# Performance of DNA barcodes for informing the subspecies controversy in North American populations of *Callophrys gryneus* (Hübner, [1819]) (Lepidoptera: Lycaenidae)

Edward Pfeiler

Department of Entomology, University of Arizona, Tucson, Arizona, 85721, USA

E-mail: pfeiler@arizona.edu

Running Title: Subspecies of the *Callophrys gryneus* complex

ABSTRACT

Analysis of genetic divergence in published mitochondrial DNA barcodes from "juniper" and "cedar" hairstreaks, *Callophrys gryneus* (Hübner) and *C. muiri* (Hy. Edwards), together with a comparison of phenotypic differences in populations from North America, have been used to address the long-standing controversy regarding subspecies and species assignments in this confusing group of hairstreaks. The most recent view holds that *C. gryneus* is a single polytypic species comprising 11 nominal subspecies, and that *C. muiri* from California is a valid species. An alternative view subdivides *C. gryneus* into three nominal species, *C. gryneus* (sensu stricto), *C. siva* and *C. nelsoni*, each with several named subspecies, and retains *C. muiri* as a full species. Haplotype networks, phylogenetic analysis, and analysis of molecular variance (AMOVA) of barcodes provided evidence supporting the subspecies scenario. However, barcodes from closely-related congeners of *C. gryneus* (sensu lato) revealed pairwise genetic divergences that would also support elevating three subspecies—*C. g. castalis*, *C. g. siva*, and *C. g. nelsoni*—to full species. The barcode data also raise the question of whether *C. muiri* warrants full species status. Larval hostplant preference as a possible driver of genetic differentiation in western populations of this group is also briefly discussed.

ADDITIONAL KEYWORDS: COI barcodes – citizen science – genetic diversity – geographic distribution –haplotype network– larval hostplants – phenotypic plasticity – population structure

## INTRODUCTION

The nominal taxon *Callophrys gryneus* (Hübner) encompasses wide-ranging populations of butterflies commonly referred to as "juniper" and "cedar" hairstreaks (Lycaenidae: Theclinae: subgenus *Mitoura* Scudder). These populations, found throughout the USA, southern Canada and northern Mexico, have historically been described as subspecies, host races, or distinct species, which has resulted in taxonomic instability that hinders an understanding of their evolutionary history and natural history. Pelham (2023) currently lists 11 subspecies-level taxa of *C. gryneus* (Table 1; Fig. 1), but with the caveat that, "There is no easy way to accommodate the diverse opinions on the specific arrangement in this complex". For example, others support subdividing the complex into the nominal species *C. gryneus* (sensu stricto), *C. siva*, and *C. nelsoni* (Pratt *et al*., 2011; Davenport, 2018). The *Mitoura* subgenus also includes *C. muiri* (Hy. Edwards), *C. loki* (Skinner), *C. hesseli* (Rawson & Ziegler) and *C. dospassosi* Clench (Zhang *et al*., 2021; Pelham, 2023) (see Table 1). Taxonomic instability in the subgenus is especially evident for *C. muiri*, which in addition to currently being considered a full species, has also been placed as a subspecies of *C. nelsoni*, *C. siva* and *C. gryneus* (Pratt *et al*., 2011; BugGuide, 2023).

Table 1; Fig. 1

Several factors have contributed to the sometimes-contentious debate on the nomenclature of juniper/cedar hairstreaks. Importantly, traits historically used in classical taxonomic studies in Lepidoptera have been unable to provide consistent diagnostic characters for determining species' or subspecies' boundaries in this group. Many of the proposed taxonomic names have relied on apparent differences in morphology of genitalia (Johnson 1976, 1978), differences which are now considered unreliable in separating proposed subspecies of *C. gryneus* (Brown, 1983; Robbins, 1990; Gervais & Shapiro, 1999; Nice & Shapiro, 2001; Forister, 2004). Also, subspecies designations based solely on wing colour (Clench, 1981) are suspect owing to substantial variability in this trait within populations. Finally, most subspecies of *C. gryneus* utilize a variety of *Juniperus* species (Cupressaceae) as a larval host, whereas far-western populations also feed on species in the genera *Calocedrus*, *Cupressus* and *Thuja* (Johnson, 1976; Nice & Shapiro, 2001; Cary & Toliver, 2022). Although some of the far-western populations show larval host preferences suggesting genetic differentiation and incipient speciation (Forister, 2004), these host preferences do not always reflect genetic relationships among populations (Nice & Shapiro, 2001; Pratt *et al*., 2011).

Only a few molecular studies have been published that might provide insights into evolutionary relationships and inform taxonomic assignments in this group of hairstreaks (Nice & Shapiro, 2001; Pratt *et al*., 2011; Zhang *et al*., 2021). Nice and Shapiro (2001) analysed allozymes and a short segment (460 base pairs [bp]) of the mitochondrial COII (or *cox2*; cytochrome *c* oxidase subunit II) gene in populations of the *C. gryneus* complex from northern California. The COII data showed that most individuals assigned to *C. nelsoni*, C. *muiri* and *C. siva* possessed the same haplotype, although *C. muiri* from coastal mountain ranges differed slightly from the common haplotype. Allozyme analysis of populations of *C. gryneus* from throughout the USA supported subdividing the complex into three nominal species, *C. gryneus*, *C. siva* and *C. nelsoni* (Pratt *et al*., 2011). Allozymes also showed *C. loki* resolving within the *C. gryneus* (sensu lato) complex, and that *C. muiri* was closely related to *C. siva* (Pratt *et al*., 2011). Finally, a recent phylogenomic study of New and Old World species of *Callophrys* supports the monophyly of the *Mitoura* subgenus, and in addition provides evidence for recognizing four additional subgenera, *Callophrys* Billberg, *Incisalia* Scudder, *Cisincisalia* K. Johnson, and *Greenie* Grishin (Zhang *et al*., 2021). The three representatives of the *Mitoura* subgenus in the genome study were: *C. gryneus* from Texas [probably subspecies *castalis*], *C. dospassosi searsi* from Sonora, Mexico, and *C. hesseli*.

Mitochondrial COI (or *cox1*; cytochrome *c* oxidase subunit I) barcodes have been used successfully in phylogenetic studies on the Lycaenidae (Nazari & ten Hagen, 2020; Talavera *et al*., 2020), although they generally perform poorly in resolving relationships in lycaenid taxa above the species level (Talavera *et al*., 2022). Mitochondrial introgression is also a potential problem to consider (Cong *et al*., 2017). Barcodes, however, have provided support for the validity of subspecies designations in a number of different families of Lepidoptera (deWaard *et al*., 2010; Wilson *et al*., 2013; Pfeiler *et al*., 2020, 2021a;) though they sometimes are unable to do so (Kodandaramaiah *et al*.,_2012; Proshek *et al*., 2015; Hill *et al*., 2018; Nazari & ten Hagen, 2020; Pfeiler *et al*., 2022). Additionally, results from the mitochondrial COI gene and the much larger protein coding nuclear genome generally are concordant in the butterfly family Hesperiidae (Li *et al*., 2019). The apparent lack of published COI barcode studies on the *C. gryneus* complex prompted the present study which aims to explore whether barcodes sourced from the Barcode of Life Data System (BOLD; Ratnasingham & Hebert, 2007) can provide insight into the contentious taxonomic arrangements proposed for this complex.

# MATERIALS AND METHODS

## SAMPLES

For genetic analyses, 69 complete barcode (COI) sequences (658 bp) for *C. gryneus* (including two for *C. muiri*), obtained from samples collected throughout the USA and southern Canada, were downloaded from BOLD and used to assess genetic diversity, and to construct haplotype networks and phylogenetic trees. Three shorter sequences, CNCBF1031–14 (549 bp), TAMIC846–10 (576 bp), and ABLCU096–09 (621 bp), were not used in the above analyses, but contained diagnostic nucleotide sites that were informative. No barcodes were available in BOLD or GenBank for *C. g. smilacis* from coastal Georgia and South Carolina, a taxon very closely related to *C. g. sweadneri* (Gatrelle, 2001), or for *C. loki* and *C. dospassosi*. Also analysed here are published COII sequences (Nice & Shapiro, 2001) downloaded from GenBank and used for comparison with the COI data set of California populations of *C. muiri* and *C. g. nelsoni*. Numerous photos of *C. gryneus* taken by citizen scientists and deposited in the iNaturalist (2023) website were examined and were instrumental in identifications of putative species and subspecies, and for assessing their geographical distributions.

