrogen).

PCR amplifications of all other fragments were performed in 50 uL volumes containing Pfu polymerase and either <10 ng of beetle DNA (*Nebria gregaria*) or 50 ng to 100 ng of beetle DNA (all others), 83 ng of each primer, 2.0 μM of each dNTP, 20 mM Tris (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 EcoRV cut pBluescript plasmid using T4 DNA ligase.

Table 35: PCR amplification primers:

Gene	Primer	Sequence
ND1		
	ND1F -	5'-GCATCACAAAAGGCTGAGGA-3'
	ND1R -	5'-ACATGATCTGAGTTGAAACC-3'
CO1		
	CO1F (mtd-6) -	5'-GGAGGATTTGGAAATTGATTAGTTCC-3'
	CO1R (mtd-9)-	5'-CCCGGTAAAATTAAAATATAAACTTC-3'
CO2b		
	mtd-13	5'-AATATGGCAGATTAGTGCA-3'
	mtd-15	5'-TCATAAGTTCARTATCATTG-3'
CO2a		
	mtd-16	5'-ATTGGACATCAATGATATTGA-3'
	mtd-18	5'-CCACAAATTTCTGAACATTGACCA-3'
Cyt B		
	mtd-26	5'-TATGTACTACCATGAGGACAAATATC-3'
	mtd-28	5'-ATTACACCTCCTAATTTATTAGGAAT-3'

Ligated plasmids were transformed into *Escherichia coli* strain XL-1, made competent by exposure to $CaCl_2$. Plasmid for sequencing was obtained by growing cultures of bacteria-containing plasmid overnight in LB media containing 100 µg/ml ampicillin at 37° C. The plasmids were isolated from the bacteria using a modification of the alkaline lysis protocol of Kraft et al. (1988). Bacteria were ruptured using a lysis solution containing 0.2 M NaOH and 1% SDS, followed by neutralization of the lysis buffer with potassium acetate, and phenol-chloroform extraction of the DNA. The DNA was then pelleted with 100% ethanol, resuspended in polyethylene glycol to remove salt, re-pelleted by centrifugation, and washed with 70% ethanol before drying in a Speed-vac. The plasmid DNA was then resuspended in either TE buffer or ddH_2O. DNA concentration was measured in a fluorimeter and the presence of an insert was tested by running a sample of plasmid DNA on an agarose gel against a plasmid standard that lacked an insert.

Sequencing:

Mitochondrial DNA inserts were sequenced on an Automated Laser Fluorescence (ALF) sequencing machine (Pharmacia) using two variations of the Sanger dideoxy sequencing method (Sanger et al. 1977) designed to make use of thermostable polymerases and fluorescene based automatic sequencing. All sequencing reactions involved the use of a bulk mixture containing 10X buffer (100 mM Tris-HCl pH 8.5, 200 mM KCl, 30 mM $MgCl_2$), 6-12 pmol of fluorescene tagged primer (Table 36), Taq polymerase, and DNA, which was aliquoted into four tubes containing the nucleotides (0.6 mM of each deoxynucleotide, and either 4 mM of dideoxyadenosine, 4 mM of dideoxythymidine, 2 mM of dideoxycytosine, or 0.4m M dideoxyguanidine). Most sequencing reactions involved a single fluorescene tagged primer and between 800 ng and 1400 ng of template DNA. However when levels of template were low, a reaction involving the addition of 5 pmol of an untagged primer located in the plasmid on the opposite side of the insert to that of the tagged primer, and the use of only 50 ng to 200 ng of template DNA was substituted. The polymerase chain reaction conditions were one cycle of 94° C for three minutes followed by 30 cycles of 30 seconds at 95° C, 30 seconds at 50° C, and 30 seconds at 72° C. Six μl of a formamide stop mix containing dextran blue was added to the reaction products before they were run on an acrylamide gel in the ALF sequencer. Pharmacia ReadyMix Gels, containing 5.7% acrylamide monomers, 3% N,N'-methylenebisacrylamide, 7.0 M urea, 100 mM Tris-borate (pH 8.3), 1 M Na_2EDTA, and 3mM N,N,N',N'-tetramethylethylenediamine were used to separate the sequencing reaction products. Polymerization of the acrylamide was initiated with the addition of 0.47 ml of 10% ammonium persulfate. Gels were run for 6 to 8 hours at 1500 volts,

(current 38 mA, power 35 watts). Gels were heated to 45° C during the run to maintain denaturation of the DNA fragments. Fluorescene tagged reaction products were detected using a laser power of 5 mW and a sampling interval of 1.5 to 2.0 seconds.

Individual sequences were put together using the Seqman program in the Lasergene package (DNASTAR, Madison, Wisconsin). 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 while cladistic analysis of the sequence data was performed using the Paup 3.1 program (Swofford, 1993).

Table 36: Sequencing primers.

M13 Universal primer: 5'-d[CGACGTTGTAAAACGACGGCCAGT]-3'

M13 -40 Forward primer: 5'-d[CGCCAGGGTTTTCCCAGTCACGAC]-3'

M13 Reverse primer: 5'-d[CAGGAAACAGCTATGAC]-3'

Results:

Five fragments were amplified and sequenced from the mitochondrial DNA of the *Nebria* beetles to produce a total of 1835 bases of sequence data for each beetle. No differences in sequence could be found between beetles taken from the same population, with the exception of *Nebria louisea* 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 respectively, while the SKA haplotype was found at both sites.

Fragments varied in both size and nucleotide content but were all characterized by a strong bias towards adenine and thymidine nucleotides, with A+T nucleotides making up from 67.2% to 78.8% of the bases in the five fragments (Table 37). Cytochrome oxidase subunit II fragment b, located at the 5'-end of the gene, showed the greatest level of A+T incorporation with an average A+T content of 78.4% across the *Nebria* species. This is in contrast to the COIIa fragment located at the opposite end of the gene which displayed an average A+T content of 72.9%, indicating that nucleotide composition varied both between and within different genes in the beetles' mitochondrial DNA.

A cladistic analysis using the branch and bound algorithm of the total data set produced two most parsimonious trees, each of 547 steps (Figure 6). Both trees agreed with Kavanaugh in the placement of the *gregaria* group as sister to the *mannerheimii*

group (as represented by *N. diversa*) and as distinct from the *metallica* lineage (represented by *N. gebleri*). However, within the *gregaria* group, both trees aligned the species *N. lituyae* as sister to *N. sahlbergii sahlbergii*, removing it from the *gregaria* infragroup. The two trees differed from each other only in the placement of *N. gregaria*, either as a sister taxon to all of the QCI *Nebria*, or as the sister taxon to *N. haida*. Very little difference existed between the members of the *gregaria* infragroup (exclusive of *N. lituyae*). In both trees, no more than two character changes separated any of the *N. louisea* haplotypes from *N. charlottae*. The greatest distance between any two of these taxa involved seven mutations out of 1835 bases (Table 38).

Both trees had a Consistency Index of 0.932, and a Retention Index of 0.711. This Consistency Index, however, decreased to 0.734 when uninformative (autopomorphic) characters were excluded. Exclusion of the three outermost species from the tree increased the Consistency Index (excluding autopomorphies) to 0.875 and the Retention Index to 0.889. Convergent base changes were concentrated between the two most distantly placed species, *D. melanogaster* and *N. gebleri*, and the remaining *Nebria* taxa, while no homoplasy was required within the *gregaria* infragroup exclusive of *N. lituyae* (Table 39) to explain the pattern of mutation. The degree of sequence divergence between members within the *gregaria* infragroup, exclusive *of N. lituyae*, ranged from 0.05% to 0.38%, while sequence divergence between the two represented lineages of the *gregaria* group reached a maximum of 1.02%, between *N. sahlbergii sahlbergii* and *N. haida* (Table 40).

A cladistic analysis using the branch and bound algorithm with retention of all trees of length 548 steps or less identified 20 trees. With the limit set to 549 steps or less, 89 trees were identified. Strict consensus of all trees produced at the 548 and 549 limits preserved both the placement of *N. lituyae* with *N. sahlbergii sahlbergii*, but could not resolve any relationships among the remaining *gregaria* infragroup taxa. Consensus trees using the 50% majority rule for both the 548 and 549 step tree lengths differed from the strict consensus trees only in the placement of *N. gregaria* and *N. haida* together as sister taxa. The two taxa occurred together in 65% of the trees produced using the 548 step limit, and 52% of the trees produced using the 549 step limit (Figure 7). An exhaustive search of the total data set evaluated 34459425 trees, and produced the two most-parsimonious trees with a length of 547 and an identical topology to the two most-parsimonious trees produced by the branch-and-bound algorithm. The graph of the overall distribution of tree lengths for the exhaustive search was skewed from that of normal distribution, with a mean length of 624.3 steps, a standard deviation of 15.6 steps, and a skew value (g1) of -1.64 (Figure 8).

A bootstrap analysis of the data, using 100 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* infragroup, and the sister relationship between the two groups of taxa (Figure 9). Within the latter clade, the group formed by *N. charlottae* and *N. louisea* was maintained as a distinct clade from *N. haida* and *N. gregaria* in 61% of the replicates.

A cladistic analysis of the data sets for each of the five individual mtDNA regions produced a mix of results, with some regions allowing a high degree of resolution and others displaying little or no resolving power (Figure 10). The COIIa and CytB regions each produced a single tree that resolved the *gregaria* group as a distinct clade and removed *N. lituyae* from the *gregaria* infragroup (although the CytB tree placed *N. lituyae* as the sister to the remainder of the *gregaria* infragroup.) Strict and 50% majority rule consensus trees of the COIIB and ND1 regions maintained the *gregaria* group but provided poor resolution within the clade. A strict consensus tree of the 17 shortest trees produced for the COI fragment could not resolve any relationships between taxa, however the 50% majority rule consensus tree distinguished the gregaria infragroup from *N. diversa* + *D. melanogaster* and paired *N. lituyae* and *N. sahlbergii* in 76% of the trees.

Table 37: Percent A+T nucleotide content of each fragment amplified.

	Size	*N.gebleri*	*N.diversa*	*N.sahlbergii*	*N.haida*	*N.charlottae*	**Average**
ND1	320	76.6%	77.6%	77.9%	78.2%	78.2%	77.7%
CO1	472	-------	67.2%	67.8%	68.2%	68.9%	68.0%
CytB	433	73.7%	71.4%	74.1%	73.7%	73.7%	73.3%
COIIa	260	72.8%	70.9%	73.2%	74.0%	73.6%	72.9%
COIIb	360	78.8%	77.1%	78.8%	78.8%	78.5%	78.4%

Table 38: Pairwise distances between taxa

1 *D. mel*	---										
2 *N.char*	353	---									
3 *N.div*	381	134	---								
4 *N.greg*	351	4	136	---							
5 *N.haid*	353	5	136	5	---						
6 *N.lou*	355	2	136	6	7	---					
7 *N.sahl*	355	16	137	16	19	18	---				
8 *N.skA*	354	1	135	5	6	1	17	---			
9 *N.skB*	353	2	134	6	7	2	16	1	---		
10 *N.lit*	357	15	135	15	18	17	6	16	17	---	
11 *N.gebl*	289	106	124	108	108	108	105	107	108	105	---
	1	2	3	4	5	6	7	8	9	10	11

Table 39a: Pairwise homoplasy matrix for tree 1.

1 *D.mel*	---										
2 *N.char*	22	---									
3 *N.div*	10	4	---								
4 *N.greg*	22	0	0	---							
5 *N.haid*	22	0	2	0	---						
6 *N.lou*	22	0	4	0	0	---					
7 *N.sahl*	20	2	2	0	0	2	---				
8 *N.skA*	22	0	4	0	0	0	2	---			
9 *N.skB*	24	0	6	0	0	0	4	0	---		
10 *N.lit*	18	2	2	0	0	2	0	2	2	---	
11 *N.geb*	0	20	10	16	16	20	16	20	20	16	---
	1	2	3	4	5	6	7	8	9	10	11

Table 39b: Pairwise homoplasy matrix for tree 2.

