Population Genetic Evidence of Restricted Gene Flow Between Host Races in the Butterfly Genus *Mitoura* (Lepidoptera: Lycaenidae)

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ABSTRACT We surveyed variation in allozymes and in the mtDNA cytochrome oxidase subunit II (COII) gene in populations of three nominal species of hairstreak butterflies in the genus *Mitoura* in northern California. These species are separated on the basis of wing pattern and larval hostplant differences: *M. nelsoni* (Boisduval) larvae feed on incense cedar, *Calocedrus decurrens* (Torrey); *M. muiri* (Hy. Edwards) larvae feed on cypress, *Cupressus macnabiana* A. Murray and *C. sargentii* Jepson; and *M. siva* (W. H. Edwards) larvae feed on juniper, *Juniperus occidentalis* Carrière. All three taxa were indistinguishable using allozyme allele frequency data. Genetic distances among populations of all three species were very small (Nei's (1978) unbiased $D = 0.000–0.007$). Percent uncorrected mtDNA divergences were similarly small ($P = 0.2–1.1\%$) and haplotypes are shared among taxa. However, analysis of molecular variation and exact tests for population differentiation indicate that populations of *M. muiri* in the coast range are differentiated from other populations. We conclude that divergence among these three taxa has occurred very recently. We further suggest the possibility that divergence is being driven by the evolution of larval host races, and that gene flow is restricted between *M. nelsoni* and at least some populations of *M. muiri.* This restriction may result from the adults' habit of mating preferentially on different host trees and through phenological differences in the timing of reproduction.

KEY WORDS *Mitoura*, phylogeography, mitochondrial DNA, allozymes, host race formation, Lepidoptera

HABITAT RACES OR host races are defined as populations that are at least partially reproductively isolated by differences in preference for particular habitats or hosts (Diehl and Bush 1984), and may be especially prevalent in phytophagous insects and parasites (Bush 1975, White 1978). Such host-races have been viewed as incipient species (Bush 1994). The best evidence for race formation comes from research on the apple-feeding and hawthorn-feeding races of the Tephritid fly *Rhagoletis pomonella* (Walsh) (see Bush 1993 and Feder et al. 1998 for recent comprehensive reviews of more than 30 yr of research). Other well-documented examples include the two host races of another Tephritid fly, the gall-forming *Eurosta solidaginis* Loew, which specialize on two species of Goldenrod (*Solidago gigantea* Aiton and *S. altissima* L.) (Craig et al. 1993, Itami et al. 1998) and the lacewing sibling species *Chrysoperla carnea* (Stephens) and *C. downesi* Banks, which are inferred to have differentiated as habitat races (Tauber and Tauber 1977, Tauber and Tauber 1989).

Our interest in the butterfly genus *Mitoura* Scudder was stimulated by their parapatric and interdigitated distributions and by the observations that the nominal taxa, *M. nelsoni* (Boisduval) and *M. muiri* (Hy. Edwards), exhibit phenological isolation (i.e., the adult flight periods do not overlap). These species also exhibit a great deal of larval host fidelity (Johnson and Borgo 1976, Scott 1986), which was defined by Feder (1998) as the tendency of phytophagous insects to mate and oviposit on the same hostplant species. These phenomena suggest that gene flow between potential host races might be low and that host recognition and assortative mating are correlated characters (because mating occurs on the host, as is often the case in phytophagous insects [Price 1980]). As in *Rhagoletis*, males of *Mitoura* form leks to which females are attracted; matings have not been seen away from the host (Johnson and Borgo 1976; unpublished data). This situation enhances the probability of successful host race formation (Rausher 1984, Wilson and Turelli 1986, Rice 1987, Rosenzweig 1987, Diehl and Bush 1989, Tauber and Tauber 1989).

We have applied a phylogeographic approach (Avise 1994) to the analysis of three California hairstreak butterflies in the genus *Mitoura* Scudder (Lycaenidae) at the population level using allozyme and mitochondrial DNA (mtDNA) data to evaluate the hypothesis that host race formation is occurring. Specifically, we tested the hypothesis that fidelity for alternative hostplant species and phenological isolation restricts gene flow among three nominal species of butterflies in the genus *Mitoura* in northern Cali-

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fornia. We analyzed the distribution of genetic variation in these butterflies to determine if variation is distributed consistently according to hostplant use or, alternatively, whether genetic variation is distributed geographically. Several tests of population differentiation were also used to detect restricted gene flow among populations.

Materials and Methods

The Butterflies. The three taxa under consideration are *Mitoura nelsoni*, *M. muiri*, and *M. siva* (W. H. Edwards). They have been considered as separate species (Garth and Tilden 1986, Tilden and Smith 1986) or as three subspecies of *M. grynea* (Hübner) (which is a polytypic species with many named subspecies in this arrangement) (Scott 1986). *Mitoura muiri* has also been considered as a well-defined subspecies within *M. nelsoni* (Johnson 1976, Miller and Brown 1981). These three entities in northern California are part of a very large and complex group of taxa that use members of the Cupressaceae (cypresses and cedars) as larval hosts and are distributed across most of temperate North America (Johnson 1976, Scott 1986). Most of these taxa are separable by wing color patterns, genitalic characters, and larval hostplant associations (Johnson 1976). In northern California, *M. nelsoni*, *M. muiri*, and *M. siva* are distinguishable by wing-color pattern, but genitalic characters tend to be unreliable (C.C.N., unpublished data; Scott 1986). The three species also differ in larval host association, adult flight phenology, and wing phenotype. Wing phenotypes and hosts are consistently associated (Garth and Tilden 1986, Scott 1986, Tilden and Smith 1986, Opler 1992).

Mitoura nelsoni (Nelson's hairstreak) occurs from British Columbia to southern California associated with its larval host *Calocedrus decurrens* (Torrey) (incense cedar), a large tree of mixed mesic forests (Garth and Tilden 1986, Scott 1986, Tilden and Smith 1986, Opler 1992). In California, *M. nelsoni* occurs in the coast ranges and at mid-elevations on the west slope of the Sierra Nevada. It is univoltine, with adults flying from April to July, varying with locality and elevation.

