

## HISTORIC CYCLES OF FRAGMENTATION AND EXPANSION IN *PARNASSIUS SMINTHEUS* (PAPILIONIDAE) INFERRED USING MITOCHONDRIAL DNA

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**Abstract.**—Climate oscillations of the Quaternary drove the repeated expansion and contraction of ecosystems. Alpine organisms were probably isolated in sky island refugia during warm interglacials, such as now, and expanded their range by migrating down-slope during glacial periods. We used population genetic and phylogenetic approaches to infer how paleoclimatic events influenced the distribution of genetic variation in the predominantly alpine butterfly *Parnassius smintheus*. We sequenced a 789 bp region of cytochrome oxidase I for 385 individuals from 20 locations throughout the Rocky Mountains, ranging from southern Colorado to northern Montana. Analyses revealed at least two centers of diversity in the northern and southern Rocky Mountains and strong population structure. Nested clade analysis suggested that the species experienced repeated cycles of population expansion and fragmentation. The estimated ages of these events, assuming a molecular clock, corresponded with paleoclimatic data on habitat expansion and contraction over the past 400,000 years. We propose that alpine butterflies persisted in an archipelago of isolated sky islands during interglacials and that populations expanded and became more connected during cold glacial periods. An archipelago model implies that the effects of genetic drift and selection varied among populations, depending on their latitude, area, and local environment. Alpine organisms are sensitive indicators of climate change and their history can be used to predict how high-elevation ecosystems might respond to further climate warming.

**Key words.**—Alpine, biogeography, Lepidoptera, nested clade, paleoclimate, Rocky Mountains.

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The glacial cycles of the Quaternary played important roles in the assembly of communities and the evolution of species by shaping the genetic structure and distribution of organisms (Huntley and Webb 1989; Pielou 1991; Webb and Bartlein 1992; Klicka and Zink 1997; Hewitt 1996, 2000). Cycles of approximately 100,000 year-long glacial periods separated by 20,000 year warm interglacials are explained largely by the behavior of the earth in its orbit around the sun (Richmond 1965). In the Rocky Mountain region of western North America, the extent of glaciation varied with latitude, creating a geographic mosaic of continental ice sheets, mountain glaciers, and suitable habitats. During glaciations, biome distributions were pushed southward and to lower elevations by a cooling climate and advancing ice. At the time of the Last Glacial Maximum—about 18,000 years ago—the Cordilleran and Laurentide ice sheets of North America blanketed most of Canada and extended south across much of the eastern United States and the Midwest (Hollin and Schilling 1981). In the Rocky Mountains, ice extended south into northern Montana, with only the highest ranges and peaks protruding above the ice sheet as nunataks (Carrara 1989). During that time, the Greater Yellowstone Ecosystem was covered in 1000 meters of ice (Pierce 1979). Drier and warmer climates of the southern Rockies shielded the ecosystems from the sheet ice, but expansion of mountain glaciers had a dominant influence on the extent and distribution of biomes (Elias 1996). Glacial periods were interrupted by warm interglacials, during which biomes were probably distributed much as they are today (Pielou 1991).

### *Models of Population Expansion*

The geographic distribution of genetic variation within a species depends on how organisms responded to the repeated cycles of habitat expansion and contraction associated with

the glacial cycles; the number, size, and location of refugia; the connectedness among populations; dispersal distances; and the rate of recolonization. Two prevailing scenarios contrast the ways that organisms might have responded to paleoclimatic oscillations—the pioneer and phalanx models.

The pioneer model, articulated by Hewitt (1996), emphasizes the strong latitudinal effect of glaciers. During cold glacial periods, biome distributions were pushed southward. Species displaced by advancing ice and cooler temperatures survived in southern refugia—ice-free areas buffered from severe environmental change because of latitude, topography, and local climate (Hewitt 2000; Tzedakis et al. 2002). As the climate warmed, populations expanded northward from refuge sites. The leading edge of expansion was marked by repeated long-distance colonization events, rapidly filling the newly available habitat, but resulting in the loss of alleles from founder effects (Hewitt 2000).

By contrast, the phalanx model describes the effects of slower expansions from refugia. In general, bottlenecks are less prevalent than in the pioneer model because many alleles are able to colonize sites over short distances (Hewitt 1996). Within population genetic diversity is maintained, and potentially generated, through this process.

### *Rocky Mountain Historical Biogeography*

The distinct climatic history and geographic heterogeneity of the Rocky Mountains present a fertile landscape for studies of historic biogeography. Unlike the well-studied mountain ranges of the western Palearctic, which follow an east–west axis and can inhibit north–south migration (see Hewitt 1996), the Rocky Mountains, like many ranges in the Nearctic, are oriented north–south. The great latitudinal range and elevation differences (<2000–4000 m) promote local variation in climate.

Most of our understanding of Rocky Mountain historic biogeography comes from studies on organisms inhabiting the low elevation biomes, such as foothills and montane forests. Two patterns of how organisms responded to glacial cycles have emerged. Several species, including plants (*Larix occidentalis*, Fins and Seeb 1986; *Pinus monticola*, Steinhoff et al. 1983; *Pseudotsuga menziesii*, Aagaard et al. 1995); mammals (*Tamiasciurus* spp, Arbogast et al. 2001; *Ochotona princeps*, Hafner and Sullivan 1995; *Microtus longicaudus*, Conroy and Cook 2000); reptiles (Zamudio et al. 1997); and insects (Nice and Shapiro 2001) show a pattern consistent with a single southern refuge during the glacial period and rapid, northward postglacial recolonization by a few haplotypes during warm periods. Species of alpine plants (Tremblay and Schoen 1999; Yates et al. 1999; Abbott et al. 2000) and alpine grasshoppers (Knowles 2000), in addition to some lower-elevation chipmunks (Good and Sullivan 2001), birds (Zink 1996), and lizards (Orange et al. 1999) show evidence of multiple glacial refugial sites throughout their distribution.

Although the Rocky Mountains harbor some of the highest diversity in North America north of Mexico, and were greatly impacted by glacial cycles throughout the Quaternary, little is known about the historic biogeography of high-elevation Rocky Mountain inhabitants. The topographic complexity of the Rocky Mountain region may have allowed for critical habitat and populations to persist in multiple areas throughout the Rockies during glaciations. In Knowles' (2000) study of alpine grasshoppers, several refugia were inferred across a relatively restricted range within the central Rockies (Wyoming and Montana). Locations of refugia for more widely distributed organisms remain to be discovered. In addition, it is unknown whether high elevation organisms primarily migrate up and down in elevation and thus follow a phalanx model of dispersal. If so, isolation on mountain tops, or sky islands, during warm interglacials could promote divergence between populations. Down-slope movement of the alpine tundra during glacial periods could broaden and connect previously fragmented habitat and allow for the exchange of alleles among populations (Elias 1996; Hewitt 2000; Fig. 1). Because the extent of the alpine probably increased during glacial periods and decreased in interglacials, opposite to the expected response of lower elevation temperate biomes, analysis of the historical biogeography of alpine organisms should provide new insights into the effects of the glacial expansion and contraction cycles on the genetic structure of populations. Such a contrast significantly highlights that general patterns of historical biogeography may be relevant only to organisms inhabiting the same biome.

Understanding the historical biogeography of the alpine is gaining urgency because of the profound effect of climate change. The alpine tundra habitat exists across a vast area of North America above the tree line. The extent of habitat above tree line can be severely limited, however, especially at lower latitudes. Decreases in snowpack, the retreat of glaciers, and the upslope advancement of forest trees associated with the increasing global temperatures shrinks alpine habitats (Dyrgerov and Meier 2000; Hewitt 2000). A broad genetic survey of high elevation species can provide an important assessment of the past effects of glacial cycles on regional biodiversity, and may identify critical habitat that

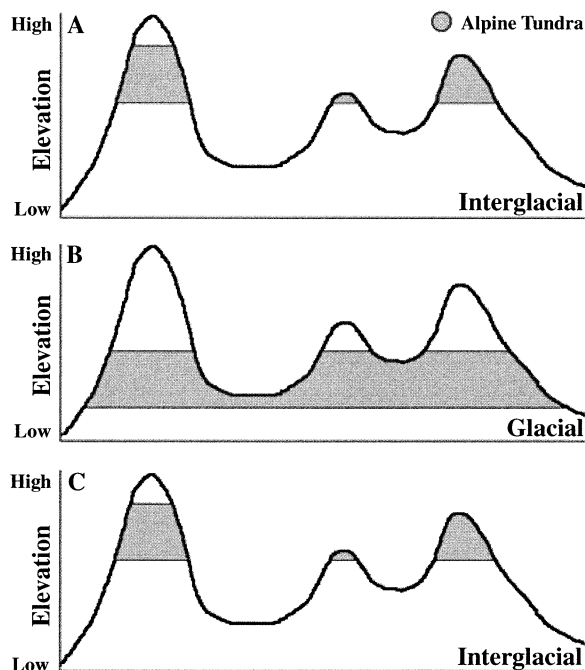


FIG. 1. Hypothetical migration of alpine tundra habitat in response to the glacial cycle of the Pleistocene. The hypothetical distribution of alpine tundra is shown in gray. (A) and (C) represent the fragmented distribution of alpine tundra during the warm interglacial or interstadial periods. (B) The connected alpine tundra of the glacial or stadial periods.

harbors unique lineages. Given that interglacials are periods of reduced distribution for alpine organisms, such a survey provides a unique view of the distribution of genetic variation in refuges where predominantly cool climate, high elevation adapted organisms persist during the "bad times" of a warm global climate.

