Patterns of Genetic Variation Between the Checkered Skippers *Pyrgus communis* and *Pyrgus albescens* (Lepidoptera: Hesperiidae)

JAMES A. FORDYCE,[1] MATTHEW L. FORISTER,[2] CHRIS C. NICE,[3] JOHN M. BURNS,[4] AND ARTHUR M. SHAPIRO[5]

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ABSTRACT We examined patterns of genetic variation between the transcontinental species *Pyrgus communis* (Grote) and *Pyrgus albescens* Plötz (Hesperiidae) to examine whether patterns of molecular variation are congruent with the taxonomy. Sequence data from mitochondrial DNA and nuclear DNA failed to distinguish the two taxa. Although substantial genetic variation is explained by the two nominal taxa, more variation is explained by geography. Specifically, our molecular data indicate that the Transverse Ranges of southern California and the Sierra Nevada are important features affecting patterns of genetic variation. The possibility of recent divergence and secondary contact is discussed. Although *P. communis* and *P. albescens* do not adhere to a phylogenetic species concept, diagnostic morphological and distributional differences exist between the two entities that merit consideration regarding their taxonomic status.

KEY WORDS genitalia, introgression, phylogeography, sibling species, Sierra Nevada

The taxonomic status of the checkered skippers *Pyrgus communis* (Grote) and *P. albescens* Plötz has been debated for a century. The designated status of *P. albescens* has ranged from a distinct species to a mere geographical form of *P. communis* (for a detailed history, see Burns 2000). The two entities can be diagnosed by the morphology of the valves (or claspers) of the male genitalia. The valves of *P. communis* are higher from dorsal to ventral margin in lateral view, more massive, and marked by a prominent posterior process that curves anterodorsad and typically ends in a pair of prongs, whereas the valves of *P. albescens*, in lateral view, are relatively low throughout (Fig. 1a) (Burns 2000: figs. 9–12, 19–20, and 23–375). Intermediate valval shapes have been reported in regions of contact between the two entities (Powell 1958, Tilden 1965, Austin 1986), but those reports were refuted by Burns (2000). Based on wing length, *P. communis* averages slightly larger than *P. albescens* [analysis of data reported in Burns (2000): $t = 8.01$, df = 115, $P < 0.001$].

P. communis ranges into southern Canada (southeastern British Columbia, southern Manitoba, Saskatchewan and Alberta, and southernmost Ontario), through most of the continental United States, and south to southern Mexico; *P. albescens* ranges across the southern United States to southern Mexico (Fig. 1b) (Layberry et al. 1998: 45; Burns 2000: figs. 21–22). Although *P. communis* and *P. albescens* are broadly sympatric in the American Southwest and montane Mexico, they narrowly replace each other elsewhere (Burns 2000: figs. 21–22).

We examine genetic variation between these two species as an initial assessment of geographic and taxonomic structure, focusing particularly on Californian portions of its range. The high endemism of other taxa in this region, coupled with the fact that the Sierra Nevada and the Transverse Ranges have been shown to be important barriers for gene flow in other systems, make this region especially interesting (Calsbeek et al. 2003, Swenson and Howard 2005, Chatzimanolis and Caterino 2007). In California, *P. albescens* occurs in the southern Coast Ranges, southern San Joaquin Valley, southern Sierra Nevada, and in the Transverse Ranges and points south and east, including desert areas, whereas *P. communis*, despite limited overlap locally, occurs primarily to the north (Fig. 1b) (Burns 2000: fig. 21). The mountains of this region have been shown to be congruent with genetic discontinuities of other butterfly species complexes (Forister et al. 2004, Nice et al. 2005, Oliver and Shapiro 2007). We examine patterns of genetic variation to assess congruence with species-level taxonomy based on morphology and geographic distribution, and to examine the effect of geography and topography on genetic variation.

[1] Corresponding author: Department of Ecology and Evolutionary Biology, University of Tennessee, Knoxville, TN 37996 (e-mail: jfordyce@utk.edu).

[2] Department of Natural Resources and Environmental Science, University of Nevada, Reno, NV 89512.

[3] Department of Biology, Population and Conservation Biology Program, Texas State University, San Marcos, TX 78666.

[4] Department of Entomology, National Museum of Natural History, Smithsonian Institution, P.O. Box 37012, MRC 127, Washington, DC 20013-7012.

[5] Section of Evolution and Ecology, Center for Population Biology, University of California, Davis, CA 95616.

