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Authors: Pratt, Gordon F., Ballmer, Gregory R., and Wright, David M.

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ALLOZYME-BASED PHYLOGENY OF NORTH AMERICAN *CALLOPHRYS* (S. L.) (LYCAENIDAE)

Gordon F. Pratt,
Department of Entomology, University of California, Riverside, CA 92521

Gregory R. Ballmer,
Department of Entomology, University of California, Riverside, CA 92521

and

David M. Wright
Department of Pathology, 100 Medical Campus Drive, Lansdale, PA 19446

Abstract. In allozyme generated trees, test populations of species of the North American *Callophrys* (sensu lato) (a group of hairstreak/elfin butterflies) clustered within the genera *Mitoura*, *Callophrys*, *Incisalia*, and *Loranthomitoura*. The pine-feeding species of *Incisalia* clustered weakly, but separately from non-pine feeding species of *Incisalia* (*Deciduphagus*). The trees present dissimilarities from recent taxonomic arrangements of *Mitoura* species and subspecies. Larval food plants (*Calocedrus, Juniperus*, and *Cupressus*), often used for distinguishing *Mitoura* taxa, do not necessarily follow the pattern of genetic relationships among populations. *Mitoura thornei* and *M. muiri* probably deserve no greater than subspecies status under *M. loki* and *M. siva* respectively. *Mitoura gryneus* and *M. siva* populations, considered conspecific by some workers, do not display a gradual geoclinal blend zone and are probably best considered separate taxa. A putative population of *M. gryneus* from Arkansas may deserve species status.

Additional key words: *Mitoura*, *Incisalia*, *Loranthomitoura*, Cupressaceae, *Juniperus, Calocedrus, Cupressus, Chamaecyparis*, allozyme, hairstreaks

Clench (1961) redefined *Callophrys* Billberg as an omnibus genus comprised of six subgenera: *Callophrys*, *Cyanophrys* Clench, *Incisalia* Scudder, *Mitoura* Scudder, *Sandia* Clench & Ehrlich, and *Xamia* Clench. Johnson (1981) used the infratribe Callophryina within the lycaenid tribe Eumaeini to encompass all components of Clench's *Callophrys*, plus the Old World *Ahlbergia* Bryk, while recognizing generic rank for Clench's subgenera. Johnson (1992a) and Ballmer & Pratt (1992) added *Cisincisalia* and *Loranthomitoura*, respectively, to the list of callophryine genera. Johnson (1992b) further split *Incisalia* by recognizing *Deciduphagus* for those species that do not feed on Pinaceae. The higher taxa within Callophryina have been variously recognized as subgenera of *Callophrys* (e.g., Ziegler 1960; Clench 1961; Harris 1972; Howe 1975; Scott 1986; Opler & Warren 2002; Pelham 2008) or separate genera (e.g., dos Passos 1964; Miller & Brown 1981; Pyle 1981; Bailowitz & Brock 1991; Bird et al. 1995). Robbins (2004) recognizes a *Callophrys* Section of the Eumaeini (equivalent to Johnson's original concept of Callophryina) containing the genera *Cyanophrys* and *Callophrys*, and assigns to the latter all taxa represented in this study without recognizing subgeneric divisions.

A mainstay for taxonomic distinctions in classical taxonomy has been differences in genital structure. The lack of consensus for taxonomic boundaries within *Callophrys* (s. l.) has been fueled by clusters of taxa with great similarity in genital structure, but with distinct differences in adult morphology, larval morphology, and ecology (especially host specificity). Similarly, the taxonomic boundaries of specific and infraspecific taxa within such clusters are often unsettled due to the absence of direct evidence of barriers to gene flow. Johnson (1976) employed both male and female genital

features to characterize *Mitoura* species, but subsequent workers have found such features to be too plastic to warrant such categorical use (Brown 1983; Ferris 1992; Layberry et al. 1998; Nice & Shapiro 2001). Alar characters, such as ground color and maculation, traditionally used to help distinguish taxonomic boundaries, are often similarly variable within populations. Thus, while Clench (1981) discriminated *C. millerorum* Clench from *C. spinetorum* on the basis of differences in wing maculation, Robbins (1990) synonymized the two, considering them as extremes of a continuum of variation within one *C. spinetorum* population. Warren (2005) also discusses variability in alar characters among some Oregon *Mitoura* populations.

The taxonomy of *Mitoura* has long been controversial. *Mitoura* species range throughout most of North America and are found nearly everywhere their cupressaceous food plants occur (Scott 1986; Opler & Wright 1999). Miller & Brown (1981) recognized ten *Mitoura* species north of Mexico: *gryneus* (Hübner), *hesseli* Rawson & Ziegler, *rosneri* K. Johnson, *barryi* K. Johnson, *byrnei* K. Johnson, *nelsoni* (Boisduval), *siva* (W. H. Edwards), *loki* (Skinner), *spinetorum* (Hewitson), and *johnsoni* (Skinner). Two of these species, *spinetorum* and *johnsoni*, were removed from *Mitoura* and placed into *Loranthomitoura* based upon food plant and autapomorphies (Ballmer & Pratt 1992). Johnson (1978), Tilden & Smith (1986), and Emmel et al. (1998) recognized a ninth species, *muiri* (Hy. Edwards), a taxon originally described as a subspecies of *M. nelsoni*. Lastly, Brown (1983) described a tenth species, *thornei*.

More recently, Guppy & Shepard (2001) treated the *Thuja* associated *M. byrnei* as a junior synonym of *M. rosneri* and Warren (2005) synonymized *M. barryi* with *M. plicataria* Johnson, originally described as a subspecies of *M. rosneri*. Warren (2005) further suggested that all *Thuja* associated populations in the Pacific Northwest might be referable to *M. rosneri*. Scott (1986) and Robbins (2004) recognized only two *Mitoura* species north of Mexico: *hesseli* and *gryneus*; their concept of *gryneus* subsumes all *Mitoura* taxa treated here other than *hesseli*. Opler & Wright (1999) recognized a similar taxonomic arrangement, but distinguished *muiri, nelsoni,* and *thornei* as separate from *gryneus*.

Two *Mitoura* taxa in the southern United States are of conservation concern due to their restricted ranges and habitat loss. *Mitoura gryneus sweadneri* F. H. Chermock is restricted to coastal stands of *Juniperus virginiana* L. var. *silicicola* (Small) J. Silba in Florida and has experienced recent habitat loss due to urbanization (Emmel 1993). *Mitoura thornei* is confined to Otay Mountain, San Diego County, and adjacent lands in southern California where its larval host plant, *Cupressus forbesii* Jepson, occurs and is threatened by increased fire frequency (Brown 1993).

The complexity of *Mitoura* populations is epitomized by the situation in the San Bernardino Mountains of Southern California, where four morphologically and ecologically distinct and narrowly allopatric *Mitoura* populations occur: *loki, nelsoni, siva juniperaria* Comstock, and what some southern California lepidopterists informally refer to as a "high elevation *nelsoni*". Each is associated with a different larval host plant. The *M. s. juniperaria* and *M. loki* populations, respectively, occur in the northern and southern portions of the range, at low to moderate elevations (mostly 3–5000') in association with what may be different chemical races of *Juniperus californica* Carr. (Vasek 1966; Vasek & Scora 1967). Both are multiple brooded in most years. Typical *M. nelsoni*, on the other hand, is univoltine and occurs at intermediate elevations (4–6000') on the coastal facing slopes in association with *Calocedrus decurrens* (Torrey) Florin. The "high elevation *nelsoni*", also univoltine, occurs above 7000' in association with *J. occidentalis australis* Vasek and differs in appearance from typical *nelsoni* in absence of pinkish overlay found in freshly eclosed *nelsoni* specimens and generally browner ventral appearance, with occasional greenish cast (Ballmer & Pratt pers. obs.).