#### Study Organism

Butterflies are particularly sensitive biological indicators of climate change (Dennis 1993). Because population survival depends on the area requirements of the organisms and small organisms need less area, Lepidoptera provide an excellent chance of locating refugia (Pielou 1991). For North America north of Mexico, lepidopteran diversity peaks in the southern Rocky Mountains, possibly due to the influence of both topographic and climatological heterogeneity (Kocher and Williams 2000). Of the North American alpine butterflies, the Rocky Mountain Apollo *Parnassius smintheus*, is well suited for analyses of historical biogeography. *Parnassius smintheus* occurs in the alpine and lower elevation meadows of the Rocky Mountains from southern Colorado to Alaska, as well as in the Sierra Nevada and Cascade Ranges, and is restricted to meadows, where it is abundant and plays an integral role in the alpine community (Scott 1992). Because movement is inhibited by forest, potential dispersal is restricted to open meadows and ridges (Keyghobadi et al. 1999). Individuals usually remain within their natal site, leading to stable, genetically differentiated populations (Keyghobadi et al. 1999).

### Study Objectives

Repeated cycles of habitat expansion and fragmentation have profound implications for maintenance and distribution of genetic diversity within and among populations. The goal of this study is to infer the biogeographic history of *Parnassius smintheus* of the Rocky Mountain alpine tundra south of the great ice sheets. To do so, several basic questions must be answered. How many refugia were there and how were they distributed across the north–south axis of the Rocky Mountains? Did the distribution of high elevation organisms contract during cold, glacial periods, as is the case for lower elevation, temperate biomes, or were populations of alpine species more affected by fragmentation during warm, interglacials and connected during cold periods? Was long-distance dispersal a major influence on the reshuffling and distribution of haplotypes, or was migration primarily short, along an elevational gradient? By answering these questions, we will begin to address how paleoclimatic oscillations of the Quaternary affected the distribution of the Rocky Mountain alpine environment and the genetic variation of its inhabitants. Based on the aforementioned studies of alpine insects and theory on how montane organisms responded to glacial cycles (Hewitt 2000), we expect that populations of *P. smintheus* survived in several interglacial refugia, expanded and migrated during glacial episodes, and exhibit a phalanx pattern of slow up-and-down migration.

In addition, we estimate the age of clade diversification to infer how historic climatological events influenced the distribution of genetic variation in *P. smintheus* of the Rocky Mountains. Our results imply that *P. smintheus* persisted in multiple refugia, and inferences of the temporal history of population expansion and contraction align closely with the record of glacial stadials and interstadials, respectively. Such a close match confirms that alpine butterflies are useful indicators of climate change.

This study infers the history of haplotypes of *P. smintheus* from Rocky Mountain populations, which constitute only a portion of the species' range. The main goal of this study is to examine how the glacial cycles affected the geographic distribution of genetic diversity within the Rockies south of the southern extent of the continental ice sheets, which required sampling many individuals from many sites within that region. A complete examination of populations throughout the entire range of the species is most desirable for phylogeographic studies. However, a recent study of the biogeographic history of the butterfly, *Oeneis chryxus*, which shares *P. smintheus*' distribution and utilizes similar habitat, showed that populations in the Sierra Nevada were derived from populations in the Rocky Mountains and thus, did not affect the distribution of haplotypes in the Rockies (Nice and Shapiro 2001). Although southern migration of individuals from a Beringian refuge is also possible, studies on other Rocky Mountain insects (Britten and Brussard 1992; Reiss et al. 1999; Nice and Shapiro 2001) demonstrated that haplotypes south of the Cordilleran and Laurentide ice sheets formed a monophyletic clade, distinct from those in the Canadian Rockies.

### METHODS

Three hundred and eighty-five individuals of *Parnassius smintheus* were collected from 20 alpine sites throughout the Rocky Mountains, ranging from southern Colorado where the effects of habitat fragmentation are most severe to northern Montana which was completely covered by ice sheets during the glacial periods. This region is only a portion of *P. smintheus*' distribution, but encompasses an area proposed to be the ancestral distribution of many western montane organisms (Wells 1983; Nice and Shapiro 2001), is geographically separated from other portions of the species' range, and is small enough to permit adequate spatial sampling of refugia. Collection sites included: Glacier National Park (permit no. GLAC-2001-SCI-0020), Yellowstone National Park (permit no. YELL-2001-SCI-0212), Grand Teton National Park (permit no. GRTE-2001-SCI-0009), Rocky Mountain National Park (permit no. ROMO-2001-SCI-0037), as well as sites in other public lands (Fig. 2, Table 1). Ten to 35 individuals were collected from each locality (Table 1). The butterflies were collected with a handnet and stored in glassine envelopes. Specimens were transported on dry ice and stored at  $-80^{\circ}\text{C}$  at the University of Colorado, Boulder.

### Molecular Methods

DNA from the thorax was extracted using the DNeasy Tissue Extraction Kit (Qiagen, Valencia, CA). Mitochondrial DNA is the best available and most commonly used genetic marker for intraspecific phylogenetic analyses of insects (Caterino et al. 2000). The small population size and rapid rate of evolution make mtDNA genealogies particularly sensitive to the processes of population subdivision (Avice 2000). Thus, a 970 bp fragment of mitochondrial DNA was amplified with the polymerase chain reaction (PCR) using specific primers for cytochrome oxidase I. Each 50 microliter reaction volume contained 2.25 mM  $\text{MgCl}_2$ , 0.02 mM dNTPs, 0.05 mM of each primer, 2.5 units of Promega (Madison, WI) B Taq polymerase, Promega 10  $\times$  reaction buffer, and approximately 100 ng of genomic DNA. Specific primers were designed from *P. smintheus* COXI sequence data that was kindly provided by F. Sperling. The primers were: Ppj: (5'-AATGGAGCAGGAAGCTGGATG-3') and Ppk: (5'-AATCGGAGTATCGTCGAGGT-3'). The thermal cycler profile was one cycle at  $94^{\circ}\text{C}$  for 4 min; 30 cycles at  $94^{\circ}\text{C}$  for 1 min;  $52^{\circ}\text{C}$  for 45 sec;  $72^{\circ}\text{C}$  for 1 min, 30 sec, and one extension cycle at  $72^{\circ}\text{C}$  for 7 min. PCR products were cleaned using the Wizard PCR Preps (Promega). Forward and reverse strands were sequenced on a Li-Cor 4200 automated sequencer (Li-Cor, Inc., Lincoln, NE). Sequencing products were generated from the cleaned PCR product with an Excel II Sequencing Kit (Epicentre Technologies, Madison, WI) and 0.09 mM of both nested primers. The nested primers were JNfwd: (5'-GCAGGAAGCTGGATGAACAG-3') and KNrev: (5'-GAGTATCGTCGAGGTATCC-3'). For the cycle sequencing reaction, the thermal cycler profile was one cycle at  $95^{\circ}\text{C}$  for 3 minutes, 30 cycles at  $95^{\circ}\text{C}$  for 1 min;  $60^{\circ}\text{C}$  for 30 sec; and  $70^{\circ}\text{C}$  for 1 min, 30 sec. Sequences were checked on AlignIR version 2.0 software (Li-Cor, Inc.) and aligned with Clustal X (Thompson et al. 1997).



