Phylogeny and Biogeography of Ice Crawlers (Insecta: Grylloblattodea): Evidence from Six Molecular Loci

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PHYLOGENY AND BIOGEOGRAPHY OF ICE CRAWLERS (INSECTA: GRYLLOBLATTODEA): EVIDENCE FROM SIX MOLECULAR LOCI

by

Karl J. Jarvis

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

Department of Integrative Biology
Brigham Young University
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GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

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This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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ABSTRACT

PHYLOGENY AND BIOGEOGRAPHY OF ICE CRAWLERS (INSECTA: GRYLLOBLATTODEA): EVIDENCE FROM SIX MOLECULAR LOCI

Karl J. Jarvis

Department of Integrative Biology

Master of Science

Ice crawlers (Insecta: Grylloblattodea) are rarely encountered insects that consist of five genera representing 26 species from North America and Asia. Asian grylloblattids are the most diverse, but North American ice crawlers (genus *Grylloblatta*) are known for their adaptation to cold conditions. Phylogenetic relationships among grylloblattid species and genera are not known. Late Pleistocene glaciations had a major effect on the current *Grylloblatta* distribution, because their specific habitat requirements restrict them to small geographical areas. Six genes were sampled in 37 individuals for 18S rRNA, 28S rRNA, histone 3, 12S rRNA, 16S rRNA, and cytochrome oxidase II (COII) from 27 populations of *Grylloblatta*, three populations from Japan (genus *Galloisiana*), and three populations from Russia (genus *Grylloblattina*). An additional 35 individuals from these
localities were sampled for COII only. Phylogenetic analysis with two mantophasmid
outgroups in POY indicates monophyletic genera, with *Grylloblatta* as sister to
*Grylloblattina*. Two major lineages exist within *Grylloblatta*: a clade in Northern
California and Oregon and a clade in Washington and Oregon. One new species and up
to six candidate species are possible based on these data. Fossils and geological events
provide little evidence for dating grylloblattid divergence times. At least six *Grylloblatta*
lineages existed before the end of the Pleistocene glaciation. Conservation status for each
lineage is proposed, based on IUCN Red List Conservation Criteria.
ACKNOWLEDGEMENTS

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1. Introduction

1.1 Background

Grylloblattids (also known as ice bugs, ice crawlers, and rock crawlers) are a poorly known group of insects that are restricted to cold and extreme habitats that are difficult to access. As adults, ice crawlers are 20-34 mm long, wingless, pale, and either nocturnal or cavernicolous insects (Fig. 1). Adults have long, 8-segmented cerci, and females have a sword-shaped ovipositor similar in shape to that of katydids (Orthoptera: Tettigoniidae). They inhabit talus slopes and ice caves in western North America, and cool temperate forests in eastern Asia and central Siberia. It is estimated that grylloblattids’ typical lifespan is five years and some could live up to ten years old, in large part due to their low metabolism (Nagashima, et al., 1982; Walker, 1937). Grylloblattids are primarily predatory or scavengers of other insects, but sometimes feed on plants, fungus, or decaying matter (Edwards and Nutting, 1950; Mills and Pepper, 1937; Pritchard and Scholefield, 1978; Rentz, 1982). Few entomologists have ever collected these cryptic insects, and little is known about their life history and biology. From a systematic standpoint they are also poorly known with no robust estimate as to their phylogeny and nothing known about their population structure.

Asian grylloblattids (4 genera, 16 species) inhabit cool temperate forests, but North American species (1 genus, 11 species) are restricted to ice caves and glacial margin habitats. As glaciers and other ice habitat recede due to the effects of global warming (Haeberli, et al., 1999), many populations and species are threatened, and some
populations that existed 100 years ago may no longer be extant (described below).

Therefore, Grylloblattodea stands as a threatened insect order, and they are in dire need of basic research and conservation. This paper provides an overview of grylloblattid biology and distribution and some critical information about the phylogeny and population structure of these little known insects.

1.2 Biology

*Grylloblatta* is a cryophilic insect that can perish simply from the heat of a collector’s hand (pers. obs.). This genus prefers 100% humidity and cold temperatures, preferring 1°C in a controlled environment with a temperature gradient (Henson, 1957). Temperatures of 15.5°C cause an increase in activity, and heat prostration occurs at 27.8°C (Mills and Pepper, 1937), and 20 minutes of exposure to 20.5°C can also cause death (Edwards and Nutting, 1950). Extreme low temperatures are also fatal, with –5.6°C causing a marked increase in activity, and prostration from cold occurs at –6.2°C. The mechanism for low temperature tolerance in *Grylloblatta* is not known, but it is not a result of significant levels of glycerol, sorbitol or erythritol in their hemolymph (Morrissey and Edwards, 1979). Death at cold temperatures occurs when ice forms in the body (Edwards, 1982).

The narrow range of temperature needs in *Grylloblatta* severely limits their vagility (Kamp, 1979). *Grylloblatta* frequently moves vertically through loose substrate in order to remain in an optimal microhabitat as temperatures at different levels in the ground and
on the surface change frequently (Edwards, 1982; Kamp, 1979; Mills and Pepper, 1937; Rentz, 1982). Consequently, North American grylloblattids are generally found at high elevations (1500-3000m) on north-facing talus slopes (Kamp, 1963; Kamp, 1979), snow patches near forest, and at low elevations (300-1000m) in caves with permanent ice. Although they move vertically through substrate, unsuitable habitat outside of their caves and high-mountain talus slopes prevents migration (Kamp, 1979).

Asian grylloblattids prefer higher temperatures than *Grylloblatta* (Storozhenko, 1996), at around 9-15°C (Nagashima, et al., 1982; Namkung, 1982; Storozhenko, 1979; Storozhenko, 1996). *Galloisiana* is found in a wide range of elevations (300-2000m), but they are not found in the ice caves that *Grylloblatta* inhabit at lower elevations (Nagashima, et al., 1982). The three sites at which *Grylloblattina* is found are at particularly low elevations, one of which is at 5 m (Pravdin and Storozhenko, 1977). *Namkungia* is found in caves, but at temperatures as high as 15°C, much higher than *Grylloblatta* (Namkung, 1982; Storozhenko and Park, 2002).

### 1.3 Glaciation events and distribution of Grylloblatta

During the Pleistocene, ice sheets enveloped most of the current distribution of grylloblattids, particularly during the Wisconsin Glaciation in the late Pleistocene. From 50 kya and until 10 kya, ice sheets covered much of the northern portion of North America. Ice sheets covered essentially all of Canada and parts of the northern-most US states (Conchon, et al., 1992). The range and species composition of pre-Pleistocene
grylloblattids is not known, but grylloblattids could not have inhabited all of the areas currently occupied by North American grylloblattids during the glaciations. Extreme conditions of ice sheets would make inhabitation by grylloblattids of any part other than the margin unlikely (Kamp, 1979). The ice sheets were a major disruption of North American grylloblattids and ecosystems in general, but as the ice sheets melted, new areas were exposed and the conditions of some old habitats changed (Kamp, 1979; Vrsansky, et al., 2001), so some populations may have moved along with the path of the melting ice sheets. On mountaintops and in ice caves, however, conditions remain similar to those near the margin of the ice sheets. The entire current distribution of *Grylloblatta scudder*, *G. occidentalis*, and *G. gurneyi* was covered by ice sheets, as was most of the distribution of *G. campodeiformis*. In British Columbia and Alberta, the only place for grylloblattids to have survived are refugia in northern British Columbia near *G. campodeiformis athapaska* and *G. campodeiformis nahanni*, and another in southern Alberta, near the current *G. campodeiformis campodeiformis* populations in Banff (Conchon, et al., 1992; Kamp, 1979). Some species inhabit areas to the south of the extent of the ice sheets – *G. chandleri*, *G. barberi*, *G. bifratrilecta*, and *G. washoa*, but local glaciers and deep seasonal frost extended across most of their range (Conchon, et al., 1992). These frosts may have rendered localities in the region of the Sierra Nevada mountains uninhabitable during the full extent of the ice sheets (20-18 kya), since temperatures were at least 10°C below the current level (Frenzel, 1992).