Ballmer & Pratt (1989, 1992) demonstrated that a suite of morphological characters of the immature stages could be used to characterize some subdivisions (as genera) of *Callophrys* (s. l.), as well as some species of *Mitoura* and *Loranthomitoura*. More recently, Nice & Shapiro (2001) showed that biochemical (allozyme) characters could yield direct evidence of gene flow (or lack of it) among some *Mitoura* populations. Here, we present allozyme-based evidence for the genetic distances and phylogenetic relationships among representatives of most taxonomic groups within North American *Callophrys* (s. l.), as well as populations of several putative *Mitoura* taxa. We use genetic distances (Nei 1972) and allozyme characters to construct a number of computer-generated phylogenetic trees, based on various analytical algorithms. We compare these trees with respect to recognized specific and generic concepts.

MATERIALS AND METHODS

Enzyme Analyses. Allozymes are enzymes that exhibit variability among individuals of a species due to genetic variability in the DNA subunits (alleles) that

TABLE 1. Enzymes used for analyses.

aconitase (ACO-1 & ACO-2)
adenylate kinase (AK)
alpha glycerophosphate dehydrogenase (αGPD)
aspartate amino transferase (AAT-1)
glucose-6-phosphate dehydrogenase (G6PD)
glucose phosphate isomerase (GPI)
isocitrate dehydrogenase (IDH-1 & IDH-2)
malic dehydrogenase (MDH-2)
NADP dependent malic enzyme (ME-1)
peptidase [2 loci: leucyl-glycine (PEP-1) and leucyl-glycyl-glycine (PEP-2) as substrates]
phosphoglucomutase (PGM)
superoxide dismutase (SOD-2)

express them. This enzyme variability is detected through electrophoretic separation of a homogenate, using an appropriate buffer and gel substrate. Individual fresh and/or frozen adult butterflies were homogenized in 50 µl of buffer (0.005 M Tris-HCl pH 7.5), electrophoresed on 10% starch gels, stained for enzymes, and scored following the procedure of Pratt (1994). Homogenates were stored in microtiter plates at -70°C and electrophoresed with a citrate-aminopropyl-morpholine continuous system (pH 8.5) (Clayton & Tretiak 1972). Fifteen enzymes (Table 1) were stained with conventional histochemical stains provided as 12 different recipes in Shaw & Prasad (1970). Some isozymes, such as IDH-1 and IDH-2, stained with the same enzyme staining recipe.

Analyses of Allelic Variation. Allelic variations of 15 presumptive loci of 400 hairstreaks were scored by distance from the origin. Genetic distances, F statistics, and trees were determined by BIOSYS-1 (Swofford & Selander 1989). Sample sizes are shown in Table 2. The various measures of genetic distances calculated by BIOSYS-1 were Nei, unbiased Nei, minimum Nei, unbiased minimum Nei, Nei identities, unbiased Nei identities, Rogers, Modified Rogers, Prevosti, Cavalli-Sforza & Edwards chord, Cavalli-Sforza & Edwards arc, and Edwards "E". Trees were constructed from genetic distances and presence/absence of allelic characters using *Strymon melinus* as an outgroup with PAUP* (Swofford 1998).

UPGMA trees were constructed by the method of Sneath & Sokal (1973) through BIOSYS-1 and PAUP* using genetic distances and the unweighted pair-group method with arithmetic averaging algorithm. Distance Wagner trees (BIOSYS-1), using the multiple addition criterion algorithm of Swofford (1981), were produced with Rogers, Modified Rogers, Prevosti, Cavalli-Sforza & Edwards chord, Cavalli-Sforza & Edwards arc, and Edwards "E" distances (Farris 1972). Only trees having the highest cophenetic correlation and low percent standard deviation for the various genetic distances were chosen for analysis. In such trees, the genetic distances between populations are closest to the actual branch lengths in the tree (Pratt & Wright 2002; Pratt et al. 2006). Edwards and Minimum Nei distances and presence/absence of allelic characters were also used to construct trees with PAUP* using neighbor-joining methodology. TreeView (Page 2009) was used to print high-resolution trees from the PAUP* generated trees.

RESULTS

The taxa in this study collectively displayed great genetic variability of 118 alleles for the 15 allozymes studied. Of the 118 allelic characters, 22 were common to all populations and 12 were found in only individual populations. All 15 loci were polymorphic at least in some taxa. The trees constructed by BIOSYS-1 and PAUP* analyses differed somewhat, yet conformed to commonly accepted concepts of subgroups within *Callophrys* (s. l.) (genera or subgenera, species and/or species groups). The trees suggest some novel hierarchical relationships.

Figure 1 illustrates a minimum Nei distance tree having the highest cophenetic correlation (0.917) and lowest percent standard deviation (17.656) among trees produced with Nei, minimum Nei, unbiased Nei, and unbiased minimum Nei distances, using the UPGMA algorithm. Although similar trees based on other genetic distances had higher cophenetic correlations and lower percent standard deviations, the tree in figure 1 is illustrated because Nei distances are often cited as measures of genetic distance between taxa (Ayala et al. 1974; Brittnacher et al. 1978; Gorman & Renzi 1979; Angevine & Brussard 1979; Berlocher & Bush 1982; Pashley 1982; Mensi et al. 1988; Pratt 1994; Paggi et al. 1998; Pratt & Wright 2002; Pratt et al. 2006) and are used for determining molecular clocks (Nei 1971; Maxson & Maxson 1979; Berlocher & Bush 1982; Mensi et al. 1988). A similar tree was constructed through PAUP* with minimum Nei distances and the neighbor-joining methodology (Fig. 2).

A tree with the highest cophenetic correlation (0.963) and second lowest percent standard deviation (6.303) was constructed using the multiple addition criterion algorithm of Swofford and Edwards "E" distances (Fig. 3). Three similar trees having a cophenetic correlation of 0.960 or greater were created with Cavalli-Sforza & Edwards chord, Cavalli-Sforza & Edwards arc, and Modified Rogers distances. In these analyses *I. eryphon* and *I. niphon* clustered together as a sister to the