The range of *M. muiri* lies entirely within the range of *M. nelsoni.* Published records (Garth and Tilden 1986, Scott 1986) indicate that *M. muiri* inhabits the inner coast range of California from Mendocino County south to Monterey County and is univoltine with the adults flying from March to June, again, varying with locality and elevation. In the northern part of this range, this butterfly uses *Cupressus sargentii* Jepson (Sargent cypress), a small tree, and *C. macnabiana* A. Murray (MacNab cypress), a large shrub, as larval host-plants. These conifers are associated with ultramafic soils and are often found on serpentine barrens; *C. sargentii* is a serpentine endemic (Kruckeberg 1984). From Mt. Diablo, Contra Costa County, CA, south, *C. macnabiana* does not occur and *C. sargentii* is relatively rare, except for several populations in San Luis Obispo County (Griffin and Stone 1967, Hickman 1993, Lanner 1999). Here *M. muiri* is associated with *Juniperus californica* Carrière (California juniper) (unpublished data; Garth and Tilden 1986), another large shrub that is calciphilic and not strongly associated with ultramafic soils. *Juniperus californica* also occurs in northern California, but no populations of *M. muiri* have been found associated with the shrub north of Mt. Diablo (unpublished data).

Mitoura nelsoni and *M. muiri* occur very near to each other in some localities. One such locality, Goat Mountain, Colusa County, CA, was a focus of this study. At Goat Mountain, *M. muiri* adults fly in April to May associated with *C. sargentii* on serpentine soils at 500 m elevation (Gervais and Shapiro 1999). *Mitoura nelsoni* adults fly in June–July in mesic forest with *Calocedrus decurrens* at ≈1,300 m and within 6–7 km of the serpentine barrens. In fact, *C. decurrens* grows within meters of *C. sargentii*, although no *M. nelsoni* have been taken from these isolated trees at low elevation. Thus, these two taxa occur in close proximity, but are potentially isolated by phenology and host-association.

During the course of this investigation, five populations of *M. muiri* in the Sierra Nevada were discovered by A.M.S. and B. Gervais (Gervais and Shapiro 1999). Before this discovery, *M. muiri* was known only from the coast range mountains. These populations are associated with McNab cypress (*C. macnabiana.*) and many are in close proximity to *M. nelsoni* populations.

The third taxon with which this investigation was concerned is *M. siva. Mitoura siva* uses *Juniperus occidentalis* Hook (western juniper) as the larval hostplant, and its range includes much of the Great Basin and the east side of the Sierra Nevada and northward to the Warner Mountains in northeastern California and into Oregon and Washington. Like *M. nelsoni* and *M. muiri*, it is single-brooded, flying in spring to early summer (April–July) in different locations. At various localities it occurs within ≈10 km of *M. nelsoni*, mainly in the northern Sierra Nevada and southern Cascade Range where the crest is low enough for the hosts to occur in close spatial proximity, albeit often at different altitudes. We included *M. siva* because its geographic proximity to Sierran *M. nelsoni* might provide an instructive comparison to the *M. nelsoni–M. muiri* situation, and its taxonomic status is similarly problematic.

Allozyme Methods. Individuals (287) of both sexes from 11 populations (seven of *M. nelsoni*, two of *M. muiri*, and two of *M. siva*) were collected for starch gel allozyme electrophoresis. (Sierran *M. muiri* populations were not sufficiently large to support sampling for allozyme electrophoresis and were only sampled for mtDNA sequence analysis. Sample sizes were generally limited by the exigencies of collecting these butterflies, which are rarely observed away from the tops of their host trees.) One population, the *M. siva* population at Sierra Valley, was sampled twice in 1994 during an extended adult flight season that was interrupted by a freeze. The "spring" and "summer" samples were collected before and after the freeze, respectively, and treated as separate populations for

Table 1. Locality and collection data for allozyme electrophoresis samples

Population Locality	n	Collection Date	Collector	County, State	Host[a]
Mitoura nelsoni	152				
Ball Mountain	18	23.vi.89	AMS	Siskiyou County, CA	*C. decurrens*
Lang Crossing	23	22,27.v.94	AMS	Nevada County, CA	*C. decurrens*
Goat Mountain	25	12.vi.94	AMS, CCN	Colusa County, CA	*C. decurrens*
Slug Gulch	25	30.v.94	CCN	El Dorado County, CA	*C. decurrens*
Mohawk Vista	24	5.vi.94	CCN	Plumas County, CA	*C. decurrens*
Red Dog	22	22.v.94	AMS, CCN	Nevada County, CA	*C. decurrens*
Deer Creek	15	22.v.94	S. Mattoon	Tehama County, CA	*C decurrens*
M. muiri	42				
Goat Mountain	12	27.iv.94, 8.v.94	AMS, CCN	Colusa County, CA	*C. sargentii*
Knoxville	30	12.iii.94	AMS, S. Graves	Napa County, CA	*C. sargentii* and *C. macnabiana*
M. siva	61				
Sierra Valley (Spring)	20	13.v.94	AMS	Sierra County, CA	*J. occidentalis*
Sierra Valley (Summer)	15	10.vi.94	AMS	sierra County, CA	*J. occidentalis*
Cedar Pass	26	4,8.vi.94	CCN	Modoc County	*J. occidentalis*

[a] Larval host plants at the localities: *Calocedrus decurrens* (incense cedar), *Cupressus sargentii* (sargent cypress), *Cupressus macnabiana* (MacNab cypress), *Juniperus occidentalis* (western juniper).

comparison with genetic distances among populations. Table 1 provides locality and sample size data for these specimens and Fig. 1 illustrates their locations. Specimens were captured and maintained alive until frozen in either a −80°C freezer or in a liquid nitrogen thermos in the field. Those frozen in liquid nitrogen were later transferred to −80°C freezers. Wings and the posterior portions of the abdomens (for males) or the whole abdomens (for females) were removed and stored as voucher specimens in numbered and dated glassine envelopes inscribed with locality and date of capture data. Voucher specimens remain in the corresponding author's possession. The remaining bodies of each individual were placed in a 0.5-ml Eppendorf tube and prepared for allozyme analysis as described in Porter and Mattoon (1989).