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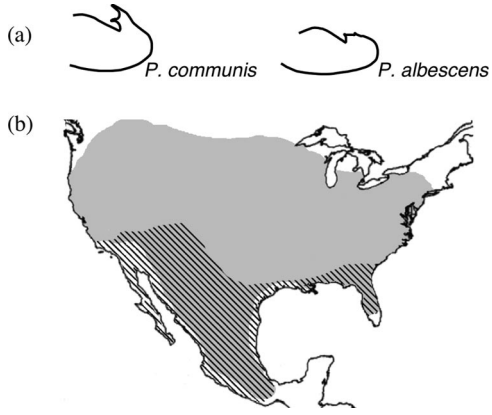

Fig. 1. (a) Male genitalia (valves) of *P. communis* and *P. albescens* in left lateral view (redrawn from Burns 2000). (b) Distribution of *P. communis* (shaded) and *P. albescens* (hatched) in the United States and Mexico (note areas of overlap in Mexico and southern United States).

Materials and Methods

Sampling, DNA Extraction, and Sequencing. Sampling consisted of 78 *P. communis* specimens and 29 *P. albescens* specimens collected from 28 locations (Table 1), and the congeners *Pyrgus scriptura* (Boisduval) ($n = 2$) and *Pyrgus philetas* W. H. Edwards ($n = 3$). We also included previously published sequence data of a *P. communis* collected in Solano Co., CA (GenBank accessions AF170857 [COII] and AF173396 [*wingless*]) (Caterino et al. 2001). Taxonomic designation was based upon geographic locality and morphology of male genitalia. Identification using male genitalic morphology was done by one of us (J.M.B.) without knowledge of collection locale. Specimens were stored at −20°C before extraction of DNA (except for a small number of pinned museum specimens).

Extractions of DNA were performed using either chloroform-phenol (Hillis et al. 1996) or Purgene DNA isolation kit (Gentra Systems, Minneapolis, MN). Precipitated DNA was dried and resuspended in 200 μl of H_2O and used in polymerase chain reactions (PCRs) with the Primers EVA (5′-GAG ACC ATT

Table 1. Localities and mtDNA haplotypes for *P. communis*/*P. albescens* specimens

Location	Collection date(s)	*n*	Morphology	SAMOVA region	mtDNA haplotypes[a]	*wingless* GenBank accessions
Cochise Co., AZ	2-XI-2002	1	*P. communis*	I	A(1)	
Del Norte Co., CA	13-VI-2002	1	*P. communis*	I	J(1)	
El Dorado Co., CA	8-VII-2002	4	*P. communis*	III	H(2), I(2)	EU327831, EU327843, EU327844, EU327850
Glenn Co., CA	19-VIII-2003	3	*P. communis*	I	A(3)	EU327836
Mono Co., CA	29-VIII-2001	1	*P. communis*	I	B(1)	EU327846
Nevada Co., CA	28-VI-1981, 24-VI-2002, 16-VII-2002	14	*P. communis*	III	A(3), C(1), D(1), H(9)	EU327832, EU327833, EU327849, EU327852, EU327855
Sacramento Co., CA	2-VII-2002	5	*P. communis*	I	A(5)	EU327830, EU327835, EU327838, EU327848, EU327854
Sierra Co., CA	10-VI-2002, 15-VII-2002, 27-X-2002	6	*P. communis*	I	A(6)	EU327834, EU327837, EU327842, EU327847, EU327851
Solano Co., CA	8-VII-1981	1	*P. communis*	I	B(1)	
Yolo Co., CA	6-VII-2001	3	*P. communis*	I	A(2), B(1)	EU327820, EU327845
Mesa Co., CO	6-V-2002	1	*P. communis*	I	A(1)	
Montgomery Co., MD	27-IX-2006	7	*P. communis*	I	A(2), M(5)	
Otero Co., NM	4-VII-2002	1	*P. communis*	I	A(1)	
Comanche Co., OK	24–27-IV-2002	7	*P. communis*	I	A(7)	EU327853
Harney Co., OR	16-V-2002	1	*P. communis*	I	A(1)	
Jackson Co., OR	8-IX-2002	1	*P. communis*	I	A(1)	
Tillamook Co., OR	22-IX-2002	4	*P. communis*	I	A(4)	EU327839, EU327840
Hays Co., TX	15-IV-2002	16	*P. communis*	I	A(16)	
Santa Cruz Co., AZ	6-XI-2002	4	*P. albescens*	I	H(4)	
Kern Co., CA	5-VII-2002	1	*P. albescens*	II	H(1)	EU327856, EU327868
Los Angeles Co., CA	17–26-VIII-2001	15	*P. albescens*	II	C(3), D(6), F(1), H(5)	EU327857, EU327858, EU327860, EU327861, EU327863–EU327867, EU327869–EU327872
Lake Co., FL	19-IV-2002	1	*P. albescens*	III	H(1)	EU327859
Richmond Co., GA	21-IV-2002	1	*P. albescens*	III	H(1)	EU327862
Mulege, Baja California Sur, Mexico	6-IV-2004	3	*P. albescens*	III	H(3)	
Saltillo, Coahuila, Mexico	9-X-2003	2	*P. albescens*	III	H(1), K(1)	
Saltillo, Coahuila, Mexico	9-X-2003	1	*P. communis*	III	A(1)	
San Carlos, Sonora, Mexico	29-I-2003	1	*P. albescens*	III	H(1)	
Alfredo V. Bonfil, Tamaulipas, Mexico	5-X-2003	1	*P. albescens*	III	H(1)	