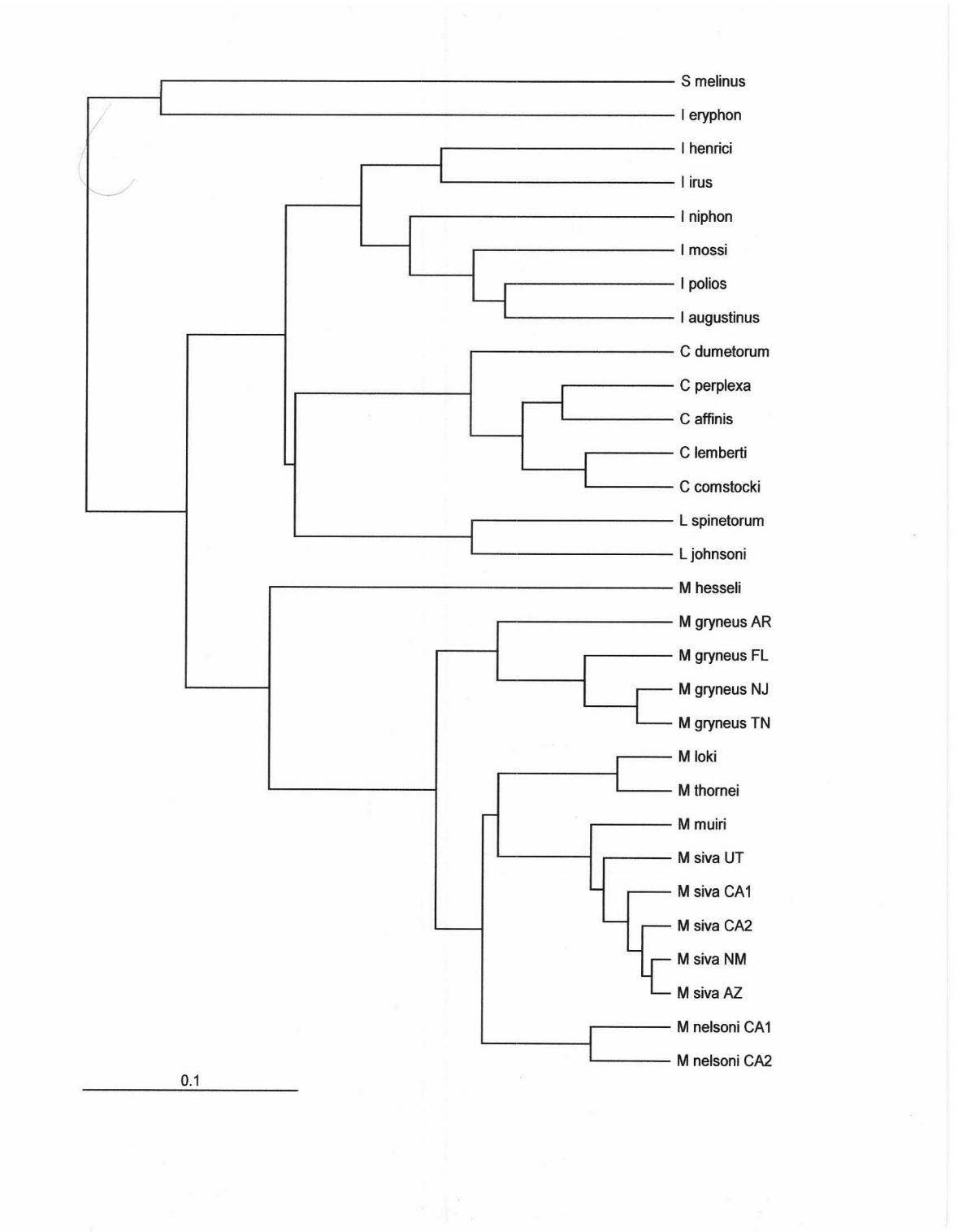

FIG. 1. Nei minimum distance tree using UPGMA algorithm of North American hairstreaks of *Callophrys* (s. l). *M gryneus* FL, *M gryneus* AR, *M gryneus* NJ, and *M gryneus* TN are *Mitoura gryneus* from Florida, Arkansas, New Jersey, and Tennessee, respectively. *M siva* CA1, *M siva* CA2, *M siva* UT, *M siva* NM, and *M siva* AZ are *Mitoura siva* from La Panza (*California mansfieldi*), New York Mountains (California), *Pintura* (Utah), Albequerque (New Mexico), and Rose Peak (Arizona), respectively. *M nelsoni* CA1 and *M nelsoni* CA2 are *Mitoura nelsoni* from Mountain Home (California) and Onyx Summit (California), respectively.

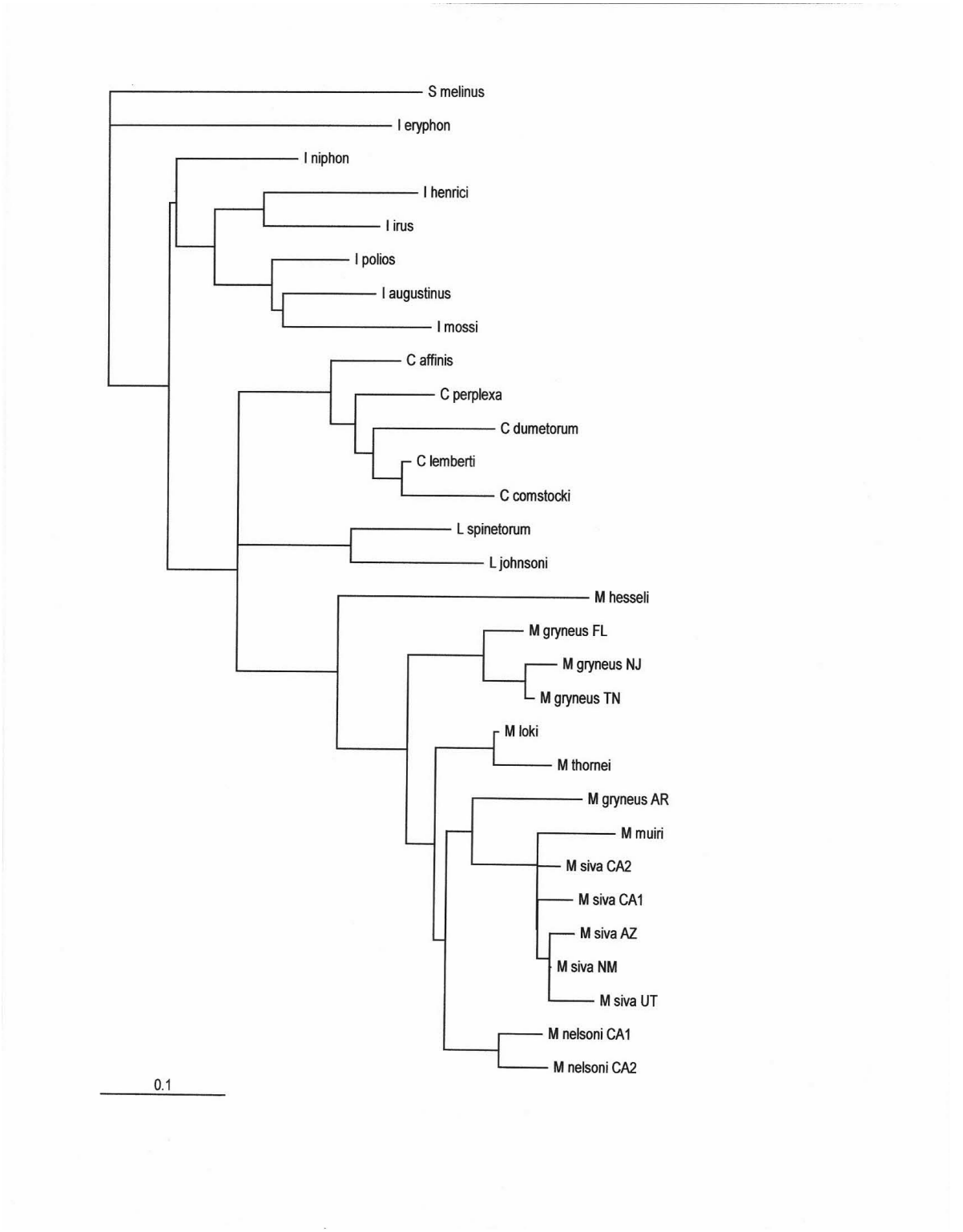

FIG. 2. Nei minimum distance tree using neighbor-joining methodology of North American hairstreaks of *Callophrys* (s. l.). *Strymon melinus* is used as an outgroup. The names of test populations are as in Fig. 1.