Twenty-four presumptive loci were surveyed, of which 13 were reliably scorable and 10 were polymorphic. These 13 loci, listed with their abbreviations and their IUBNC Enzyme Commission numbers (Shaklee et al. 1990), are as follows: adenylate kinase (AK) (2.7.4.3), aspartate aminotransferase (AAT) (2.6.1.1), fructose-bisphosphate aldolase (FBALD) (4.2.1.13), glucose-6-phosphate isomerase (GPI) (5.3.1.9), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1.2.1.12), glycerol-3-phosphate dehydrogenase (G3PDH) (1.1.1.8), hexokinase (HK) (2.7.1.1), isocitrate dehydrogenase (IDH-1) (1.1.1.42), malate dehydrogenase (2 loci: MDH-1 and MDH-2) (1.1.1.37), malic enzyme (NAD+) (ME) (1.1.1.38), malic enzyme (NADP+) (MEP) (1.1.1.40), and phosphoglucomutase (PGM) (5.4.2.2). Electromorphs were assigned letter designations arbitrarily. Potentially ambiguous electromorphs were rerun in adjacent lanes to confirm scoring. Genotype data for each individual for the 13 scorable loci were analyzed using the BIOSYS computer program (Swofford and Selander 1981) and the Genetic Data Analysis (GDA) computer program version 1.0 (Lewis and Zaykin 1996). Hierarchical *F*-statistic estimators (Wright 1951, Crow and Aoki 1984) were calculated using the formulae of Weir and Cockerham (1984) and Weir (1996). Genetic distances between populations and measures of genetic variability were calculated. Maximum likelihood analysis was performed with the program CONTML of the PHYLIP software package (Felsenstein 1993) to construct a phylogeny based on population allele frequencies. Nonmetric multidimensional scaling (Lessa 1990) using the NCSS 97 computer statistical package was also used to illustrate the relationships among populations.

MtDNA Methods. A 480-bp region of the mtDNA cytochrome oxidase subunit II (COII) region was sequenced for 54 specimens of *Mitoura* from northern California, three specimens of *Mitoura grynea* from Wisconsin, plus specimens of *Incisalia iroides* (Boisduval), which was used as an outgroup. Table 2 provides locality data for these specimens. Samples of *Mitoura* used in the mtDNA sequence survey included specimens from two sources. Twenty-one specimens were previously processed for allozyme electrophoresis (see above) and the material from these homogenates was extracted. Thirty-three freshly caught specimens were kept alive until placed in −80°C freezers or frozen in liquid nitrogen in the field and transported to −80°C freezers. Forty-nine specimens (including outgroup specimens) underwent chloroform-phenol extraction. Ten specimens underwent extraction using DNAzol Genomic DNA Isolation Reagent (Molecular Research Center, Cincinnati, OH).

Wings and abdomens were removed in the same fashion as described in the allozyme electrophoresis methods, and the remaining body was placed in an autoclaved 1.5-ml Eppendorf tube. For those samples that were originally prepared for electrophoresis, an unmeasured portion of the electrophoresis homogenate was transferred to a new autoclaved 1.5-ml Eppendorf tube. Chloroform-phenol extractions were performed following the methods of Hillis et al. (1996). DNAzol extractions, following Chomczynski et al. (1997), were performed for 10 specimens and extracted DNA was rehydrated with 300 μl of H_2O. These solutions from each extraction protocol were used in polymerase chain reactions (PCR) (Kocher et

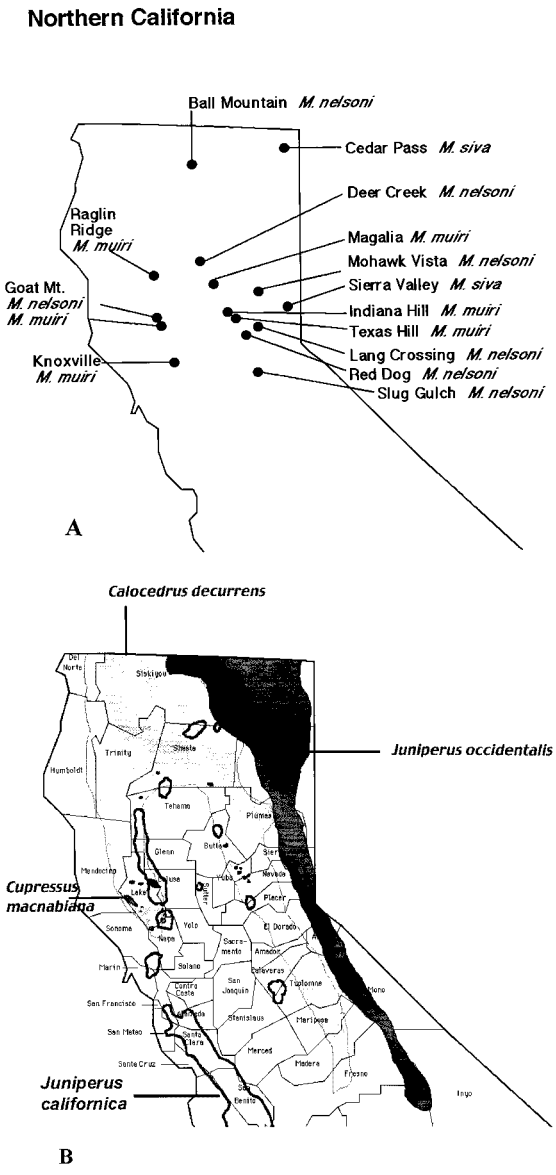

Fig. 1. Distribution of *Mitoura* and larval hostplants. (A) Northern California locations of *Mitoura* populations surveyed for allozyme and mtDNA sequence variation. (B) The approximate ranges of *Calocedrus decurrens, Juniperus occidentalis, J. californica,* and *Cupressus macnabiana.* The range of *Cupressus macnabiana* is indicated as dark shapes within the range of *C. decurrens.* The range of *Cupressus sargentii* is not shown but corresponds closely to the western half of the range of *Cupressus macnabiana* (i.e., in the coast range). Isolated populations of *C. sargentii* in the central coast range (San Luis Obispo County) are not shown.