## GENETIC DIVERSITY

Nucleotide sequences for *Callophrys* were uploaded to MEGA version 5.0.5 (Tamura *et al*., 2011). To confirm apparent diagnostic COI nucleotide substitutions in aligned sequences of putative subspecies of *C. gryneus*, the original trace files (electropherograms) available from the BOLD website were examined. This was especially important for the five sequences assigned to *C. g. castalis* in which one second codon position mutation was present (see Results). Genetic distances (*d*) among populations of *C. gryneus* were calculated in MEGA using the uncorrected *p*-distance model. Haplotype networks for both genes were constructed based on statistical parsimony implemented in TCS version 1.21 (Clement *et al*., 2000) using the default haplotype connection limit of 95%. Analysis of molecular variance (AMOVA) carried out in ARLEQUIN ver. 3.5.2.2 (Excoffier & Lischer, 2010) was used to test for population structure among COI haplotype groups identified in the TCS network. Calculations of COI genetic diversity indices were performed in DnaSP version 5.10.01 (Librado & Rozas, 2009).

## PHYLOGENETIC RELATIONSHIPS

Phylogenetic relationships among COI barcode sequences of named subspecies of *C. gryneus*, including *C. muiri*, were assessed by constructing phylogenetic trees based on the neighbour-joining (NJ) method carried out in MEGA using a matrix of *p*-distances, and Bayesian inference implemented in MrBayes v.3.1 (Huelsenbeck & Ronquist, 2001). The best fit model of nucleotide substitution in the data set obtained from MODELTEST v.3.7 (Posada & Crandall, 1998), based on the Akaike information criterion, was TrN + G. Bayesian analyses were run for 1,000,000 generations, sampled every 250th generation (4,000 trees sampled), using the default random tree option to begin the analysis. Clade support, expressed as posterior probabilities, was estimated using a Markov chain Monte Carlo (MCMC) algorithm. Log-likelihood values from four simultaneous (MCMC) chains (three hot and one cold) stabilized at about 15,000 generations, resulting in the first 60 trees being discarded from the analysis (burnin = 60). Outgroups were selected based on results of relationships found from genomic analysis of *Callophrys* subgenera (Zhang *et al*., 2021), and included *C. hesseli* (subgenus *Mitoura*), and *C. johnsonii* (Skinner) and *C. spinetorum spinetorum* (Hewitson) from the subgenus *Cisincisalia*.

## RESULTS

In the barcode data set of *C. gryneus* (including *C. muiri*), there were 24 variable sites and 19 haplotypes. Haplotype diversity ($h \pm$ SD) was 0.913 ± 0.015; nucleotide diversity ($\pi \pm$ SD) was 0.00869 ± 0.00040. The TCS network (Fig. 2A) shows that all COI haplotypes resolved in a single network, which was subdivided into five haplotype groups (groups I–V) based on apparent diagnostic nucleotide differences, as described below. Fig. 2

Haplotype group I from eastern USA and south-eastern Canada corresponds to the currently recognized nominal subspecies *C. g. gryneus* (type locality [TL]: Virginia). Group II from Florida corresponds to the closely related *C. g. sweadneri* (TL: St. Augustine, Florida). Mean *p*-distance (*d*) between groups I and II was 0.006 (Table 2). Groups I and II are also separated by three apparent fixed nucleotide point mutations in the barcode segment at sites 274, 550 and 658 (Table 3). Table 2 Table 3

Haplotype group III is assigned to *C. g. castalis* (TL: near Waco, TX), a subspecies found throughout Texas and north-eastern Mexico, and occasionally recorded from eastern New Mexico (Cary & Toliver, 2022; iNaturalist, 2023). Group III shows the largest genetic divergence between groups I, II, and V (mean $d = 0.013–0.014$), but is closely related to group IV (mean $d = 0.004$) (Table 2). Individuals in group III, however, differ phenotypically from those in Group IV—*C. g. siva* (TL: Wingate, AZ), *C. g. chalcosiva* (TL: Tooele County, UT), *C. g. juniperaria* (TL: Los Angeles County, CA) and *C. g. mansfieldi* (TL: San Luis Obispo County, CA)—in the presence of two postbasal spots on the ventral hindwing (Fig. 1D), and possess an apparent diagnostic second codon non-synonymous nucleotide substitution (Table 3). Although no complete barcode was available for *C. g. juniperaria*, a short sequence (576 bp) in BOLD (TAMIC846–10) for a specimen of *C. gryneus* collected near the type locality of *C. g. juniperaria* (Fig. 1G) shows the phenotype of this subspecies and resolves in haplotype group IV (not shown). The common haplotype in group IV is widely distributed in the USA, occurring in Texas, New Mexico, Arizona, Nevada, California, Wyoming and North Dakota (Fig. 2A).

Group V comprises the western subspecies *C. g. nelsoni* (TL: California), *C. g. rosneri* (TL: Kootenay Lake, B.C., Canada), *C. g. plicataria* (TL: Vancouver Island, B.C., Canada), and *C. muiri* (TL: Mendocino County, CA). Most samples for this group (27 of 31) were collected in British Columbia. Six of these individuals, labelled in BOLD as *C. g. plicataria* and *C. gryneus*, shared a haplotype with a specimen from Monterey County, California labelled *C. muiri*. The group V haplotype in two California samples included one specimen labelled *C. muiri* and one labelled *C. gryneus*, both collected from north-eastern California. The remaining California haplotype is from a specimen labelled *C. g. nelsoni* from Tulare County.

Owing to the low sample size available for COI haplotype group V from California ($n = 4$), a haplotype network was also prepared using published sequences from a segment of the mitochondrial COII gene (Nice & Shapiro, 2001) in populations assigned to *C. g. nelsoni, C. g. siva* and *C. muiri* from northern California ($n = 54$), and *C. g. gryneus* from Wisconsin (Fig. 2B). Three of the five COI haplotypes groups were recovered in the COII data set. Samples of *C. g. gryneus* (haplotype G of Nice and Shapiro) were separated from the common haplotype from California (haplotype D) by four mutational steps. Haplotype D consisted mainly of sequences assigned to *C. muiri* and *C. g. nelsoni*. Haplotypes G and D corresponded to COI groups I and V of the present study. One *C. g. siva* sample (haplotype B) was separated from haplotype D by

three mutational steps, and is consistent with COI haplotype group IV. The other three *C. g. siva* possessed haplotype D. Specimens of the juniper-feeding *C. g. siva* from northern California, referred to informally as "brown *siva*" (Gervais & Shapiro, 1999), or *C. g. chalcosiva*, are very similar morphologically to *C. g. nelsoni* (cf. Figs. 1E and I), suggesting that they may have been misidentified. All of the *C. g. nelsoni*, and most of the *C. muiri*, possessed the same COII haplotype (haplotype D). Overall, the COI and COII haplotype networks were generally concordant, especially in showing a close genetic relationship between *C. muiri* and *C. g. nelsoni*.

Neighbour-joining (NJ) analysis (Fig. 3A) shows clustering of haplotypes of *C. gryneus* and *C. muiri* into the five groups seen on Figure 2A. Relatively strong bootstrap support was found for four of the five clades (80–86%), but no support (<40%) was found for the clade comprising group IV sequences.