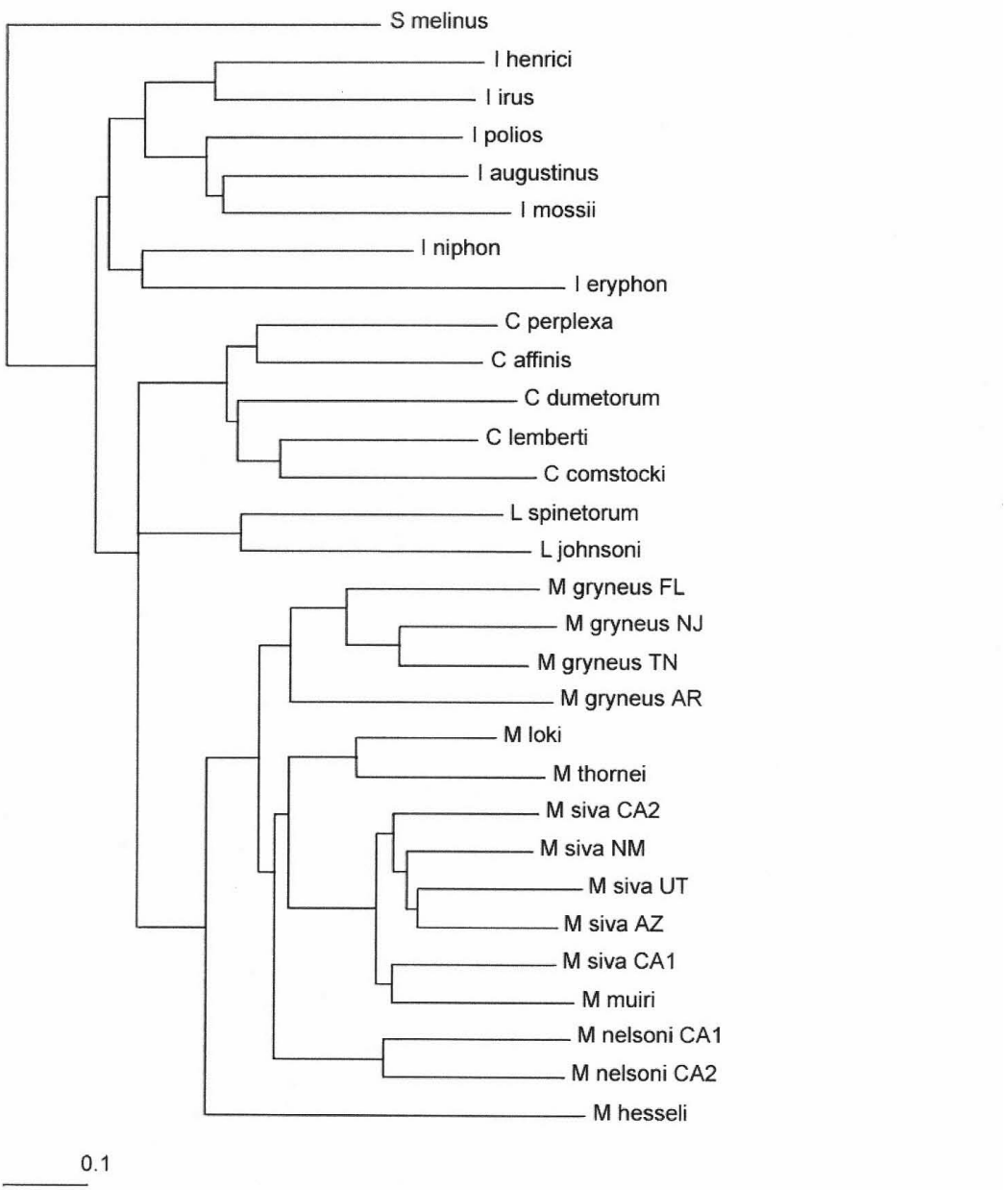

FIG. 3. Distance Wagner tree of North American hairstreaks of *Callophrys* (s. l.) group using the multiple addition criterion algorithm of Swofford and Edwards "E" distance. Total length of the tree is 8.792. The tree has a cophenetic correlation of 0.963 and percent standard deviation of 6.303. The names of test populations are the same as given in Fig. 1.

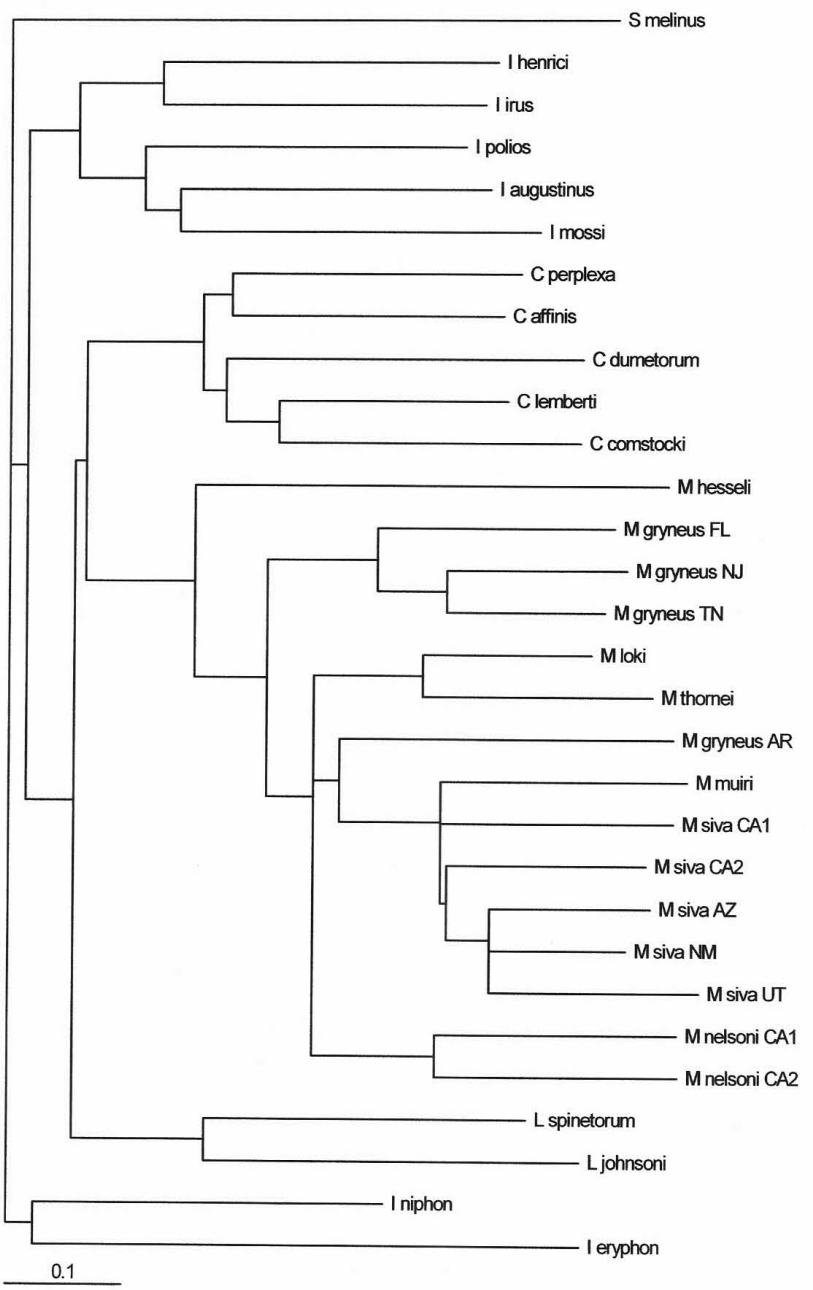

FIG. 4. Edwards distance tree using neighbor-joining methodology of North American hairstreaks of *Callophrys* (s. l.). *Strymon melinus* is used as an outgroup. The names of test populations are as in Fig. 1.