1.4 Taxonomy
In June of 1913, T.B. Kurata and E.M. Walker collected two specimens of a new insect at 2000m elevation on Sulphur Mountain, Banff, Alberta. This unique species had a similar body form to earwigs (Dermaptera), head and legs similar to cockroaches (Blattodea), antennal position similar to that of stoneflies (Plecoptera), cerci similar to *Campodea* (Diplura) and mouthparts and ovipositor similar to katydids (Orthoptera) (Walker, 1914). Walker dubbed the species *Grylloblatta campodeiformis* and placed it in a new family Grylloblattidae (Walker, 1914). Crampton (1915), Bruner (1915), and Brues and Melander (1915) noted the unique combination of morphological characters of Grylloblattidae and all placed it in its own order, which they termed Notoptera, Grylloblattaria, and Grylloblattoidea, respectively. Brues and Melander (1932) and Essig (1942) called the order Grylloblattodea, which is the most commonly used name.

Taxonomically, ice crawlers consist of a single order (Grylloblattodea), a single family (Grylloblattidae), 5 genera, and 26 described species. Asian grylloblattids consist of 16 species in four genera: *Galloisiana*, *Grylloblattina*, *Grylloblattella*, and *Namkungia*. *Galloisiana* is the most species-rich, containing 11 species from Japan, Korea, China and Russia. Six of the *Galloisiana* species are found on the Japanese islands: *G. yezoensis* on Hokkaido, *G. nipponensis*, *G. yuasai*, and *G. kiyosawai* on Honshu, *G. chujoi* on Shikoku, and *G. notabilis* on Kyushu. The other *Galloisiana* species are on the mainland but not far away: *G. kosuensis* in South Korea, *G. sofiae* in North Korea, *G. sinensis* in far northeastern China, and *G. ussuriensis* and *G. olgae* in the Maritime Province of far eastern Russia. The remaining three genera each consist of one or two species. *Grylloblattina* consists of a single species, *G. djakonovi*, which is divided into two
subspecies, *G. djakonovi djakonovi* and *G. djakonovi kurentzovi*, both found in the far eastern Maritime Province of Russia (Pravdin and Storozhenko, 1977; Storozhenko, 1979). *Namkungia* contains a single species, *N. biryongensis*, which is found in South Korean caves (Namkung, 1982; Storozhenko and Park, 2002). The large individuals of *Grylloblattella* are found in western to central Siberia in two species: *G. pravdini* in the Altai Mountains, and *G. sayanensis* in the Sayan Mountains.

In North America, *Grylloblatta* is the only genus of ice crawlers, comprised of 11 described species. Most grylloblattid species are found in the Cascade and Sierra Nevada ranges from southern British Columbia to central California: *G. scudder*, *G. occidentalis*, *G. chirurgica*, *G. scullen*, *G. rothi*, *G. gurneyi*, *G. chandleri*, *G. barberi*, *G. bifratrilecta*, and *G. washoa*. The latter four species are known from a single locality only, while the other species are found in relatively small geographic areas (within 100 km). *G. campodeiformis* is the only species found outside of the Sierra Nevada and Cascade mountain regions, and is the most widespread species, having three subspecies distributed in multiple populations across the northern Rockies. *G. campodeiformis campodeiformis* is distributed from southwestern Montana northward to Banff, Alberta, *G. campodeiformis athapaska* in north central British Columbia, and *G. campodeiformis nahanni* in northwestern British Columbia. *G. campodeiformis athapaska* and *nahanni* are several hundred kilometers northward from any other grylloblattid population. In all cases, these species are closely associated with Pleistocene glacier remnants, ice caves, talus slopes, and similar habitats.
From a morphological standpoint, *Grylloblatta* species are quite similar and the actual species diversity of this group is poorly known. Species are difficult to delimit because of the lack of available specimens, the lack of adult males among those that are collected (upon which species descriptions are based), and the lack of distinguishing characters among adults. Most of the alpha-level taxonomic work on *Grylloblatta* species was performed more than 40 years ago and based exclusively on morphology (Gurney, 1937; Gurney, 1948; Gurney, 1953; Gurney, 1961; Kamp, 1963; Kamp, 1979; Walker, 1914). Since this time, additional isolated populations of grylloblattids have been found with individuals intermediate in form to the described species (Crawford, pers. comm.; Gurney, 1961; Lisowski, pers. comm.; Rentz, 1982; Zack, pers. comm.). Migration among populations is almost certainly severely limited or nonexistent in current conditions due to grylloblattids’ habitat specificity, limited geographical size of populations, and winglessness (Kamp, 1979; Rentz, 1982).

1.5 Interordinal Phylogeny

Ice crawlers have been hypothesized to be sister group to many different insect orders of directly-developing winged insects in “Polyneoptera” (Boudreaux, 1979; Wheeler, et al., 2001). The tettigoniid-like ovipositor and general orthopteroid appearance led Walker (Walker, 1914) to place them initially as a family within Orthoptera, but Sharov (1966) demonstrated that the grylloblattid and tettigoniid ovipositors have independent origins. Ordinal status was proposed for Grylloblattidae as early as 1917, and other authors have supported this, although relationships to other orders were still uncertain (Crampton,
1917; Giles, 1963; Imms, 1927; Snodgrass, 1937). Kamp (1979) analyzed the data of Giles (1963) and Blackith and Blackith (1968), which indicated that Grylloblattodea and Dermaptera share cylindrical paraglossae, confluent antennal sulcus and anterior tentorial pit, the position of the episternum and epimeron as nearly horizontal, and both orders lack ocelli and a sternellum. Kamp’s study also indicated a close relationship of grylloblattids to Phasmida, Blattaria and Orthoptera (Kamp, 1979). Hennig (1981) placed grylloblattids as sister to Dermaptera + Dictyoptera, and Kristensen (Kristensen, 1991) placed them at an unresolved node of several polyneopterous orders. Wheeler et al. (2001) presented a combined molecular and morphological analysis which indicated an unresolved Dermaptera + Zoraptera + Grylloblattodea + Dictyoptera within “Polyneoptera.”

Recent analyses suggest that Grylloblattodea is sister group to the recently described order Mantophasmatodea (Engel and Grimaldi, 2004; Klass, et al., 2002; Terry and Whiting, 2005). Terry and Whiting (2005) analyzed a molecular and morphological data set of 18S rRNA, 28S rRNA, histone 3 (H3), and 125 morphological characters across all insect orders with a focus on Polyneoptera (Terry and Whiting, 2005). Their results robustly support the placement of Grylloblattodea as sister group to Mantophasmatodea. Klass et al. (2002) noted several possible morphological synapomorphies that support this relationship; 1) the proventriculus of the gut share a similar arrangement of sclerites and lobes, 2) the caecae of the midgut share a “similar condition,” and 3) a “process-bearing sclerite” is present on the tenth ventral segment. In addition, grylloblattids and
mantophasmids resemble each other in a gross morphological sense, having similar body lengths, with long legs and antennae.

1.6 Fossil Record

Some workers have included a wide range of orthopteran fossils within Grylloblattodea. Storozhenko (1998) described a wide diversity of fossil taxa and placed them within a group he equated with Grylloblattodea ("Grylloblattida"), increasing the size of the order to 44 families, 165 genera, and 295 fossil species (Storozhenko, 1998). Fossils of these families have been found on all of the continents except Antarctica and extend temporally from the Carboniferous through the mid-Cretaceous deposits (345-100 Ma) (Storozhenko, 1998). Unlike extant grylloblattids, fossil grylloblattids are all winged, and were likely capable of flight (Rasnitsyn, 1980), though no fossils have been described for the modern family Grylloblattidae. Storozhenko (1998) presented a cladistic analysis of families within "Grylloblattida", but no putative synapomorphies support the monophyly of this group (Béthoux, et al., 2005), and it is likely a paraphyletic assemblage of orthopteran-like insects.

1.7 Intraordinal Phylogeny

Little is known of grylloblattid genus and species phylogeny. Morphological features distinguishing genera and species are few, difficult to identify, and often based on adult males, which are rarely collected. No formal phylogenetic analysis has ever been
performed on grylloblattids, and no species-level phylogenies have ever been presented. This is further complicated by the tendency of some authors to name new species based on one or two specimens found in new locations, but which lack any discrete characters distinguishing them from other described species.