Fig. 3

The Bayesian tree (Fig. 3B) showed similar topology and statistical support for clades as the NJ tree, including the lack of resolution for a group IV clade. Also, the group I clade, was not supported (posterior probability = 0.62). One barcode from Texas labelled *C. g. gryneus* (BBLSX694-09) did not cluster with any group on the NJ and Bayesian trees owing to a non-diagnostic nucleotide substituion at site 340 (Table 3), but this specimen possessed the remaining diagnostic site combinations which placed it with haplotype group IV. Clades of haplotype groups II and III were highly supported (1.00 and 0.99, respectively). The clade comprising haplotype group V was also highly supported (0.99). The name *C. barryi* in this clade is considered a synonym of *C. g. plicataria* (Warren, 2005). BOLD photos of the 25 of the 27 specimens from British Columbia in group V show ventral hindwing colouration and maculation similar to *C. g. nelsoni*, especially in the presence of a weak, or poorly developed, white postmedian line in most specimens (cf. Fig. 1 [I, K and L]).

The AMOVA based on the barcode data set of combined populations of *C. gryneus* and *C. muiri* showed significant population structure (overall $\Phi_{ST} = 0.830$; $P < 0.001$). Pairwise comparisons of $\Phi_{ST}$ among the five haplotype groups indicated significant population structure among all groups (Table 4).

Table 4

## DISCUSSION

Analysis of mitochondrial DNA barcodes presented here has contributed to our understanding of the evolution of "juniper" and "cedar" hairstreaks, but these molecular markers provided no clear resolution of the currently unstabilized taxonomy of populations comprising the *C. gryneu*s complex, including *C. muiri*. Results that would appear to support the subspecies scenario for the *C. gryneu*s complex include: (1) haplotypes of *C. gryneus* (and *C. muiri*) resolved in a single network (Fig. 2A), as would be predicted for a single species; (2) five geographically separated haplotype groups (groups I–V) were identified within the network (except for groups III and IV in eastern New Mexico which were sympatric [Fig. 4]); and (3) significant pairwise population structure was seen among all group comparisons (Table 4). These results also suggest that *C. muiri* be reassigned as a subspecies of *C. gryneus*, as discussed in more detail below.

Fig. 4

The barcode results, however, are also consistent with the alternative hypothesis that the complex comprises additional species-level taxa. Although exceptions occur, barcode genetic divergences in the range of 2–3% often delimit separate species (Hebert *et al*., 2003). Phylogenomic studies on subgenera of the genus *Callophrys* have confirmed the species status of a number of closely-related taxa (Zhang *et al*., 2021). Barcode sequences for many of these species are also available in BOLD and can be used to calculate pairwise genetic distances among them. However, only three species of the *Mitoura* subgenus were studied by Zhang *et al.* (2021), including *C. gryneus* and *C. hesseli* which show a barcode genetic divergence of 3.4–4.1% (Table 2). Barcodes were not available for the third species, *C. dospassosi*, but a single private sequence in BOLD resolved in the same Barcode Index Number (BIN AAB2586) as *C. gryneus* and *C. muiri* and showed a divergence value (K2P distance) of 2%, only slightly higher than the pairwise values among *C. gryneus* haplotype groups (Table 2). Calculations of pairwise divergence values in nominal species of other subgenera of *Callophrys* revealed *d* values of 1.1% (*C. dumetorum*/*C. affinis*), 1.2% (*C. lanoraieensis*/*C. polios*), and 1.5% (*C. niphon*/*C. eryphon*), which agree well with most pairwise divergences among haplotype groups of *C. gryneus* (Table 2), suggesting species-level differentiation. Low pairwise genetic divergence (*d* <2%) also has been found between phenotypically similar buckeye butterflies (Nymphalidae), *Junonia coenia* Hübner and *J. grisea* Austin & J. Emmel (Pfeiler *et al*., 2012: Lalonde & Marcus, 2019), which have been confirmed as distinct species by whole genome analysis (Cong *et al*., 2020).

Haplotype groups of *C gryneus* that warrant consideration as subspecies (groups I and II), and those that may represent full species (groups III, IV and V) are discussed in more detail below.

## HAPLOTYPE GROUPS I AND II

Barcode analyses provide support for recognizing *C. g. gryneus* (group I) and *C. g. sweadneri* (group II) as valid subspecies. The two taxa resolve as monophyletic sister taxa on phylogenetic analysis (Fig. 3), show a mean COI genetic distance (*d*) of 0.6%, and possess three apparent diagnostic nucleotide differences (Tables 2, 3). They are also geographically separated and show minor phenotypic differences (Gatrelle, 2001). Although there are no clear guidelines for establishing genetic divergence values that delimit subspecies (Haig *et al*., 2006; Braby *et al*., 2012), mean pairwise *d* values of 0.3–0.6% are often found among subspecies of Lepidoptera and other insects (Chapuis *et al*., 2016; Chen *et al*., 2016; Pfeiler *et al*., 2020). Gatrelle (2001) also argued that because the southern *C. g. sweadneri* blends into the northern populations of *C. g. gryneus*, they should not be considered distinct species.

Because barcodes were not available for *C. g. smilacis* (TL: Chatham County, Georgia) (Gatrelle, 2001), its subspecies status requires further study, but is provisionally recognized here. Adults of *C. g. smilacis*, are found in a restricted coastal habitat ranging from southern Georgia to South Carolina, are phenotypically very similar to *C. g. sweadneri* and utilize the same larval hostplant, *Juniperus silicicola* (Gatrelle, 2001).

## HAPLOTYPE GROUPS III AND IV

Putative subspecies *C. g. castalis* (group III) and *C. g. siva* (group IV) were both found in Texas and New Mexico (Figs. 2A and 4). Both subspecies typically show green ventral hindwings, with *C. g. castalis* characterized by two postbasal white marks (Edwards, 1871) which are absent in *C. g. siva* (cf. Figs. 1D and 1E). Although barcode samples for Texas are limited ($n = 9$), hundreds of photos available in iNaturalist (2023) show that the *C. g. castalis* phenotype predominates in Texas, with only several records for the *C. g. siva* phenotype from western Texas. Increased barcode sampling in Texas will be required to confirm if the *C. g. castalis*

phenotype continues to show the apparently diagnostic second codon nucleotide composition that characterizes this subspecies (Table 3). In New Mexico, the *C. g.siva* phenotype is common, with only scarce records for *C. g.castalis*, all of which are from the eastern portion of the state (Cary & Toliver, 2022). Interestingly, the two *C. g.siva* and one *C. g.castalis* from New Mexico (Fig. 4) were collected at the same locality, and on the same date, in Lincoln County. The *C. g. castalis* phenotype is also present in Oklahoma, and in the state of Coahuila, Mexico (iNaturalist, 2023). Because of morphological similarities between *C. g. castalis* and *C. g. gryneus*, the north-eastern distribution limit of *C. g. castalis* shown in Figure 4 is only an approximation, and requires confirmation. Allozyme data, however, have confirmed the presence of a phenotype similar to *C. g. castalis* in Arkansas, and indicate that it is very closely related to *C. g. siva* (Pratt *et al*., 2011). Although *C. g. castalis* and *C. g. siva* are closely related ($d$ = 0.4%; Table 2), the divergence values of *C. g. castalis* among all other groups ($d$ = 1.3–1.4%), together with the unique nucleotide point mutation and the observation that *castalis* and *siva* can occur sympatrically, argue for reinstating *C. g. castalis* as a full species (Edwards, 1871). Genetic divergences between *C. g. siva* and groups I, II, and V ($d$ = 1.0–1.2%) and geographical distribution (Fig. 4) also argue for reinstating *siva* as a full species (Edwards, 1874).

The other members of group IV, *C. g. chalcosiva*, *C. g. juniperaria*, and *C. g. mansfieldi*, were originally described as subspecies of *C. siva* (Comstock, 1925; Tilden, 1951; Clench, 1981). Although *C. g. juniperaria* and *C. g. mansfieldi* typically show green ventral hindwings (Figs. 1G and H), as in *C. g. siva* (Fig. 1E), *C. g. chalcosiva* was described based mainly on having coppery brown ventral hindwings (Fig. 1F). Because this trait is variable, with greenish specimens sometimes seen (Clench, 1981), it raises doubt whether *C. g. chalcosiva* should be considered a valid subspecies. But given that data are limited, the names *C. g. chalcosiva*, as well as *C. g. juniperaria* and *C. g. mansfieldi*, should be retained until more information is available, but if *C. g. siva* is elevated to a full species, they would be considered subspecies of *C. siva* as originally described.