1 *D.mel*	---										
2 *N.char*	20	---									
3 *N.div*	10	0	---								
4 *N.greg*	24	0	0	---							
5 *N.haid*	22	0	0	0	---						
6 *N.lou*	20	0	0	0	0	---					
7 *N.sahl*	22	0	2	2	0	0	---				
8 *N.skA*	20	0	0	0	0	0	0	---			
9 *N.skB*	22	0	2	0	0	0	2	0	---		
10 *N.lit*	20	0	2	2	0	0	0	0	0	---	
11 *N.geb*	0	18	10	18	16	18	18	18	18	16	---
	1	2	3	4	5	6	7	8	9	10	11

Table 40: Percent sequence distance between taxa (adjusted for missing data)

1 *D. mel*	---										
2 *N.char*	19.02	---									
3 *N.div*	20.53	7.22	---								
4 *N.greg*	18.91	0.22	7.33	---							
5 *N.haid*	19.02	0.27	7.33	0.27	---						
6 *N.lou*	19.13	0.11	7.33	0.32	0.38	---					
7 *N.sahl*	19.13	0.86	7.39	0.86	1.02	0.97	---				
8 *N.skA*	19.07	0.054	7.27	0.27	0.32	0.054	0.92	---			
9 *N.skB*	19.02	0.11	7.22	0.32	0.38	0.11	0.86	0.054	---		
10 *N.lit*	19.23	0.81	7.27	0.81	0.92	0.92	0.33	0.86	0.92	---	
11 *N.gebl*	20.88	7.66	8.96	7.80	7.80	7.80	7.59	7.73	7.80	7.59	---
	1	2	3	4	5	6	7	8	9	10	11

a.

271
0
2
1
1
1
0
6
1
3
52
1
3
56
5
3
78
61

D. melanogaster
N. charlottae
N. louisea (Nlou)
N. louisea (NskA)
N. louisea (NskB)
N. haida
N. gregaria
N. sahlbergii sahl.
N. lituyea
N. diversa
N. gebleri

b.

271
0
1
1
1
0
1
5
2
1
52
3
3
57
6
3
77
61

D. melanogaster
N. charlottae
N. louisea (Nlou)
N. louisea (NskA)
N. louisea (NskB)
N. haida
N. gregaria
N. sahlbergii sahl.
N. lituyea
N. diversa
N. gebleri

Figure 6. Two most parsimonius trees produced from the total data set. Numbers above the branches indicate the number of synapomorphies supporting each clade.

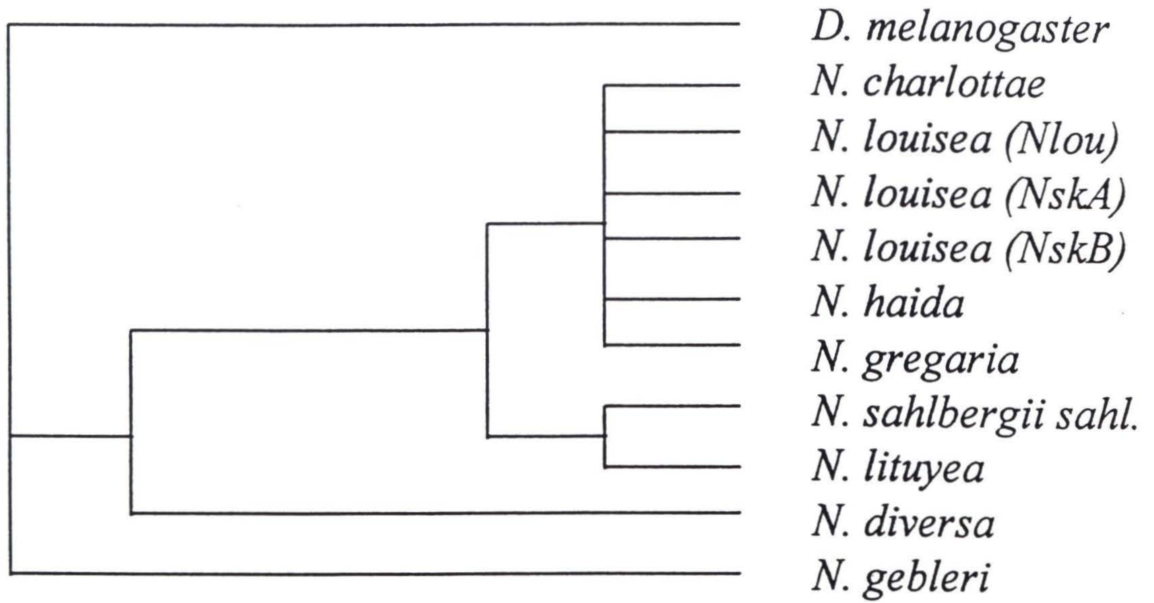


Figure 7. Consensus of all trees of length eaqual to or less then 548 steps using the total data set.

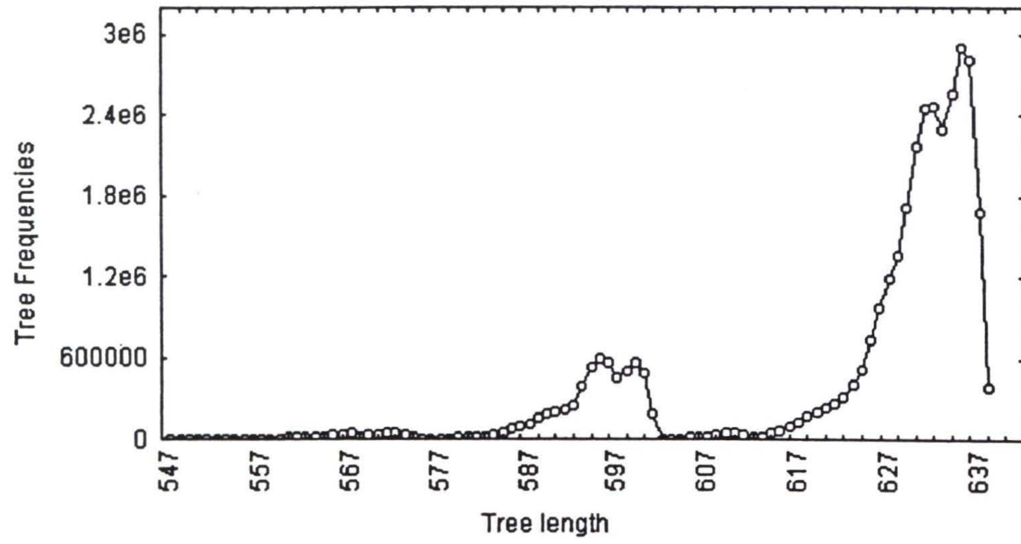


Figure 8. Frequency distribution of tree lengths.

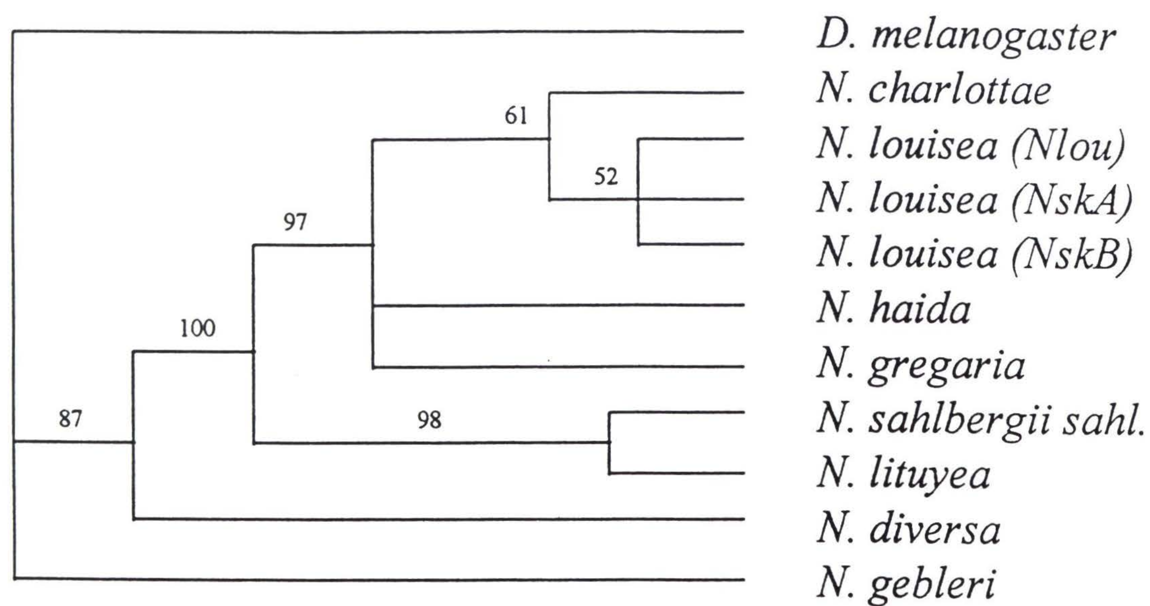
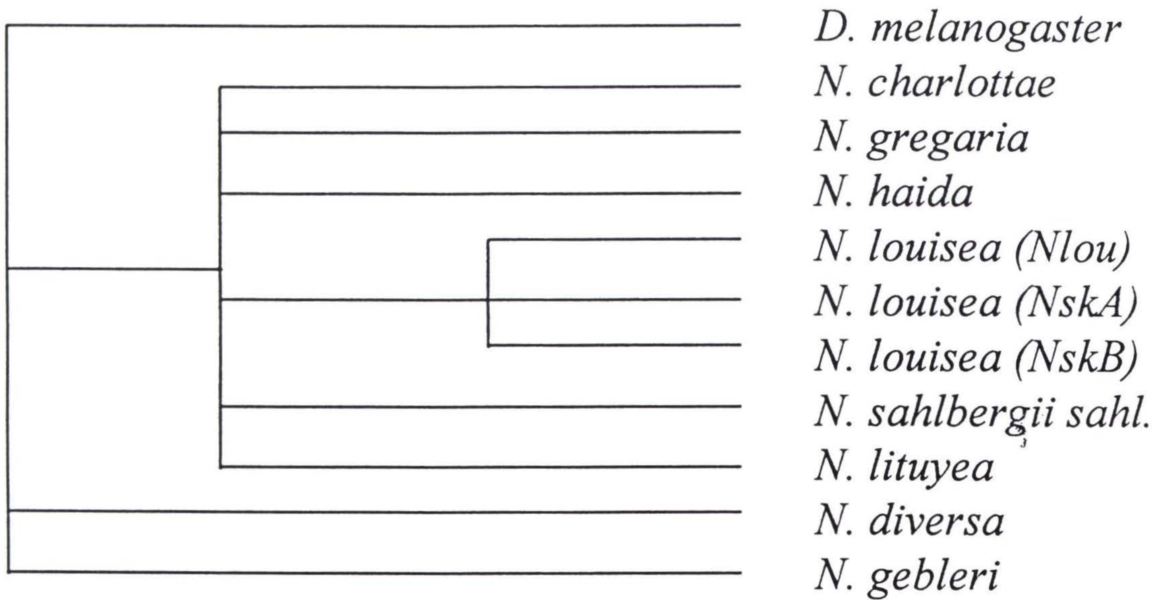


Figure 9. Bootstrap tree of total data set.

a.



b.

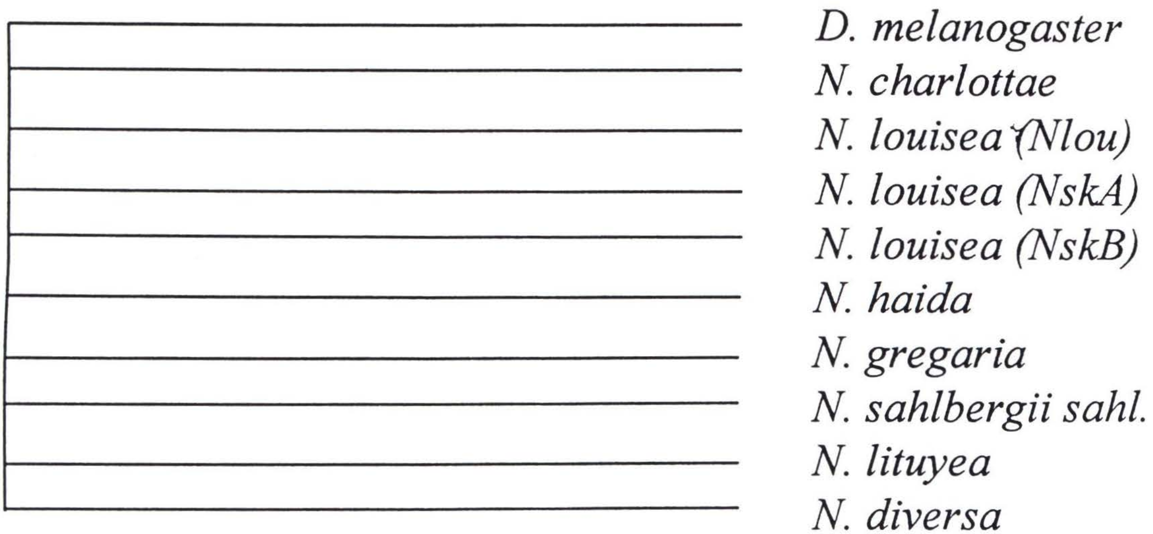
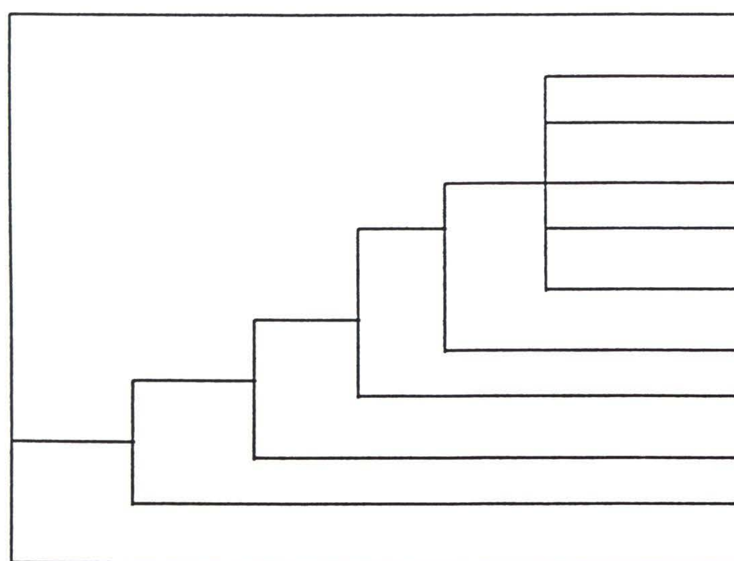


Figure 10 a-b. Strict consensus trees.
a. Consenus of 4 trees produced using ND1 region sequences
b. Consensus of 17 trees produced using CO1 region sequences.

c.



d.