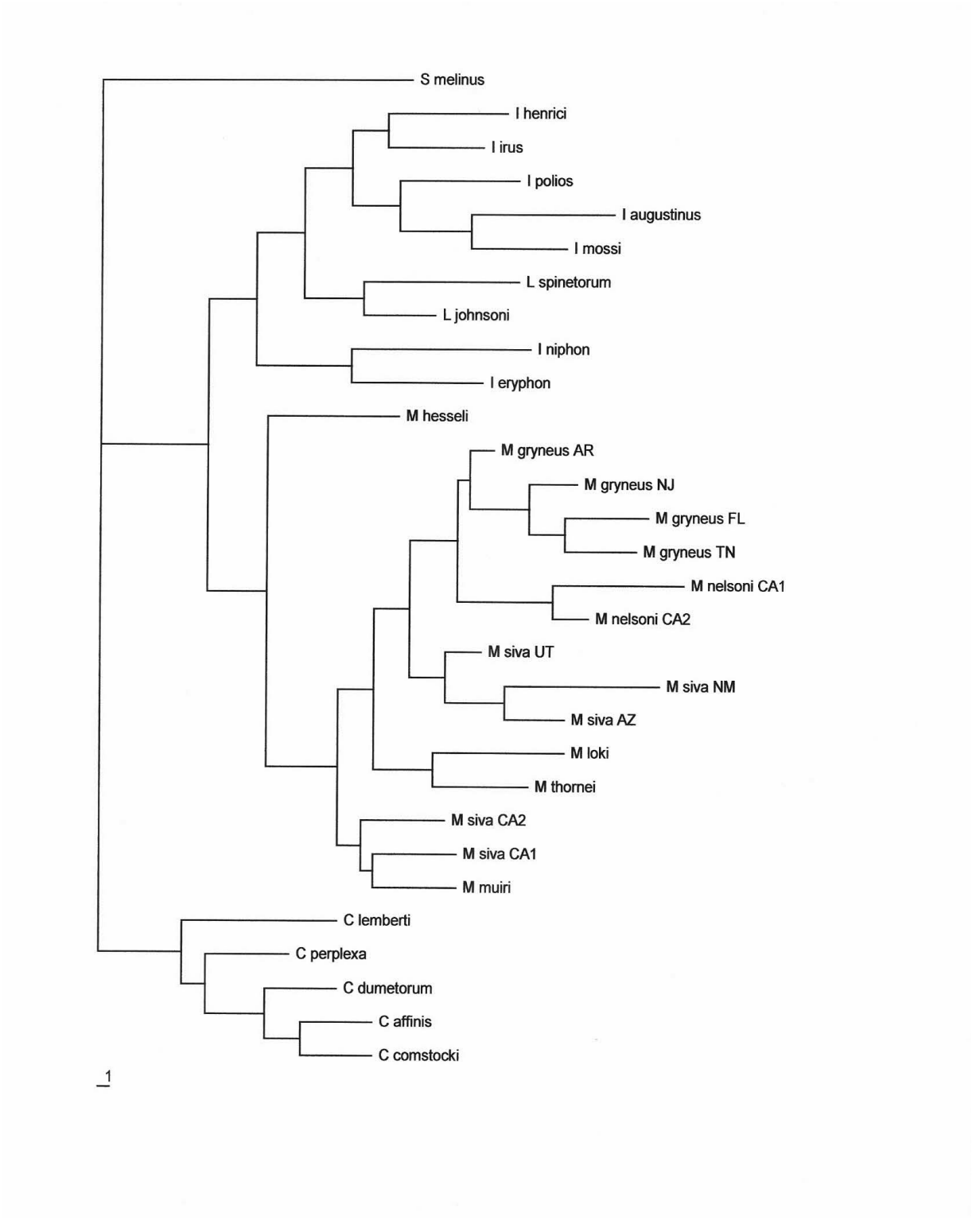

FIG. 5. Tree created using presence/absence of allelic characters and the neighbor-joining methodology of North American hairstreaks of *Callophrys* (s. l.). *Strymon melinus* is used as an outgroup. The names of test populations are as in Fig. 1.

Table 2. Sample sizes and locations of hairstreak populations used for enzyme analyses

Genus	*species/subspecies*	N	Location
Strymon	*melinus*	11	Albuquerque, NM
Incisalia	*augustinus*	10	Chatsworth, NJ
	mossii	12	Forest Falls, CA
	polios	11	Chatsworth, NJ
	irus	10	Mizpah, NJ
	henrici	34	Nanticoke, DE
	niphon	17	Chatsworth, NJ
	eryphon	4	Sugarloaf Mt., CA
Callophrys	*affinis*	5	Rose Peak, AZ
	perplexa	10	Riverside Co., CA
	dumetorum	5	Marina Dunes, CA
	lemberti	6	Sierra Nevada, CA
	comstocki	3	Providence Mts., CA
Loranthomitoura	*spinetorum*	13	Lassen County, CA
	johnsoni	2	Lassen County, CA
Mitoura	*hesseli*	19	Chatsworth, NJ
	nelsoni nelsoni	10	Mountain Home, CA1 (assoc. w/ *C. decurrens*)
	nelsoni ssp.	11	Onyx Summit, CA2 (assoc. w/ *J. occidentalis*)
	muiri	32	Cuesta Ridge, CA
	gryneus gryneus	24	Vineland, NJ
	gryneus gryneus	11	Rome, TN
	gryneus gryneus	5	Magazine Mountain, AR
	gryneus sweadneri	6	Yankeetown, FL
	loki loki	27	Long Canyon, Riverside Co., CA
	thornei	17	Otay Mountain, CA
	siva siva	27	Albuquerque, NM
	siva siva	10	Rose Peak, AZ
	siva siva	11	Pintura, UT
	siva mansfieldi	25	La Panza, CA1
	siva siva	12	New York Mts., CA2

TABLE 3. Heterozygosities for 15 loci and food plant families

	Heterozygosity		
Species/Population	**Direct count**	**H-W expected***	**Food plant families**
Strymon melinus	0.234	0.289	many families
	(0.058)	(0.069)	
Incisalia augustinus	0.238	0.230	Ericaceae, Rosaceae, Rhamnaceae, & others
	(0.060)	(0.057)	
Incisalia mossii	0.097	0.161	Crassulaceae
	(0.051)	(0.062)	
Incisalia polios	0.193	0.275	Ericaceae
	(0.041)	(0.063)	
Incisalia irus	0.184	0.193	Fabaceae
	(0.044)	(0.045)	
Incisalia henrici	0.164	0.160	Fabaceae, Rosaceae, Aquifoliaceae, & others
	(0.050)	(0.048)	
Incisalia niphon	0.273	0.277	Pinaceae
	(0.056)	(0.053)	
Incisalia eryphon	0.067	0.133	Pinaceae
	(0.030)	(0.054)	
Callophrys affinis	0.187	0.166	Polygonaceae & Rhamnaceae
	(0.072)	(0.063)	
Callophrys perplexa	0.107	0.106	Polygonaceae & Fabaceae
	(0.041)	(0.042)	
Callophrys dumetorum	0.153	0.138	Polygonaceae
	(0.052)	(0.047)	
Callophrys lemberti	0.211	0.285	Polygonaceae
	(0.053)	(0.071)	
Callophrys comstocki	0.133	0.133	Polygonaceae
	(0.063)	(0.064)	
Loranthomitoura spinetorum	0.086	0.191	Viscaceae
	(0.033)	(0.057)	
Loranthomitoura johnsoni	0.300	0.256	Viscaceae
	(0.095)	(0.074)	

TABLE 3. Continued

	Heterozygosity		
Species/Population	**Direct count**	**H-W expected***	**Food plant families**
Mitoura hesseli	0.257	0.198	Cupressaceae
	(0.086)	(0.059)	
Mitoura nelsoni nelsoni	0.349	0.425	Cupressaceae
	(0.090)	(0.084)	
Mitoura nelsoni (high elev.)	0.319	0.333	Cupressaceae
	(0.074)	(0.054)	
Mitoura muiri	0.137	0.176	Cupressaceae
	(0.050)	(0.064)	
Mitoura gryneus (NJ)	0.119	0.122	Cupressaceae
	(0.058)	(0.061)	
Mitoura gryneus (TN)	0.113	0.125	Cupressaceae
	(0.044)	(0.050)	
Mitoura gryneus (AR)	0.213	0.190	Cupressaceae
	(0.072)	(0.063)	
Mitoura gryneus (FL)	0.178	0.183	Cupressaceae
	(0.044)	(0.045)	
Mitoura loki loki	0.249	0.296	Cupressaceae
	(0.050)	(0.066)	
Mitoura thornei	0.176	0.190	Cupressaceae
	(0.044)	(0.047)	
Mitoura siva (NM)	0.251	0.269	Cupressaceae
	(0.052)	(0.059)	
Mitoura siva (AZ)	0.200	0.217	Cupressaceae
	(0.051)	(0.058)	
Mitoura siva (UT)	0.193	0.195	Cupressaceae
	(0.057)	(0.053)	
Mitoura siva (CA2)	0.263	0.264	Cupressaceae
	(0.064)	(0.059)	
Mitoura siva (CA1)	0.215	0.219	Cupressaceae
	(0.059)	(0.060)	