## HAPLOTYPE GROUP V

Of the five haplotype groups, the taxonomic status of members of group V—*C. g. nelsoni*, *C. g. rosneri*, *C. g. plicataria* and *C. muiri*—is arguably the most contentious. Two other taxa

previously proposed for the western group, *C. barryi* and *C. byrnei* (Johnson, 1976) are now considered junior subjective synonyms of *C. g, plicataria* (= *C. barryi*) (Warren, 2005) and *C. g. rosneri* (= *C. byrnei*) (Guppy & Shepard, 2001, p, 213). Genetic divergence between haplotype group V and the other four groups ($d$ = 1.0–1.4%; Table 2) is consistent with species-level separations within the genus *Callophrys*, as mentioned above. Also, Warren (2005) suggested the possibility of placing *C. g. plicataria* as a synonym of *C. g. rosneri* given that: (a) male and female genitalia of the holotypes of *C. g. rosneri* and *C. g. plicataria* are very similar (Johnson, 1976); (b) the two taxa are sympatric in British Columbia; and (c) no consistent phenotypic differences in ventral wing colour and maculation are apparent in the two taxa (Figs. 1K and L). Also, 25 BOLD photos of both taxa show weak, or poorly developed, white postmedian lines, typical of *C. g. nelsoni*. The oldest name available for group V taxa is *C. g. nelsoni* (Boisduval, 1869; pp. 43–44). If *C. g. nelsoni* is reinstated to a full species, *C. g. rosneri* would be placed as a subspecies of *C. nelsoni.*

*Callophrys g. nelsoni* is found throughout mid-elevation mountains, from southern California to Oregon, where larvae utilize incense cedar (*Calocedrus decurrens*) as a host. It was also previously found on Isla Guadalupe off the Pacific coast of Baja California, Mexico, probably using the endemic cypress, *Hesperocyparis guadalupensis*, as a larval host, but that population may now be extirpated owing to extensive environmental degradation (Pfeiler *et al*., 2021b). In captivity, *C. g. nelsoni* larvae have also been reared on cultivated arborvitae (*Thuj*a [probably *occidentalis*]) (Comstock & Dammers, 1932), suggesting that western red cedar (*Thuja plicata*) could also be used, as it is in *C. g. rosneri* (Johnson, 1976).

*Callophrys muiri* feeds on Sargent's cypress (*Cupressus sargentii*) and MacNab cypress (*Cupressus macnabiana*) in coastal mountains of California and *C. macnabiana* in the Sierra Nevada, and is sometime found in close association with *Callophrys g. nelsoni* (Nice & Shapiro, 2001; Forister, 2004). *Callophrys muiri* and *C. g. nelsoni* also show seasonal differences in flight times, suggesting they may be genetically isolated (Gervais & Shapiro 1999; Nice & Shapiro, 2001). In the laboratory, however, the two taxa show no consistent genitalic differences, and can interbreed and produce fertile offspring (Nice & Shapiro, 2001; Forister, 2005). The possible role of natural hybridization and mitochondrial introgression in shaping genetic divergence and evolution among populations of the *C. gryneus* complex and *C. muiri* (e.g. Shapoval *et al*., 2021) will require further study. Also, information is lacking on the

possible role of bacterial infection by the endosymbiont *Wolbachia*, especially prevalent in some groups of Lepidoptera (Silva-Brandão *et al*., 2021, and references therein) on evolutionary relationships among the members of the complex needs study.

Oviposition preference and larval host performance suggest an early stage of host-associated reproductive isolation may be occurring between *Callophrys* collected on the two species of cypress and those from incense cedar (Forister, 2004), which are assumed to represent *C. muiri* and *C. g. nelsoni*, respectively (Forister, 2005). Variation in larval performance, however, occurred among hosts (Forister, 2004), questioning the overall contribution of host preference in incipient speciation. For example, survival of larvae from wild-caught females collected on incense cedar in northern California was similar whether fed on cedar, juniper, or the two species of cypress. Survival of larvae from females collected on coastal cypress, however, was low when fed on cedar, as would be predicted, but high when fed on both species of cypress (also as predicted), but interestingly was the highest when fed on juniper. *Callophrys muiri* is also known to be associated with *Juniperus californica* farther south in San Luis Obispo County (Nice & Shapiro, 2001), which complicates an understanding on a possible relation between larval host and reproductive isolation in the two taxa.

In the original description of *C. muiri* (as *Thecla muiri*), Edwards (1881) stated that this species was similar to *C. g. nelsoni* but differed ventrally in having the postmedian white band on the forewings placed closer to the wing margin and that hindwings were darker than in *C. g. nelsoni* (see Figs. 1I and 1J). The postmedian white band on ventral hindwings is usually better developed than in *C. g. nelsoni*, but this character is variable and intermediate phenotypes are found (Nice & Shapiro, 2001). The very low COII genetic divergence between *C. g. nelsoni* (haplotype A) and *C. muiri* from coastal mountains in California (haplotypes C and D), $d$ = 0.002–0.004 (Nice & Shapiro, 2001), the lack of divergence in Sierra populations of the two taxa (as also suggested by the limited COI data), and the relatively minor phenotypic differences between the two taxa suggest that *C. muiri* does not warrant full species status and should be placed as a subspecies of either *C. nelsoni*—if reinstated to a full species—or *C. gryneus*.

## CONCLUSIONS

Overall, mitochondrial DNA barcodes offered little new insight into the subspecies controversy in the *Callophrys gryneus* complex. Barcode analysis provided support for the subspecies status of *C. g. sweadneri*, but left open the question of whether three additional subspecies, *C. g. castalis*, *C. g. siva*, and *C. g. nelsoni* should be elevated to full species. Also, similarities in phenotype and barcodes among western populations question whether *C. muiri* should be considered a distinct species. Additional studies using phylogenomic, morphological and life history data from a larger data set will be required to confidently resolve these taxonomic uncertainties in this confusing group of hairstreaks. Finally, additional phylogenomic studies also offer promise for understanding the adaptive radiation of the *C. gryneus* complex in North America. The scant information currently available for the subgenus *Mitoura* places *C. dospassosi* from Mexico basal to the sister species *C. gryneus* and *C. hesseli* (Zhang *et al*., 2021). The similarity of ventral wing surfaces of *C. dospassosi* and *C. g. siva* (Clench, 1981), together with the results of Zhang *et al*. (2021), suggests a Mexico origin for the ancestor of the *C. gryneus* complex, an hypothesis that needs to be tested with a more complete genomic dataset.

## ACKNOWLEDGEMENTS

I thank Richard C. Brusca for providing suggestions and corrections on an earlier draft of this manuscript. The assistance of Wendy Moore and Trini Hernández Mendoza during the preparation of this paper is greatly appreciated. I also wish to thank the many citizen scientists who have deposited photographs of *C. gryneus* and corresponding locality data on the iNaturalist website.

## DATA AVAILABILITY

Identification numbers for BOLD COI sequences are given on Figure 3B. The COII sequences were from Nice and Shapiro (2001) and are deposited in GenBank (Accession numbers: AF180502–AF180516, AF307330).

# REFERENCES

**Boisduval JB. 1869**. Lépidoptères de la Californie. *Annales de la Société Entomologique de Belgique* **12**: 5–94.

**Braby MF, Eastwood R, Murray N. 2012**. The subspecies concept in butterflies: has its application in taxonomy and conservation biology outlived its usefulness? *Biological Journal of the Linnean Society* **106**: 699–716.

**Brown JW. 1983**. A new species of *Mitoura* Scudder from southern California (Lepidoptera: Lycaenidae). *The Journal of Research on the Lepidoptera* **21**: 245–254. ("1982").