Storozhenko (1996) proposed a phylogeny of four of the extant grylloblattid genera, *Grylloblatta, Galloisiana, Grylloblattina*, and *Grylloblattella*, based on an intuitive analysis of ten morphological and two habitat characters. According to Storozhenko (1996), a single character supports the monophyly of the Asian genera (presence of four to eight setae on the edges of the cervical sclerites), rendering the Asian genera as sister group to *Grylloblatta*. However, since no outgroups were used in his analysis, it appears likely to us that the shared presence of this character state is symplesiomorphic. Among the Asian genera, Storozhenko places *Grylloblattina* as sister to *Galloisiana* + *Grylloblattella*, supported by the presence of a narrow elongated right coxopodite of the male in *Galloisiana* and *Grylloblattella* rather than a short thickened one in *Grylloblattella* (Storozhenko, 1996). While Storozhenko’s efforts to recover some sort of phylogenetic hypothesis for grylloblattids was valiant, the failure to find a robust solution to grylloblattid phylogeny highlights the difficulty of relying exclusively on morphology to decipher the evolution of these morphologically similar insects.

1.8 Goals
The focus of this paper is to use DNA sequence data from six genes to reconstruct the phylogenetic relationships among grylloblattid genera, species, and populations, with an emphasis on the North American taxa. Parsimony and likelihood approaches are implemented and the robustness of this phylogeny is assessed using Partitioned Bremer Support and non-parametric bootstrapping. The Shimodaira-Hasegawa test is used to assess the significance of the difference among the resulting topologies. We review geological events that could be used to calibrate molecular divergence time estimates and discuss problems with the use of these particular events. We place the major lineages into clades and hypothesize current *Grylloblatta* species on the basis of phylogeny, support, population distribution, and morphology. Historical lineages are inferred based on expected generalized patterns. Conservation status of species is designated based on IUCN Red List Conservation Criteria (IUCN, 2001).

2. Methods

2.1 Collecting Methods and Taxon Sampling

Collecting was focused on sampling localities as recorded in grylloblattid literature (Asahina, 1959; Gurney, 1937; Gurney, 1948; Gurney, 1953; Gurney, 1961; Kamp, 1963; Kamp, 1979; Nagashima, et al., 1982; Pravdin and Storozhenko, 1977; Rentz, 1982; Storozhenko, 1979; Visscher, et al., 1982; Walker, 1914). These data were augmented with the locality labels of specimens from the University of Washington (51 localities),
Washington State University (5 localities), Western Washington University (1 locality), Brigham Young University (1 locality), and University of California at Davis (1 locality). Individuals with experience collecting grylloblattids also provided information about additional localities.

The sampling strategy of this study was to sample as many individuals from as wide a distributional range as possible. Ninety localities were surveyed, and 67 specimens in 19 localities were successfully collected. It is difficult to determine where grylloblattids will be active and on the surface at a given time, since ice crawlers’ optimal microhabitat changes throughout the year. Most specimens were collected by turning over rocks in ice caves or on talus slopes, or by surveying the surface of ice fields at night. An alternative method consisted of placing sticky traps baited with Limburger cheese (=“stinky traps”) in several caves and other surface localities, as there is some anecdotal evidence to suggest that “stinky cheese” attracts grylloblattids. Grylloblattids were found on or near the stinky traps in three caves. Local collectors checked traps at two localities over one to six months and sent specimens to the authors. In addition to the specimens collected in the field, 18 specimens preserved in ethanol representing 15 populations were obtained from museum collections or individual collectors. Of these, we successfully amplified and sequenced 12 specimens from 11 populations.

Specimens obtained for this analysis include 37 grylloblattid individuals sampled from 27 populations, representing eight species and three genera (Table 1). Twenty-nine of these individuals are from 21 Grylloblatta (North America) localities, five individuals from
three *Galloisiana* (Japan) localities, and five individuals from three *Grylloblattina* (Russia) localities. This represents 36% of *Grylloblatta* species, and 27% of ice crawler species worldwide. All but two of the sampled *Grylloblatta* populations were collected in and around the Sierra Nevada and Cascade mountains (Fig. 2b), distributed from Carpenter Ridge in central California to near Banff, Alberta. Seven individuals from five populations were sampled in California, five individuals from four populations in Oregon, 11 individuals from ten populations in Washington, and one individual each from Montana and Alberta (Fig. 2). Two mantophasmid individuals were included as outgroups in this analysis: *Tyrannophasma gladiator* and a *Mantophasma zephyra*, both from Namibia.

This sampling is dense across much of the westernmost portion of *Grylloblatta* distribution in the United States. This analysis lacks *G. campodeiformis nahanni* and *G. campodeiformis athapaska* of northern British Columbia, *G. scudder* of southern British Columbia and *G. occidentalis* of northern Washington. Southward from the sampled localities are *G. washoa*, *G. bifratrilecta*, *G. chandleri*, and *G. barberi*, all described before 1969, but possibly not collected since this time.

We targeted cave and surface populations throughout the sampling localities. Nineteen individuals from fourteen locations were sampled from caves, all in North America. Eight of these populations are located in southern Washington, two are in Oregon, and four are in California. The 12 surface populations consist of all six Asian populations, two populations each in Oregon and Washington, and one population each in California,
Montana, and Alberta. Troglophilic grylloblattids are found in both North America and Asia, but seem to be more widespread in North American caves, as ice crawlers have been collected from at least 43 caves in California, Oregon, and Washington (Burke Museum, University of Washington). However, one author documented 28 cave localities in South Korea (Namkung, 1982). We attempted to sample the one known Japanese cave population (Gurney, 1961) but were unsuccessful.

2.2 Identification

Current taxonomic keys for grylloblattid identification require adult specimens (Storozhenko, 1988), but the majority of individuals collected were nymphs or incomplete specimens. Nearly all of the distinguishing characteristics among species are only applicable to adults, such as male or female genitalic characters, body length, pronotal ratio and size, number of antennal segments, and ratio of eye length to width. Of the specimens collected, only 21% were identifiable to species, since 69% were immature, and another 10% were incomplete or female. Unidentifiable specimens were labeled sp. indet. Russian specimens were collected with and identified by Sergei Storozhenko; Hiroshi Ando identified Japanese specimens.

2.3 Molecular sampling

Grylloblattid and mantophasmid specimens were collected into 100% ethanol and preserved at –80°C or frozen immediately in liquid nitrogen. Eight of the 12 grylloblattid
specimens that were borrowed from other collections had been preserved in 70% ethanol at room temperature, and the remaining four had been collected recently into 100% ethanol. Three *Grylloblatta chirurgica* specimens Skamania Co., Washington were collected longest from the time of sequencing: New Cave in 1973, Surprise Cave in 1983, and Ape Cave in 1983. The *G. sculleni* specimens from Mary’s Peak, Oregon, were collected between 1993 and 1996, and the *G. sp. indet.* from Carpenter Ridge, Nevada Co., California was collected in 2000. All other specimens were collected within two years of extraction. Vouchers are deposited in the Brigham Young University Insect Genomics Collection.

DNA was extracted from the thorax or leg in adult specimens. In immature specimens, the abdomen was removed, and the rest of the body was used for extraction to avoid contamination from the gut. DNA was extracted according to the specifications of the Qiagen DNeasy® Tissue Extraction Kit (Qiagen Inc., Valencia, CA). Gene regions were amplified via Polymerase Chain Reaction (PCR) on DNA Engine Dyads® (MJ Research, Waltham, MA) with AmpliTaq Gold® or AmpliTaq Platinum® DNA Polymerases (Applied Biosystems, Foster City, CA).

Six genes were sequenced: 18S rRNA (~2000 bp), 28S rRNA (~2300 bp), and histone H3 (H3) (~300 bp) from the nuclear genome, and 12S rRNA (~400 bp), 16S rRNA (~500 bp), and cytochrome oxidase II (COII) (~700 bp) from the mitochondrial genome. The 18S and 28S primers are given in Whiting (2002), and the 12S, 16S, COII, and H3 primers are as in Svenson and Whiting (2004). PCR temperature profiles for 18S, 28S,
and H3 were the same as in Jarvis et al. (2004) and profiles for 12S, 16S, and COII were the same as the H3 profile. COII was sequenced for the 37 sampled individuals and additional specimens from the same localities, in which uncorrected pairwise distances averaged 23.9% and were as high as 31.4% between mantophasmids and grylloblattids. Among individuals within populations, divergence levels ranged from 0.0% to 15.7%, with an average of 2.1% and a median divergence of 0.3%. Taxa that exhibited a 0.00-0.01% divergence in COII from other members of their respective populations were excluded from further sequencing (n = 21 individuals).