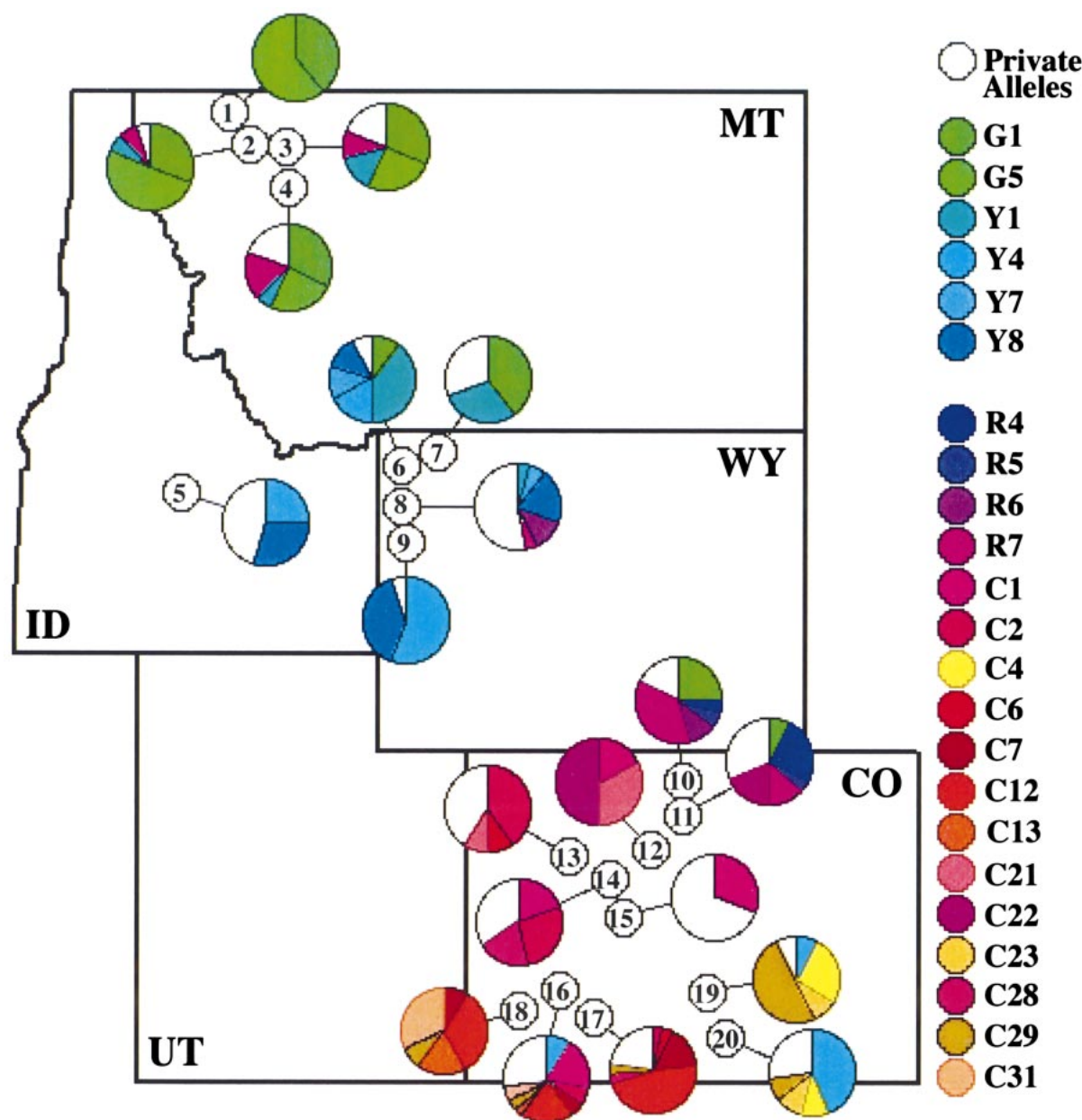


FIG. 2. Map showing the collection sites (numbered within circles) and the distribution of haplotypes. Population numbers correspond to those in Table 1. The distributions of wide-ranging haplotypes are denoted by color. Private alleles are shown in white (see Table 1 for list of alleles). Pie charts show the proportions of haplotypes within each population. See Table 1 for the frequency of private alleles within each population.

*Genetic Variation*

The average pairwise sequence divergence, theta ( $\pi$ ), and the nucleotide polymorphism, theta ( $S$ ) were used to estimate genetic diversity within each population. Calculations were made on Arlequin 2.0 (Schneider et al. 2000). Regressions of theta ( $\pi$  and  $S$ ) versus latitude were performed to test whether diversity decreased with increasing latitude as is expected under a model of rapid, postglacial expansion from southern refugia, or had no relation with latitude. Analyses were carried out for all populations, and for the northern populations 1–9 and southern populations 10–20 separately.

To test for isolation-by-distance, we performed a regression of standardized pairwise population  $F_{ST}$  values [ $=F_{ST}/(1-F_{ST})$ ] against the logarithm of geographic distance (Rousset 1997) for all populations within the Rocky Mountain study area, as well as within the northern and southern regions separately. Hierarchical population structure was tested with an Analysis of Molecular Variance (AMOVA). Variation was partitioned at three levels: among regions (northern and southern), among populations within regions, and within populations. These calculations were performed using Arlequin 2.0 (Schneider et al. 2000).

TABLE 1. Collection sites, genetic diversity (theta), and distribution of private alleles. Site numbers correspond to those in Figure 2 and allele numbers to those in Figure 4. Abbreviations are as follows: GNP, Glacier National Park; GTNP, Grand Teton National Park; RMNP, Rocky Mountain National Park; ID, Idaho; and CO, Colorado. Private alleles only occur within one population, by definition. % Private is defined as the percentage of all individuals within a population that exhibit private alleles.

Site No	Site name	n	Latitude	Longitude	Theta ( $\pi$ )	Theta (S)	Private alleles	% Private
1	GNP Numa Peak	12	48.8963 N	114.1691 W	1.5909	0.9934	—	0.0
2	GNP Gunsight Pass	27	48.6111 N	113.7333 W	1.9145	1.8161	G9	3.7
3	GNP Triple-Divide Pass	21	48.5694 N	113.5139 W	2.2857	1.9457	G4, G7, G8	23.8
4	GNP Dawson Pass	13	48.4833 N	113.4667 W	2.6838	2.6621	G2, G3, G6	23.5
5	ID Hyndman Peak	32	43.7381 N	114.1394 W	1.7241	1.7824	I1, I2, I3, I4	48.3
6	YNP Mt. Washburn	27	44.7999 N	110.4256 W	1.7949	1.8161	Y3, Y11	7.4
7	YNP Amethyst Mtn.	20	44.8194 N	110.2306 W	1.8526	1.4093	Y2, Y5, Y6	30.0
8	GTNP Moose Pass	20	43.9528 N	110.8444 W	2.6667	2.2718	Y9, Y10, Y12, Y13, Y14, Y16, Y17	53.3
9	GTNP Static Peak	15	43.6797 N	110.8178 W	1.1810	0.9226	Y15	6.7
10	RMNP Sundance Mtn.	13	40.4041 N	105.7019 W	2.1754	2.0028	R11, R12	15.8
11	RMNP Long's Peak	35	40.2583 N	105.6167 W	3.5397	3.0837	R1, R2, R3, R8, R9, R10	32.1
12	CO Quandary Peak	10	39.3947 N	106.0981 W	0.9111	0.7070	—	0.0
13	CO Maroon Bells	10	39.0417 N	106.9878 W	1.6667	1.7674	C3	40.0
14	CO Mt. Elbert	15	39.1191 N	106.4287 W	1.8476	1.5377	C5, C18	33.3
15	CO Mt. Shavano	21	38.6167 N	106.2417 W	2.0762	2.5016	C17, C24, C25, C26, C27, C32, C33	71.4
16	CO American Basin	33	37.9139 N	107.5139 W	3.7727	2.9568	C19, C20	27.3
17	CO San Luis Peak	28	37.9875 N	106.9292 W	2.6995	2.5464	C8, C10, C11, C14, C30, C34	24.1
18	CO Wilson Peak	10	37.8583 N	107.9875 W	2.3556	1.7674	—	0.0
19	CO Humboldt Peak	13	37.9750 N	105.5611 W	2.9848	2.6491	C9	8.3
20	CO Iron Nipple	10	37.5917 N	105.4542 W	3.1273	2.7313	C15, C16	27.3

### Nested Clade Analysis

Nested clade analysis (NCA) infers population history by examining a haplotype network in a geographic context. Statistical parsimony methods (Templeton et al. 1992) implemented in the TCS program (Clement et al. 2000) were used to infer the topology of relationships among haplotypes. The relationship between the inferred phylogeny and geography was assessed using the GeoDis software (Posada et al. 2000), which implements nested clade analysis (Templeton et al. 1995). Nested clade analysis can be used to postulate past events such as historical fragmentation, restricted gene flow, range expansion, and dispersal that influenced the population structure of the species. Inferences of historical processes made through NCA should be viewed with caution because the inference key (Templeton et al. 1995) makes qualitative assessments and is unable to estimate error or statistically examine alternative interpretations (Knowles and Maddison 2002).