**BugGuide. 2023**. Available at: https://bugguide.net/node/view/866764

**Cary SJ, Toliver ME. 2022**. *Butterflies of New Mexico: The Gossamerwings II: The Hairstreaks (Lycaenidae: Theclinae)*. Available at: https://peecnature.org/butterflies-of-new-mexico/hairstreaks-lycaenidae-theclinae/

**Chapuis M-P, Bazelet CS, Blondin L, Foucart A, Vitalis R, Samways MJ. 2016**. Subspecific taxonomy of the desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae), based on molecular and morphological characters. *Systematic Entomology* **41**: 516–530.

**Chen F, Luo Y, Keena MA, Wu Y, Wu P, Shi J. 2016**. DNA barcoding of gypsy moths from China (Lepidoptera: Erebidae) reveals new haplotypes and divergence patterns within gypsy moth subspecies. *Journal of Economic Entomology* **109**: 366–374.

**Clement M, Posada D, Crandall KA. 2000**. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**: 1657–1659.

**Clench HK. 1981**. New *Callophrys* (Lycaenidae) from North and Middle America. *Bulletin of the Allyn Museum* **6**: 1–31.

**Comstock JA. 1925**. Studies in Pacific Coast Lepidoptera. *Bulletin of the Southern California Academy of Sciences* **24**: 36–38.

**Comstock JA, Dammers CM. 1932**. The metamorphoses of six California Lepidoptera. *Bulletin of the Southern California Academy of Sciences* **31**: 88–100.

**Cong Q, Shen J, Borek D, Robbins RK, Opler PA, Otwinowski Z, Grishin NV. 2017**. When COI barcodes deceive: complete genomes reveal introgression in hairstreaks.

*Proceedings of the Royal Society of London B* **284**: 20161735. Available at https://doi.org/10.1098/rspb.2016.1735

**Cong Q, Zhang J, Shen J, Cao X, Brévignon C, Grishin NV. 2020**. Speciation in North American *Junonia* from a genomic perspective. *Systematic Entomology* **45**: 803– 837.

**Davenport K. 2018**. *Butterflies of southern California in 2018: updating Emmel and Emmel's 1973 Butterflies of southern California.* Lepidoptera of North America 15. Fort Collins, CO: Contributions of the C.P. Gillette Museum of Arthropod Diversity, Colorado State University. Available at: https://docslib.org/doc/6483469/updating-emmel-and-emmels-1973-butterflies-of-southern-california

**deWaard JR, Mitchell A, Keena MA, Gopurenko D, Boykin LM, Armstrong KF, Pogue MG, Lima J, Floyd R, Hanner RH, Humble LM. 2010**. Towards a global barcode library for *Lymantria* (Lepidoptera: Lymantriinae) tussock moths of biosecurity concern. *Public Library of Science One* **5**; e14280. Available at: https://doi.10.1371/journal.pone.0014280

**Edwards WH. 1871**. Descriptions of new species of diurnal Lepidoptera found within the United States. *Transactions of the American Entomological Society* **3**: 205–216.

**Edwards WH. 1874**. Descriptions of new species of diurnal Lepidoptera found within the United States. *Transactions of the American Entomological Society* **5**: 103–111.

**Edwards H. 1881**. On some apparently new forms of diurnal Lepidoptera. *Papilio* **1**: 50–55.

**Excoffier L, Lischer HEL. 2010.** Arlequin suite ver. 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources* **10**: 564–567.

**Forister ML. 2004**. Oviposition preference and larval performance within a diverging lineage of lycaenid butterflies. *Ecological Entomology* **29**: 264–272.

**Forister ML. 2005**. Independent inheritance of preference and performance in hybrids between host races of *Mitoura* butterflies (Lepidoptera: Lycaenidae). *Evolution* **59**: 1149–1155.

**Gatrelle RR. 2001**. An examination of the *Mitoura* (Lycaenidae) in the southeastern United States: with the description of a new subspecies of *Mitoura hesseli*. *The Taxonomic Report of the International Lepidoptera Survey* **3**: 1–10.

**Gervais BR, Shapiro AM. 1999**. Distribution of edaphic-endemic butterflies in the Sierra Nevada of California. *Global Ecology and Biogeography* **8**: 151–162.

**Guppy CS, Shepard JH. 2001**. *Butterflies of British Columbia. Including western Alberta, southern Yukon, the Alaska panhandle, Washington, northern Oregon, northern Idaho, northwestern Montana*. Vancouver: University of British Columbia Press.

**Haig SM, Beever EA, Chambers SM, Draheim HM, Dugger BD, Dunham S, Elliott-Smith E, Fontaine JB, Kesler DC, Knaus BJ, Lopes IF, Loschl P, Mullins TD, Sheffield LM. 2006**. Taxonomic considerations in listing subspecies under the U.S. Endangered Species Act. *Conservation Biology* **20**: 1584–1594.

**Hebert PDN, Cywinska A, Ball SL, deWaard JR. 2003**. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B* **270**: 313–321.

**Hill RI, Ganeshan M, Wourms L, Kronforst MR, Mullen SP, Savage WK. 2018**. Effectiveness of DNA barcoding in *Speyeria* butterflies at small geographic scales. *Diversity* **10**: 130. Available at: https://doi:10.3390/d10040130

**Huelsenbeck JP, Ronquist F. 2001**. MrBayes: Bayesian inference of phylogeny. *Bioinformatics* **17**: 754–755.

**iNaturalist. 2023**. Available at: https://www.inaturalist.org/

**Johnson K. 1976**. Three new Nearctic species of *Callophrys* (*Mitoura*), with a diagnostis [sic] of all Nearctic consubgeners (Lepidoptera: Lycaenidae). *Bulletin of the Allyn Museum,* **38**: 1–30.

**Johnson K. 1978**. Specificity, geographic distributions, and foodplant diversity in four *Callophrys* (*Mitoura*) (Lycaenidae). *Journal of the Lepidopterists' Society* **32**: 3–19.

**Kodandaramaiah U, Weingartner E, Janz N, Leski M, Slove J, Warren A, Nylin S. 2012**. Investigating concordance among genetic data, subspecies circumscriptions and hostplant use in the nymphalid butterfly *Polygonia faunus*. *Public Library of Science One* **7**: e41058. Available at: https://doi.org/10.1371/journal.pone.0041058

**Lalonde MML, Marcus JM. 2019**. Getting western: biogeographical analysis of morphological variation, mitochondrial haplotypes and nuclear markers reveals cryptic species and hybrid zones in the *Junonia* butterflies of the American southwest and Mexico. *Systematic Entomology* **44**: 465–489.

**Li W, Cong Q, Shen J, Zhang J, Hallwachs W, Janzen DH, Grishin NV. 2019**. Genomes of skipper butterflies reveal extensive convergence of wing patterns. *Proceedings of the National Academy of Sciences, USA* **116**: 6232–6237.

**Librado P, Rozas J. 2009**. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25:** 1451–1452.

**Nazari V, ten Hagen W. 2020**. Molecular taxonomy of *Tomares* hairstreaks (Lepidoptera, Lycaenidae, Theclinae). *Deutsche Entomologische Zeitschrift* **67**: 19–33. Available at: https://doi.10.3897/dez.67.50252

**Nice CC, Shapiro AM. 2001**. Population genetic evidence of restricted gene flow between host races in the butterfly genus *Mitoura* (Lepidoptera: Lycaenidae). *Annals of the Entomological Society of America*, **94**: 257–267.

**Pelham JP. 2023**. *A catalogue of the butterflies of the United States and Canada*. Available at: http://butterfliesofamerica.com/US-Can-Cat.htm

**Pfeiler E, Johnson S, Markow TA. 2012**. DNA barcodes and insights into the relationships and systematics of buckeye butterflies (Nymphalidae: Nymphalinae: *Junonia*) from the Americas. *Journal of the Lepidopterists' Society* **66:** 185–198.

**Pfeiler E, Barba-Acuña I, Hernández-Cervantes PL, Markow TA. 2021a**. Butterfly biodiversity on Isla Socorro, Mexico, with comments on the taxonomic status of the endemic hairstreak *Strymon istapa socorroica* (Lycaenidae). *Journal of the Lepidopterists' Society* **75**: 165–173.