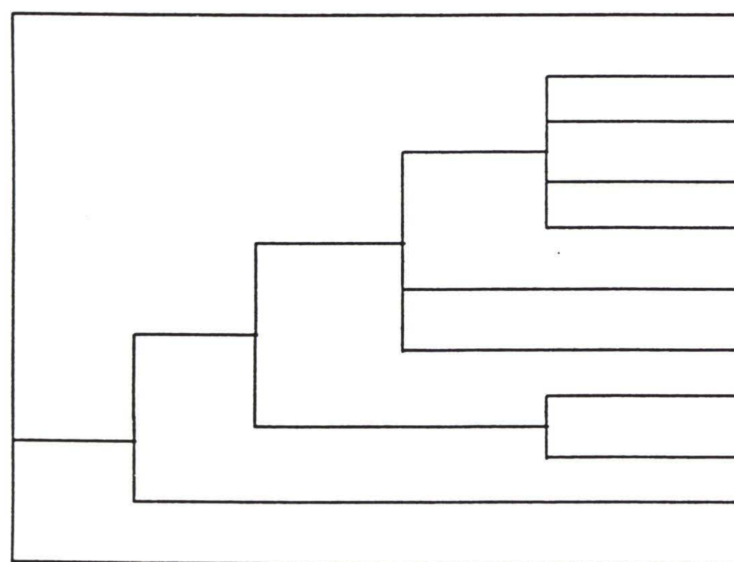


Figure 10 c-d. Strict consensus trees.
c. Single most parsimonius tree produced using CytB region sequences
d. Single most parsimonius tree produced using COIIa region sequences.

e.

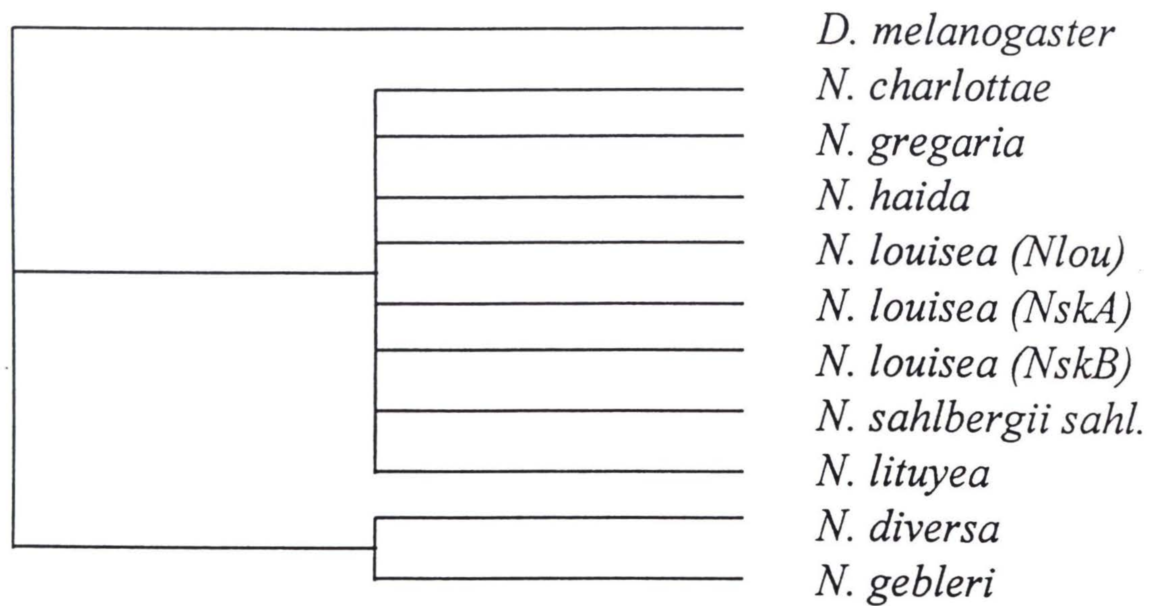


Figure 10 e. Strict consensus trees.
e. Consensus of 5 trees produced using COIIb region sequences

Discussion

The results of the sequence analyses contradict the predictions of Kavanaugh's hypothesis on the evolution of the *gregaria* infragroup. Kavanaugh's hypothesis holds that the *gregaria* infragroup radiated from an ancestral species over a period of hundreds of thousands of years, the result of repeated isolation during glacial cycles. In contrast, the sequence data suggests that the *gregaria* infragroup, as defined by Kavanaugh, is both polyphyletic and far younger than predicted. One species, *N. lituyae*, falls outside the *gregaria* infragroup entirely, and is, instead, linked to the riparian beetle *N. sahlbergii sahlbergii*. The four remaining species of the *gregaria* infragroup appear to be the product of a recent radiation given the small degree of sequence divergence between populations. The predicted division of the *gregaria* infragroup along lines of habitat choice did not occur. Instead, the phylogenetic tree shows that four of the five species can be grouped into two clusters, one formed by *N. charlottae* and the *N. louisea* populations, and the second linking *N. gregaria*, a beach dwelling beetle, with the mountain dwelling *N. haida*. The two species, *N. charlottae* and *N. louisea,* are barely distinguishable from each other, with only a single mutation separating *N. charlottae* from the SKA haplotype.

By applying mutation rates estimated from arthropod species with known speciation dates, a rough approximation of the time at which the members of the *gregaria* infragroup radiated can be generated. The greatest degree of sequence divergence found within the *gregaria* infragroup is 0.378% between *N. haida* and the *N. louisea* haplotype

KV. The smallest degree of divergence between nominal species of this infragroup is 0.054%, the single mutation found between *N. charlottae* and the SKA haplotype of *N. louisea*. While there is no agreement on the rate of mutation in mtDNA in insects, or even whether a universal insect rate of mutation can be hypothesized, estimates of the time of separation between the mitochondrial lineages of the *gregaria* infragroup can be made based on the two extremes of mutation rate mentioned in Brower (1994) and the estimate given for Japanese Carabinii given in Su et al. (1996a). Using Brower's '*Artemia*' mutation rate of 2.3% sequence divergence per million years, the *N. haida* haplotype would have separated from the KV haplotype approximately 164,000 years ago, while the *N. charlottae* and the SKA haplotypes would have diverged 23,000 years ago. By applying Brower's 'insect' mutation rates of 1.7%, the time span of radiation of the QCI *Nebria* is extended to the period between 222,000 years and 32,000 years B.P.. With the Japanese Carabinii mutation rate of 1.2%, the radiation spans 315,000 to 45,000 years.

The separation between the *N. lituyae*, *N. sahlbergii sahlbergii*, and the remainder of the *gregaria* infragroup can be dated with a somewhat greater degree of confidence. Using Browers mutation rates, estimates of the date for the separation of the former two species from the latter clade range from 389,000 years B.P. to 525,000 years B.P., with an extreme of 745,000 years B.P. using Su et al.'s Carabini mutation rate. The separation dates for *N. lituyae* from *N. sahlbergii sahlbergii* lie within the range of 143,000 to 194,000 years B.P. (Brower) or 275,000 years B.P. (Su et al.).

The speciation dates resulting from the application of the molecular clock to the *gregaria* group sequence data are almost certainly overestimates of the timing of the actual speciation events. Almost all of the mutations within the *gregaria* group occur in the third position of the codon and therefore do not change the amino acid sequence of the protein the sequences code for. The rate of change in non-coding regions of the DNA can be as fast as 10% sequence divergence per million years in mammals (Irwin, et al. 1991). A similarly rapid rate of change in insects would place almost all of the divergence dates within the time range of the last glacial event. Secondly, dating the speciation events using the molecular clock is unreliable in a situation like that encountered with the *gregaria* infragroup, where radiation of the different populations has occurred within a very short span of time. The divergence time calculated for non-recombining or mitochondrial DNA will produce an estimate of the time at which a mutation caused a single form of a gene in a population to become two different haplotypes, but will not necessarily signify the separation of a single population into two. Because multiple forms of mitochondrial DNA will frequently exist within a single species and because genetic isolation will usually result in each new species becoming fixed for a different version of the existing genetic haplotypes, the speciation date produced by calculation of sequence divergence will tend to overestimate the actual date that two populations became genetically isolated.

Whether this overestimate is important depends on the overall span of time separating the two species. If two species have been separated for a period of millions of years, this overestimation is unlikely to be significant. However, if the species in

question has only recently diverged or is only partially genetically isolated, the presence of multiple forms of a fast evolving gene such as mtDNA may only reflect the natural variation that exists within a cluster of populations and can give no reliable estimate of the time at which genetic isolation has been achieved between two neighbouring populations. The degree of genetic variation found within the *gregaria* infragroup, exclusive of *N. lituyae,* is within what could be expected to be found in a single species with multiple, semi-isolated populations. The discontinuity between *N. haida* and *N. louisea* + *N. charlottae* is more likely to be an artefact of sampling then an accurate portrayal of historical events in the radiation of these populations. The close proximity of all three species, the small genetic difference between populations, and the lack of significant morphological changes among species supports this viewpoint. While the number of mutations separating *N. sahlbergii sahlbergii* and *N. lituyae* is only slightly greater than that separating *N. haida* from the beach dwelling species, *N. lituyae* is brachypterous, unlike the fully winged *N. salbergii sahlbergii*, and lives in close proximity to the latter species. This lends support to the status of *N. lituyae* as a distinct species from *N. sahlbergii sahlbergii*, although it does not rule out the possibility that *N. lituyae* is descended from a now extinct *N. sahlbergii sahlbergii* haplotype and that a recent switch in habitat preference in *N. lituyae* is all that prevents the two species from hybridizing.

CHAPTER 4: RAPD analysis

Introduction

Analysis of the mtDNA sequence divergence between the members of the *gregaria* infragroup suggests that four of the five species assigned to this taxon are very closely related and that the fifth species, *N. lituyae,* has been misassigned and should be placed close to or within the *N. sahlbergii* species group. However, the low degree of sequence divergence between the QCI members of the infragroup makes it difficult to draw conclusions about the status of the beach dwelling *N. louisea* + *N. charlottae* in relation to the alpine dwelling *N. haida*. Although the sequence data indicate that there is a genetic difference between the two populations, the mtDNA sequences for *N. haida* were obtained from a single location on Graham Island that was well separated by both distance and geography from all of the surveyed beach dwelling populations. As well, most populations of *Nebria* had only a single mtDNA haplotype, which prevented any estimation of the amount of genetic diversity in each population.

By comparing the DNA fingerprints produced by the RAPD procedure, a greater degree of resolution can be obtained for the phylogeny of the QCI populations of the *Nebria gregaria* infragroup. In addition, estimates of genetic diversity for the populations of this infragroup can be used to discriminate between different hypothesis for the colonization of the QCIs by these beetles, based on the predicted pattern of genetic variation that each method of colonizing would produce.

Materials and Methods:

The beetles used in the 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 Dr. Kavanaugh. Ten specimens of *gregaria* infragroup *Nebria* from Toe Hill, nine from Kaisun village, twelve from Graham and Moresby mountain ranges, five from Reef Island and five from Skedans Island were used in the analysis. Five specimens of *N. sahlbergii sahlbergii* obtained from low-altitude streams in Manning Park, B.C. were used for an outgroup.

DNA for RAPD analysis was extracted from the beetles using the QIAamp tissue kit using the manufacturers protocol (Qiagen, 1996) 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.