We estimated the timing of inferred historical population events from nested clade analysis assuming that the butterfly COI gene evolves at a rate of 2.3% per million years (Brower 1994). Estimated dates for fragmentation events (or restricted gene flow) were based on the pairwise sequence divergence between clades (Edwards and Beerli 2000). Thus, for example, the initial fragmentation between clades 4-1 and 4-2/4-3 was dated based on the average divergence between haplotypes comprising each clade (i.e., the divergence between all 4-1 and 4-2/4-3 haplotypes). Range expansion events were dated by calculating tau ( $=2\mu t$ ), as well as its upper and lower 95% confidence limits, based on mismatch distributions generated using Arlequin 2.0 (Schneider et al. 2000).

### RESULTS

A 789-bp region of mitochondrial cytochrome oxidase I was sequenced for 385 individuals sampled from 20 populations. The 76 unique haplotypes were deposited in Genbank with accession numbers AY251311 through AY251385. Of the 54 variable sites, 15 (27.8%) occurred in the first codon position, six (11.1%) in the second position, and 33 (61.1%) were found in the third position. Nucleotide frequencies were strongly AT biased (freq. A = 0.302, C = 0.141, G = 0.141, T = 0.416) as for other insects (Simon et al. 1994).

### Distribution of Genetic Variation

Genetic variation, estimated as the average pairwise sequence divergence within populations, ranged from 0.971 to 3.773 across the 20 localities surveyed (Table 1). The sites with the lowest and highest diversity were Quandary Peak and American Basin, respectively. Both of these populations were at relatively low latitudes. Quandary Peak is isolated in the fragmented Mosquito range of northern Colorado. The American Basin, surrounded by many nearby patches of alpine habitat, is located in the large massif of the San Juan Mountains in southwestern Colorado. Genetic diversity estimates, theta ( $\pi$ , S), were similar regardless of latitude ( $P = 0.17$  for both regressions). Southern latitude sites exhibited a higher variance in theta than northern latitude sites; more-

TABLE 2. AMOVA results for tests of genetic subdivision between populations from northern and southern regions.

Source of variation	Variance components	% of variation
Among regions	0.88***	33.37
Among populations within regions	0.59***	22.15
Within populations	1.18***	44.48
Overall $F_{ST}$	0.56***	

\*\*\*  $P < 0.001$ .

over, of the 10 most variable populations, five were from the most southern localities.

The geographic distribution of haplotypes suggests two geographically distinct sets of populations, north and south of the Wyoming Basin Dispersal Barrier (Noonan 2001), with little gene flow between them (Fig. 2). In addition, most populations harbored private alleles (Table 1), or alleles restricted to a single location, which imply isolation (Slatkin 1985) of sky-island habitat. Most pairwise population comparisons (using  $F_{ST}$ ) were significant. The few exceptions were comparisons involving geographically close populations (see Appendix). A signal of isolation-by-distance was evident for all northern populations ( $r = 0.30$ ,  $P < 0.001$ ), southern populations ( $r = 0.13$ ,  $P = 0.008$ ), and for all populations combined ( $r = 0.18$ ,  $P < 0.001$ ). Similarly, AMOVA revealed significant genetic structure at all hierarchical levels (Table 2). Thirty-three percent of the molecular variation was between the northern and southern populations, 22% of the variation was explained among populations within each region, and an additional 44% was found within populations. These analyses suggest restricted gene flow and geographic isolation of populations.

Nested Clade Analysis

Relationships among haplotypes were inferred using statistical parsimony. A nested cladogram was constructed based on the 95% parsimony network generated by TCS (Clement et al. 2000). Twenty unsampled, ancestral haplotypes were inferred. The simplified cladogram (Fig. 3) shows three 4th level clades; a southern clade (4-1), a geographically intermediate, ancestral clade (4-3), and a predominantly northern clade (4-2). Although we did not determine the ancestral root of the cladogram and thus the geographic origin of *P. smintheus* in the Rocky Mountains, we did infer from the geographic transitions in the cladogram (Wares et al. 2001) that haplotypes from the northern clade colonized the south at least twice (A, B), with one migration from the south to the north (C). Haplotype G1 is geographically widespread and thus confounds inferences of migration direction for those haplotypes associated with it. In addition, the northern haplotype, I1, probably occurred in both regions because of its phylogenetic position at a transitional node between southern and northern populations; our inability to find this haplotype in the south may be a consequence of incomplete sampling. The few geographic transitions emphasize the geographic structure identified from analysis of variance.

The 76 distinct haplotypes were grouped into 41 1st-level clades, 16 2nd-level clades, six 3rd-level clades, three 4th-level clades, and one 5th-level clade (the whole tree) (Fig.

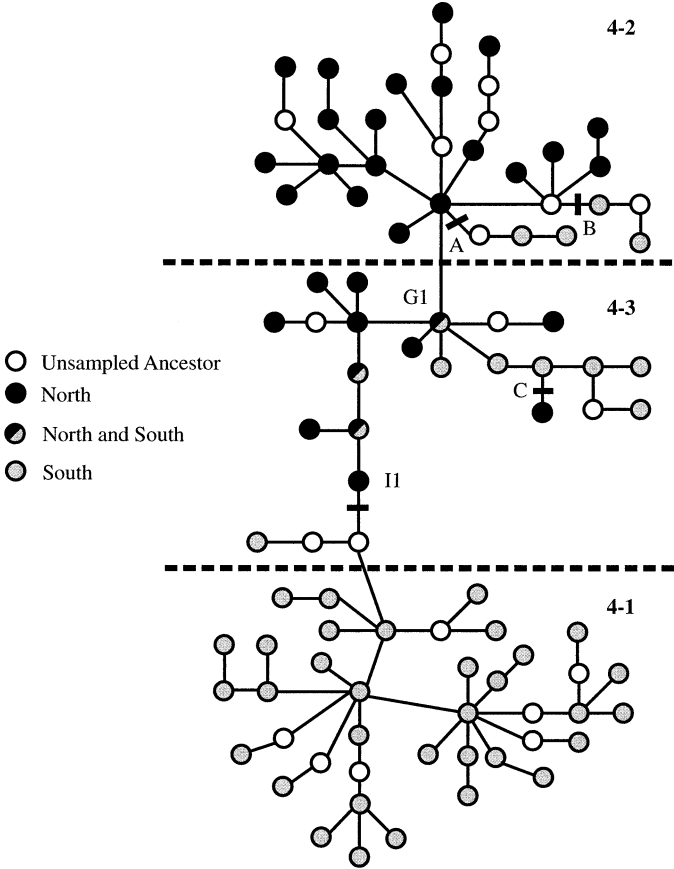


FIG. 3. Simplified phylogenetic network from the nested clade analysis (NCA; Fig. 4). Open circles represent unsampled ancestors, black circles represent haplotypes found in the north, gray are haplotypes found exclusively in Colorado, and black/gray circles are haplotypes occurring in both regions. Hatch marks crossing the lines connecting haplotypes denote possible migration/colonization events between the northern and southern populations. Haplotypes G1 and I1 and possible geographic transition events are referred to in the text. Dashed lines delineate clades 4-1, 4-2, and 4-3 from the NCA.

4). In the nested clade analysis (NCA), significance was determined using  $\alpha = 0.05$ . In Figure 5, significantly large (L) and small (S) clade distances indicate significantly widespread or restricted distributions of haplotypes/clades, respectively. The within clade distance ( $D_c$ ) are the distance of haplotypes/clades from the geographic center of the clade. Nested clade distances ( $D_n$ ) refer to the geographic distribution of the haplotype/clade with respect to other haplotypes/clades with which it is nested. Finally, significant interior-tip contrasts for both  $D_c$  and  $D_n$  values denote a displaced (L) or restricted (S) distribution of young versus old haplotypes/clades. In our nested clade analysis, based on the inferred phylogenetic relationships among haplotypes, we identified 23 clades in which there was a significant match between genealogy and geography (Figs. 4, 5). Based on the inference key (Templeton et al. 1995), the history of *P. smintheus* in the Rocky Mountains was punctuated by several cycles of allopatric fragmentation or restricted gene flow followed by contiguous (phalanx) range expansion or long-distance (pioneer) colonization (Table 3).