PCR products were visualized by ultraviolet light on an agarose gel after electrophoresis and were then purified using GeneClean® filter plates (Millipore, Billerica, MA). Gene regions were sequenced with the same primers as in PCR using BigDye v.3.0 chemistry (Applied Biosystems, Foster City, CA). Sequencing reactions were purified by centrifuging through Sephadex™ G-50 columns (Amersham Biosciences Corp., Piscataway, NJ) and fractionated on an ABI 3730 sequencer (Applied Biosystems, Foster City, CA).

Raw sequence data and contigs were viewed and assembled in Sequencher™ 4.1 (Gene Codes Corporation, Ann Arbor, MI). Forward and reverse primers were used to sequence each region in both directions, and the larger genes (18S and 28S) were amplified in multiple overlapping regions. Primer sequences were removed from the beginning of each sequence. Sequence data were checked for accuracy by matching forward and reverse sequences for each gene region and checking base calls.
2.4 Analysis

Protein-coding genes were aligned based on conservation of the amino acid sequence. The ribosomal genes were partitioned along conserved regions to facilitate efficient heuristic searching in POY (Giribet, 2001). The 18S dataset was partitioned at seven sites, 28S at 12 sites, 12S at two sites, and 16S at five sites by cutting contigs in regions with no variation among taxa. These conserved regions surround variable regions, thereby allowing POY to spend more time optimizing variable regions. There were no polymorphisms within at least 10 base pairs of each partition, except in four cases, where there were no more than three polymorphisms within 10 base pairs of the partition. The 18S partitions correspond to the following regions (De Rijk, et al., 1992): partition 1 contains regions V1 and V2, partition 2 contains V3, partitions 3-5 contain V4, V5 is in partition 5, partition 6 is flanked by V5 and V7, partition 7 contains V7, and partition 8 contains both V8 and V9. The 28S partitions correspond to the following regions (De Rijk, et al., 1994): partitions 1 and 2 precede D2, partitions 3 and 4 contain D2, partition 5 contains D3, partition 6 is flanked by D3 and D4, partition 7 contains D4, partition 8 contains D5, partition 9 is flanked by D5 and D6, partition 10 contains D6, partition 11 contains D7a, partition 12 contains D7b, and partition 13 is flanked by D7b and D8.

Uncorrected pairwise distances were calculated in PAUP* on the implied alignment from POY. Phylogenetic analyses were performed via Direct Optimization (DO) under parsimony optimality criteria as implemented in POY. This allows pairwise alignments
among actual sequences and inferred ancestral sequences to be optimized simultaneously with tree topology reconstruction. Transitions, transversions and indels were weighted equally (Grant and Kluge, 2003). Commands in POY were as follows: “-minterminals 70 -fitchtrees -leading -norandomizeoutgroup -impliedalignment -sprmaxtrees 1 -tbrmaxtrees 1 -maxtrees 2 -holdmaxtrees 2 -slop 2 -checkslop 2 -buildspr -buildmaxtrees 1 -replicates 50 -stopat 100 -treefuse -fuselimit 2 -fusematrix 2 -fusemaxtrees 2 -numdriftchanges 5 -driftspr -numdriftspr 2 -drifttbr -numdrifttbr 2 -slop 2 -checkslop 2 -molecularmatrix 111.txt -seed –1.”

Likelihood scores of a variety of models were computed via in ModelTest 3.6 (Posada and Crandall, 1998) on the implied alignment from POY. A model was selected based on a hierarchical Likelihood Ratio Test (hLRT) and the Akaike Information Criterion (AIC). A Maximum Likelihood (ML) analysis (Felsenstein, 1981) implementing the model with associated ML-estimated parameters found in ModelTest was subsequently performed in PAUP* (Swofford, 2002). The maximum number of branch-length smoothing passes was set to 20, with smoothing passes stopping when branch length changed by less than 10^{-6}. Starting branch lengths were calculated using the Rogers-Swofford method, trees were rejected if their approximate likelihood exceeded the target by more than 5% with dynamic readjustment. Starting values for other parameters were found using parsimony-based approximations. Branch lengths of effectively zero length were collapsed. Ten random addition sequences were performed in a heuristic search. Starting trees for TBR branch swapping were obtained by stepwise addition, with swapping performed on the 100 best trees.
A topology test was performed in order to obtain confidence limits on topologies found by DO and ML. The Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 1999) performs well when the number of topologies tested is small (Goldman, et al., 2000; Shimodaira, 2002), as in this study. Both trees were tested for significant differences using the implied alignment from POY. Partial likelihoods were resampled 1000 times, as implemented in the Reestimated Log Likelihood (RELL) method in PAUP.

Nodal support was evaluated using nonparametric bootstraps (Felsenstein, 1985) and Partitioned Bremer Supports (PBS) (Baker and DeSalle, 1997) in order to indicate relative support that dataset partitions have for each node. The implied alignment from POY was analyzed for both of these nodal support calculations with gaps treated as a 5th state and characters coded as having multiple states (i.e. “N”) interpreted as uncertain. The bootstrap analysis was performed in PAUP* (Swofford, 2002), resampling all of the characters in 1000 replicates of 10 random additions each. Constraints for PBS scores in a PAUP* command file were developed in TreeRot (Sorenson, 1999) based on the optimal tree from POY. Tree scores for the partitions based on the constraints were found heuristically in PAUP* and the resulting log file was parsed in TreeRot. Sums of PBS scores for each gene were normalized by dividing them by the number of parsimony-informative characters in each gene to indicate support per character for the topology (see Jarvis et al, 2004).
Species delimitation

Species are designated in an approach similar to that of Wiens and Penkrot (2002), a phylogeny-based method that incorporates multi-locus DNA sequence data, morphological, ecological, and distributional data (Sites and Marshall, 2003). Sexual species are united by gene flow among their members (Mayr, 1957; Templeton, 1989; Wiens and Penkrot, 2002). Total evidence phylogenetic analyses are the basis for species determination, and congruent individual gene phylogenies are considered additional evidence of these relationships. Gene flow is detected by non-monophyly of populations. A population within a species is considered to be all of the individuals from a single locality or from localities lacking major barriers to gene flow between them (e.g. the large rivers, long distances) and whose habitat is similar. Non-monophyly of a population is considered to be evidence of gene flow. Lack of differences in morphology and habitat and presence of gene flow among individuals suggest a single species. Monophyletic populations within a species that are concordant with geographical distribution are considered to be evidence of cryptic species. Within a species, we consider the failure of individuals from a putative species at a given locality to form monophyletic groups to be evidence of gene flow. If well-supported clades of haplotypes within a species are not concordant with geography, this is considered to be evidence of gene flow among localities (Wiens and Penkrot, 2002). The pitfall of ancestral polymorphism cannot be entirely avoided with this method, as with all phylogenetic methods, but by including multiple genes, its effects are minimized.
2.6 Dispersal Hypotheses

Specific patterns in phylogenetic and population-level relationships are to be expected for populations that inhabit regions that were covered by the ice sheets. These patterns would depend on the number of species, their distribution, and population structure within them.

1) If an ancestral panmictic population from a single species inhabited the margin of an ice sheet, the population would be expected to follow the ice sheet margin as the ice sheet receded (Fig. 3 a-d). Since the melting of the ice sheets took thousands of years, it would not have been difficult for grylloblattids to stay close to the margin as the ice sheets melted. This process would leave behind relictual populations in ice caves and on mountains tops that still supported the glacier habitat. These populations would then be entirely isolated in “sky islands” (Heald, 1967), with little chance for gene exchange. A phylogeny of such populations would be expected to be a pectinate tree of monophyletic populations, with the populations in the farthest southern extent of the ice sheet being most basal, and populations in the most recent position of the ice sheet being nested more apically in the tree.

2) Another possibility is that prior to the Pleistocene glaciations there were multiple *Grylloblatta* species along the ice sheet margins (Fig. 3a-c,e). These species may have been in different regions along the ice sheet margin, but some may also have been in sympatrically or parapatrically distributed along the ice sheet margins. Since much of the
margin would be expected to be good grylloblattid habitat, they may have dispersed along the ice sheet margins. If there were multiple pre-Pleistocene species, each of these species would be expected to leave behind relictual populations in “sky islands” as the ice sheets receded. The resulting distributional pattern would be multiple populations scattered across the path of the receding ice sheets, which is similar to the pattern left by a single species. However, modern populations originating from the same ancestral species would nest together in a phylogeny, reflecting pre-Pleistocene relationships. The result would be multiple pectinate clades, each with the most southern populations being basal and the most northern populations being apical.