The Pharmacia Ready-to-go RAPD analysis bead kit was used to perform the RAPD reactions. Each reaction contained 6 ng of genomic DNA and 25 pmol of a 10mer oligonucleotide primer, along with a RAPD bead consisting of AmpiTAQ and Stoffel fragment thermostable polymerases, 0.4 mM each of 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. Reactions were overlaid with mineral oil to prevent evaporation. The thermocycler program used for the RAPD reactions was as follows: 1 cycle at 95° C for 5 minutes,

followed by 45 cycle of 95° C for 1 minute, 36° C for 1 minute, and 72° C for 2 minutes. 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 (Table 41) in separate reactions. Rooted neighbour joining trees were constructed for the 47 samples using the Jaccard, Nei and Li, and Sokal and Sneath options of the RAPDistance package V1.04 (Armstrong et al. 1994) to calculate genetic distance. 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 V.1.04 program (Excoffier, 1992) was used to compare variation among the six observed clusters.

Table 41: RAPD analysis primers

Primer 1 - (5'-d[GGTGCGGGAA]-3')
Primer 2 - (5'-d[GTTTCGCTCC]-3')
Primer 3 - (5'-d[GTAGACCCGT]-3')
Primer 4 - (5'-d[AAGAGCCCGT]-3')

Results:

The 77 bands produced by the RAPD procedure are summarized by population in Table 42. Across the 47 samples, only one band was uniformly present although twelve 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. louisea* and *N. charlottae* populations, each with fixation rates exceeding 70% and average heterozygosity of less then 0.090. The Kaisun village *N. louisea* showed a greater degree of heterozygosity with only 60.0% of the bands present in all individuals and an average heterozygosity of 0.132. *N. haida*, when considered as a single population, contained the greatest heterozygosity, with only 30% of the bands fixed across all twelve individuals and an average heterozygosity of 0.210, comparable to the outgroup species *N. sahlbergii sahlbergii*.

All three coefficients of genetic distance produced similar neighbour-joining trees relating the 47 samples (Figure 11). The five specimens of *Nebria sahlbergii sahlbergii* all grouped together to form an outgroup cluster with a genetic distance of either 0.166 (Jaccard), 0.134 (Nei and Li) or 0.152 (Sokal and Sneath) from the cluster containing the members of the *gregaria* infragroup. Members of the Kaisun village population of *N. louisea*, *N. charlottae*, and the eastern *N. louisea* population each formed distinct clusters in all three trees, while *N. haida* either formed a single cluster with *N. lituyae* as sister group (Jaccard, Sokal and Sneath) or was divided into two clusters, one of which included *N lituyae* as a member (Li and Nei). Moresby Island and Graham Island

populations of *N. haida* could not be differentiated in any of the trees. The four populations of the *gregaria* infragroup from the QCIs were linked in all three trees by a simple hierarchy of descent, with *N. haida* forming the sister group to the Kaisun village population. The Kaisun village population, in turn, represented the sister population to the clusters of *N. charlottae* and the eastern *N. louisea.*

Branching patterns within clusters varied slightly between the three different neighbour-joining trees but did not affect the general pattern of radiation among the 47 samples. The degree that the trees differed from random was assessed using a permutation tail probability test with 20 random trees. The three neighbour-joining trees (Jaccard, Nei and Li, and Sokal and Sneath) differed from the mean length of the random trees by 41.94, 34.31, and 39.75 standard deviations respectively, indicating that the neighbour-joining trees were not the product of randomized data (Table 43).

An analysis of molecular variance (AMOVA) using the genetic distances calculated from the three coefficients, divided the variation fairly evenly between populations and individuals (Table 44). Using the genetic distances obtained from the Jaccard coefficient, the AMOVA attributed 53% of the variance to among population while allotting the remaining 47% of the variance to within populations. For the Sokal and Sneath distance matrix, 47% of the variance was contributed from among populations while the Nei and Li distance matrix had 57.5% of the variance derived from among populations. The PHI-statistic representing the correlation of random haplotypes within populations relative to that of pairs of haplotypes drawn randomly from the whole

species, is essentially equal to the variance among populations, and was calculated as 0.534 for the Jaccard distance matrix, 0.575 for Nei and Li, and 0.468 for Sokal and Sneath. The PHI-statistics were each significant to the 99% level.

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

Table 42: Summary of RAPD statistics

Population	Population size	Fixed bands	Variable bands (2+)	Single bands	Absent	Percentage unfixed bands	Average Hetero-zygosity
Eastern *N. louisea* (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
Toe 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 Isl.)	8	19	26	8	24	64.2%	0.217
N.haida (Moresby Isl.)	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

Table 43: Permutation-Tail Probabilities

	Original tree length	Mean length random trees	SD random trees	PTP value
Jaccard	4.0480	6.5968	0.0608	41.94
Nei and Li	2.3680	3.8873	0.0443	34.31
Sokal and Sneath	6.3820	10.2030	0.0961	39.75

Table 44: Analysis of Molecular Variance:

a) Jaccard

Variance component	Variance	% Total	PHI-statistic
Between populations	0.0997	53.38	PHIst=0.534
Within populations	0.0871	46.62	

b) Nei and Li

Variance component	Variance	% Total	PHI-statistic
Between populations	0.0681	57.50	PHIst=0.575
Within populations	0.0503	42.50	

c) Sokal and Sneath

Variance component	Variance	% Total	PHI-statistic
Between populations	0.1248	46.84	PHIst=0.468
Within populations	0.1416	53.16	

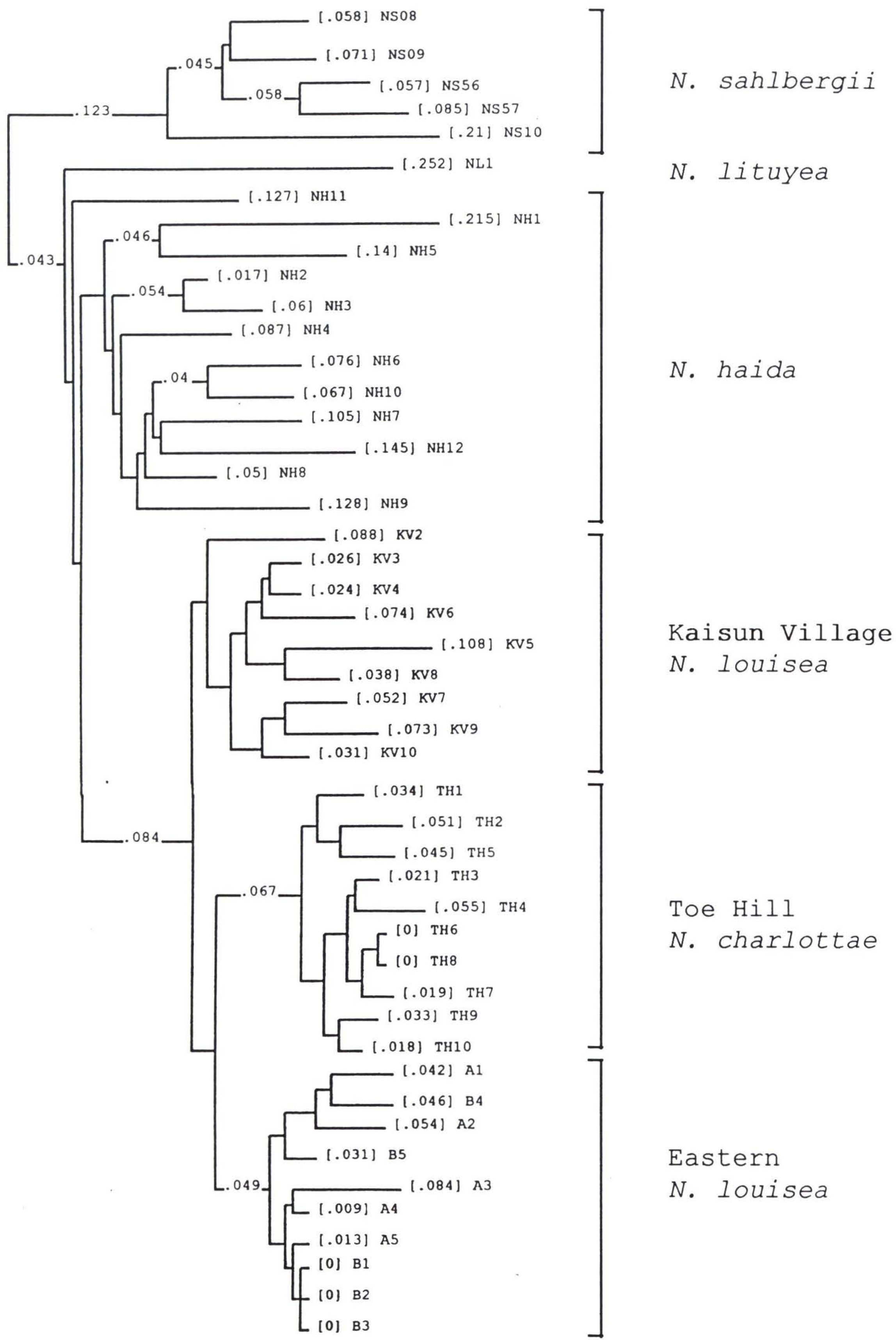


Figure 11a. Neighbour-joining tree from RAPD data using Jaccard coefficients of relatedness.

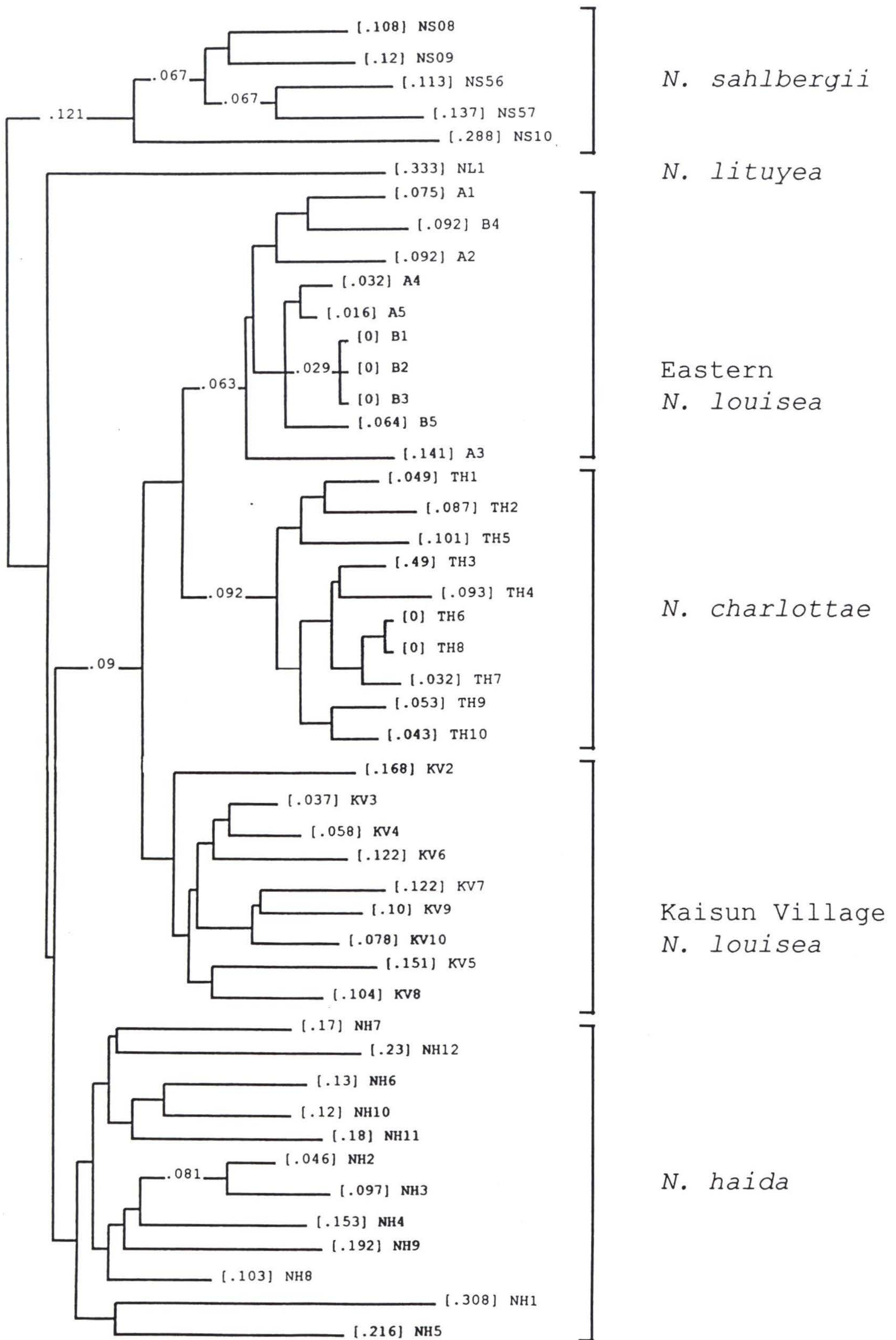


Figure 11b. Neighbour-joining tree from RAPD data using Sokal and Sneath coefficients of relatedness.