* Unbiased Hardy-Weinberg equilibrium estimate (see Nei, 1978)

remaining *Incisalia* populations (subgenus *Deciduphagus*). But using Edwards distances and neighbor-joining methodology, PAUP* constructed a tree in which *I. eryphon* and *I. niphon* clustered together as a sister to all other callophryine taxa (Figure 4). The *Loranthomitoura* species (*johnsoni* and *spinetorum*) clustered separately from all *Mitoura* populations in all BIOSYS-1 and PAUP* trees (Figures 1–5).

Within *Mitoura*, five distinct clusters above 0.1 minimum Nei distance are discriminated: *M. loki* (including *M. thornei*), *M. siva* (including *M. muiri*), *M. nelsoni* (including the "high elevation" *M. nelsoni* population associated with *Juniperus occidentalis*), *M. hesseli*, and *M. gryneus* (including *M. g. sweadneri*). *Mitoura hesseli* is the most distinct member of the *Mitoura* taxa, based on minimum Nei distances, which ranged from 0.297 (*M. loki*) to 0.430 (*M. gryneus* [Arkansas]), while the distances among the other *Mitoura* populations ranged from 0.017 to 0.324. In comparison, the minimum Nei distances between the various *Mitoura* populations and *Incisalia augustinus* ranged from 0.438 to 0.555.

The Arkansas population [perhaps referable to *M. g. castalis* (W. H. Edwards) and hereafter referred to as *M. g.* near *castalis*] displays an unusual relationship to other *Mitoura* populations. This population branched above 0.1 minimum Nei distance from the other *M. gryneus* populations (Table 4), but clustered with them in all the UPGMA trees created with unbiased Nei, minimum Nei, and unbiased minimum Nei distances plus genetic and unbiased genetic identities (as in Fig. 1). The same cluster pattern was produced by the PAUP* tree constructed through Edwards distances (Fig. 3) and by presence/absence of allelic characters (Fig. 5). The other PAUP* trees placed *M. g.* nr. *castalis* within the *M. siva* cluster, as sister to all other *siva* populations and quite distant to them (Figs. 2 & 4). The somewhat ambiguous placement of the *M. g.* near *castalis* population could be due to the low sample size, greater genetic isolation of this population, a combination of both, being a distinct species, or perhaps an intermediate population in a clinal blend zone between eastern *M. gryneus* and western *M. siva* populations.

The minimum Nei distances between *M. muiri* and *M. siva* populations (0.067 to 0.085) was within the range of genetic distances among the other *M. siva* populations and is less than the minimum Nei distance between *M. muiri* and the nominate *M. nelsoni* population (0.174). Similarly, the minimum Nei distance between *M. loki* and *M. thornei* (0.050) is within the range of observed minimum Nei distances among the *M. siva* populations (Table 5).

TABLE 4. Genetic distances between *Mitoura gryneus* populations. Locations are the same as those from table 2.

		NJ	TN	AR
1.	FL	0.099	0.063	0.207
2.	NJ		0.032	0.144
3.	TN			0.136

The mean F(ST)s between loki and thornei was 0.096 and was significant based on contingency chi-square analyses across all 15 loci (P<0.00001). The mean F(ST)s between the two putative San Bernardino Mountains *M. nelsoni* populations was only 0.032 at 11 loci (Table 6) and was not significant based on contingency chi-square analyses (P=0.13707); the other four loci were represented by only a single individual in *M. nelsoni* from Mountain Home. Although the sites of these two *Mitoura nelsoni* populations are separated by 26 kilometers, their ranges may converge where their respective host plant distributions narrowly overlap. The mean F(ST)s of the remaining western southern California *Mitoura* populations ranged from 0.155 to 0.264 (Table 6) and were all significantly different based on contingency chi-square analyses (P<0.00001).

DISCUSSION

Allozyme analyses have been used for over thirty years to illuminate the evolutionary relationships between and within species of Lepidoptera (Brittnacher et al., 1978; Angevine & Brussard 1979; Pashley 1982; Mensi et al. 1988; Brussard et al. 1989; Pratt 1994). Although DNA studies are more frequent in the recent taxonomic literature, allozyme research remains a useful alternative tool for understanding phylogenetic relationships (Nice & Shapiro 2001; Pratt & Wright 2002; Vandewoestijne & Baguette 2002; Pratt et al. 2006; Habel et al. 2008; Habel & Schmitt 2009). Vandewoestijne & Baguette (2002) examined the amount or value of information from Random Amplified Polymorphic DNA (RAPD) compared to allozymes. Not surprisingly they found RAPDs correlated better with geographic differences among Cranberry Fritillary populations than did allozymes. They also found greater genetic diversity in these Fritillary populations with RAPDs than with allozymes. But these comparisons may be somewhat unfair since there were far more polymorphic loci for RAPDs (18) than for allozymes (4).

Recently Pratt & Wright (2002) and Pratt et al. (2006) used allozyme analyses to construct trees that show the evolutionary relationships between North American species of coppers and blues, respectively. Genetic studies using allozymes were used to examine the phylogeography of the marbled white butterfly in

TABLE 5. Matrix of minimum Nei distances of the five *Mitoura siva* and *M. gryneus* (AR) populations. Locations are the same as those from table 2.

	CA2	AZ	NM	UT	AR
CA1	0.050	0.035	0.033	0.090	0.184
CA2		0.028	0.024	0.075	0.127
AZ			0.017	0.047	0.147
NM				0.037	0.136
UT					0.201

TABLE 6. Matrix of minimum Nei distances of the four *Mitoura* taxa found in the San Bernardino Mountains of southern California. Locations are the same as those from table 2.

	M. nelsoni (CA1)	*M. loki*	M. *siva* (CA2)
M. nelsoni (CA2)	0.074	0.169	0.159
M. nelsoni (CA1)		0.161	0.164
M. loki			0.159

TABLE 7. Mean F(ST) across 15 loci.

M. thornei	*siva* CA1	*nelsoni*	CA2	*muiri*
M. loki	0.096	0.205	0.220	0.249
M. *siva* CA1	0.253	XXXX	0.250	0.155
M. CA2	0.264	0.250	XXXX	0.247
M. CA1[1]	0.236	0.178	0.032*	0.194

[1] across 11 loci

*not significant based on chi-square contingency tests

Northern Africa through Europe (Habel et al. 2008). Allozymes were also used to show that genetic differences between populations of two lycaenid species reflect the dispersal differences between populations (Habel & Schmitt 2009).

Analytical considerations. Because allozymes are proteins that differ in size, shape, and charge at a specific pH, they travel at different relative rates through a gel. For consistent results, gels are made with the same percent starch and buffer. Following addition of enzyme substrates (as in recipes of Shaw & Prasad 1970), stained precipitated products result in visible banding corresponding to the differential mobilities of the allozymes. Sometimes more than one enzyme can be identified by the same stain system, since different enzymes may perform the same enzymatic reaction. These allozymes can be differentiated by the banding patterns in the gel due to 1) subunit differences, 2) distances traveled, and 3) stain intensities. Geiger (1990) discusses much of this methodology in more detail.