al. 1989, Palumbi 1996) employing the mtDNA COII primers STROM (5′ TAA TTT GAA CTA TYT TAC CNG CA 3′) and EVA (5′ GAG ACC ATT ACT TGC TTT CAG TCA TCT 3′) (Caterino and Sperling 1999) yielding an ≈480 bp PCR product, which was sequenced. Fluorescently labeled dideoxy terminators were used for single stranded sequencing reactions according to Applied Biosystems, Foster City, CA, specifications. Labeled extension products were separated on a gel and analyzed with an automated DNA sequencer (model 377, Applied Biosystems) at the DNA sequencing facility, Division of Biological Sciences, University of California, Davis. Forward sequences were obtained for all 59 *Mitoura* specimens plus outgroup specimens and 12 arbitrarily chosen samples were sequenced in the reverse direction as a check of sequencing errors. From these sequences, 460 continuous nucleotides could be reliably read for all specimens. This portion of the COII region of the mtDNA genome is approximately homologous to positions 3292 through 3755 of the *Drosophila yakuba* Burla reference sequence (Clary and Wolstenholme 1985). Parsimony and maximum likelihood analysis and phylogeny reconstructions based on these sequences were performed with the PAUP* program (Swofford 1998).

Genetic variation and the extent of population subdivision at the mtDNA COII locus were evaluated with nested analyses of molecular variance (AMOVA; Excoffier et al. 1992). Three nested AMOVA analyses were performed by partitioning the total sum of squares into components representing variation among individuals within populations, among populations within groups, and among groups using the ARLEQUIN version 2.0 software (Schneider et al. 2000). The AMOVA analyses were used to determine if mtDNA variation was distributed along taxonomic lines (which corresponds with hostplant association) or by geographic region. The three nesting designs grouped populations: (1) by nominal taxonomic designation, (2) by regions: Sierra Nevada populations versus coast range populations, and (3) coast range *M. muiri* populations versus Sierra Nevada *M. muiri, M. siva,* and *M. nelsoni* populations. Conventional pairwise *F*-statistics and pairwise *F*-statistic analogs (ϕ-statistics) using Kimura 2-parameter distances were also calculated. For these *F*-statistic calculations, the Red Dog and Slug Gulch populations of *M. nelsoni* were pooled to increase sample size ($n = 4$). Significance of the AMOVA ϕ-statistics and pairwise *F*-statistics and analogs was determined by 1,000 permutations of haplotypes among populations and among groups under the null hypothesis of panmixia. The ARLEQUIN version 2.0 software (Schneider et al. 2000) was also used to test for population differentiation using the test of Raymond and Rousset (1995). This procedure employs a Markov chain method to obtain an unbiased estimate of the exact test of Fisher (1935). It is computationally more practical than Fisher's exact test for data sets containing more than a few populations (Raymond and Rousset 1995).

Results

Allozymes. Allele frequencies of the allozyme loci (Nice 1998) and measures of population genetic variability (Table 3) were similar in all populations. Genetic distances between populations of *M. nelsoni, M.*

Table 2. Locality and collection data for mtDNA samples

Population Locality	N	Collection date	Collector	County, State	Haplotype
Mitoura nelsoni	12				
Goat Mountain	8	12.vi.94	AMS, CCN	Colusa County, CA	A
Slug Gulch	2	30.v.94	CCN	El Dorado County, CA	A
Red Dog	2	22.v.94	AMS, CCN	Nevada County, CA	A
M. muiri	38				
Goat Mountain	10	27.iv.94	AMS, CCN	Colusa County, CA	A(1),C(1),D(8)
Knoxville	2	12.iii.94	AMS, S.Graves	Napa County, CA	D
Knoxville	1	20.iii.96	AMS	Napa County, CA	D
Texas Hill[a]	1	8.v.96	AMS, B. Gervais	Yuba County, CA	A
Texas Hill[a]	1	20.iv.96	AMS	Yuba County, CA	A
Texas Hill[a]	1	3.iv.96	AMS	Yuba County, CA	A
Indiana Hill[a]	9	8.v.96	AMS, B. Gervais	Yuba County, CA	A
Indiana Hill[a]	4	3.iv.96	AMS	Yuba County, CA	A
Magalia[a]	8	4.v.96	AMS, B.Gervais	Butte County, CA	A
Raglin Ridge[a]	1	29.iv.96	AMS, B. Gervais	Tehama County, CA	A
M. siva	4				
Sierra Valley (Spring)	2	13.v.94	AMS	Sierra County, CA	A(1),B(1)
Sierra Valley (Summer)	1	10.vi.94	AMS	Sierra County, CA	A
Sierra Valley	1	26.v.96	AMS	Sierra County, CA	A
M. grynea	3				
Walking Iron, WI[b]	3	5.v.00	CCN	Dane County, WI	G
Outgroup taxa					
Incisalia iroides	1	22.v.94	CCN	Nevada County, CA	1

[a] Associated with *Cupressus macnabiana* (MacNab Cypress).
[b] Associated with *Juniperus virginiana* (eastern red cedar). Hosts otherwise as in Table 1.

siva, and *M. muiri* were very small. The range of genetic distances (Nei's (1978) unbiased $D = 0.000–0.007$) fell within the range of distances observed for conspecific populations of other invertebrates (Thorpe 1983), indicating extremely low levels of differentiation among all of the populations surveyed, regardless of their specific identification.