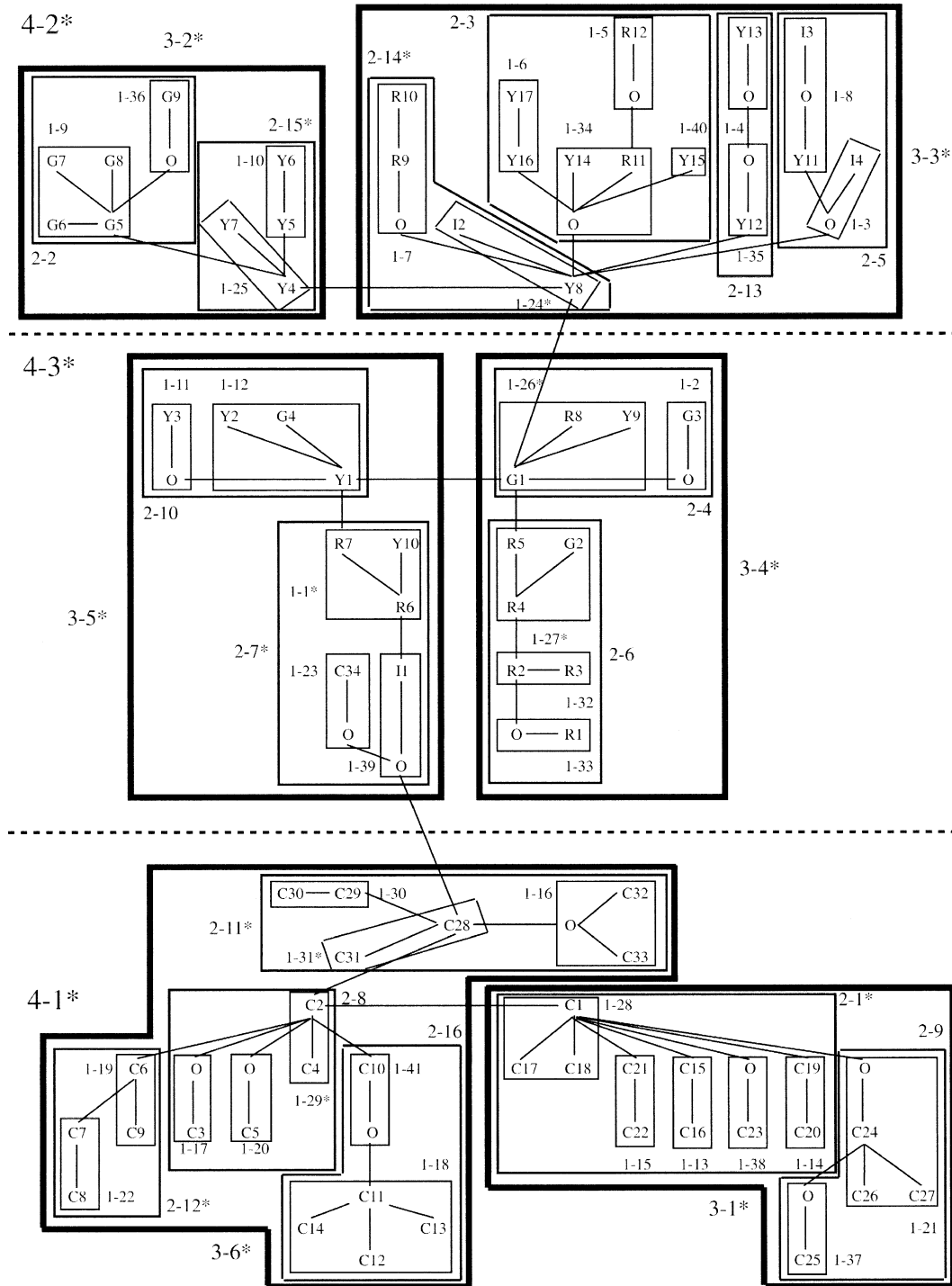


FIG. 4. Haplotype network for nested clade analysis. The 76 unique haplotypes of *Parnassius smintheus* are shown. Hypothetical ancestral haplotypes are denoted by circles. Lines connecting haplotypes represent one mutational step. Clade numbers with an asterisk show significant geographic association. All haplotypes are nested within Clade 5-1. Note that the southern clade (4-1) is separated by the northern clades (4-2, 4-3) by two steps. The dashed line shows divisions between the 4th level clades.

The first NCA-inferred fragmentation event occurred between mostly southern haplotypes (predominantly clustered within clade 4-1) and northern haplotypes (in clades 4-2 and 4-3). Two of these clades, the southern clade (4-1) and a northern clade (4-2), underwent contiguous range expansion. In the south (4-1), the range expansion was followed by frag-

mentation events or restricted gene flow resulting in the two 3rd-level clades (3-1 and 3-6) and three of the 2nd-level clades (2-1, 2-11, and 2-12). More recently, two clades show a signal of long-distance colonization (1-29) and range expansion (1-31). Similar results were evident for the northern clades. Within 4-3, clade 2-7 fragmented. This was followed



## Results of nested clade analysis

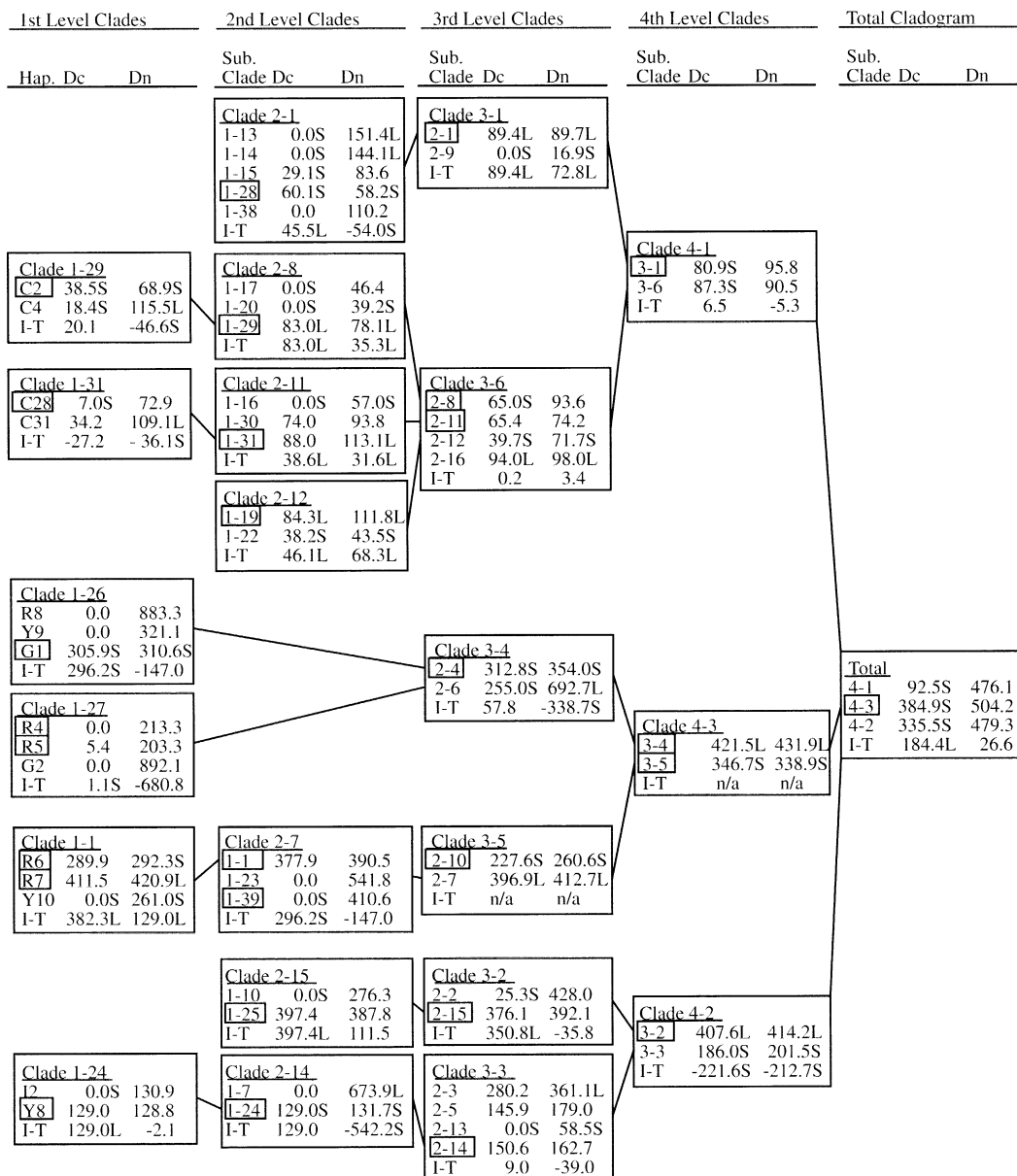


FIG. 5. Results for the nested clade analysis for clades with significant geographic structure. Nesting hierarchy proceeds from the most recent 1st level clades on the left to the deeper clades on right. Within in each clade (boxed), columns indicate haplotypes/subclades or interior-tip contrasts, clade distance (Dc), and nested distance (Dn). Individual haplotypes/clades that are boxed are interior, whereas tip clades are left unboxed. Significantly small and large statistics are denoted by S and L, respectively.

by two recent contiguous range expansions (1-26 and 1-27). It should be noted that clade 3-4 exhibited long distance colonization, but that the uncertainty in the estimate of the age of that event is very large. Following the early contiguous range expansion of clade 4-2, clades 3-2, 2-15, and 1-24 underwent fragmentation or showed signs of restricted gene flow. Clade 2-14 expanded more recently.