Sample size can affect the calculation of genetic distances from allozymes, due to differences in allele frequencies (Nei 1972; Avise 1974; Ayala et al. 1974) and the chance that rare alleles may be included in small samples. It is generally assumed that the larger the sample size the more likely the test sample will accurately represent the frequencies of alleles in the wild population. Another complication can arise when different alleles have indistinguishable mobilities on a given buffer/gel combination. Employing a buffer that can separate as many alleles as possible may reduce this effect. Because we have found that a higher pH selects for greater charge differences in enzyme proteins for

butterflies, we tested a number of buffer systems that could be used at high pH and found Clayton Tretiak pH 8.5 buffer elucidated more alleles with lycaenids than did other buffer systems (Pratt 1994; Pratt & Wright 2002; Pratt et al. 2006).

The use of genetic distance as a measure of phylogenetic distinction and justification for taxonomic decisions is not without controversy. Even the time needed for the speciation process to occur is controversial, with estimates ranging from four to less than a million years (Johnson et al. 1996; Klicka & Zink 1997; Avise & Walker 1998; Avise et al. 1998). Similarly, there has been much controversy over the molecular clock and the amount of time needed to produce a given amount of genetic change in various organisms. Estimates for one Nei unit range from three (Nei 1971) to five (Mensi et al. 1988) and up to 14 millions of years of genetic isolation (Maxson & Maxson 1979; Berlocher & Bush 1982). Certainly, reported differences in Nei distance correlations could be due to different rates of evolution for different organisms under different selection pressures. A Nei distance of 0.1 has been used to distinguish species from subspecies-level differentiation (Ferguson 2002), yet interspecific Nei distance can be well below this number (Berlocher & Bush 1982; Brittnacher et al. 1978; Pratt 1994), while Nei distances among conspecific populations sometimes can be much higher (Mensi et al. 1988). The use of Nei distance in taxonomic/systematic research should be used with caution, especially when applied as a yardstick across more distantly related taxonomic groups.

Because genetic distance does not necessarily equate to genetic incompatibility, the Nei distance alone is not proof of speciation. Nevertheless, genetic distance may be useful in helping resolve phylogenetic relationships and, ultimately, taxonomy among closely-related populations. Where undisputed species-level differentiation of some members of a taxonomic group exists, Nei distances may provide guidance for interpretation of phylogenetic relationships among other populations.

Phylogenetic considerations. In this study, populations within most trees generally clustered within their recognized generic, subgeneric, and/or species groups. Most species within generic clusters, other than *Mitoura*, branched above 0.1 Nei distance from their congeners (Fig. 1). One exception pertains to *Incisalia eryphon*, which either separated near the base of the tree (Figs. 1 & 2) or branched with *I. niphon* separately from other *Incisalia* (Figs. 3, 4, & 5). These differences could be an artifact of low sample size or the western pine elfin (*I. eryphon*) being more genetically divergent. Because the phylogenetic tree in figure 3 has a higher cophenetic correlation (0.963 vs. 0.917) and a lower percent standard deviation (6.303 vs. 17.656) than the tree in figure 1, it may portray the evolution of the group more accurately. In the Distance Wagner Trees, *Incisalia* was often the most basal branch and, within the genus, the pine elfins (*I. eryphon* and *I. niphon*) may be the most basal ("primitive") subgroup, correlating with their subgeneric separation (Johnson 1992b).

The relative minimum Nei distances suggest some interesting relationships amongst the Callophryina. For instance, the genetic distance between *C. lemberti* and *C. comstocki* (minimum Nei distance 0.081) is the lowest of all *Callophrys* (s. str.) pairs in this study (Table 4), while *C. dumetorum* is sister to both of them (Figs. 1–4). The former two taxa are considered by some workers to represent subspecies of *C. sheridanii* (W. H. Edwards) (e.g. Howe 1975; Scott 1986; Pelham 2008), while Warren (2005) considers *C. dumetorum* and *C. sheridanii* to be conspecific. Also, the minimum Nei distances for the pairs *M. loki* & *M. thornei* (0.050) and *M. siva* & *M. muiri* (0.067–0.085) are quite low compared to other species pairs in this study, suggesting perhaps only infraspecific level distinction.

Loranthomitoura appears to have closer affinities with *Callophrys* (s. str.) (Figs. 1, 2, 4) or *Incisalia* (Fig. 5), or separate from all other genera (Figure 3), than with *Mitoura*, the genus (or subgenus) in which its members were once placed. The systematic position status of *Loranthomitoura* was recently confused by the formal placement of *Callophrys guatemalena* (Clench, 1981) into *Cisincisalia* (Johnson 1992a) and the subsequent recognition by some workers (e.g. Robbins 2004) that *C. guatemalena* is conspecific with *Cisincisalia moeki* Johnson, the type species for *Cisincisalia*. The relationship between *Cisincisalia* and *Loranthomitoura* remains murky, because the description of the former is based largely on plesiomorphic adult alar and genital characters, while that of the latter is based primarily on a suite of autapomorphies of the immature stages including larval host plant usage. Furthermore, the life history and morphology of *C. moecki* immatures remain undescribed; when those factors become known, and/or when the phylogenetic relationship of that taxon to other Callophryina is more directly quantified, its taxonomic status with respect to *C. guatemalena* and the propriety of synonymizing *Cisincisalia* and *Loranthomitoura* will likely be clarified.

Within the *Mitoura* cluster *M. hesseli* exhibited the greatest minimum Nei distances from other populations, supporting at least a minimal two-species genus interpretation consisting of *M. hesseli* and *M.*

gryneus. However, if one used a genetic distance yardstick based on the Nei distances among other callophryine taxa, then one could justify additional species within the *Mitoura* cluster. The genetic distances between sometimes narrowly allopatric and parapatric populations of western taxa (*M. loki*, *nelsoni*, and *siva*), which have been lumped under *M. gryneus* by some workers (e.g. Scott 1986), are often as great as the genetic distances that discriminate each of them from geographically more distant populations of *M. gryneus* (east of the Great Plains).

The presence of a geoclinal blend zone between eastern *M. gryneus* and western *M. siva*, with *M.* g. nr. *castalis* as an intermediate, is not supported by the genetic distances observed. While the test population of *M g.* near *castalis* is geographically intermediate between the nearest test populations of *M. g. gryneus* (Tennessee) and *M.* s. *siva* (New Mexico), it is not part of a smooth geocline between the latter two taxa. The geographic distance between the *M.* g. near *castalis* population and the nearest test population of *M.* g. *gryneus* (Tennessee) is around 830 kilometers and the minimum Nei distance is 0.136, while the geographic distance between the Tennessee and New Jersey test populations of *M.* g. *gryneus* is 790 kilometers and the minimum Nei distance between them is only 0.032 (Table 4). Similarly, the geographic distance between the *M.* g. near *castalis* and New Mexico *M.* s. *siva* test populations is approximately 1320 kilometers and the minimum Nei distance between them is 0.136, while the distance between the same *M.* s. *siva* and the *M.* s. *mansfieldi* test populations is 1250 kilometers and the minimum Nei distance between them is only 0.033 (Table 5). Thus, the *M.* g. near *castalis* population presents a disjunction in the east-west pattern of genetic distances (relative to geographic distances) among populations of *M.* g. *gryneus* and *M. siva*, and appears to be equally genetically distant from both taxa. This suggests a degree of genetic isolation that could justify conferring equal taxonomic status on all three.