No clear pattern of relationships among populations was evident in the allozyme data. All three taxa were indistinguishable and very closely related. Hierarchical *F*-statistics demonstrated the nearly complete lack of differentiation among *Mitoura* populations. The overall F_{ST}, calculated using the estimator of Weir and Cockerham (1984), was not different from zero ($F_{ST} = 0.005$; 95% confidence interval: $-0.001–0.010$). An F_{ST} based estimation of gene flow assuming an island model, indicated that there was substantial gene flow between populations ($Nm = 497.5$; Wright 1951, Weir and Cockerham 1984, Slatkin 1987) or that substantial gene flow has occurred in the recent past and there is currently a low level of divergence between populations. This is also illustrated in the maximum likelihood phylogeny based on allozyme allele frequencies (Fig. 2). Multidimensional scaling may be more appropriate than hierarchical analyses when divergence is recent or in cases of reticulation and clinal variation (Lessa 1990). However, no clear taxonomic or geographical patterns, including clines, were evident in the results of nonmetric multidimensional scaling (Fig. 3).

Table 3. Genetic variability statistics for the 11 *Mitoura* populations for 13 loci surveyed electrophoretically

Population	Mean $_{alleles}$[a]	P[b]	H_{obs}[c]	H_{exp}[d]
Mitoura nelsoni				
Ball Mountain	1.9 (0.3)	46.2	0.133 (0.049)	0.137 (0.052)
Lang Crossing	2.1 (0.3)	38.5	0.134 (0.056)	0.138 (0.054)
Goat Mountain	1.9 (0.4)	30.8	0.139 (0.065)	0.143 (0.066)
Slug Gulch	2.0 (.03)	38.5	0.150 (0.055)	0.154 (0.056)
Mohawk Vista	2.5 (0.4)	30.8	0.140 (0.049)	0.146 (0.049)
Red Dog	2.2 (0.5)	38.5	0.151 (0.056)	0.154 (0.058)
Deer Creek	2.0 (0.3)	46.2	0.129 (0.054)	0.130 (0.049)
M. muiri				
Goat Mountain	1.9 (0.3)	53.8	0.090 (0.033)	0.165 (0.048)
Knoxville	2.1 (0.4)	38.5	0.154 (0.063)	0.152 (0.059)
M. siva				
Sierra Valley (Spring)	2.2 (0.3)	46.2	0.167 (0.058)	0.168 (0.055)
Sierra Valley (Summer)	1.9 (0.3)	38.5	0.139 (0.056)	0.132 (0.052)
Cedar pass	2.0 (0.4)	38.5	0.100 (0.037)	0.115 (0.043)
Means	2.06 (0.18)	40.4 (6.6)	0.136 (0.022)	0.144 (0.015)

[a] Mean $_{alleles}$ = mean number of alleles per locus.
[b] P = percent of polymorphic loci
[c] H_{obs} = observed heterozygosity.
[d] H_{exp} = Hardy–Weinberg expected heterozygosity. Standard errors in parentheses.

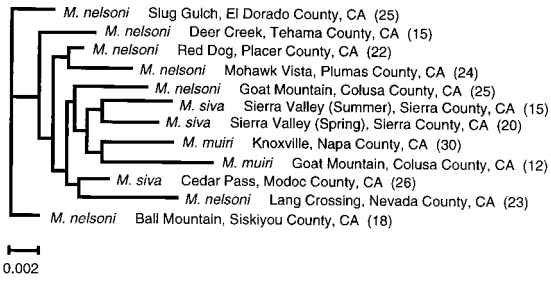

Fig. 2. Maximum likelihood phylogeny of allozyme allele frequencies. Sample sizes are given in parentheses.

Table 4. Mean (uncorrected) distances (above diagonal) and absolute distances (below diagonal) are given for all five haplotypes present in sequences of COII mtDNA gene of *Mitoura* specimens

Haplotypes	A	B	C	D	G
A	—	0.007	0.004	0.002	0.009
B	3	—	0.011	0.009	0.015
C	2	5	—	0.002	0.013
D	1	4	1	—	0.011
G	4	7	6	5	—

Haplotpe G is confined to *M. grynea* from Wisconsin (see Table 2 for locality, collection, and haplotype data).

MtDNA. Five haplotypes were detected in these analyses of the 460-bp mtDNA sequences of 57 *Mitoura* sequences (Genbank accession numbers AF180502-AF180517). Mean A-T content for the five haplotypes and the outgroup haplotypes was 77.5%. This value is commonly observed in insect mtDNA sequences (Simon et al. 1994). In the rest of these results we will consider *Mitoura* from northern California exclusively. Five (1.01%) of the 460 nucleotides sequenced from California *Mitoura* specimens were found to be variable. The most common haplotype, haplotype A, was present in 41 specimens (Table 2 provides haplotypes for all specimens). Uncorrected sequence divergence between the four haplotypes (=100 × [No. nucleotides different/No. nucleotide sites]) ranged from 0.2 to 1.1% (Table 4).

Most of the sequences of *M. muiri* from the coast range of California, except at Raglin Ridge (i.e., the previously recognized range of *M. muiri*, not including the newly discovered populations), possessed a private nucleotide change (haplotype D). One of these coast range *M. muiri* had a unique sequence with one additional nucleotide change (haplotype C) and one individual from the Goat Mountain *M. muiri* population possessed haplotype A. Among the 40 other individuals with haplotype A (the most common haplotype) were specimens of *M. nelsoni* and *M. siva* and the specimens of *M. muiri* from the Sierra Nevada and Raglin Ridge; these groups were indistinguishable using these COII mtDNA sequences. One specimen of *M. siva* had a unique sequence with one additional change (haplotype B). A maximium likelihood analysis (Fig. 4) of the mtDNA sequences showed that sequences from members of the coast range populations of *M. muiri* form a distinct clade, though bootstrap support was somewhat weak (67%). (Neighbor-joining and maximum parsimony analyses resulted in the same phylogeny.) No other geographical patterns were evident.