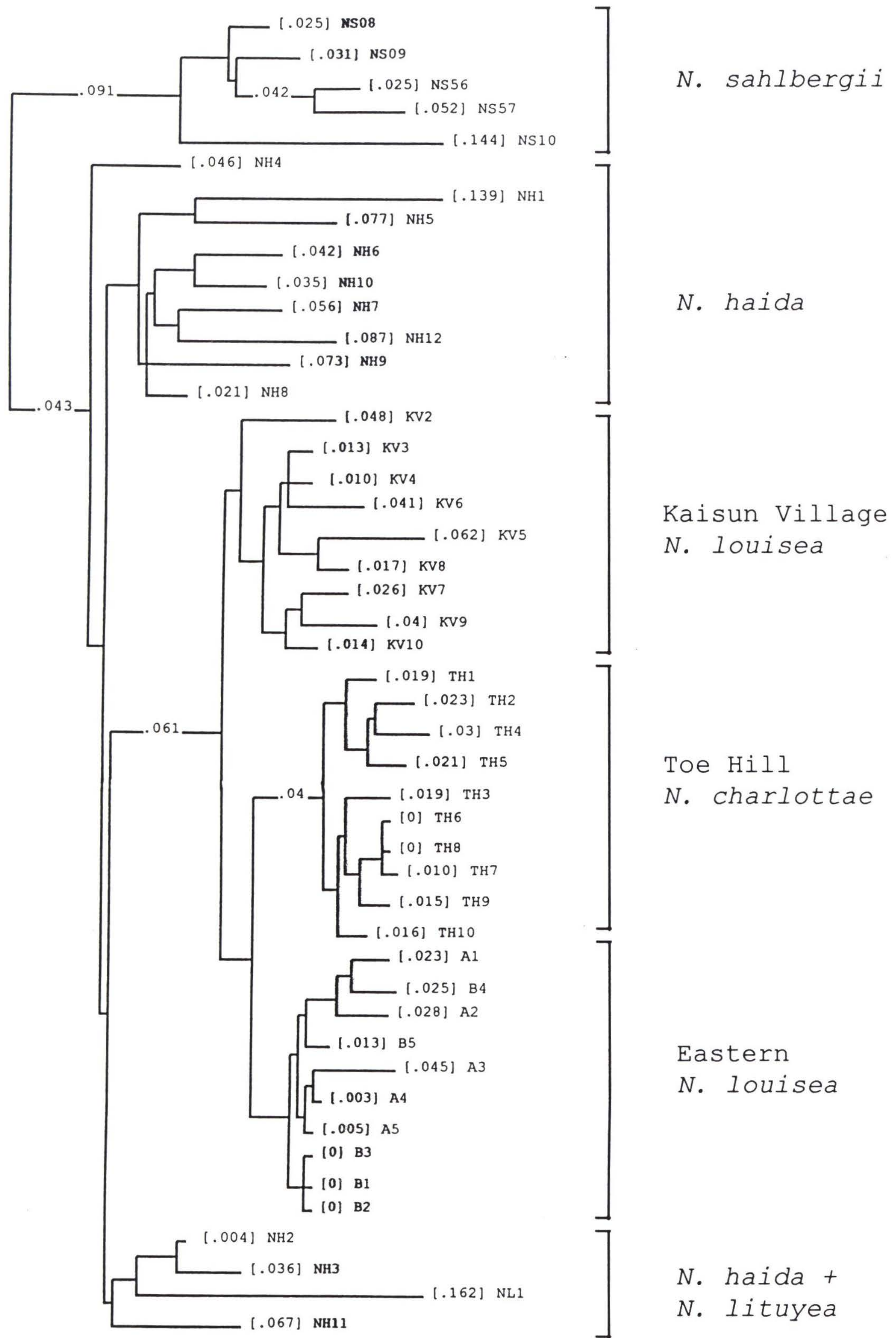


Figure 11c. Neighbour-joining tree from RAPD data using Nei and Li coefficients of relatedness.

Discussion:

The picture of radiation obtained from the RAPD data shows a greater degree of detail than could be obtained from mtDNA sequence analysis. Although the nature of RAPD bands does not allow an estimation of the timing of separation events between haplotypes, as is possible with sequence data, it does allow conclusions to be drawn on the pattern of variation present within the nuclear DNA of a species and the mechanisms that may be acting at a population level that would produce the observed patterns. Where a population of haplotypes displays a low degree of genetic differentiation between members, it can be assumed that the population is interbreeding with few barriers to reproduction. Where higher values of genetic distance exist that clearly cluster individuals into groups, it can be concluded that a barrier to gene flow exists at some level between the populations or species surveyed. When such a genetic division exists within a single population, mechanisms of partitioning based on microhabitat, mating preference, or selection against hybrids can be envisioned. However, when genetic divisions exist between closely related yet physically separated populations, as is observed within the *gregaria* infragroup, it is reasonable to conclude that the immediate barrier to interbreeding is the physical obstruction and may not necessarily represent the existence of a post-mating barrier to gene-flow should individuals within the two populations ever come into contact.

The three neighbour-joining trees produced from the RAPD data set show a nested hierarchy of populations within the *gregaria* infragroup and a relationship between

populations similar to that found in the sequence data. The *gregaria* infragroup forms a distinct cluster clearly separate from its near relative, *N. sahlbergii sahlbergii* with *N. lituyae* as the sister to the QCI populations. Within the QCI cluster, *N. haida* emerges as the sister to the beach dwelling species, with *N. charlottae* and the eastern population of *N. louisea* the most derived populations. No differentiation could be made between Skedans Island and Reef Island populations of the eastern *N. louisea*, indicating that the inlets and passages between these islands are not a barrier to gene flow. In contrast, a barrier to gene flow does exist between eastern and western populations of *N. louisea*, demonstrating that little if any gene flow exists across the land-mass of Moresby Island. Unexpectedly, the *N. haida* samples did not cluster according to the mountain chain from which they were obtained, despite the significant barrier that the intervening lowland and Skidegate channel would present to an apterous alpine insect. The clustering of all *N. haida* samples within a single population implies a very recent division into the present Moresby and Graham Island populations, with little time for the accumulation of genetic differences. The placement of *N. charlottae* within the species *N. louisea* supports the contention of the sequence data that the population designated *N. charlottae* should not be considered as distinct from those populations labelled *N. louisea,* despite the small morphological difference between them.

There are two possible explanations for the pattern of branching observed among the populations of the *gregaria* infragroup on the QCIs. The first is that a single ancestral population became subdivided during the deglaciation period at the end of the most recent ice age into a large alpine population containing the majority of the genetic

diversity and a number of small isolated populations that occupied beach habitats. The effects of genetic drift on the small beach populations would quickly reduce their genetic diversity while allowing the fixation of alleles that were uncommon in the original population, producing the distinctive eastern *N. louisea*, Kaisun *N. louisea*, and *N. charlottae*. The alpine population, in contrast, would be able to maintain much of its genetic diversity if it were sufficiently large and would therefore not show a division between populations recently isolated on separate mountains. The most likely mechanism for such a subdivision would be the movement of the cold-adapted ancestral population into the mountains in response to warming temperatures, with smaller populations washed down to or trapped in the lowland glacial till during the deglaciation of the QCIs.

What is not clear from this explanation is why the Kaisun village population occupies an intermediate position, both on the neighbour joining trees and in terms of genetic diversity, considering its isolated location on the west side of Moresby Island. One possible explanation is that the Kaisun village *Nebria* are not as isolated as they appear to be, and that other populations of beach dwelling *Nebria* belonging to the *gregaria* infragroup exist along the west coast of Moresby. Gene-flow between the populations would serve to maintain the genetic diversity at a level greater than that possible in an isolated population like *N. charlottae*. Limited unidirectional gene flow could also theoretically exist between the mountain populations and the beach populations in the form of beetles washed down by streams or rock-slides onto the beaches below. This would not only boost the genetic diversity of the lowland populations, but also account for the intermediate position of Kaisun village *N. louisea*

between *N. haida* and the eastern *Nebria* populations. The existence of other west-Moresby *N. louisea* populations and downward gene-flow from the mountains is only hypothetical - sampling from the western beaches and the mountains of Moresby Island has been minimal due to the isolation and the difficulties entailed in surveying these areas (Kavanaugh, 1992 and pers. observations).

The second possible explanation for the pattern observed in the neighbour joining trees is that the differentiation of the alpine from the beach dwelling populations occurred separately and preceded the differentiation of the three beach dwelling populations. With this scenario, the accumulation of differences over time replaces genetic drift acting on small populations as the mechanism by which the populations become genetically distanced from each other, and the pattern of branching represents the chronological order in which the populations became isolated from each other. The lack of a genetic separation between Moresby and Graham Island *N. haida* populations serves both as a calibration and a drawback for this theory, indicating that the various populations became isolated well before the end of the ice age (the presumed time at which *N. haida* became split between the two mountain chains), but producing a chronology for the infragroup that is both excessively old and not in accordance with the observed sequence difference. The weight of the combined evidence would therefore favour a recent splitting off of the beach-dwelling *Nebria* from the alpine populations and a consequent reduction in genetic diversity among the former populations compared to the latter.

CHAPTER 5: Discussion

Since Lindroth (1961) first described *Nebria charlottae* from a single specimen collected on Graham Island, the existence of the *gregaria* infragroup on the Queen Charlotte Islands has been a doubly unusual phenomenon. The postulated existence of a group of five predacious ground beetle species that seemed to have evolved rapidly during the Pleistocene period stood in stark contrast to the accepted wisdom put forward by Matthews (1980) and others, that almost all speciation amongst northern carabid beetles had occurred during the Tertiary, and that the Pleistocene period, with its glacial advances and associated upheavals, had resulted in population range shifts, rather than speciation, as the insects tracked their preferred habitats in the wake of glacial movements. That three of these five *Nebria* species existed on an isolated cluster of islands only recently relieved from the weight of the glaciers further deepened the mystery. With the publication by Kavanaugh (1984) of an interpretation of the five species and their relationship to each other, the mystery seemed to be solved. The beetles had radiated gradually, in a process of repeated movement from the genetic isolation of the mountain tops to the lowland hills where populations could quickly expand their ranges, driven by the rhythm of glacial advance and retreat. Their presence on the Queen Charlotte Islands signified a glacial refugium and highlighted the destructiveness of an ice age that had apparently wiped out much of a far-ranging *Nebria* species group, leaving only a few scattered remnants along the north-west coast of North America.

This study, however, has produced data that require a modification of

Composition of the *gregaria* Infragroup

Under the biological species concept (Dobzhansky, 1937) a population or group of populations can only be considered a species if they are reproductively isolated from all other population groups. While in practice, limited gene flow between nominal species can occur, most geneticists will accept a population as a valid species if the transgression of reproductive isolation is sufficiently limited or localized to only a fraction of the organism's range and the species is otherwise well established as being an old or discrete lineage (Mayr, 1969; Avise, 1994). The difficulty that most morphological taxonomists face is that there is often no clear knowledge of the extent of gene flow or the genetic distance between populations. In applying the biological species concept, the traditional taxonomist uses differences in morphology to estimate the degree of genetic divergence, and applies the labels of species, subspecies, and race based on his or her own concept of the rate of morphological evolution. Amongst some taxonomists, there is a tendency to ascribe a large amount of significance to single character differences between populations, and to define species on the basis of subtleties. This can result in a huge number of described 'species' in biological species that display local variants or ecological morphs. Examples are the *Heliconius* butterflies, wherein 700 named species and subspecies (Neustetter 1929) have been over time reduced to 54 valid species due to both careful re-evaluation of morphological characters and to genetic analysis (Brower, 1996); and the tiger beetles, where in Canada alone 135 specific and subspecific names have been applied to what are only 27 species (Bousquet, 1991). The opposite mistake, that of grouping together two or more biological species under the

same specific name, can occur in those instances where a complex of species has undergone little morphological divergence despite millions of years of reproductive isolation. Examples within the invertebrates are the cryptic species complexes of the blackflies (Rothfels, 1979), Sicilian stick insects (Bullini and Nascetti, 1987), and bark beetles (Bright, 1976).