These hypotheses are oversimplified in assuming a uniform pattern of glacier recession, which is probably not always the case, since the topography, climate, and geology of local regions caused some ice sheets to melt more quickly in some areas than others (Conchon, et al., 1992). In areas where ice sheet melting was drastically uneven, some populations could have dispersed into newly created “peninsulas” of habitat. This could confound hypotheses 1 and 2 if populations that are at the northern end of the distribution came to that point because of uneven melting. Nonetheless, in the absence of a fine-scale knowledge of the extent of the ice sheets, glaciers, and regions of deep seasonal frost for the entire grylloblattid distribution throughout the melting period, these simplistic hypotheses provide a model against which the reconstructed phylogeny can be compared to detect any general pattern of glacier recession.
2.7 Conservation Status

The grylloblattid species included in this analysis are assessed for conservation status according to IUCN criteria. *Grylloblatta chirurgica* was previously listed as a “Vulnerable” species on the IUCN Red List in 1983 (Wells, et al., 1983), and remains on the list today under the criteria of the IUCN Red List ver. 2.3 (1994). The loss of habitat and occupancy area, combined with the projected loss of habitat and the highly restricted size of their habitat area are the criteria under which this designation was made. All grylloblattids have low vagility and likely have small population size (Kamp, 1979), which are Red List criteria for classifying species at risk for extinction (Baillie, et al., 2004).

3. Results

3.1 Analyses

We sequenced a 2021-2077 bp piece of 18S, a 2171-2430 bp piece of 28S, 394-398 bp of 12S, 509-514 bp of 16S, 327 bp of H3, and 690 bp of COII. 93% of the sequences were present for the sampled taxa. All of the taxa were sampled for 18S and 28S, 95% for 12S, 98% for 16S, 76% for H3, and 88% for COII (Table 1). Average uncorrected pairwise distances were 6.6% across the entire combined molecular dataset, and 5.8% across the grylloblattid ingroups. Distances among species within genera were 3.7%
within *Grylloblatta*, 0.9% within *Grylloblattina*, and 2.1% within *Galloisiana*. The minimum and maximum pairwise distances between any two taxa was 0.0% and 17.4%, respectively.

Length differences of unaligned sequences due to indels among ingroup taxa were highest in 18S and 28S. Within grylloblattid 18S, there is only a 3 bp difference between the longest and shortest sequence with the exception of partition 3, with a difference of 28 bp. Of the 13 partitioned regions of 28S, seven had length variation on the order of 10-34 bp. The majority of length difference was between ingroups and outgroups, where mantophasmids were 118 bp longer for 18S partition 7 and 51-164 bp shorter in 18S partition 3 and 28S partitions 5 and 12. Sequence length variation was smaller in 12S (4 bp difference among all taxa) and 16S (5 bp difference among all taxa). The amino acid sequences in both H3 and COII were similar (100% amino acids invariant in H3, 62% invariant in COII) with no length variation.

The POY analysis resulted in a single most parsimonious tree of length 4057 with a consistency index (CI) of 0.7705 and a retention index (RI) of 0.9156 (Fig. 4). The implied alignment of 18S was longer than the unaligned sequences by 6.8-9.8% (143-199 bp), 28S increased by 9.4-22.5% (229-488 bp), 12S increased by 2.3-3.3% (9-13 bp) and 16S increased by 4.3-5.3% (22-27 bp). Implied alignments from POY converted to NEXUS format are available at: http://inbio.byu.edu/faculty/mfw2/whitinglab/index.htm.
Deeper nodes tended to have the highest bootstrap (BP) values, as could be expected in clades isolated for long periods of time – one clade with 11 terminals had a bootstrap of 74, but all other clades with eight or more terminals had values of 100. Bremer Support (BS) values were also highest on deeper nodes, reaching as high as 826 supporting a monophyletic Grylloblattodea, and 198, 191 and 170 for the monophyly of the grylloblattid genera. PBS values indicate that the largest percentage of nodal support originated from the nuclear ribosomal genes (Table 2). When normalized by the number of parsimony-informative (PI) characters, 28S provided 18.6% and 18S provided 32.8% of the signal. Within *Grylloblattina*, the majority of the signal is derived from 18S and 28S, while within *Grylloblatta*, there was a relatively even distribution of signal from each gene partition. All six genes agreed on supporting the majority of nodes on the topology (26 of 33), and where genes disagreed, on six nodes only one gene disagreed, and on one node two genes disagreed. These results suggest that the topology is very robust with congruent support coming from multiple loci.

ModelTest indicated the most optimal –ln Likelihood value for all genes combined was 25591.5859 under the General Time Reversible model with invariant sites and Gamma distribution (GTR+I+Γ). The hLRT rejects other models (all at p<0.000001), and the AIC value of 51203.1719 also supports the selection of the GTR+I+Γ model. Base frequencies were found to be A = 0.2558, C = 0.2173, G = 0.2545, and T = 0.2724, and base substitution rates under this model were $A\sqrt{C} = 1.3138$, $A\sqrt{G} = 2.9255$, $A\sqrt{T} = 1.8538$, $C\sqrt{G} = 0.6843$, $C\sqrt{T} = 5.9479$, and $G\sqrt{T} = 1.0000$. The proportion of invariable sites was 0.5281 and the Gamma distribution shape parameter was 0.5862. The number
of discrete Gamma classes was set to 4. A ML analysis under these parameters resulted in a single tree with a \(-\ln \) Likelihood of 25571.37153.

The ML tree differed from the parsimony analysis only slightly: \textit{G.} sp. indet. individuals from Dead Horse Cave and Little Red River Cave were sister group within a polytomy of Ice Caves Picnic Area, New Cave, and Dry Creek Cave individuals, and the clade containing \textit{G.} sp. indet. from Chinook Pass and Goat Peak Trail in the Chirurgica clade also included \textit{G. chirurgica} from Ape Cave. Both of the conflicting clades in the parsimony analysis were supported by bootstrap values of 74 or less. The Shimodaira-Hasegawa indicated that the POY- parsimony topology was not significantly less likely than the likelihood topology (\(p=0.4227\)). The similarity of these topologies indicates that gaps are not the principal driving factor in a DO analysis, and that the reconstructed topology is robust to particular analytical methodologies.

3.2 Phylogenetic Relationships

The monophyly of Grylloblattodea (BP=100, BS=826) and the genera \textit{Galloisiana}, \textit{Grylloblattina}, and \textit{Grylloblatta} (BP=100 for all; BS=198, 191, 170, respectively) is well supported by these data, with indels supporting the monophyly of each genus. \textit{Grylloblattina} and \textit{Grylloblatta} are supported as sister groups (BP=100, BS=54), and the monophyly of \textit{Grylloblatta} is well supported (BP=100, BS=170).
Within *Galloisiana*, the *G. yuasai* from Tokugo Pass and the indet. Kakuma Valley populations are sister taxa (BP=100, BS=54), and this clade is in turn sister to the Lake Chuzenji *G. nipponensis* population. Within *Grylloblattina*, the Beriozovii Stream and Mt. Krinitshnaya populations are reciprocally monophyletic (BP=100, BS=34) and sister to the Petrov Island individuals (BP=100, BS=191). All partitions support all of these relationships with the exception of the Tokugo Pass + Kakuma Valley clade, in which H3 only weakly contradicts this relationship with a PBS score of –1.

Two major clades within *Grylloblatta* are supported on the topology: 1) a clade containing *G. sculleni*, *G. rothi*, *G. gurneyi*, and *G*. sp. indet. individuals, from all of the California populations and three Oregon populations (hereafter Sierra Clade, after the Sierra Nevada mountain range that covers much of their distribution, 15-21 of Fig. 2b); and 2) a clade containing *G. campodeiformis*, *G. chirurgica*, and *G*. sp. indet. individuals, from one Oregon population, all of the Washington populations, and Montana (hereafter Cascade Clade, after the Cascade mountain range that covers much of their distribution, 1-14 of Fig. 2b). Both groups are present in both POY and ML analyses, and are well supported (Sierra: BP=100, BS=52; Cascade: BP=100, BS=16).