An AMOVA indicated that the coast range *M. muiri* populations are differentiated from the other populations. The largest partitioning of molecular variance existed between the coast range *M. muiri* populations (at Goat Mountain and Knoxville) and all other sampled populations. 82% of the total variance existed among these groups (Fig. 5C; ϕ_{ct} = 0.8227, P = 0.0137). None of the total genetic variation was distributed along taxonomic lines (Fig. 5A; ϕ_{ct} =

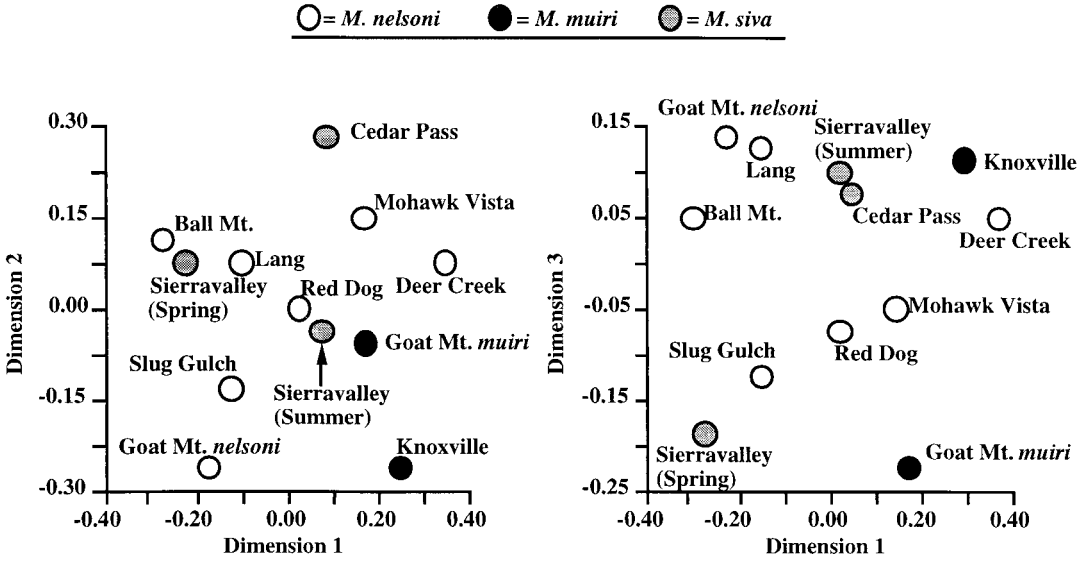

Fig. 3. Multidimensional scaling of 12 population samples of the *Mitoura* complex from northern California.

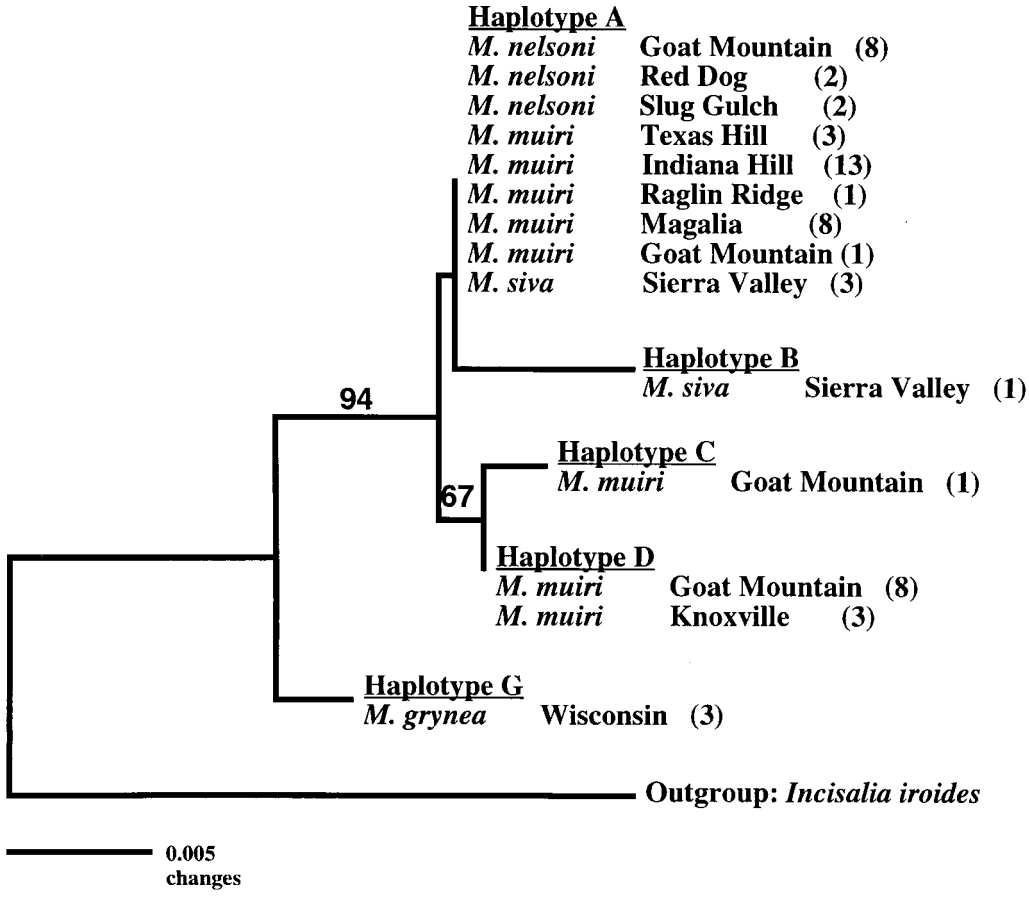

Fig. 4. Maximum likelihood phylogeny of *Mitoura* COII haplotypes. Bootstrap values from 100 bootstrap replicates are above branches. Sample sizes are given in parentheses.