Estimates of the timing of fragmentation and expansion events come with broad confidence intervals (Arbogast et al. 2002). Nevertheless, for both fragmentation (or isolation-by-distance) and expansion events, the estimated dates suggests three distinct episodes of expansion and contraction (Table

3, Fig. 6). Nested clade analysis indicated that clades 1-27, 1-31, and 2-14 exhibited a signal of recent population expansion, about 40,000 years ago. Clades 1-26, 3-5, and 4-2 suggest an expansion occurred about 140,000 years ago, and clade 4-1 appears to have expanded over 200,000 years ago. Similarly, several clades (1-24, 2-1, 2-8, 2-11, 2-12, 2-15, and 3-2) appeared to have recorded fragmentation events sandwiched between the inferred expansion events at 50,000 and 150,000 years ago, and clades 2-7, 3-1, and 3-6 suggest a range contraction/fragmentation at about 200,000 years. Although we recognize that the confidence interval on the estimates of time are broad, the repeated juxtaposition of



TABLE 3. Inferences of historical processes. Clades, steps in inference key, and inferences (Templeton et al. 1995) are shown. Tau (min and max) and average number of pairwise differences ( $\pm$  standard deviation) were used to estimate the ages (Kya) of clade divergences for expanding and fragmenting clades, respectively.

Clade	Steps in inference key	Inferred event	tau	tau min	tau max	Average no. differences	SD differences	Estimated Age (kya) of divergence	Age (kya)	
									min	max
Total	1-2-3-4-9-10-yes	Allopatric fragmentation	4.024	2.332	5.524	6.18812	2.5040	341.0	203.0	479.0
4-1	1-2-11-12-no	Contiguous range expansion	2.711	0.872	3.826	—	—	221.7	128.5	304.4
4-2	1-2-11-12-no	Contiguous range expansion	—	—	—	—	—	149.4	48.1	210.8
4-3	1-2-?	Inconclusive	—	—	—	—	—	—	—	—
3-1	1-2-3-4-no	Restricted gene flow with isolation by distance	—	—	—	3.56383	1.8950	196.4	92.0	300.8
3-2	1-2-3-4-9-no	Past fragmentation or isolation by distance	—	—	—	2.15152	1.4700	118.6	37.5	199.5
3-3	1-2-11-17-no	Inconclusive	—	—	—	—	—	—	—	—
3-4	1-2-11-12-13-14-yes	Long distance colonization	0.000	0.000	5.687	—	—	0.0	0.0	313.4
3-5	1-2-?	Inconclusive	—	—	—	—	—	—	—	—
3-6	1-2-3-5-15-no	Past fragmentation	—	—	—	3.69138	1.9285	203.4	97.1	309.6
2-1	1-2-3-5-15-no	Past fragmentation	—	—	—	2.70560	1.6479	149.1	58.0	239.6
2-7	1-2-3-4-9-no	Past fragmentation	—	—	—	3.96637	1.9999	218.6	108.3	328.8
2-8	1-2-3-4-no	Restricted gene flow with isolation by distance	—	—	—	2.84445	1.6915	156.7	63.5	249.9
2-11	1-2-3-4-9-no	Past fragmentation	—	—	—	2.79167	1.6753	153.8	61.4	246.1
2-12	1-2-3-4-9-no	Past fragmentation	—	—	—	1.44444	1.2035	79.6	13.3	145.9
2-14	1-2-11-12-no	Contiguous range expansion	0.594	0.000	3.224	—	—	32.7	0.0	177.7
2-15	1-2-3-4-9-no	Past fragmentation	—	—	—	2.00000	1.4172	110.2	32.1	188.3
1-1	1-2-3-5-6-7-yes	Restricted gene flow with long distance colonization	—	—	—	2.00000	1.4172	110.2	32.1	188.3
1-24	1-2-3-4-no	Restricted gene flow with isolation by distance	—	—	—	1.00000	1.0011	55.1	0.0	110.3
1-26	1-2-11-12-no	Contiguous range expansion	3.000	0.549	4.250	—	—	165.3	30.3	234.2
1-27	1-2-11-12-no	Contiguous range expansion	0.651	0.000	1.791	—	—	35.9	0.0	98.7
1-29	1-2-11-12-13-yes	Long distance colonization	0.610	0.000	1.474	—	—	33.6	0.0	81.2
1-31	1-2-11-12-no	Contiguous range expansion	0.901	0.000	2.279	—	—	49.7	0.0	125.6

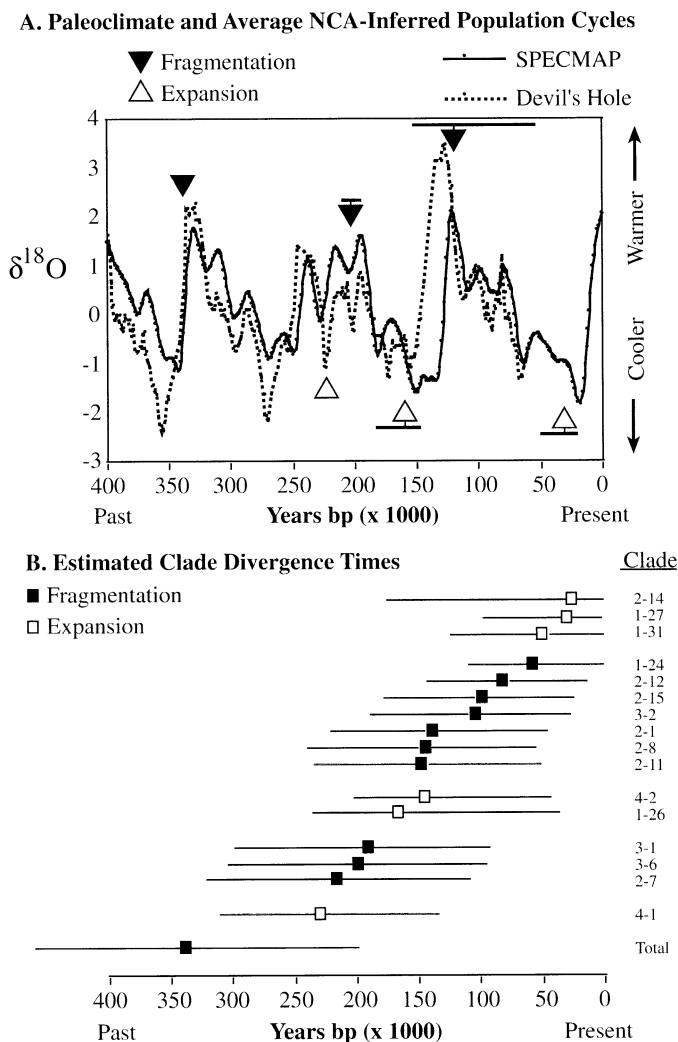


FIG. 6. Comparison of molecular age estimates of clade divergence with paleoclimatic chronologies. Timelines are given along the x-axis in thousand years before present (by). (A) Paleoclimate and average NCA-inferred population cycles. Devil's Hole (Landwehr et al. 1997) and SPECMAP (Imbrie et al. 1989) data are shown in dashed and solid lines, respectively. Delta  $^{18}\text{O}$  is given in standard deviations and serves as a proxy for past climates (cooler and warmer). Molecular ages of fragmentation/restriction (black filled triangle) and expansion (open triangle) events were averaged from clades of similar depth within the phylogeny and are shown on the chart with their ranges (black bars). (B) Estimated clade divergence times. All molecular age estimates of clades along with  $\pm$  standard deviation are shown. Again, fragmentation events are shown in black and expansions are white.

expansion and contraction further back in time suggests that the estimated temporal scale of events may be reasonably accurate.