Morphologically, *M.* g. near *castalis* presents a mosaic of adult and larval features of eastern *M. gryneus* and western *M. siva*. While *M.* g. near *castalis* closely resembles eastern *M. gryneus* populations in alar characters, first instar chaetotaxy allies it more with western *M. siva*. The first instar larvae of the *M.* g. *gryneus* and *M.* g. *sweadneri* test populations have a single well-developed subdorsal (SD) seta, presumably SD1 (Ballmer & Pratt 1992; Ballmer & Wright 2008) on each of segments T3–A7, while *M.* g. near *castalis* has none (G. Ballmer pers obs.). The absence of a well-developed SD1 seta on T3–A7 is shared with populations of *M.* g. *castalis* from Texas and Durango, Mexico, as well as all western test populations of *Mitoura* in this study (G. Ballmer & D. Wright pers obs.).

Some uncertainty remains as to the overall relationships among *Mitoura* species because of low sample sizes for some populations and absence of some taxa from the data set. The relatively great range of genetic distances among conspecific populations of recognized species reported here further complicates the picture. This genetic divergence within species is probably due largely to their low vagility and close association with isolated populations of their larval hosts. Because the test sample of *M.* g. *sweadneri* was the progeny of a single female, the degree of its genetic separation from *M g. gryneus* populations remains uncertain. The absence of representatives of such taxa as *M. s. chalcosiva* Clench and *Thuja*-associated *Mitoura* could also affect the trees generated. Further allozyme studies of additional populations of *Mitoura* taxa are needed to determine reliability of the frequency differences between them and the effects of including additional taxa. Similarly the inclusion of populations representing additional higher categories within Callophryina [e.g. *Ahlbergia*, *Sandia*, *Xamia*, and *Cyanophrys*] could help clarify the status of such groups, as well as their evolutionary relationships. Studies of additional loci, such as from DNA markers that help distinguish species of the Callophryina may further elucidate relationships.

Some of the *Mitoura* taxa treated here were also subjects of an allozyme study by Nice & Shapiro (2001). In that study unbiased minimum genetic distances from 0.000 to 0.007 were reported for northern California populations of *M. muiri*, *nelsoni*, and *siva.* In contrast, we found unbiased minimum genetic distances for southern California populations of these taxa ranged from 0.009 to 0.164. The differences between the studies could be due to factors such as: (1) our buffer system may have unmasked more alleles, (2) genetic differentiation may be greater among the Southern California test populations, (3) taxonomic decisions based on host plant usage may be fallible, (4) relatively low sample sizes were used in both studies, or (5) some combination of these four factors. A greater diversity of alleles unmasked by the buffer system would increase the measured genetic distances between taxa. Allele diversity could also be affected by different evolutionary histories for the different test populations of the same putative taxa in terms of bottlenecks and/or evolution rates (Soulé 1976). Because both studies employed relatively small sample sizes representing few localities, the reported Nei distances for the relevant taxa may not be definitive. A study using larger sample sizes and

representing additional localities could clarify the genetic variability within and relationships among these taxa.

Larvae. *Callophrys* (s. l.) larvae collectively utilize a wide range of host plants, while host specificity is often a useful correlate of taxonomic boundaries within the group. Thus, the pattern of larval host plant usage among test populations mirrors their allozyme-based cluster patterns (Fig. 2, Table 3). Old World *Callophrys* (s. str.) larvae [e.g. *C. rubi* (L.) and *C. avis* Chapman] collectively utilize at least 17 plant families (Robinson et al. 2010); and, while all North American taxa utilize *Eriogonum* (Polygonaceae), *C. affinis* and *C. perplexa*, respectively, also use *Ceanothus* (Rhamnaceae) and *Lotus* (Fabaceae) (Brown & Opler 1967; Gorelick 1968, 1971; Ferris 1973; Scott 1986; Ballmer & Pratt 1989; Allen et al. 2005). The test populations of *C. affinis* and *C. perplexa* exhibit the lowest minimum Nei and Edwards distances and usually cluster together and apart from those that use only *Eriogonum* (see Figs. 1, 3, & 4).

Incisalia larvae collectively feed on members of at least 11 plant families (Aquifoliaceae, Caprifoliaceae, Cuscutaceae, Crassulaceae, Ebenaceae, Ericaceae, Fabaceae, Liliaceae, Pinaceae, Rhamnaceae, and Rosaceae) (Ballmer & Pratt 1989; Howe 1975; Opler & Wright 1999; Pratt & Pierce 2001; Pyle 1981; Scott 1986; Tilden & Smith 1986). Yet, while a few species have a broad host range (e.g. *I. augustinus* is reported to use at least eight plant families), others are much more host-specific. Thus *I. mossii* uses only Crassulaceae and *I. polios* uses only Ericaceae, while *I. eryphon* and *I. niphon* utilize only Pinaceae (Scott 1986; Robinson et al. 2010). Similarly, taxa assigned to *Loranthomitoura*, *Mitoura*, *Sandia*, and *Xamia* use only Viscaceae, Cupressaceae, Agavaceae, and Crassulaceae, respectively (Bailowitz & Brock 1991; Ballmer & Pratt 1989; Howe 1975; Opler & Wright 1999; Pyle 1981; Scott 1986; Tilden & Smith 1986).

It is noteworthy that larvae of taxa assigned to *Deciduphagus*, *Incisalia*, Loranth*omitoura*, *Sandia*, and *Xamia*, which do not use fabacaeous hosts in nature, could be successfully reared to maturity on *Lotus scoparius* (Fabaceae) in the lab, suggesting that they may have a retained ancestral ability to feed on legumes (Pratt & Ballmer 1991). However, *Mitoura* larvae, which feed exclusively on Cupressaceae, cannot utilize *Lotus scoparius* (Pratt & Ballmer 1991) and speciation or subspeciation within *Mitoura* corresponds with host shifts strictly within the Cupressaceae (Johnson 1978; Gifford & Opler 1983; Ballmer & Pratt 1989, 1992).

Although host plant specificity in nature is a frequent criterion for recognizing *Mitoura* species (Johnson 1972, 1976, 1978; Ferris 1992) (e.g. *M. hesselli* on *Chamaecyparis* and typical *M. nelsoni* on *Calocedrus*), some taxa are more polyphagous in nature [e.g. *M. siva* on various western *Juniperus* species (Johnson 1978) and *M. loki* on *Juniperus californica* and *Cupressus forbesii* (Ballmer pers. obs.)]. Furthermore, larvae of many *Mitoura* species can be reared on cupressaceous hosts not ordinarily available to them in nature (Comstock & Dammers 1932; Layberry et al. 1998; Guppy & Shepard 2001; Pyle 2002; Forister 2004; Ballmer, Pratt & Wright pers. obs.). In this regard, we report the previously unreported use by *M. loki* (in Orange County, CA) of *Cupressus forbesii*, thought to be used exclusively by *M. thornei* in San Diego County. This observation consisted of numerous phenotypically typical adult *M. loki* in direct association with *C. forbesii* in the upper drainage of Coal Canyon during July 1993 (Ballmer pers. obs.), over a mile distant from the nearest *J. californica* (itself an isolated specimen several miles distant from any other known *J. californica*). The utilization of *C. forbesii* by both taxa further supports their genetic proximity and an interpretation of infraspecific status for *M. thornei*.

Incisalia henrici and *I. irus*, according to all phylogenies (Figs. 1–5) cluster together and appear as sister species. *Incisalia irus* larvae feed only on closely related plants in the Fabaceae (Scott 1986; Albanese et al. 2007), while *I. henrici* larvae utilize many plant species in a number of families, including Fabaceae (Scott 1986; Pratt & Pierce 2001). This suggests that Fabaceae may be the primitive food plant for the ancestor of these two elfins, unless obligate use of Fabaceae is a secondary specialization from a polyphagous ancestor. In this regard it is notable that larvae of a population of *I. henrici* known to use *Ilex opaca* Ait. (Aquifolicaeae) in nature had significantly greater pupal weight and percent survival to adult when reared in the lab on *Cercis canadensis* L. (Fabaceae) than on *I. opaca* and *Prunus serotina* J.F. Ehrh. (Rosaceae) (Pratt & Pierce 2001).

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