−0.0801, $P = 0.4604$). Genetic diversity was also not strongly partitioned between the Sierra Nevada and coast range mountains in northern California (Fig. 5B; $\phi_{ct} = 0.3827$, $P = 0.0655$). Pairwise F_{st} and ϕ_{st} values confirmed the AMOVA results with most of the significant and largest values occurring between coast range *M. muiri* populations and all other populations (Table 5). Undoubtedly the small sample sizes for some populations limited the power of the permutation test of significance; however, the patterns from both AMOVA and *F*-statistic analyses were the same: the coast range *M. muiri* populations were the most differentiated, while the Sierra Nevada *M. muiri* were indistinguishable from *M. nelsoni* or *M. siva* populations. A nonzero F_{st} also occurred between the Sierra Valley *M. siva* population and the Goat Mt. *M. nelsoni*, and between Sierra Valley and two Sierran *M. muiri* populations (Indiana Hill and Magalia) (Table 5). The exact test of population differentiation using the methods of Raymond and Rousset (1995), also indicated significant differences between the coast range *M. muiri* populations and most other populations (Table 6). The Indiana Hill and Sierra Valley populations were also significantly different in terms of haplotype frequencies in this analysis. Again, the limited sample sizes for some populations decreases the power of the test, but the patterns were congruent with the other analyses reported above.

Discussion

Results from the electrophoretic survey of allozyme allele frequencies in populations of *M. nelsoni*, *M. siva*, and *M. muiri* in northern California indicated that these taxa are very closely related to each other. Genetic distances from pairwise comparisons of allele frequencies in populations were extraordinarily small and comparable to levels of divergence observed for phenotypically undifferentiated, intraspecific populations of other butterflies (Shapiro and Geiger 1986, Porter and Geiger 1988, Porter and Mattoon 1989) and other invertebrates (Thorpe 1983). Furthermore, the genetic distance calculated between "spring" and "summer" samples of the Sierra Valley population was greater than more than half of all other pairwise genetic distances. This seems to indicate that there is almost no differentiation between most populations included in the allozyme survey. These results indi-

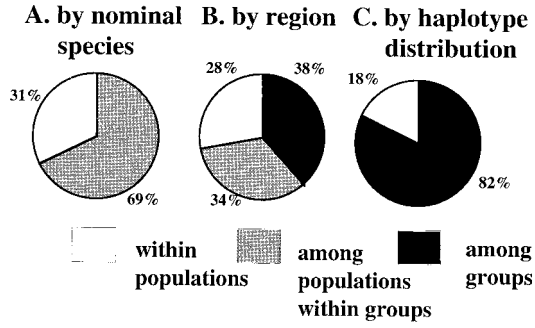

Fig. 5. Results of hierarchical AMOVA of the mtDNA COII sequence data. Molecular variance was partitioned in three ways: (A) by nominal species, (B) by region: Sierran populations versus coast range populations, and (C) by haplotype distribution (see text). This analysis excludes outgroup and *M. grynea* sequences. See Table 2 and Fig. 1 for locality data.

cate that the three taxa identified by wing phenotypes and larval host-plant associations have diverged very recently or there is significant gene flow occurring between them, or both. The low level of mtDNA sequence divergence is congruent with the allozyme results.

This low level of differentiation and the apparent recency of divergence suggests that Pleistocene-age climate changes (Hewitt 2000) may have significantly affected the historical distribution of *Mitoura* in northern California. Evidence from the Great Basin (east of the Sierra Nevada) indicates that Piñon pine–juniper woodlands were displaced by limber pine–bristlecone pine habitats during the Pleistocene (Wells 1983). Junipers migrated southward into what is now the Mojave desert during the Pleistocene and recolonized the Great Basin after the end of the glacial maximum (Van Devender 1977, Van Devender and Spaulding 1979). If a similar migration of Cupressaceous trees occurred on the west side of the Sierra Nevada, recolonization of northern California may have occurred within the last 12–15,000 yr. The colonization of incense cedar (*Calocedrus decurrens*) or cypresses (*Cupressus* spp.) by *Mitoura* may have occurred within this time as well.

Low levels of genetic divergence armong populations, subspecies, and species have been documented for other California butterflies (Porter and Geiger 1988, Tong and Shapiro 1989, Porter and Shapiro 1991, Nice and Shapiro 1999; J. A. Fordyce, personal communication). This pattern may be indicative of the recency of Pleistocene-associated changes in the distribution of northern California butterflies or their hostplants (Shapiro 1996). The low level of divergence may then reflect a high degree of ancestral polymorphism and the limited time available for lineage sorting among *Mitoura* populations using different host trees (Niegel and Avise 1986). The pattern observed in *Mitoura* is also congruent with evidence of rapid ecological and morphological differentiation documented in butterflies (Nice and Shapiro 1999) and other taxa (Orr and Smith 1998).

Despite the low level of divergence and the presence of shared haplotypes among taxa, there is significant genetic differentiation between the coast range *M. muiri* (excluding the Raglin Ridge population) and all other populations included in our mtDNA survey. *Mitoura muiri* in the coast range appears to be differentiated from *M. siva* and *M. nelsoni* despite the close physical proximity of the latter to *M. muiri* in locations like Goat Mountain. The incongruence between allozyme and mtDNA data sets may reflect differences in the evolutionary dynamics of these markers. MtDNA should be more sensitive than allozyme markers to the effects of drift because of its inherently smaller effective population size (Avise 1994). The AMOVA, F_{st}, and population differentiation analyses of mtDNA variation indicate that gene flow is restricted between coast range *M. muiri* and other populations.

One possible explanation for the lack of differentiation in the Sierra Nevada is that *M. nelsoni* is not strictly monophagous but may occasionally switch opportunistically to cypress. We reject this hypothesis because it cannot account for the phenotypic differences between *M. nelsoni* and the Sierran cypress-associated specimens (which appear to be phenotypically *M. muiri*) without ad hoc invocation of

Table 5. Pairwise comparisons among nine populations of *Mitoura* based on mtDNA variation