**Pfeiler E, Barba-Acuña I, Luna-Mendoza L, Oberbauer TA, Gallo-Reynoso JP. 2021b**. Preliminary assessment of Lepidoptera biodiversity on Isla Guadalupe, Mexico, a critically threatened ecosystem currently recovering from extensive habitat degradation. *Journal of the Lepidopterists' Society* **75**, 135–148.

**Pfeiler E, Nazario-Yepiz NO, Hernández-Cervantes PL, Markow TA. 2020**. Mitochondrial DNA barcodes provide insight into the phylogeography and subspecies controversy in the widespread Neotropical white peacock butterfly, *Anartia jatrophae* (Nymphalidae: Nymphalinae). *Biological Journal of the Linnean Society* **130**: 700–714.

**Pfeiler E, Nazario-Yepiz NO, Hernández-Cervantes PL, Markow TA. 2022**. Phenotypic diversity, population genetics and interspecific relationships of the Neotropical banded

peacock butterfly *Anartia fatima* (Nymphalidae: Nymphalinae). *Biological Journal of the Linnean Society* **136**: 135–144.

**Posada D, Crandall KA. 1998**. Modeltest: Testing the model of DNA substitution. *Bioinformatics* **14**: 817–818.

**Pratt GF, Ballmer GR, Wright DM. 2011**. Allozyme-based phylogeny of North American *Callophrys* (s. l.) (Lycaenidae). *Journal of the Lepidopterists' Society* **65**: 205–222.

**Proshek B, Dupuis JR, Engberg A, Davenport K, Opler PA, Powell JA, Sperling FA. 2015**. Genetic evaluation of the evolutionary distinctness of a federally endangered butterfly, Lange's Metalmark. *BMC Evolutionary Biology* **15**: 73. Available at: https://doi.10.1186/s12862-015-0354-9

**Ratnasingham S, Hebert PDN. 2007.** BOLD: The Barcode of Life Data System (www.barcodinglife.org). *Molecular Ecology Notes* **7**: 355–364.

**Robbins RK. 1990**. The *Mitoura spinetorum* complex in New Mexico and the validity of *M. millerorum* (Lycaenidae: Theclinae). *Journal of the Lepidopterists' Society* **44**: 63–76.

**Shapoval NA, Yakovlev RV, Kuftina GN, Lukhtanov VA, Knyazev SA, Romanovich AE, Krupitsky AV. 2021**. Identification of natural hybrids between *Ahlbergia frivaldszkyi* (Lederer, 1853) and *Callophrys rubi* (Linnaeus, 1758) (Lepidoptera, Lycaenidae) using mitochondrial and nuclear markers. *Insects* **12**: 1124. Available at: https://doi.org/10.3390/ insects12121124

**Silva-Brandão KL, Cirino M, de Moraes Magaldi L, Eyng Gueratto P, Mattos RG, Freitas AVL. 2021**. Subspecies limits and hidden *Wolbachia* diversity in *Actinote pellenea* butterflies. *Systematics and Biodiversity* **19**: 1012–1025.

**Talavera G, Kaliszewska ZA, Heath A, Pierce NE. 2020**. Recent diversification of *Chrysoritis* butterflies in the South African Cape (Lepidoptera: Lycaenidae). *Molecular Phylogenetics and Evolution* **148**: 106817. Available at: https://doi.org/10.1016/j.ympev.2020.106817

**Talavera G, Lukhtanov V, Pierce NE, Vila R. 2022**. DNA barcodes combined with multilocus data of representative taxa can generate reliable higher-level phylogenies. *Systematic Biology* **71**: 382–395.

**Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011**. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and

maximum parsimony methods. *Molecular Biology and Evolution* **28**: 2731–2739.

**Tilden JW. 1951**. A new subspecies of *Mitoura siva* Edwards. *Bulletin of the Southern California Academy of Sciences* **50**: 96–98.

**Warren AD. 2005**. *Butterflies of Oregon. Their taxonomy, distribution, and biology. Lepidoptera of North America 6*. Fort Collins, CO: C.P. Gillette Museum of Arthropod Diversity, Colorado State University.

**Wilson JJ, Sing KW, Sofian-Azirum M. 2013**. Building a DNA barcode reference library for the true butterflies (Lepidoptera) of Peninsula Malaysia: What about the subspecies? *Public Library of Science One* **8**: e79969. Available at: https://doi.org/10.1371/journal.pone.0079969

**Zhang J, Cong Q, Shen J, Opler PA, Grishin NV. 2021**. Genomics-guided refinement of butterfly taxonomy. *The Taxonomic Report of the International Lepidoptera Survey* **9**: 1–54.

LEGENDS TO FIGURES

**Figure 1**. Photos of live individuals of *Callophrys hesseli*, putative subspecies of *C. gryneus*, and *C. muiri* from North America downloaded from the iNaturalist (2023) website (except photo 1G, a pinned BOLD voucher TAMIC846–10) showing variability in ventral wing colour and maculation. Permissions to use these copyrighted photos provided by Creative Commons licensing CC BY 4.0 or CC BY-NC 4.0, unless otherwise noted. (**A**) *C. hesseli* Norfolk County. MA, (c) Nick Block; (**B**) C. *g. gryneus* Ontario, Canada, (c) Samuel Brinker (the two postbasal marks and postmedian line on ventral hindwing are labelled); (**C**) *C. g. sweadneri* Dixie County, FL, (c) Bart Jones; (**D**) *C. g. castalis* Coryell County, TX, (c) Liam Wolff; (**E**) *C. g. siva* Boulder, CO, (c) Steven Mlodinow; (**F**) *C. g. chalcosiva* Tooele County, UT, (c) Andrey Zharkikh; (**G**) *C. g. juniperaria* Los Angeles County, CA, (c) CBG Photography Group, Centre for Biodiversity Genomics (CC BY-NC-SA 3.0); (**H**) *C. g. mansfieldi* San Luis Obispo County, CA, (c) gilbertj; (**I**) *C. g. nelsoni* San Diego County, CA, (c) B. J. Stacey; (**J**) *C. muiri* Sonoma County, CA, (c) icosahedron; (**K**) *C. g. rosneri* British Columbia, Canada, (c) Paul Prappas; (**L**) *C. g. plicataria* Galiano Island, British Columbia, Canada, (c) Andrew Simon.

**Figure 2**. (A) TCS haplotype network of the 658 bp COI barcode segment sourced from BOLD ($n$ = 69) in populations of *Callophrys gryneus* (including two sequences labelled *C. muiri*) from the USA and southern Canada showing relationships among 19 haplotypes and the five main haplotype groups (I–V). Numbers within circles represent the number of individuals with that haplotype. Each line segment between haplotypes represents a single mutation. Inferred intermediate haplotypes that were not sampled are shown as black dots. Size of the circles is scaled to haplotype frequency. Geographical locations of samples are given in the coloured key. (B) Haplotype network of the 460 bp segment of the mitochondrial COII gene in *C. gryneus* subspecies mainly from northern California constructed from sequence data given in Nice and Shapiro (2001) using their haplotype identifications (haplotype G was obtained from Wisconsin). The key to subspecies is given at right. Roman numerals indicate corresponding haplotype groups from the present study.

**Figure 3.** (A) Neighbour-joining (NJ) tree of the COI data set in populations of *Callophrys gryneus* and *C. muiri* showing clustering of the five main haplotype groups (groups I–V). The three outgroup taxa are not shown. Numbers on the branches are bootstrap support values obtained using 1000 pseudoreplicates (values <80% not shown). The scale bar indicates sequence divergence. (B) Bayesian 50% majority rule consensus phylogenetic tree of COI barcode sequences of *C. gryneus*. Taxa are labelled with BOLD identification codes, followed by specific or subspecific epithets and geographic region as they appear in the BOLD record. For the name *C. gryneus*, only the three sequences from Tennessee (TN) in group I were assigned specifically to the nominate subspecies in BOLD; *C. gryneus* was used at the species level, without subspecies assignments, for the remainder of the entries. The name *C. barryi* is now considered a junior synonym of *C. g. plicataria* (Warren, 2005). Outgroups are *C. hesseli* (subgenus *Mitoura*), and *C. johnsoni* and *C. spinetorum* (subgenus *Cisincisalia*). Standard abbreviations are used for USA states; Canadian province abbreviations are B.C. (British Columbia) and Ont. (Ontario). Statistical support for clades (posterior probabilities) is shown at the nodes. The scale represents expected substitutions per site.