This does not mean that morphology cannot indicate either relationship or degree of genetic divergence, only that caution must be taken when interpreting morphological evidence if there is reason to believe that other factors may be influencing the rate and direction of morphological evolution. Within the *Heliconius* butterflies, Mullerian mimicry rings maintain strong selection for a different, easily recognizable wing pattern in each of the various regions of South America, resulting in as many as 21 highly divergent 'races' in a single species of butterfly (Brower, 1996). In the whitefish (*Coregonus clupeaformis*) and in the rainbow smelt (*Osmerus mordax*), selection for different trophic niches has produced divergent 'dwarf' and 'normal' forms despite less than 12,000 years of reproductive separation (Pigeon et al. 1997; Bernatchez, 1997). Similarly, rapid changes in behaviour and host preference have been recorded in Goldenrod gall flies as a response to the host-searching behaviour of its parasite, *Eurytoma obtusiventris* (Brown et al. 1996) and in QCI stickleback size and lateral plate numbers in response to predation pressures (Reimchen 1994).

Compared to the above examples, the degree of morphological variation within the three QCI *gregaria* infragroup species is very small. *N. charlottae* is the most easily

differentiated of the three, due to the nearly right-angle formed at the basal corners of the pronotum and the corresponding short, deep basal sinuation of the lateral margin. Other defining characters include slightly broader elytra, flat elytral intervals, and shorter legs. *N. louisea* possesses a more obtuse basal corner on the pronotum and a shallower basal sinuation, longer apical pronotal angles, slightly rounded elytral intervals, slightly narrower elytra, and slightly longer legs. *N. haida* is very similar to *N. louisea* but has shorter apical pronotal angles and a slight colour difference in the tibia (Kavanaugh, 1992). The difference in habitat preference appears to be the most crucial factor in separating the latter two species.

Determining species on the basis of morphological subtleties is a common practice in insect taxonomy and the shape of the pronotum is one of the primary sources of characters for separating species within the larger genera of Carabids (Lindroth, 1961). Species definitions, however, generally hinge on characters of the male genitalia, under the assumption that the genitalia operate under a 'lock-and-key' mechanism. It is the requirement of the female insect that the male genitalia have a particular shape that provides for taxonomists the 'guarantee' of reproductive isolation necessary for the biological species concept. However, neither Kavanaugh nor Lindroth have mentioned differences in the genitalia of the *gregaria* infragroup that could be used to define species. Without the presence of shape variations in the genitalia between nominal species among the *gregaria* infragroup, only geographic isolation, differential habitat preference, and small morphological differences exist to subdivide the infragroup into five species. Of the three, only habitat preference is likely to signify an actual case of

long term reproductive isolation.

With the addition of the genetic data, the position put forward by Kavanaugh that five distinct species exist within the *gregaria* infragroup is no longer tenable. Excluding for the moment consideration of *N. lituyae*, which does not appear to belong in the *gregaria* infragroup, the RAPD data indicate that the populations of beetles are to some degree genetically distinct. However both the degree of similarity between the RAPD profiles of different populations and the almost identical mitochondrial sequences imply that should the populations ever be brought back into contact with each other, there would be little reason to expect that they could not fully interbreed without detrimental effect on the 'hybrid' progeny. The isolation of individual populations belonging to the same species of *Nebria* on separate mountain tops for thousands of years during interstadial periods has been a recurring feature of this genus' history (Kavanaugh, 1978) and has certainly contributed to the speciation of this group. However, the very fact that this genus contains only 55 species and not a hundred times that figure indicates that during cooler climatic periods, populations of beetles from different mountain tops are capable of freely intergrading at lower altitudes with minimal reproductive isolation. Thus, the most conservative interpretation of the data is that four of the five species originally designated within the '*gregaria*' infragroup belong instead to a single species, *Nebria gregaria,* and that variation in mitochondrial sequence RAPD bands is a result of post-glacial segregation of the existing mitochondrial and nuclear haplotypes into separate and now semi-isolated populations.

A more liberal interpretation of the data would be that the genetic separation between *N. haida / gregaria* and *N. charlottae / louisea*, while small, is too great to consider these populations of the *gregaria* infragroup as a single monolithic species and instead a second species, *N. charlottae*, should be retained into which should be placed the populations formerly designated *N. louisea*. The evidence for such an interpretation would be the estimated date of separation of the two haplotypes (160 - 220 thousand years) and the pattern of nuclear genetic relationships revealed by the RAPD data which places *N. charlottae / louisea* as a distinct yet subordinate cluster of populations to *N. haida*.

This question of whether the *gregaria* infragroup comprises one or two species hinges on the reasons behind the difference in preferred habitats between *N. haida* and the beach dwelling populations. The pattern of response observed in carabid beetles to changes in the environment produced by the Fraser glaciation has been to shift their distribution or to become extinct. This is a pattern that has been described for a wide range of Carabid species including other *Nebria* (Morgan and Morgan, 1980; Schwert and Ashworth, 1988; Kavanaugh, 1988), and is believed to have contributed to the lack of speciation that has occurred within the northern members of this family during the entirety of the Pleistocene (Matthews, 1980). Against this background of species that have remained static in their environmental preferences for a period of two to three million years, the appearance of a population of beetles that have evidently undergone a recent shift in habitat from alpine to beach-dwelling is highly unusual. Alpine and montane river banks are assumed to be the ancestral habitat preference, as they are the

habitats of the sister species *N. sahlbergii*, *N. acuta*, and *N. arkansana*.

If this shift in habitat is part of an underlying genetic change in preference and adaptation, then there may be some grounds for supporting two different species within the *gregaria* infragroup on the QCIs. However, it has been previously mentioned that habitat choice in *Nebria* is primarily a question of optimal temperature, not of optimal altitude, and high-altitude species have been found at lower then normal elevations where conditions have conspired to produce suitably cool microclimates (e.g.., in the shade of bridges over streams) (Kavanaugh, 1978). If the cobble-beach environment of the QCIs, under the cooling influence of the ocean, can produce a suitably cold environment to allow the beetles to survive, then the presence of *Nebria* at such a low altitude may not be unusual at all and the difference in habitat between *N. haida* and the *charlottae-louisea* populations cannot be used to uphold these two populations as separate species. Until a better understanding of the environmental requirements of these beetles can answer whether the beach dwelling species are physiologically reproductively isolated, the most parsimonious conclusion would be that the members of the *gregaria* infragroup should be considered a single species displaying local population-level variances in morphology and genetics.

Colonization of the Queen Charlotte Islands

The assimilation of the various disparate populations of the *gregaria* infragroup exclusive of *N. lituyae,* into a single *Nebria gregaria* species greatly simplifies but does not itself answer the question of whether the QCIs served as a refugium for these beetles during the last glacial advance. If a post-glacial colonization of the QCIs by the *gregaria* species is to be assumed, there are three possible sources from which the beetles may have arrived on the islands. Beetles may have migrated down the coast from refugia in the Aleutians and Beringia, somehow bypassing the beach and mountain habitat of the Alaskan panhandle in favour of crossing the Hecate Strait to colonize the QCIs. Alternatively, beetles may have migrated northward following the retreating glaciers, from refugia either in the Cascade mountains or in the Rocky mountains. If the latter, the beetles must have at some point crossed over into the Coastal Range at or before Prince Rupert before continuing their northward migration. Finally, the member populations of *N. gregaria* may have survived the ice age in refugia located in the vicinity of the QCIs and on the unglaciated Aleutian Islands.

To evaluate the possible theories of the *gregaria* presence on the Queen Charlotte Islands, the patterns of post-glacial colonization of North America by other Carabid species must be examined. Comparisons of fossil and extant ranges of northern Carabid beetles by Danks (1981), Schwert and Ashworth (1988), and Morgan and Morgan (1980) indicate that the carabid fauna of Canada up to the northern-most boreal forest are composed of species that had sheltered south of the ice sheets during the last glacial

advance. Few of the carabid beetles that sheltered in the Beringia refugium have been capable of extending their range east of the MacKenzie River, and none have been demonstrated to have headed any significant distance southwards. The few arctic species located in more southerly latitudes of Canada represent survivors of a large community of tundra dwelling beetles existing at the edge of the ice sheet which were largely obliterated during the process of climate warming and glacial retreat. More temperate species of beetles dwelling in comparatively sheltered areas in Beringia are believed to have become assimilated into their sister populations advancing northwards behind the ice-sheet, and not to have contributed greatly to the re-colonization of North America (Elias, 1992). Thus, a southward land-based invasion of the coastal Alaska mountains and the QCIs from a refugium in the Aleutian islands (the only current location of these beetles that is known to have been free of glacial ice) is very unlikely given the trends in post-glacial Carabid migration, and can be excluded from serious consideration. This does not mean, however, that populations of *N. gregaria* could not have passed the last ice age within the Aleutian Islands - given the absence of *N. gregaria* representatives in southern Alaska, the existence of an Aleutian population during the glacial advance would explain the disjunct distribution of this group.

A postulated southern origin of the *N. gregaria*, while more likely than a northern invasion, raises other questions. A comparison of the response of beetles adapted to lowland conditions (Morgan and Morgan, 1980) with that of *Nebria* sp. (Kavanaugh, 1979b) to glacial retreat demonstrates two very different patterns in the expansion of their respective ranges. With the onset of climate warming and the shift of optimal habitats

northwards, lowland beetles abandoned their southern localities and migrated north. *Nebria*, in contrast, responded to climate warming and glacial retreat by extending their ranges both northwards along the mountain ranges and vertically up the mountainsides. Thus, while the glacial range of lowland beetles must be inferred from the discovery of fossil specimens, populations of *Nebria* sp. will continue to occupy their original glacial locations, albeit at higher altitudes than they would have been found during the glacial maximum. From this information, the glacial age refugia of the three other *gregaria* group species occurring in Canada can be located. Based on the distribution maps of Kavanaugh (1979c), *N. acuta* can be clearly traced to a refugium in the Cascade Mountain range of northern Washington State, *N. arkansana* can be traced to a refugium in the Rocky Mountains, while *N. sahlbergii sahlbergii* has populations in both refugial areas, and may have spread from one, the other, or both. Despite extensive collecting in both the Cascades, southern Coastal Mountains, and the southern Rocky Mountains, no specimens belonging to the *gregaria* infragroup have been recorded from these regions in the literature. It is possible that *N. gregaria* existed at the glacial edge and moved northwards following the retreat. This however ignores both the absence of any known correlation between *N. gregaria* and glacier environments and the presence of other *Nebria* species that are associated with glaciers and which have extended, not shifted, their ranges northwards as a response to ice retreat. A second possibility is that *N. gregaria* was extirpated by climate change or eliminated by competition from ancestral habitats. This would again be highly unusual, considering that *N. gregaria* is not at the extreme end of environmental tolerance, nor has competition been found to display a significant role in determining the subdivision of habitats within this genus (Gereben,

1995). The absence of populations of *N. gregaria* from any of the potential southern ice age refugia remains a major difficulty in postulating a post-glacial occupation of the QCIs and the Alaska panhandle.

A lack of evidence for the post glacial migration of *N. gregaria* onto the QCIs does not in and of itself validate the hypothesis that there existed a western coastal refugium for this species during the last glaciation. As has been reviewed previously, the geology and climate of the QCIs created a very inhospitable environment during the height of the Fraser glaciation. Very little of the current surface area of the islands would have been free of ice, and much of that would have been too cold and dry to support these insects. While a single site of plant deposits on eastern Graham Island has been dated to 16,000 years B.P., these deposits overlay thick layers of glacial till, presumably left during the early deglaciation of the Queen Charlotte mountains. However, geological evidence on the changes in sea levels associated with the glacial period has indicated that the offshore banks of the Hecate Strait would have been emergent above sea level and could have served as a habitat for various forms of life (Barrie et al. 1993; Josenhans et al., 1995). Further supporting evidence that a refugium may have existed in the now submerged Hecate Strait comes from the molecular work of Byun et al. (1997) who found distinct coastal lineages in two different species of mammals ranging up to but no further north then the QCIs and the southern Alexander Archipelago. Rapid colonization of the QCIs by the coastal lineages before the inundation of the Hecate Strait would indicate that the coastal bears and marten had been residing in a coastal refugium and not in north-western Washington State.

In terms of the *gregaria* infragroup, the unusual distribution of *N. gregaria* on the QCIs, north of Prince Rupert, and on the Aleutian islands would indicate that an ancestral population with a wider distribution had been extirpated from much of its mainland habitat and had sheltered during the ice age in refugia at the extreme north-western and southern ends of its range. The distribution of *N. gregaria* on both the QCIs and the mountains of the adjacent mainland would be the expected distribution if the southern refugium had been situated in the Hecate Strait, allowing populations to migrate into the mountains on either side as the glacial refuge became inundated by the ocean during deglaciation.