	1	2	3	4	5	6	7	8	9
M. nelsoni									
1 Red Dog/Slug Gulch	—	0.000	0.000	0.071[a]	1.000[a]	0.000	0.000	0.000	0.000
2 Goat Mt.	0.000	—	0.186	0.769[a]	1.000[a]	0.000	0.000	0.000	0.000
M. siva									
3 Sierravalley	0.000	0.186[a]	—	0.548[a]	0.707[a]	−1.000	−0.091	0.316[a]	0.186
M. muiri (Coast Range)									
4 Goat Mt.	0.719[a]	0.779[a]	0.569[a]	—	0.136	0.580	0.682[a]	0.815[a]	0.769[a]
5 Knoxville	1.000[a]	1.000[a]	0.506[a]	0.180	—	1.000	1.000	1.000[a]	1.000[a]
6 Raglin	0.000	0.000	−1.000	0.600[a]	1.000	—	0.000	0.000	0.000
M. muiri (Sierran)									
7 Texas Hill	0.000	0.000	−0.090	0.696[a]	1.000	0.000	—	0.000	0.000
8 Indiana Hill	0.000	0.000	0.316	0.824[a]	1.000[a]	0.000	0.000	—	0.000
9 Magalia	0.000	0.000	0.186	0.779[a1]	1.000[a]	0.000	0.000	0.000	—

F_{st} values shown above the diagonal, ϕ_{st} values, calculated using Kimura 2-parameter distances, are shown below the diagonal.
[a] Statistically significant values (at the $P < 0.05$ level; calculated from 1,000 random permutations).

Table 6. Results of exact test for population differentiation

	1	2	3	4	5	6	7	8	9
M. nelsoni									
1 Red Dog/Slug Gulch	—								
2 Goat Mt.	—	—							
M. siva									
3 Sierravalley	1.000	0.186	—						
M. muiri (Coast Range)									
4 Goat Mt.	0.000[a]	0.000[a]	0.001[a]	—					
5 Knoxville	0.000[a]	0.000[a]	0.000[a]	0.416	—				
6 Raglin	—	—	1.000	0.218	0.000[a]	—			
M. muiri (Sierran)									
7 Texas Hill	—	—	0.434	0.000[a]	0.085	—	—		
8 Indiana Hill	—	—	0.000[a]	0.000[a]	0.001[a]	—	—	—	
9 Magalia	—	—	0.3323	0.001[a]	0.000[a]	—	—	—	—

The probability method of Raymond and Rousett (1995) provides *P*-values for pairwise population comparisons. — indicates that a comparison was not possible because populations are fixed for the same haplotype.

[a] Significant *P*-values (<0.05) also indicate significant differentiations between populations.

phenotypic plasticity or a host-dependent (or host-induced) phenotype, neither of which seems likely: *M. nelsoni* reared in the laboratory to adulthood on incense cedar, Sargent cypress, or ornamental junipers exhibit normal *M. nelsoni* phenotypes (C.C.N., unpublished data).

Alternatively, the Sierran cypress-associated populations may represent a dispersal event from the coast range to the Sierra Nevada or a vicariance event that has isolated disjunct *M. muiri* populations in these two areas. However, the Sierran populations do not share the most frequent coast range *M. muiri* mtDNA haplotype. It is possible that very recent mutations have occurred locally in the coast range and have not spread via gene flow to the Sierran populations due to their recent origin or because the coast range and Sierran populations are now disjunct. However, we might also expect to observe population differentiation arise through this process in other populations or regions and we have found no evidence for this.

It is also possible that the Sierran cypress-associated populations are a separately evolving host race, whose formation may not be complete in as much as the sharing of identical haplotypes between *M. nelsoni* and these new cypress-feeding populations is indicative of continuing or recent gene flow. In other words, host race formation may be occurring in parallel in several geographically distinct localities simultaneously. The apparent convergence in phenotype among the cypress-associated populations across the Central Valley of California might be a product of parallel selection, perhaps reflecting thermoregulatory exigencies of an earlier flight period dictated by cypress phenology. *Mitoura muiri* is distinguished from the other taxa by its strong melanization of the ventral hindwing, precisely the surface most effective for thermoregulation in other cold-adapted butterflies (Heinrich 1993). More thorough examination of these Sierran populations and the origins of individuals with intermediate phenotypes will be necessary to test these alternatives. Further investigations to include *M. muiri* populations from the central and southern coast ranges may also provide significant information bearing on the origin of the Sierran *M. muiri* populations.

Although the subject of sympatric speciation has generated controversy for decades (Mayr 1963, Maynard Smith 1966, Bush 1969, Feder et al. 1998), recent theoretical advances (Diehl and Bush 1989, Tauber and Tauber 1989, Dieckmann and Doebeli 1999, Kondrashov and Kondrashov 1999) and empirical investigations (Feder et al. 1998, Itami et al. 1998) have contributed to the recognition that nonallopatric modes of speciation are plausible and possibly important in some groups of animals. Phytophagous insects may be one such group. Our investigations of *M. nelsoni*, *M. siva*, and *M. muiri* in northern California demonstrate that these taxa are very recently diverged. In fact, *Mitoura nelsoni* and *M. siva* and some *M. muiri* populations in the Sierra Nevada are indistinguishable using COII sequences and 13 allozyme loci. Their habit of mating on their specific host trees and the observation that *M. nelsoni* and *M. muiri* are phenologically isolated suggests that gene flow between these taxa may be restricted despite their being broadly sympatric and nearly adjacent in some localities such as at Goat Mountain. Genetic differentiation was detected between the *M. muiri* populations in the coast range and all others sampled. This is especially clear at Goat Mountain where phenologically isolated populations of *M. nelsoni* and *M. muiri* use different hosts and show significant differentiation with mtDNA markers.

The case of *M. muiri* in the Sierra Nevada is especially intriguing. These newly discovered populations are more similar genetically to *M. siva* and *M. nelsoni*, but are ecologically related to coast range *M. muiri.* All phenotypically "*muiri*" populations, regardless of mtDNA haplotypes, have a host association with *Cupressus* sp., a flight period that is earlier than nearby *M. nelsoni* and a shared, darker "*muiri*" wing pattern (Garth and Tilden 1986, Scott 1986, Tilden and Smith 1986, Opler 1992). Parallel evolution of a MacNab cypress-associated, "*muiri*"-like race may be occurring now in the Sierra Nevada. Sympatric divergence of host-races is a plausible explanation for these data, but other possibilities, including dispersal and vicariance, cannot be excluded without further phylogeographic investigation.

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