There is a remarkable agreement between the inferred timing of population expansions or contraction and climate. We plotted averages for the estimated dates of NCA inferred continuous expansion and fragmentation/restricted gene flow events against the Devil's Hole (Landwehr et al. 1997) and SPECMAP (Imbrie et al. 1989) delta  $^{18}\text{O}$  paleoclimatic records (Fig. 6). Both paleoclimatic histories record climate oscillations over the same time span as our inferred molecular

history of *P. smintheus* (the past 400,000 years). Also, recent evidence from Devil's Hole suggests that western North America was subjected to regional climate processes and historical inferences should use regional paleoclimatic records (Herbert et al. 2001). As can be seen in Figure 6, there is a great deal of variation between the Devil's Hole and SPECMAP chronologies (Winograd et al. 1997). Standard deviations around the molecular age estimates span about 200,000 years and increase with age (Table 3, Fig. 6). The uncertainty in the molecular and paleoclimatic estimates makes comparisons between the chronologies difficult—a temporal shift could offset the two records. Nonetheless, NCA-inferred expansion events correspond to cold, wet periods when the alpine was more broadly distributed, and periods of population contraction coincide with three of the last four major interglacials (Winograd et al. 1997), when alpine habitats were severely restricted.

## DISCUSSION

### Cycles of Expansion and Contraction during the Last 350,000 Years

Our inference of the maternal genealogy for *Parnassius smintheus* extends back through the late Pleistocene, a period covering several glacial-interglacial cycles. During this period, the alpine habitat must have expanded and contracted in concert with the changing climate (Wolfe 1987; Huntley and Webb 1989; Pielou 1991; and Elias 1996). Our inference of several cycles of population fragmentation and range expansion derived from nested clade analysis of the DNA data suggests that alpine butterfly populations responded to climate change in a predictable way. In fact, the inferred timing of population fragmentation and expansion events matched the climate record reasonably well. Fragmentation events aligned with warm periods when the alpine tundra was restricted in extent and range expansions occurred during cooler periods favorable to the spread of alpine habitats. The close match between climate and inferences of population processes led us to develop a detailed hypothesis for the evolutionary history of *P. smintheus* in the Rocky Mountains over the last 400,000 years (Fig. 7). The hypothesized model assumes that climatic oscillations propelled the expansion and contraction of the alpine tundra, and that *P. smintheus* occupied the same geographic distribution as determined in this study. Moreover, because genealogies coalesce, ultimately to a single common ancestor, our hypothesis necessarily involves progressively less haplotype variation with increasingly more ancient inferences of history. Finally, we recognize that the estimated timing of inferred population processes and the estimates of climatic events based on paleoclimatic data are accompanied by uncertainty. In particular, a large variance is associated with the estimated dates based on molecules. Nonetheless, despite these caveats, our inference of history gleaned from the analysis of molecular variation provides a general model for alpine phylogeography of the Rocky Mountains (south of the southernmost limit of the Cordilleran and Laurentide ice sheets).

The mitochondrial genealogy of *P. smintheus* coalesces about 340,000 years ago. This marks the timing of the earliest fragmentation of populations during a long warm, interglacial

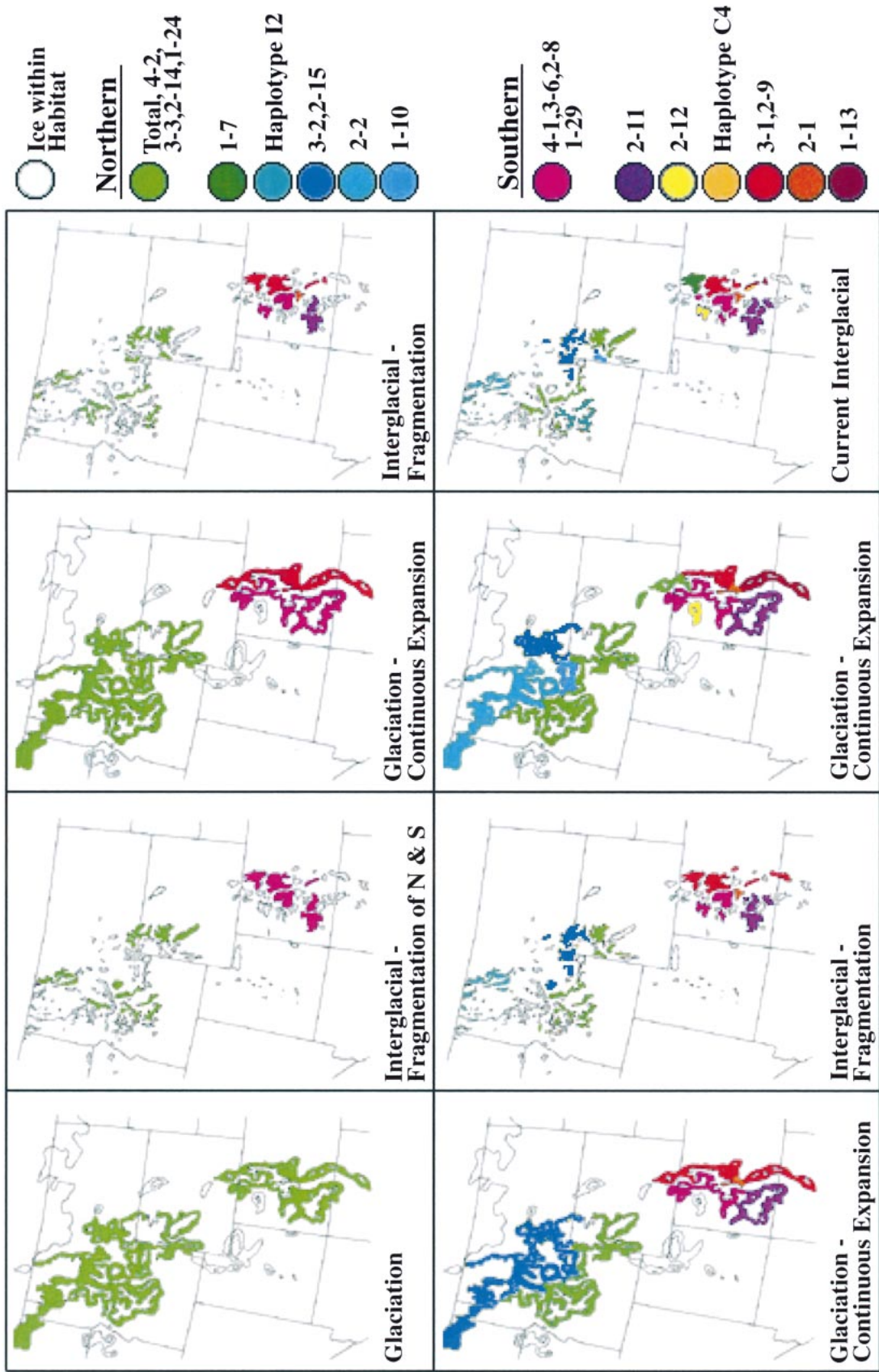


FIG. 7. Hypothetical chronology and distribution of clades from the estimated divergence times and nested clade inferences. The distribution of ice during the glacial periods was adopted from Hollin and Schilling (1981). The distribution of alpine and subalpine habitats during the glacial periods was inferred given a 600–1000 m drop in elevation (Elias 1996). The Distribution of alpine/subalpine habitat during interglacials and interstadials was estimated from their current distribution (Küchler 1985). Clades nested within clade 4-3 are not represented because of the inconclusive inferences about the historical processes affecting those clades. The first panel shows the hypothetical distribution of the ancestral clade before the initial fragmentation of the north and south. Historical distributions of clades were estimated given the current distribution of haplotypes within that clade, the hypothetical distribution of habitat at that time, and an arbitrary loss of populations from the more contracted/fragmented habitat during interglacials.

of the middle Pleistocene. The glaciation preceding this interglacial was a period of extensive sheet and montane ice, when alpine habitat was widely distributed and interconnected across much of the Rockies. Although many distinct haplotypes may have existed at this time, the common ancestral haplotype of all sampled *P. smintheus* individuals could have existed anywhere across the range of available habitat (Fig. 7A). Warming, accompanied by the retreat of glaciers and upward migration of alpine habitat, fragmented *P. smintheus* into distinct northern and southern populations during the interglacial (Fig. 7B). The significant break between northern and southern clades is geographically associated with the Wyoming basin dispersal barrier (Noonan 2001). This region has been shown to inhibit gene flow between high elevation insects because of its low elevation, low precipitation, and desert types soils and vegetation (Noonan 2001). Coinciding with the onset of glacial expansion (about 300 kya; Richmond 1986; Winograd et al. 1997), the northern and southern populations underwent contiguous range expansions. In addition, the southern populations were split in two by the continental divide (Fig. 7C). During the next glacial retreat, the southern populations became more fragmented (Fig. 7D). Unique haplotypes appeared in both regions that underwent range expansions with the advance of glaciers (Fig. 7E). This pattern of fragmentation and expansion occurred two more times (Figs. 7F–H). The most recent expansion of glaciers (lasting until ~12,000 years ago) probably registered as a population expansion for some of the lowest level clades and may explain the wide distribution of several haplotypes (including Y7, C12, C13, C22, C23, and C31).