### 3.3 Sierra Clade

Within the Sierra Clade, the topology supported the monophyly of the Sisters Area Clade (BP=100; BS=21), the Wilson Ice Cave Clade (BP=100; BS=10), and the Lava Beds National Monument (LBNM) Clade (BP=99, BS=4). The LBNM Clade is sister to
Wilson Ice Cave Clade, forming the California Caves Clade (BP=100, BS=14). The individual from Carpenter Ridge (G. sp. indet.) was poorly supported as sister to the California Caves Clade (BP=56, BS=2). The Sisters Area Clade is supported as a monophyletic group (BP=100, BS=21) and is marginally supported (BP=73, BS=4) as sister group to a clade composed of the lineages described above. The Oregon Cave Grylloblatta sp. indet. individual was sister to the remainder of the Sierra Clade (BP=100, BS=52).

3.4 Cascade Clade

The Cascade Clade consists of three major groups: the Campodeiformis Clade (BP=99, BS=17), the Chirurgica Clade (BP=100; BS=32), and the Trout Lake Clade (BP=100, BS=31). One indet. individual from Sawyer Ice Cave, Oregon was placed as sister to the Trout Lake Clade (BP=100, BS=62). The Chirurgica Clade is well supported as sister to the Trout Lake Clade (BP=100, BS=74). The Campodeiformis Clade includes two Eastern populations (from Montana and Alberta) and one individual from Sawyer Ice Cave, Oregon, and one individual from Chinook Pass, Washington. These results provide the first evidence of multiple grylloblattid lineages living in sympatry, as discussed below.

4. Discussion
4.1 Phylogeny

The high Bremer and bootstrap values throughout the topology, and the overall congruence among independent genes, suggests that the molecular data are providing robust signal across the tree, and that the topological results are robust to multiple methods of tree reconstruction. The basal relationships on the tree are particularly well supported, with each genus supported by very high Bremer support values. Overall, these data provide further evidence that grylloblattid populations are small and restricted in gene flow (Kamp, 1979), which is expected to lead to rapid sorting to monophyly (Shao, et al., 2001).

Our finding that *Grylloblatta* is sister group to *Grylloblattina* disagrees with Storozhenko’s hypothesis (1996) which placed *Grylloblatta* as sister to monophyletic Asian genera. The Shimodaira-Hasegawa test rejected Storozhenko’s hypothesis (p<0.021). The sister-group relationship with the Siberian *Grylloblattina* is consistent with the hypothesis that *Grylloblatta* colonized North America via the Beringian Land Bridge (Storozhenko, 1998).

4.2 Divergence times

No fossils of modern ice crawlers have been found, and the monophyly of fossil “Grylloblattida” has not been demonstrated, so inferring divergence times of the grylloblattid genera and species is difficult. However, some partial clues can be gathered
from mantophasmid fossils and geologic events. Two mantophasmid genera have been found in Baltic amber, both from 40-50 Ma (Engel and Grimaldi, 2004). Therefore, mantophasmids and grylloblattids must have diverged before this point. Engel and Grimaldi suggested that this may have happened during the breakup of Pangaea during the Jurassic (ca. 200 Ma), similar to the plecopteran suborders (Engel and Grimaldi, 2004). If, however, modern grylloblattids are part of the “Grylloblattida,” then this divergence must have taken place before the origin of these fossils, in the Carboniferous. Since no comprehensive phylogenetic analysis of “polyneopteran” fossils has been completed, it can only be inferred that this divergence took place some time between the origin of the earliest neopterans (Carboniferous, 360-286 Ma) and the mantophasmid fossils (Eocene, 40-50 Ma).

Vrsansky et al. (2001) suggested that the modern Grylloblattidae may have originated in the mid-Cenozoic (65 Ma-present) from the fossil family Blattogryllidae (Storozhenko, 1998). Storozhenko also postulated that the modern grylloblattid genera originated at the end of the Oligocene and beginning of the Miocene (ca. 30-20 Ma) while becoming accustomed to newly formed regions of high altitude in Asia (Storozhenko, 1998). However, these hypotheses are based on fossils of flora found in the same forests as grylloblattids, and no direct fossil evidence supports these hypotheses.

The colonization of North America by grylloblattids may have happened via the Beringian land bridge (Storozhenko, 1998). During the Plio-Pleistocene transition (5-1.8 Ma), forests were continuous across the land bridge and it was a time of major migration
of insects and other animals between North America and Asia (Matthews Jr. and Telka, 1997). The land bridge was breached by the ocean around 3 Ma, and has been uncovered only intermittently from that time until the present due to Pleistocene marine transgressions (Matthews Jr. and Telka, 1997). Glacial advances and retreats also caused major changes in the flora on and around the land bridge, replacing the coniferous forests with tundra around 2.5 Ma (Repenning and Brouwers, 1992). Therefore the ancestors to *Grylloblatta* may have migrated across the land bridge between 5 and 3 Ma, and subsequently adapted to cold conditions during the ice ages (Storozhenko, 1998). The wood cockroach *Cryptocercus* likely underwent a similar migration in the Pliocene (Grandcolas, 1999; Vrsansky, et al., 2001), as may have other insects (Matthews Jr. and Telka, 1997). However, there is no evidence to refute the possibility that a migration occurred earlier on the land bridge, or that it resulted from a vicariant event. Ice sheet melting periods could provide additional calibration points for grylloblattids, but since the Wisconsin glaciation was the last and the most extensive, matching nodes with glaciation events beyond the last glaciation would be speculative.

4.3 *Grylloblatta* Species

Since grylloblattid populations are highly fragmented and because vagility is limited (Kamp, 1979), gene flow among populations is expected to be rare. However, throughout the Pleistocene, glaciations and multiple cooling periods have often changed the climate of North America (Frenzel, et al., 1992; Matthews Jr. and Telka, 1997), allowing populations to move more freely than is currently possible. In addition, many
grylloblattid populations are in lava caves that are part of large systems of lava beds. Many of the caves that grylloblattids inhabit may be connected via underground networks, and near many known caves, loose lava exists for miles. Cool air is emitted from some portions of these lava beds, indicating caves, many of which have no human-accessible entrance. Grylloblattids may find small caves suitable, if not preferable to larger caves, since grylloblattids typically are found under rocks and in tight spaces. Therefore, migration among interconnected caves and deep among rocks in lava fields may have provided multiple opportunities for grylloblattid dispersal during and directly after the Pleistocene, but it is unlikely for migration to occur in most other situations.

4.4 Lineages within the Sierra Clade

California Cave Clade:

Our results support the recognition of two major cave lineages within California. The first is the LBNM populations that inhabit a large network of lava fields. The three localities sampled in LBNM are all located within 20 km of each other, and Merrill and Cox Ice Caves are less than five km from each other. The lack of monophyly within individual caves is to be expected throughout LBNM, since within this large complex loose lava, cold temperatures and small passageways likely exist that would enable grylloblattids to migrate between caves. Cool air is emitted from some portions of these lava beds, indicating caves, many of which have no human-accessible entrance. Grylloblattids may find small caves suitable, if not preferable to larger caves, since grylloblattids typically are found under rocks and in tight spaces.
The second ice cave lineage is in Wilson Ice Cave, which is located 160 km from LBNM. Individuals from these two cave complexes are morphologically indistinguishable and current taxonomic keys identify both populations as *G. gurneyi*. However, the geographic distance between these two cave systems, and the lack of intermediate habitat, makes it unlikely that they are connected via a continuous series of underground lava tubes. The phylogeny gives no evidence of gene flow between Wilson Ice Cave and LBNM populations, and the high bootstrap and bremer values indicate that these are isolated lineages with distinct haplotypes. We therefore suspect that these taxa are cryptic species in need of further investigation. The holotype for *Grylloblatta gurneyi* is from LBNM, so the Wilson Cave population is likely to be an undescribed species.