From the pattern of branching in the neighbour-joining tree constructed from the RAPD data, it can be seen that the beach dwelling populations of *N. gregaria* are all derived from the mountain populations, and that the beach dwelling populations appear to have diversified from a limited subset of the total gene-pool available in the '*haida*' populations. If the QCIs had been colonized from refugia south of the ice sheet, it would be expected that either the beach-dwelling populations would represent the basal cluster from which the mountain species had been derived, or the different populations of *Nebria gregaria* on the QCIs would show a more random pattern of associations between individuals without the distinct clustering observed in the neighbour-joining tree.

The neighbour-joining tree instead points towards a large population of beetles surviving on an offshore refugium and existing at the glacial edge. As the ice retreated, the beetles migrated up the mountain sides behind the glaciers, with small populations

either left behind on the cobble beaches, or washed down as the glaciers melted. Those beetles that migrated into the mountains would form the beetle populations designated '*N. haida*' while the populations that remained at sea level would become the '*N. charlottae*' and '*N. louisea*'. The high heterozygosity of the alpine population relative to the beach dwelling population and the lack of a genetic break between Moresby and Graham Islands populations indicates that the mountains still contain the bulk of the ancestral genetic diversity, of which only a small subset remains on the cobble beaches.

The role of the *N. gregaria* infragroup as evidence of a Hecate Straight refugium is aided by the presence of *N. haida* on both the QCIs and the adjacent mainland. However, the presence of only alpine dwelling members of the *gregaria* infragroup on the mainland adjacent to the QCIs is unusual, given that the distributions of most other refugial taxa typically include northern Vancouver Island and the adjacent mainland in addition to the QCIs. The lack of *gregaria* populations on the beaches of the mainland may simply reflect a lack of sampling in this region, low amounts of suitable cobble-beach habitats (D. Kavanaugh, pers. com.), or historical differences in the deglaciation and subsequent colonization of the mainland and QCIs. However, the possibility also exists that the mainland *N. haida* represents a different genetic lineage not closely related to the QCI *Nebria*. If the mainland *N. haida* has undergone a separate origin and the *gregaria* infragroup, as considered in this study, is in fact restricted to the QCIs, it would indicate that suitable habitat existed in the vicinity of the QCI archipelago, likely at the glacier edges, but would say little about the nature, extent, or even existence of a larger Hecate Straight refugium. A molecular examination of the mainland *N. haida* would

clarify the phylogenetic position of this population and allow a more accurate assessment of the ancestral range of the *gregaria* infragroup.

The preceding discussion has assumed that any hypothetical colonization by the members of the *N. gregaria* infragroup of the QCIs would have involved terrestrial migration. There are, however, two other possible modes by which these *Nebria* may have reached the QCIs: long distance transport of colonists on floating logs and human mediated transport onto the islands. With regards to the first scenario, the genetic homogeneity of the beetle populations on the islands east of Moresby Island indicate that the beetles can be transported across water for short distances. However, the long-distance overseas transport of colonizing *Nebria* must take into account the location of a source population, the distance that must be traversed (which will affect the potential for the colonists survival), and the direction of ocean currents.

The nearest extant source population for the *N. gregaria* infragroup is on the Aleutian Islands, requiring that the beetles survive an ocean journey of at least 1900 km to reach the QCIs. No evidence exists of source populations to the south of the QCIs, nor is it likely that a beach dwelling source population may have existed and later gone extinct, given the number of other beach-dwelling insects that have successfully migrated northwards following the glacier retreat. Summer ocean currents along the northern Pacific coast of North America typically travel in a north-westerly direction, while currents in the vicinity of the Aleutians move either westward or north between the islands (Couper, 1983). The effect of these currents would be to increase the effective

distance that *Nebria* would have to travel to reach the QCIs, while favouring Kamchatka as the likely landing area for any rafting Aleutian beetles. Instead, it is more likely that the Aleutian Islands populations of *N. gregaria* are the result of long-distance dispersal of beetles from the QCIs, following the direction of the prevailing ocean currents. Such a colonization of the Aleutians by populations of QCI *Nebria* would explain both the disjunct distribution and the genetic similarity of these two populations, although the lack of other *N. gregaria* populations along the south Alaska coast would have to be accounted for.

The possibility of human assisted transport of *N. gregaria* to the islands by the native Haida is difficult to assess. Given the location of most native villages close to the shoreline and the importance of canoe travel to their culture, inter-island and island to mainland transport of beetles is a possibility. The absence of *N. gregaria* populations on the mainland, despite no obvious barriers to establishments, would indicate, however, that this has been an unsuccessful route of transport. Although further sampling of the mainland coast might discover *N. gregaria* populations, determining whether these populations originated from human transport or by migration from a refugium in the Hecate Strait would be difficult. Human transport of *N. gregaria* to the QCIs from the Aleutians is very unlikely, given the lack of human contact between the two archipelagos, and would not explain the existence of the mountain dwelling '*N. haida*' populations.

Nebria lituyae

The DNA sequence evidence places the species *N. lituyae* outside the *N. gregaria* infragroup and allies it with the sister species *N. sahlbergii sahlbergii*. Brachyptery, the difference in preferred habitat, and the lack of evidence for hybridization between the two species despite living in close proximity, all stand as evidence that *N. lituyae* can be considered a distinct species despite the small degree of sequence divergence between taxa. The origin of this species, however, is somewhat more enigmatic. Hypotheses involving the migration of *N. lituyae* into the Lituyae Bay region from a source in either Beringia or the United States are beset with the same problems as the hypothesis that the Queen Charlotte Islands were colonized by *N. gregaria* in the post-glacial period. The most parsimonious conclusion is that this species is the remnant of a population of *N. sahlbergii sahlbergii* that was trapped in a refugium on the Alaska pan-handle during the ice age and, as a result of this isolation, developed differences in morphology and habitat preference sufficient to allow this population to remain genetically distinct once *N. sahlbergii sahlbergii* re-colonized the area. Geological evidence used in the reconstruction of maximum glacier extent in the Lituyae Bay region indicate that ice free land could have existed in this region during the ice age (Mann and Hamilton, 1995). Although endemic species other than *N. lituyae* that could be used in support of this hypothesis are lacking, this may simply be a reflection of poor sampling in this relatively isolated region.

Summary and Conclusions

Until the publication of Moodie and Reimchen's (1976a) study documenting the range of variability in the stickleback of the QCIs, the general consensus among biologists was that the variation observed between mainland and island populations of the same species had arisen as a result of long periods of isolation in a glacial refugium. However, the variation that existed between the populations of QCI stickleback had clearly arisen in post-glacial time (Moodie and Reimchen, 1976b), opening the possibility that the differences between mainland and island populations of other animals had a similarly recent origin. Molecular studies of endemic mammals of the QCIs by Byun et al. (1997 and pers. com.) confirmed that for three species, both island and adjacent mainland forms had evolved following the retreat of the glaciers. Byun et al. were also able to identify for each of the species a distinct north-western coastal lineage centred on the archipelago that appeared to be refugial in origin.

The *Nebria gregaria* infragroup has been upheld as evidence that the QCIs served as a glacial refugium, based on their location, restricted distribution, and on differences in morphology and habitat between the five species, that were presumed to have arisen over a period of several hundred thousand years (Kavanaugh, 1989). The genetic evidence, however, indicates that the species described as *N. haida, N. charlottae*, and *N. louisea* from the QCIs are, in fact, conspecifics of the species *N. gregaria*, first described from the Aleutian Islands. The most parsimonious explanation for the unusual distribution of the members of this species is that the ancestral *N. gregaria* population survived the ice

age in refugia located on the Aleutian Islands and on the now submerged banks in the Hecate Strait. The shift in habitat preference arose as a response to the changing climatic conditions both during and after the Fraser glaciation, while the small morphological differences are most likely the result of genetic drift following the post-glacial isolation of the populations. A similar shift in habitat preference coupled with small morphological changes likely occurred independently in the *N. sahlbergii sahlbergii* species group, producing the species *N. lituyae*.

The survival of *N. gregaria* on the QCIs supports the biogeographic evidence of Byun et al. (1997) that there existed a coastal refugium separate from the refugium located in Washington State, but can provide little indication of whether conditions on such a refugium would have been sufficiently tolerable to support large omnivorous mammals. The *Nebria* as a whole are a group well adapted for survival in harsh and barren environments and can at best serve only as an indicator of the minimal conditions that would have existed on the refugium during the glacial period.

Further molecular work is required on the taxonomy of the *N. gregaria* species group in order to include the two other widely distributed species, *N. acuta,* and *N. arkansana*. This would confirm the alignment of *N. lituyae* with *N. sahlbergii* and determine more accurately the age and phylogenetic relationships surrounding the species *N. gregaria*. In relation to the question of west coast refugia, a wider range of invertebrate species needs to be surveyed for possible endemism and evidence of survival in refugia, both on the QCIs and in the Lituyae Bay region of Alaska. Previously

published surveys of insects have focused on species with a high mobility that would allow the rapid colonization of the QCIs with mainland representatives that could potentially replace endemic sister species. In contrast, many of the plant and animal lineages for which there is the most evidence of refugial survival are those that lack the ability for long range dispersal (Kavanaugh, 1992; Ogilvie, 1989). Certain species of ground spiders (e.g,, *Pardosa diuturna,* Dondale and Redner, 1990) show patterns of distribution that are endemic to the west coast and would be in keeping with a refugial origin, but have not been studied further beyond the casual accumulation of collection information. A wider survey of such fauna and flora, showing restricted ranges and low vagility, combined with the continued examination of the top predators of the QCIs, would provide a clearer picture of the kind of environment that existed during the ice age on the now-submerged banks off the north-west coast of North America.

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Appendix: Sequence Data

NADH subunit 1 fragment

```
N. gregaria         ATCCCTATATAACCTACCTTATTAGGACCTTTACGAATTTGAATATAACCTAAAACTTTA
N. haida            ............................................................
N. charlottae       ............................................................
N. louisea (KV)     ............................................................
N. louisea (SKA)    ............................................................
N. louisea (SKB)    ............................................................
N. sahlbergii       ............................................................
N. lituyea          ............................................................
N. diversa          ..A...................................C.....G...............
N. gebleri          ..A........C..C............GCC...............A..............

N. gregaria         CGTTCTAATAATGTTAAAAAAGCAACCCCCACTAAAACACAAATTAATAATAATAATATA
N. haida            ............................................................
N. charlottae       ............................................................
N. louisea (KV)     ............................................................
N. louisea (SKA)    ............................................................
N. louisea (SKB)    ............................................................
N. sahlbergii       ...............................................C............
N. lituyea          ...............................................C............
N. diversa          ...........C.................T....T......G.....C....T.......
N. gebleri          ..........................T..T..............................

N. gregaria         CAAATTAAAGAAAATATAATATCTATATAAAACAAGTATTATTTGTAATAAAAATTACAT
N. charlottae       ............................................................
N. haida            ............................................................
N. louisea (KV)     A.......................G...................................
N. louisea (SKA)    A...........................................................
N. louisea (SKB)    A...........................................................
N. sahlbergii       ............................................................
N. lituyea          ............................................................
N. diversa          .................T..........................................
N. gebleri          ..........GG.....T..................G....C..................

N. gregaria         TTATAAATTCTAAATTTACTGCACTAATCTGCCAAAATAATTAATAATAATCAAAATATA
N. haida            ............................................................
N. charlottae       ............................................................
N. louisea (KV)     ............................................................
N. louisea (SKA)    ............................................................
N. louisea (SKB)    ............................................................
N. sahlbergii       ............................................................
N. lituyea          ............................................................
N. diversa          A.................T......................................T..
N. gebleri          ....G...........C.T.........................................

N. gregaria         AATATT--TATATTAAATAATTGGTCCTTTCGTACTGAAATATTTTAATTTACTAAAGAT
N. haida            ......--....................................................
N. charlottae       ......--....................................................
N. louisea (KV)     ......--....................................................
N. louisea (SKA)    ......--....................................................
N. louisea (SKB)    ......--....................................................
N. sahlbergii       ......--....................................................
N. lituyea          ......--....................................................
N. diversa          ......--..A.................................................
N. gebleri          ......AA.....C......................A.......................
```

```
N. gregaria          AGAAACCGACCTGGCTTACACC
N. haida             ......................
N. charlottae        ......................
N. louisea (KV)      ......................
N. louisea (SKA)     ......................
N. louisea (SKB)     ......................
N. sahlbergii        ......................
N. lituyea           ......................
N. diversa           ......................
N. gebleri           ......................
```