#### *An Archipelago Refuge System*

The NCA inference of early fragmentation between predominantly northern and southern lineages implies the existence of at least two geographic centers of diversity. During interglacials, the topographic heterogeneity and continued moisture availability of the Rocky Mountains probably generated many localized areas of relatively stable, isolated habitat (Hewitt 2000; Knowles 2000). Assuming contemporary conditions provide a reasonable assessment of warm periods, numerous alpine “refugia” probably existed. These refugia were most likely distributed in an archipelago of isolated sky islands throughout the latitudinal range of the species. Based on current conditions, refuge habitats probably varied for many features that can influence population dynamics, including area, degree of isolation (or proximity to other populations), and the alpine community composition (e.g., presence or absence of parasites).

The hypothesized archipelago model corresponds with Hewitt’s (1996) phalanx model in the sense that the expansion and contraction of suitable habitat for butterflies occurred locally and involved migration along an elevation gradient rather than wholesale shifts in latitude. The inevitable loss of genetic variation by genetic drift accompanying range contraction primarily occurred locally; consequently, the genetic diversity of the species was probably relatively unaffected. This is in contrast to species that underwent latitudinal range shifts in response to climatic cycles. Long distance

latitudinal migration greatly reduces genetic diversity of a species through founder effects and because only one or a few southern refugia persist.

It is unclear whether an archipelago model might be generally true for other Rocky Mountain alpine organisms and for organisms inhabiting lower elevation, temperate habitats. Our inference is based on a large sample size (about 750 bps for 385 individuals from 20 populations), a fact that permitted high-resolution inference of historical population biology. In our study, had we surveyed fewer individuals, we would have detected the strong genetic break between the northern and southern populations, but would have missed the signal of repeated population expansion and contraction and evidence for genetic structure among populations within the same region. Previous studies have generally surveyed far fewer individuals, and only one, another montane study (Veith et al. 2003), revealed evidence for repeated cycles of population fragmentation and expansion. Studies on other widely distributed Rocky Mountain alpine insects, including butterflies (Britten and Brussard 1992; Nice and Shapiro 2001), and beetles (Reiss et al. 1999; Noonan 2001) show some phylogenetic divergence between the northern and southern Rockies of the U.S. In addition, Knowles’ (2000) study of alpine grasshoppers inferred several refugia within the Rocky Mountains of Montana, Idaho, and Wyoming. These combined studies suggest that alpine organisms in the Rocky Mountains may have responded similarly to the repeated cycles of habitat expansion and fragmentation throughout the Quaternary.

#### *Engine of Diversification or Erosion by Extinction*

Imagine a large population shrinking in size and becoming fragmented into many, small, isolated populations inhabiting different areas with differing prevailing environmental conditions. This is probably what happened to *P. smintheus* during transitions from cool to warm climates. The limited migratory capacity of the species (Keyghobadi et al. 1999) maintained the isolation of each population during the interglacials and interstadials. Such a scenario would promote local adaptation and diversification when populations are confined to fragments of alpine habitat persisting on mountain tops. Assuming many populations persisted during the warm interglacials, alpine species (and others conforming to this model) might be expected to harbor tremendous genetic diversity. Why isn’t the coalescent time more ancient than the estimated 340,000 years?

Increasing fragmentation comes with an increased extinction risk, especially if population sizes were much reduced (Nieminen et al. 2001). Many species of butterflies exist as metapopulations (Hanski and Gilpin 1997). Populations that may be relatively large and appear stable eventually will crash and go extinct. Thus, glacial cycles may have acted as an engine of diversification by promoting population genetic structure, or as a mechanism of genetic erosion through an increased risk of population extinction. Whether the scale tips in favor of diversification or extinction depends ultimately on the magnitude of climate change. Relatively cool and short interglacial periods might favor diversification whereas transition to an extremely warm and long interglacial



would tip the scale in favor of extinction. The earliest interglacial at about 300,000 years ago (see Fig. 6) may have tipped the balance towards extinction and explain the relatively shallow coalescent time for the species.

### *Implications of Future Climate Change*

Because the geographic distribution and genetic variation of alpine populations are most impacted by warm interglacials, like the contemporary conditions, high elevation organisms are uniquely situated as indicators of future climate warming. Through understanding how these organisms responded to past warming trends, we can make predictions about how they will respond to similar changes in the future. Recent climate models predict that the response of alpine tundra to climate change will differ between the southern and northern US Rocky Mountains (Kittel et al. 2002). Historical data from our study supports that conclusion. The southern Rockies are expected to undergo greater drying and local losses of native species and high elevation ecosystems than the north (Kittel et al. 2002). If the model's differential temperature and precipitation predictions are realized, southern habitat will be impacted more severely than that of the north. Increased habitat fragmentation may lead to increased population extinction and greater loss of diversity in the south. A similar historical effect has been inferred for the endangered *Boloria acrocneuma*, the Uncompahgre Fritillary (Britten and Brussard 1992). This species only exists as a relictual population on a single peak in Colorado, being historically isolated from congeners to the north. Currently, glaciers are receding at a drastic rate (Arendt et al. 2002) in response to climate change. This, coupled with the findings from our study, demonstrates that alpine organisms are precariously positioned as early indicators of how ecosystems may respond to further climate warming.

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APPENDIX  
 $F_{ST}$  values for all pairwise population comparisons. All comparisons were significant except those in bold.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1																			
2	<b>-0.0487</b>																		
3	<b>0.0102</b>	<b>-0.0065</b>																	
4	<b>0.0321</b>	<b>0.0349</b>	<b>-0.0060</b>																
5	<b>0.2945</b>	<b>0.2755</b>	<b>0.2569</b>	<b>0.2386</b>															
6	0.2248	0.1766	0.1182	0.1743	0.2169														
7	0.1689	0.1448	0.0790	0.0790	0.2085	0.0624													
8	0.1543	0.1520	0.0804	0.0420	0.2060	0.1458	0.0344												
9	0.3399	0.2835	0.2609	0.2791	0.1044	0.1519	0.2553	0.2610											
10	0.2827	0.2646	0.1734	<b>0.0756</b>	0.3152	0.3319	0.2016	0.0644	0.4210										
11	0.2959	0.3045	0.2448	0.1736	0.2736	0.3386	0.2661	0.1980	0.3561	0.1113									
12	0.7763	0.7166	0.6826	0.6646	0.7215	0.7236	0.7392	0.6538	0.7980	0.7028	0.4737								
13	0.7392	0.7024	0.6665	0.6446	0.6902	0.7020	0.7163	0.6431	0.7437	0.6799	0.5057	0.5760							
14	0.6900	0.6588	0.6232	0.6025	0.6484	0.6647	0.6704	0.6015	0.7073	0.6319	0.4504	0.4552	0.2103						
15	0.6706	0.6482	0.6147	0.5993	0.6411	0.6514	0.6596	0.6034	0.6823	0.6262	0.4628	0.4027	0.4076	0.2643					
16	0.5032	0.5167	0.4839	0.4637	0.4780	0.5145	0.5070	0.4798	0.4990	0.4729	0.3566	0.2942	0.1962	0.1186	0.2083				
17	0.6893	0.6927	0.6701	0.6454	0.6586	0.7000	0.6805	0.6428	0.7000	0.6499	0.5362	0.5953	0.4292	0.4059	0.5261	0.2164			
18	0.7217	0.7092	0.6773	0.6409	0.6769	0.7210	0.7004	0.6333	0.7473	0.6578	0.4965	0.6525	0.5408	0.4699	0.5241	<b>0.1713</b>	<b>0.0461</b>		
19	0.5280	0.5244	0.4803	0.4437	0.4625	0.5284	0.5116	0.4514	0.5374	0.4709	0.2951	0.4357	0.3009	0.2003	0.2955	0.0927	0.3290	0.3317	
20	0.3518	0.3359	0.2849	0.2853	0.2701	0.2743	0.3455	0.3180	<b>0.2272</b>	0.3768	0.2604	0.4902	0.4079	0.3795	0.3840	0.2429	0.5401	0.5282	0.2140