*Sisters Area Clade:*

A total of 12 individuals of *G. rothi* were sampled from McKenzie Pass, Oregon, which is a high elevation volcanic mountain pass with permanent ice fields. The sister group to the McKenzie Pass population is *G. sculleni*, which inhabits Mary’s Peak. Mary’s Peak is ca. 140 km to the east of McKenzie Pass, 30 km from the Oregon coast, and these mountains are all part of the Sisters Area mountain range. This sister group relationship is well supported (BP=100, BS=21). These current allopatric populations were likely sympatric during the late Pleistocene, as migration between these sites would have been possible along glaciers.
Other Sierra Taxa:

One indet. specimen was obtained from Carpenter Ridge, located ca. 150 km southeast of Wilson Ice Cave. The individual from this locality was collected on the surface, in contrast to its sister taxon, where all individuals live in ice caves of northern California. A *Grylloblatta* sp. indet. from Oregon Cave was placed as sister group to the remainder of the Sierra taxa. Oregon cave is a large ancient marble cave in southwestern Oregon, ca. 250 km southwest from the Sisters Area lineage and ca. 250 km northwest from the California ice caves. While it is clear that both of these taxa belong within the Sierra Clade, and that neither of them belong within the Sisters Area clade or California Ice Cave clade, their position relative to these latter clades is more ambiguous, with relatively low bootstrap and Bremer values. In addition, the greatest degree of conflict among data partitions occurs in the placement of these individuals. PBS values indicate that two genes agree and two genes disagree with the Sisters Clade + Carpenter Ridge + California Caves Clade relationship (12S=5, COII=2, 28S=-2, H3=-1) and that one gene agrees and another disagrees with the Carpenter Ridge + California Caves Clade relationship (12S=2, 16S=-1). All other disagreements on the tree are accompanied by the support of more genes than in these cases. While these lineages are distinct and unique, their actual phylogenetic position is still ambiguous.

Dispersal of Sierra taxa:

The dispersal pattern of the Carpenter Ridge, Wilson Ice cave, and LBNM lineages follow the expectations of Hypothesis 1 (Fig. 3d), with its most basal taxon to the south and more apical taxa to the north. This suggests that the ancestor to this clade was at or
south of the farthest south population (Carpenter Ridge) prior to the ice age, and migrated north during the melting period. However, the phylogenetic position of the Carpenter Ridge population is uncertain (see above). In addition, this pattern does not continue beyond the California populations, since the phylogenetic relationships of *G. gurneyi* to *G. sculleni* and *G. sp. indet.* (Oregon Caves, OR) do not follow the pectinate pattern expected by a direction northward migration by a single ancestral lineage (Fig. 3e). This could be expected in this region, since Oregon Caves, northern Oregon populations and California populations were all south of the major ice sheets, and ancestral populations would likely have inhabited the margin of local glaciers. *G. sculleni* populations could have migrated during the melting period. Therefore at least three lineages in the Sierra Clade were present during the late Pleistocene, since the Oregon Cave population, *G. sculleni*, and *G. gurneyi* would not be expected to be found in their present distribution based on northward migration.

Temperatures and ice were not as extreme along the coast of California, Oregon, Washington, and British Columbia, and much of the coast and nearby inland areas may have been free of ice sheets throughout the Pleistocene (Conchon, et al., 1992). Therefore the *Grylloblatta* sp. indet. population in Oregon Caves likely could have inhabited this locality throughout the glaciation without little change in habitat.

4.5 Lineages within the Cascade Clade
Hundreds of lava tubes are found in southern Washington, many of which maintain permanent ice. The principal concentration of caves in southern Washington is in two localities: near Mt. St. Helens and 50 km to the east in the town of Trout Lake. These caves are not at high elevation (300-400m), but they are numerous and often surrounded by exposed and unexposed lava beds.

**Trout Lake Clade:**

A total of 16 individuals were sampled from six populations near the Trout Lake region in Washington, and all localities are within 20 km of each other, except for Little Red River Cave, which is ca. 40 km away from the other caves. All of these individuals form a well-supported monophyletic group (BP=100, BS=31). Within this clade, there are two well supported lineages: one comprised of taxa from Dead Horse Cave, Little Red River Cave, Ice Caves, Dry Creek Cave, and New Cave (Clade A: BP=100, BS=15); and the other with taxa from Ice Caves and Cheese Cave (Clade B: BP=99, BS=). There were seven individuals sampled from the Ice Caves Picnic area: the six immature specimens group with clade B while the single adult groups with clade A. This result suggests that there is migration among these populations, and that the adult collected in Ice Caves Picnic Area originated from a clade A rather than clade B population. Given the proximity of these populations, and the network of lava tubes in this region, we expected to observe some migration patterns.
**Chirugica Clade:**

The Chirugica Clade contains populations from the Mt. St. Helens and Mt. Rainer area, and this is a well-supported monophyletic group (BP=100, BS=32). Both individuals from the Mt. St. Helens area are from caves (Ape Cave and Surprise Cave), and both individuals from the Mt. Rainier area are surface populations (Chinook Pass and Goat Peak Trail). Mt. Rainier is ca. 80 km to the north of Mt. St. Helens, with high elevation and year-round cold temperatures in the region between the two areas, so as in the other clades, some migration may be expected. The haplotype diversity within this group is very low (0.0-0.1% pairwise distance), so these taxa are all considered *G. chirugica*.

**Campodeiformis Clade:**

The adult *G. campodeiformis* specimen from Alberta is well supported as sister taxon to the two *G. sp. indet.* immature specimens from Montana (BP=100, BS=58, Fig. 3b, localities 1&2). These populations are likely conspecific, as *G. campodeiformis* has been documented in the vicinity of the Montana locality, and these sister taxa are very similar (0.2% pairwise distance across all six genes). This clade is in turn sister to a clade comprised of an individual from Sawyer Ice Cave in Oregon and an individual from Chinook Pass in Washington (Fig. 3b, localities 4&14. While the closely related Montana and Alberta populations are both likely *G. campodeiformis*, the Sawyer Ice Cave and Chinook Pass individuals are phylogenetically distinct and may represent an isolated lineage.
**Sawyer Ice Cave Taxa:**

The polyphyly of the Sawyer Ice Cave and Chinook Pass *G. sp. indet.* populations is well supported in each case (BP=100 for each, BS=32-62). These rather unexpected relationships indicate that multiple species exist sympatrically, which has never been suggested previously. Phylogenetic analyses of each gene in POY indicate that none of the genes support monophyly of either the Sawyer Ice Cave or the Chinook Pass population. In the Campodeiformis Clade, both of the individuals from these populations were adults, whereas the Chinook Pass individual in the Chirurgica Clade was a nymph and the two Sawyer Ice Cave individuals sister to the Trout Lake Clade were nymphs. The relationships among grylloblattid populations may be much more complex than previously assumed.

**Cascade Clade dispersal:**

Northern Oregon ice crawlers provide a unique story in the evolution of grylloblattids, with three species inhabiting a relatively small geographical area. The Sawyer Ice Cave individuals of the Cascade Clade are only 30 km from the McKenzie Pass population of the Sierra Clade. Although the major ice sheets did not completely cover Oregon, large portions of Oregon experienced deep seasonal frosts and large glaciers, so the ancestors to the modern grylloblattid populations may have lived at lower elevations, at the edge of these glaciers, or in caves with relatively constant temperatures. This region may have been an ideal place for multiple lineages to inhabit in the Pleistocene. The result is the highest grylloblattid diversity for an area with a diameter of 30 km.
The range of *Grylloblatta campodeiformis campodeiformis* is congruent with an early split in the ice sheets that extended into Alberta before the rest of the ice sheets receded as far northward. This would cause one to expect close relationships among this subspecies, since this species could have migrated northward more quickly than other populations as this “peninsula” of habitat formed. This coincides with our finding of *G. campodeiformis* from the region of Bozeman, Montana as a closely related sister group to the Banff, Alberta *G. campodeiformis* population.

4.6 Conservation Status in Gryllolbattids

The continual warming of the earth since the last glaciation, compounded by human-induced global warming in recent years is causing the rapid loss of glaciers and ice sheets (Haeberli, et al., 1999; Houghton, 2001). In the next few decades, the rate at which glaciers are melting is projected to increase by two to four times from their already high rate, largely due to anthropogenic causes (Haeberli, et al., 1999). Gryllolbattids’ dependence on glacial margin habitats means that global warming is a direct threat to their future.