**Figure 4**. Map showing sampling locations for *Callophrys gryneus* in USA and southern Canada, as defined by this work. Roman numerals I to V indicate the five main COI haplotype groups from Fig. 2A. Coloured shading represents approximate boundaries of haplotype groups based on diagnostic nucleotide sites (see Table 3) and inferred from photographs available in iNaturalist (2023); not shown is the distribution of haplotype group V subspecies *C. g. nelsoni* in the higher mountain ranges of coastal southern California. Coloured dots represent numbers of barcodes analysed from each locality and are scaled to reflect sample size.

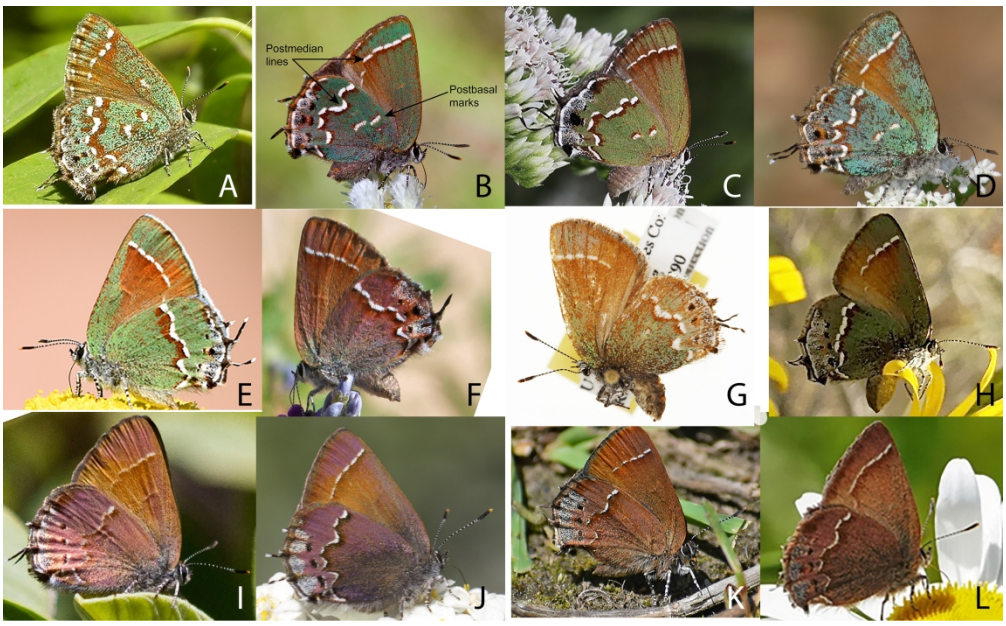


Figure 1

254x156mm (300 x 300 DPI)

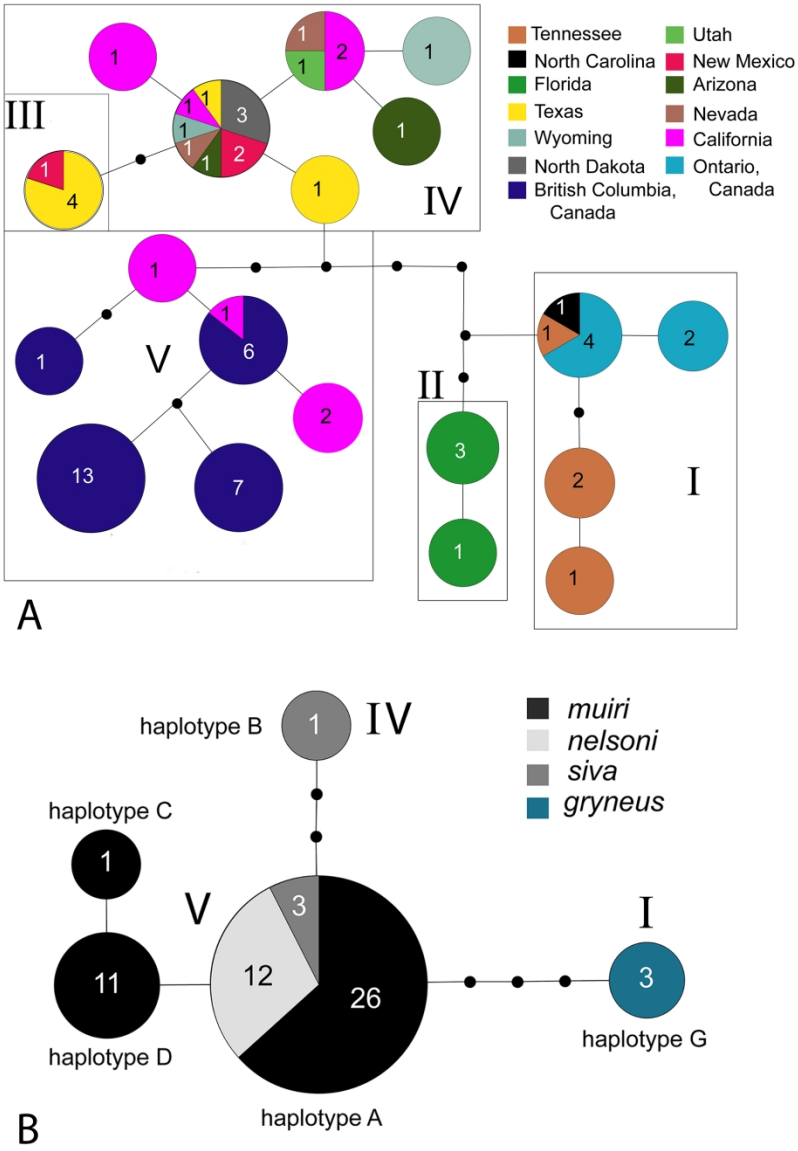


Figure 2

162x230mm (300 x 300 DPI)