Cytochrome oxidase subunit 1 fragment

```
N. gregaria        TTTAATATTAGGAGCTCCTGATATAGCATTTCCTCGAATAAATAATATAAGTTTTTGAT
N. haida           ...........................................................
N. charlottae      ...........................................................
N. louisea (KV)    ...........................................................
N. louisea (SKA)   ...........................................................
N. louisea (SKB)   ...........................................................
N. sahlbergii      ...................................................C.......
N. lituyea         ...........................................................
N. diversa         A...........G.................C.......................C....

N. gregaria        TATTACCTCCTTCTTTAACTCTTCTTTTAATAAGTTCAATAGTAGAAAGAGGAGCAGGAA
N. haida           ............................................................
N. charlottae      ............................................................
N. louisea (KV)    ............................................................
N. louisea (SKA)   ............................................................
N. louisea (SKB)   ............................................................
N. sahlbergii      ...................C........................................
N. lituyea         ...................C........................................
N. diversa         .......A.....A.....T........................................

N. gregaria        CAGGATGAACAGTATACCCTCCTTTATCATCTAGAATTGCTCATAGAGGAGCATCTGTAG
N. haida           ............................................................
N. charlottae      ............................................................
N. louisea (KV)    ............................................................
N. louisea (SKA)   ............................................................
N. louisea (SKB)   ............................................................
N. sahlbergii      .......................C....................................
N. lituyea         .......................C....................................
N. diversa         ....T........T.....A.............AT..C..C...................

N. gregaria        ATTTAGCTATTTTTAGCTTACATCTAGCTGGAGTATCATCAATCTTAGGTGCAGTAAATT
N. haida           .....................................G......................
N. charlottae      ............................................................
N. louisea (KV)    ............................................................
N. louisea (SKA)   ............................................................
N. louisea (SKB)   ............................................................
N. sahlbergii      ............................................................
N. lituyea         ............................................................
N. diversa         ..........C.....A......T.......G.....T.....TC....G..........

N. gregaria        TTATTACTACAATTATTAATATACGATCAATTGGAATAACATTCGATCGAATACCTTTAT
N. haida           ............................................................
N. charlottae      ............................................................
N. louisea (KV)    ............................................................
N. louisea (SKA)   ............................................................
N. louisea (SKB)   ............................................................
N. sahlbergii      ............................................................
N. lituyea         ............................................................
N. diversa         ....C..............................G.......T..C.............
```

```
N. gregaria         TTGTCTGATCAGTTGGAATTACAGCATTATTATTATTATTATCATTACCTGTTTTAGCTG
N. haida            ............................................................
N. charlottae       ............................................................
N. louisea (KV)     ............................................................
N. louisea (SKA)    ............................................................
N. louisea (SKB)    ............................................................
N. sahlbergii       ...................................C....G...................
N. lituyea          ........................................G...................
N. diversa          ....A.................T.........C.....C.T.................A.

N. gregaria         GAGCTATTACTATACTTTTAACTGATCGAAATTTAAATACATCATTTTTTGACCCTGCAG
N. haida            .G..........................................................
N. charlottae       ............................................................
N. louisea (KV)     ............................................................
N. louisea (SKA)    ............................................................
N. louisea (SKB)    ............................................................
N. sahlbergii       ............................................................
N. lituyea          ............................................................
N. diversa          .........................C......C..........T........T.....T.

N. gregaria         GAGGAGGAGATCCTATTTTATACCAACATTTATTTTGATTCTTTGGCCACCCA
N. haida            .....................................................
N. charlottae       .....................................................
N. louisea (KV)     .....................................................
N. louisea (SKA)    .....................................................
N. louisea (SKB)    .....................................................
N. sahlbergii       .....................................................
N. lituyea          .....................................................
N. diversa          ..........C...........T.......................A......
```

Cytochrome B fragment

```
N. gregaria         ATTTTGAGGAGCTACAGTAATTACAAATTTATTATCAGCAATTCCTTATTTAGGAACAAT
N. haida            ............................................................
N. charlottae       ............................................................
N. louisea (KV)     ............................................................
N. louisea (SKA)    ............................................................
N. louisea (SKB)    ..................T.........................................
N. sahlbergii       ............................................................
N. lituyea          ............................................................
N. diversa          ............C........C....................C.....C........T..
N. gebleri          ........................T....................A...........T..

N. gregaria         AATAGTACAATGAGTATGATGAGGATTTGCTGTTGATAATGCTACTTTAACACGATTTTT
N. haida            ............................................................
N. charlottae       ............................................................
N. louisea (KV)     ............................................................
N. louisea (SKA)    ............................................................
N. louisea (SKB)    ............................................................
N. sahlbergii       ............................................................
N. lituyea          ..................G.........................................
N. diversa          .................................A..........................
N. gebleri          ......T...................................A..............C..

N. gregaria         TACTATTCATTTCTTATTACCATTTATTGTTACAGCTATAGTTATAATTCACCTTTTATT
N. haida            ................................................C...........
N. charlottae       ................................................C...........
N. louisea (KV)     ................................................C...........
N. louisea (SKA)    ................................................C...........
N. louisea (SKB)    ................................................C...........
N. sahlbergii       ............................................................
N. lituyea          ............................................................
N. diversa          ..................G...........A.................C......C....
N. gebleri          ............T...C...........A........T..A...........T.AC....

N. gregaria         TCTACATCAAACAGGATCTAATAACCCATTAGGAATTAATAGAAATATTGATAAAATTCC
N. haida            ............................................................
N. charlottae       ............................................................
N. louisea (KV)     ............................................................
N. louisea (SKA)    ............................................................
N. louisea (SKB)    ............................................................
N. sahlbergii       ............................................................
N. lituyea          ............................................................
N. diversa          ......C...........A.....T..TC...............................
N. gebleri          .T.............G..A..............GG.........................

N. gregaria         TTTTCACCCATATTTCTCTTATAAAGATATTATAGGATTTATTATTTTAATAATAACTTT
N. haida            ............................................................
N. charlottae       ............................................................
N. louisea (KV)     ............................................................
N. louisea (SKA)    ............................................................
N. louisea (SKB)    ............................................................
N. sahlbergii       ............................................................
N. lituyea          ............................................................
N. diversa          ...C..T...........A............T.............C..............
N. gebleri          ...C...........T..A...............................C.....T...
```

```
N. gregaria          AACTATTTTAACTCTTTTAAATCCATACTATTTAGGAGATCCAGATAATTTTACACCTGC
N. haida             ............................................................
N. charlottae        ............................................................
N. louisea (KV)      ............................................................
N. louisea (SKA)     ............................................................
N. louisea (SKB)     ............................................................
N. sahlbergii        ...................G........................................
N. lituyea           ............................................................
N. diversa           ...............A.....C.....T..CC...................C........
N. gebleri           ......C..............C.....T...........C..T.................

N. gregaria          AAATCCATTAGTAACCCCAATTCATATTCAACCTGAATGATATTTTTTATTTGCTTACGC
N. haida             ............................................................
N. charlottae        ............................................................
N. louisea (KV)      ............................................................
N. louisea (SKA)     ............................................................
N. louisea (SKB)     ............................................................
N. sahlbergii        ...............T............................................
N. lituyea           ...............T............................................
N. diversa           .......C.......T.....C................................A..T..
N. gebleri           T..............T......................................A..T..

N. gregaria          TATTCTACGATCT
N. haida             .............
N. charlottae        .............
N. louisea (KV)      .............
N. louisea (SKA)     .............
N. louisea (SKB)     .............
N. sahlbergii        ....T........
N. lituyea           ....T........
N. diversa           ....T.......A
N. gebleri           A...T.......A
```

Cytochrome oxidase subunit 2, fragment a

```
N. gregaria         AGATATGAATATTCTGATTTTAACAAACTTGAATTTGATTCTTATATAATTCCTATTAAT
N. haida            ............................................................
N. charlottae       ............................................................
N. louisea (KV)     ............................................................
N. louisea (SKA)    ............................................................
N. louisea (SKB)    ............................................................
N. sahlbergii       ...................................C........................
N. lituyea          ...................................C.......................C
N. diversa          .......................T...........C..............C..C......
N. gebleri          ..T....................T...T.A.....C..............C.....A...

N. gregaria         GAAATACAAATAAATAATTTTCGATTATTAGATGTAGATAACCGTATTATTCTACCTTTT
N. haida            ............................................................
N. charlottae       ............................................................
N. louisea (KV)     ............................................................
N. louisea (SKA)    ............................................................
N. louisea (SKB)    ............................................................
N. sahlbergii       ............................................................
N. lituyea          ............................................................
N. diversa          ......A.............C........G........C..T.....CC.C..T......
N. gebleri          ......A.................C..C....C........T..A......T........

N. gregaria         AATTCACAAATTCGTATTTTAGTTTCAGCAACTGATGTACTTCACTCTTGAACAATCCCA
N. haida            ............................................................
N. charlottae       ............................................................
N. louisea (KV)     ............................................................
N. louisea (SKA)    ............................................................
N. louisea (SKB)    ............................................................
N. sahlbergii       ............................................................
N. lituyea          ............................................................
N. diversa          .......................G..............T.....T..A...........T
N. gebleri          .......................A.....T........T.....T........T.....T

N. gregaria         ACTTTAGGAGTAAAAATTGATGCTACACCTGGCCGTCTAAATCAAACTAGATTTTTTATA
N. haida            ............................................................
N. charlottae       ........G...................................................
N. louisea (KV)     ........G...................................................
N. louisea (SKA)    ........G...................................................
N. louisea (SKB)    ........G...................................................
N. sahlbergii       G...........................................................
N. lituyea          G...............................T...........................
N. diversa          ....................C...........A....................C......
N. gebleri          .G......G....................C..A.....T........C............

N. gregaria         AATCGTTCAGGATTATTTTT
N. haida            ....................
N. charlottae       ....................
N. louisea (KV)     ....................
N. louisea (SKA)    ....................
N. louisea (SKB)    ....................
N. sahlbergii       ....................
N. lituyea          ....................
N. diversa          .....G.....T........
N. gebleri          .....G.....C........
```

Cytochrome oxidase subunit 2, fragment b

```
N. gregaria         ATGAATTTAAGCTTCATAAATAAAGTTTTTCTTTTATTAGAATAATGGCAACATGGTCTA
N. haida            ............................................................
N. charlottae       ............................................................
N. louisea (KV)     ............................................................
N. louisea (SKA)    ............................................................
N. louisea (SKB)    ............................................................
N. sahlbergii       ............................................................
N. lituyea          ............................................................
N. diversa          ........G.........T.......................A............A...T
N. gebleri          .........................A.............................A..A.

N. gregaria         ATTTTAGATTACAAGACAGAGCTTCCCCATTAATAGAACAATTAATATTTTTTCATGATCA
N. haida            .............................................................
N. charlottae       .............................................................
N. louisea (KV)     .............................................................
N. louisea (SKA)    .............................................................
N. louisea (SKB)    .............................................................
N. sahlbergii       .............................................................
N. lituyea          .............................................................
N. diversa          ......AT..........T......................C...................
N. gebleri          ......AT..........T...........................G..C...........

N. gregaria         TACTTTAATAATTTTAGCAATAATTACAATTTTAGTAAGATATTTAATATTTTCATTATT
N. haida            ............................................................
N. charlottae       ............................................................
N. louisea (KV)     ............................................................
N. louisea (SKA)    ............................................................
N. louisea (SKB)    ............................................................
N. sahlbergii       ................A...................T.......................
N. lituyea          ................A...................T.......................
N. diversa          ...AC.......C...AT.............A..........C.................
N. gebleri          ...A............AT..................T...............A.......

N. gregaria         TTATAATAAAAATATTAATCGATACTTACTTGAAGGACAAATAATTGAAATTATTTGAAC
N. haida            ............................................................
N. charlottae       .................................................G..........
N. louisea (KV)     .................................................G..........
N. louisea (SKA)    .................................................G..........
N. louisea (SKB)    .................................................G..........
N. sahlbergii       .................................................G..........
N. lituyea          .................................................G..........
N. diversa          ..................C....TA........................G.A........
N. gebleri          .........................C..........T............G.A........

N. gregaria         AATTTTACCAGCTATTACTTTATTTTTATTGCTCTTCCTTCATTACGATTATTATATTTA
N. haida            ............................................................
N. charlottae       ............................................................
N. louisea (KV)     ............................................................
N. louisea (SKA)    ............................................................
N. louisea (SKB)    ............................................................
N. sahlbergii       ............................................................
N. lituyea          ............................................................
N. diversa          ........................................C......C.T..........
N. gebleri          ......................................T.A.....TC.T..........
```

```
N. gregaria        TTAGATGAAATTAGAAATCCATCTCTTACATTAAAATCTATTGGACAC
N. haida           ................................................
N. charlottae      ................................................
N. louisea (KV)    ................................................
N. louisea (SKA)   ................................................
N. louisea (SKB)   ................................................
N. sahlbergii      ................................................
N. lituyea         ................................................
N. diversa         .................C..............................
N. gebleri         .................C..........................T...
```

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Title of Thesis:

Molecular reevaluation of the *Nebria gregaria* infragroup and the implications for the existence of an ice age refugium on the Queen Charlotte Islands

Author

Thomas E Clarke
August 19, 1998