Conservation Status of Asian genera:

Both *Gryllolbattina djakonovi djakonovi* and *G. djakonovi kurentzovi* merited Vulnerable (VU, Criterion D2) status by IUCN Red List Criteria (IUCN, 2001). A generous estimate of an area of occupancy is ca. 1 km$^2$ for each lineage, much less than the 20 km$^2$ required for this designation. *Gryllolbattina djakonovi djakonovi* is only known from a single
locality, and *G. djakonovi kurentzovi* is known from two localities (Pravdin and Storozhenko, 1977). There is no imminent threat to these localities, but the habitat is specific enough that stochastic events could jeopardize these grylloblattids so future changes in this designation are likely.

The *Galloisiana* population at Kakuma Valley was designated as Data Deficient (DD), whereas *G. yuasai* and *G. nipponensis* were designated as Near Threatened (NT). The Kakuma Valley population is a unique lineage relative to the other species included in this study, but little is known about its relationships to the other eight *Galloisiana* species, so conservation status could not be designated with certainty. *Galloisiana yuasai* and *nipponensis* have been found at upwards of ten localities each (Asahina, 1959; Gokan, et al., 1982; Gurney, 1948; Kishida, 1929; Nagashima, et al., 1982; Storozhenko, 1996), and are found at disparate locations across Japan. Thus it seems likely that these species are even more widespread than current records indicate, and thus are not under as immediate a threat as other grylloblattids. However, the nature of their habitat indicates that they are not of Least Concern (LC).

**Sierra Clade Conservation Status:**

*Grylloblatta gurneyi* and the Wilson Ice Cave Clade were designated as Critically Endangered (CR, Criteria B.2.a&b), as they are each found in a small and fragmented extent of occupancy (<10 km$^2$) with declining habitat. The ice is melting in Merrill Ice Cave and other LBNM caves (Kelly Fuhrmann, pers. comm.), and thus visitors are not allowed in some caves. We observed that Wilson Ice Cave is a small cave (<200m$^3$) that
is not gated or protected otherwise, and that is frequented by visitors and thus is highly disturbed, underscoring the importance of carefully managing these populations to prevent their extinction.

*Grylloblatta sculleni* and *G. rothi* both fit the criteria of Endangered (EN, Criteria B.2.a & b), with their relatively small extent of occupancy (<5000 km$^2$), presence of less than five populations, and decline of their ice field habitat. Populations of these surface species may have a larger area of occupancy than cave populations, since the ice fields on McKenzie Pass are large for most of the year and there is much space between lava rocks on the pass. Thus the area of occupancy for populations of both *G. sculleni* and *G. rothi* may be as large as 1 km$^2$, but there are only three known populations of *G. sculleni* and two known populations of *G. rothi*.

Data Deficient status was designated for the Oregon Cave and Carpenter Ridge populations. Since the Oregon Cave and Carpenter ridge populations are separate lineages from other members of the Sierra Clade, these lineages were found to be in extreme need of further research. Only single specimens were available from each location, and nothing else is known of the populations, so designating conservation status is not yet warranted, and therefore we suggest that conservation measures be taken urgently until further research is completed on these populations. Oregon Cave, which is in Oregon Cave National Park, is under careful management presently, but Carpenter Ridge population is found on US Forest Service land and not under protection currently.
Cascade Group Conservation Status:

*Grylloblatta* populations from the Trout Lake Clade and Chirurgica Clade both fit Vulnerable status (VU Criterion D2). *Grylloblatta chirurgica* is the only grylloblattid currently listed in the IUCN Red List (Groombridge, 1994). This species contains at least 14 populations (University of Washington Burke Museum Collection), and this analysis indicates that populations in the Mt. Rainier area are also *G. chirurgica*, but the area of occupancy for each population is smaller than for surface populations, and the caves are threatened by human destruction, global warming, and the eruptions of the Mt. St. Helens volcano. The Trout Lake Clade includes six populations in a small geographical area, and may be more widespread than this analysis indicates, since there are many similar caves that are likely connected in this region. The Trout Lake area populations face the same threats as *G. chirurgica*.

*Grylloblatta campodeiformis* was designated as Near Threatened. This species has a wide range across the Rocky Mountains. Most of the data on *G. campodeiformis* distribution are decades old (Kamp 1979). Current specific habitat conditions are unknown, but it is known that glaciers and ice sheets in general are declining, which are the primary habitat for *G. campodeiformis*. The area of occupancy of each population is small (~1km$^2$), as with all grylloblattids, but ca. 30 populations (Kamp 1979) exist, and the extent of occupancy is great. The habitat is certainly declining, however, and we expect that future conservation designations will be warranted in the future.

The Sawyer Ice Cave populations in both the Campodeiformis Clade and the sister to the Trout Lake lineage are in unique phylogenetic positions, but little is understood about
these novel relationships, so we these were designated as Data Deficient. This designation was also applied to the Chinook Pass individual in the Campodeiformis Clade. We propose that special attention will be given to these regions until more is understood about the populations and the relationships among them. The Sisters region of Oregon contains a high concentration of grylloblattid diversity, and Sawyer Ice Cave may contain the most phylogenetically diverse group of grylloblattids. It would behoove land managers of the area to carefully monitor this cave to prevent its destruction, particularly since they are located in easily accessible regions that can be damaged by careless visitors.

Conservation of Other Central California Populations

*Grylloblatta washoa*, *G. bifratrilecta*, *G. chandleri*, and *G. barberi* of central California warranted Critically Endangered status (CR criteria B1a+biii, B2a+biii). *Grylloblatta washoa* is only found near Echo Peak, by Lake Tahoe, California. Willis Gertsch and Vincent Roth collected four specimens there in early September 1959. If this species is still extant, it is likely very few in number; attempts by Karl Jarvis and six others to collect *G. washoa* in August 2001, June 2002, and November 2003 were unsuccessful, although they dug through rotten logs as in the type description (Gurney, 1961). They also set sticky traps baited with Limburger cheese during each collecting attempt and spent 40 collective hours searching in and under logs and under rocks. *Grylloblatta barberi*, *bifratrilecta*, and *chandleri* are all in similar situations, each are found only in a single location and not collected since 1959, 1968, and 1959, respectively. The authors have visited the type localities of each of these species and attempted to collect these
species on multiple occasions, but without success. When D.C.F. Rentz collected *G. bifratrilecta* at the type locality (Sonora Pass, Tuolumne Co., California, near Yosemite National Park) he collected them on snowfields in July. The authors found no snow within 5km of Sonora Pass in August 2001. The combination of *Grylloblatta*’s unique cryophilic characteristics and humanity’s effect on the environment may prove deadly for these insects.

5. Conclusions

This study presents the first-ever formal phylogenetic hypothesis of grylloblattid genera and species. The topology confirms the monophyly of the three genera included in the analysis: *Grylloblatta*, *Galloisiana*, and *Grylloblattina*. This analysis indicates that the eastern Asian genus *Galloisiana* is sister to a monophyletic group of the east Siberian *Grylloblattina* and the North American *Grylloblatta*. Two major clades are recovered within *Grylloblatta*: the Cascade Clade, which is principally in the northern Pacific Northwest, and the Sierra Clade, which is principally in the southern Pacific Northwest. Well-supported clades within the Cascade Clade include the California Caves Clade, composed of *Grylloblatta gurneyi* in the LBNM Clade and the Wilson Ice Cave Clade, as well as the Sisters Area Clade, containing *G. rothi* and *G. sculleni*. Phylogenetic relationships within the Cascade Clade include the Trout Lake Clade containing a new species, the Chirurgica Clade with *G. chirurgica*, and the Campodeiformis Clade with *G. campodeiformis* and two *G. sp. indet.* specimens from Oregon and Washington.
*Grylloblatta* populations generally do not follow a pattern expected if they were part of a single species prior to the end of the Pleistocene glaciation. Fossils do not provide many clues to the origin of modern grylloblattids. Shrinking ice fields and ice caves are an imminent threat to this species. Most grylloblattids species were designated as Vulnerable, *G. rothi* and *G. sculleni* were designated as Endangered, and the California cave species are designated as Critically Endangered.
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<td>1.61</td>
<td>1.14</td>
<td>1.56</td>
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</tr>
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<td>Normalized Support</td>
<td>32.8%</td>
<td>18.6%</td>
<td>13.1%</td>
<td>18.1%</td>
<td>8.9%</td>
<td>8.5%</td>
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<td><strong>Grylloblatta</strong></td>
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<tr>
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<td>46.8%</td>
<td>10.6%</td>
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<td>1.02</td>
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<td>4.2%</td>
</tr>
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Figure 4.
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