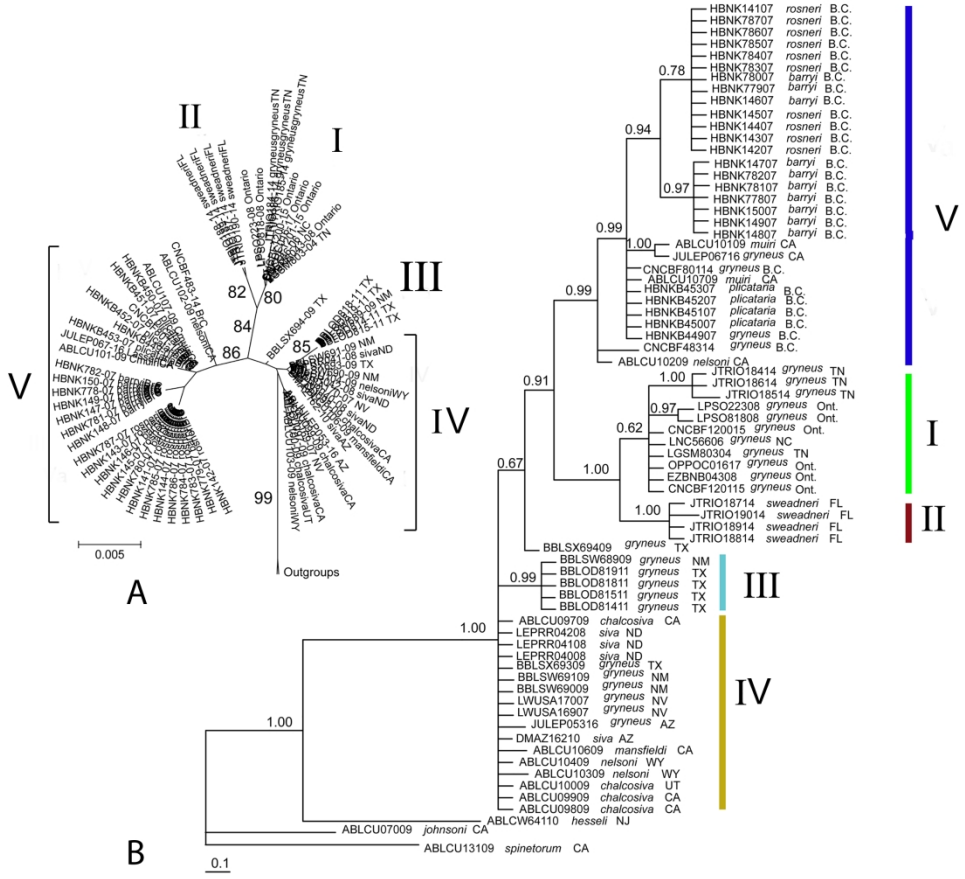


Figure 3

237x229mm (300 x 300 DPI)

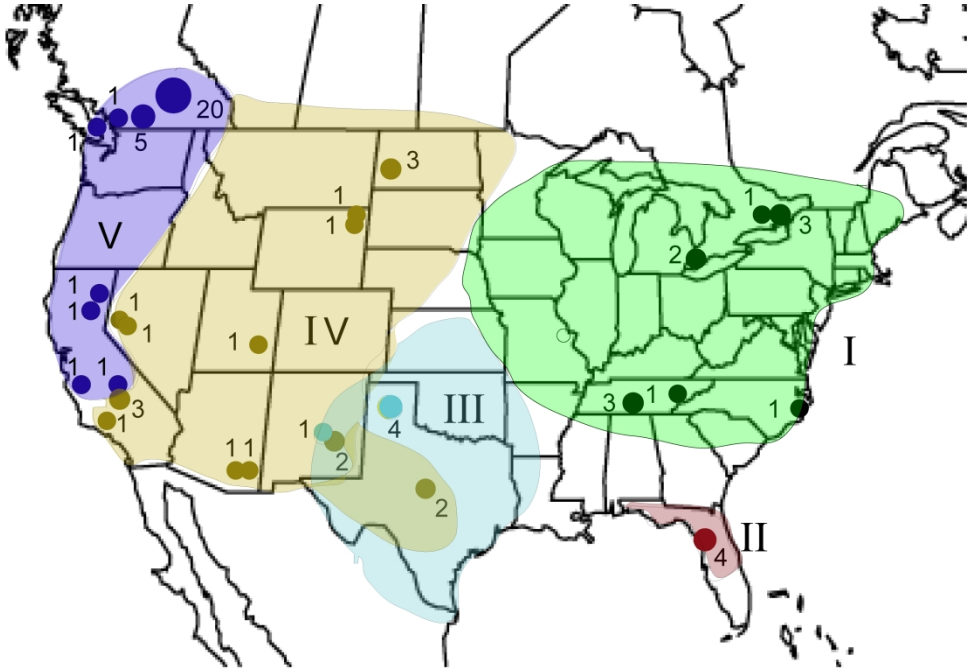


Figure 4

297x205mm (300 x 300 DPI)

**Table 1.** Species of *Callophrys* (subgenus *Mitoura*) and COI haplotype group assignments in *C. muiri* and putative subspecies of *C. gryneus*.

| Species/subspecies | Haplotype Group |
|---|---|
| *C. gryneus gryneus* (Hübner, [1819]) | I |
| *C. g. smilacis* (Boisduval & Le Conte, [1835]) | ? |
| *C. g. sweadneri* (F. Chermock, 1945) | II |
| *C. g. castalis* (W.H. Edwards, 1871) | III |
| *C. g. siva* (W.H. Edwards, 1874) | IV |
| *C. g. chalcosiva* Clench, 1981 | IV |
| *C. g. juniperaria* (J.A. Comstock, 1925) | IV |
| *C. g. mansfieldi* (Tilden, 1951) | IV |
| *C. g. nelsoni* (Boisduval, 1869) | V |
| *C. g. plicataria* K. Johnson, 1976 | V |
| *C. g. rosneri* K. Johnson, 1976 | V |
| *C. muiri* (Hy. Edwards, 1881) | V |
| *C. hesseli* (Rawson & Ziegler, 1950) | |
| *C. loki* (Skinner, 1907) | |
| *C. dospassosi* Clench, 1981 | |

**Table 2**. Mean (below the diagonal) and minimum (above the diagonal) pairwise *p*–distances (*d*) among barcode sequences in haplotype groups of *Callophrys gryneus* (including *C. muiri*) and the putative sister species *C. hesseli*. Within group distances are highlighted along the diagonal.

| Haplotype group[a] | I ($n$ = 11) | II ($n$ = 4) | III ($n$ = 5) | IV ($n$ = 18) | V ($n$ = 31) | *C. hesseli* ($n$ = 3) |
|---|---|---|---|---|---|---|
| I | 0.002 | 0.005 | 0.011 | 0.008 | 0.009 | 0.040 |
| II | 0.006 | 0.001 | 0.014 | 0.009 | 0.009 | 0.040 |
| III | 0.013 | 0.014 | 0.000 | 0.003 | 0.009 | 0.035 |
| IV | 0.011 | 0.012 | 0.004 | 0.001 | 0.005 | 0.032 |
| V | 0.014 | 0.014 | 0.013 | 0.010 | 0.003 | 0.036 |
| *C. hesseli* | 0.041 | 0.040 | 0.035 | 0.034 | 0.040 | 0.000 |

[a] Group assignments: **group I**, *C. g. gryneus*; **group II**, *C. g. sweadneri*; **group III**, *C. g. castalis*; **group IV**, *C. g. siva*, *C. g. chalcosiva*,, *C. g. juniperaria*, *C. g. mansfieldi*; **group V**, *C. g. nelsoni*, *C .g. rosneri*, *C. g. plicataria* (and *C. muiri*).

**Table 3**. Apparent fixed nucleotide differences in COI barcodes among haplotype groups of *Callophrys gryneus*.

| | Nucleotide Site | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Haplotype Group | 5 | 81 | 85 | 274 | 340 | 520 | 550 | 589 | 595 | 658 |
| I (*n* = 11) | C | C | T | C | C | A | A | C | C | C |
| II (*n* = 4) | C | C | T | T | C | A | G | C | C | T |
| III (*n* =5) | T | T | T | T | T | T | A | T | T | C |
| IV (*n* = 18 ) | T | C | T | T | T* | T | A | T | T | C |
| V (*n* = 31) | T | C | C | T | C | T | A | T | C | C |

* One group IV specimen from Texas possessed a "C" at site 340.

A second codon position substitution (site 81) results in an amino acid change (threonine to isoleucine) in samples from group III. All other nucleotide changes (one first position change at site 5, and all others at the third position) are synonymous substitutions.

**Table 4**. Pairwise comparisons of $\Phi_{ST}$ among haplotype groups of *Callophrys gryneus* based on COI sequences (below the diagonal), with corresponding *P*-values (above the diagonal).

| Haplotype group | I (11) | II (4) | III (5) | IV (18) | V (31) |
|---|---|---|---|---|---|
| I | ----- | 0.009 | <0.001 | <0.001 | <0.001 |
| II | **0.721*** | ----- | 0.009 | <0.001 | <0.001 |
| III | **0.886*** | **0.977*** | ----- | <0.001 | <0.001 |
| IV | **0.849*** | **0.891*** | **0.733*** | ----- | <0.001 |
| V | **0.823*** | **0.830*** | **0.829*** | **0.793*** | ----- |

The number of individuals analysed from each haplotype group is shown in parentheses.

*All pairwise comparisons of $\Phi_{ST}$ were significant (bold type) at the 0.05 level.