[a] GenBank accessions of mtDNA haplotypes: A, EU327820; B, EU327823; C, EU327824; D, EU327828; F, EU327825; H, EU327822; I, EU327826; J, EU327821; K, EU327827; and M, EU327829.

ACT TGC TTT CAG TCA TCT-3′) and STROM (5′-TAA TTT GAA CTA TYT TAC CNG CA-3′), yielding a 450-bp product of the mitochondrial DNA (mtDNA) cytochrome oxidase subunit II (COII) region (Caterino and Sperling 1999) for all 107 *P. communis*/*P. albescens* individuals and the outgroups. Nuclear sequences (*wingless*, Brower and DeSalle 1998) were obtained for 43 individuals (17 *P. albescens* and 26 *P. communis*) by using the primers wg1 (GAR TGY AAR TGY CAY GGY ATG TCT GG) and wg2 (ACT ICG CRC ACC ART GGA ATG TRC A), yielding a 417-bp PCR product. Sequencing of additional individuals for *wingless* was not carried out once it became obvious that the information contained within this data set would not be useful for addressing the questions motivating this study (see Results). PCR conditions were as follows: 94°C for 2 min; 39 cycles of 94°C for 1 min, 42–55°C for 1 min, 72°C for 1 min 30 s; and finally, 72°C for 10 min. Annealing temperatures varied among reactions to optimize amplification. PCR products were sequenced in both directions at the DNA sequencing facility, Division of Biological Science, University of California, Davis, or at the Molecular Biology Resource Facility, Division of Biology, University of Tennessee, Knoxville. Sequences were aligned visually with the aid of the Sequencher, version 4.6 (Gene Codes Corporation, Ann Arbor, MI) computer program. Unique sequences of COII and *wingless* from *P. communis* and *P. albescens* are available from GenBank (Table 1). Voucher specimens will be placed in the Bohart Museum of Entomology (University of California, Davis, CA).

Genetic data were approached as both a phylogenetic problem (the initial motivation of the study) and a phylogeographic problem. If *P. albescens* and *P. communis* represent two species with a long history of reproductive isolation, the COII and *wingless* gene trees should possess a structure consistent with the hypothesis of ancient divergence, indicated by reciprocal monophyly of the two nominal taxa. Similarly, if the mountain ranges of the Southwest are important factors determining the distribution of unique lineages, as has been reported for other butterfly species groups (e.g., Forister et al. 2004, Nice et al. 2005), it is expected that gene trees will indicate lineages congruent with these barriers. Conflicting patterns between gene trees based on mitochondrial and nuclear data might indicate previous introgression between lineages, or current gene flow.

Phylogenetic hypotheses were generated using neighbor-joining, maximum parsimony, and maximum likelihood methods using PAUP* version 4.0b10 (Swofford 2002). Genes were not combined for analysis (see Slowinski and Page 1999, Degnan and Rosenberg 2006, Kubatko and Degnan 2007, Knowles and Carstens 2007 for a discussion of potential problems of concatenated analysis). Models of sequence evolution for maximum likelihood were evaluated with the program Modeltest 3.7 (Posada and Crandall 1998) by using Akaike information criterion (AIC). The trees were rooted using *Polites sabuleti* (Boisduval) (AY700701) and *Hesperia juba* (Scudder) (AY700702) (both Hesperiidae) as outgroups. We also included the congeners *Pyrgus scriptura* (collected in Solano Co., CA) and *P. philetas* (collected in Hays Co., TX) in the analysis (GenBank accessions EU327818, EU327819, respectively). Relationships among haplotypes also were examined by generating a statistical parsimony network using the program TCS (Clement et al. 2000). Unlike tree-based approaches, networks allow for extant ancestral haplotypes.

Analysis of molecular variation (AMOVA) (Excoffier et al. 1992) was performed to determine the proportion of the total genetic variation for COII and *wingless* that was distributed according to the nominal taxonomic designation using the computer program ARLEQUIN version 2.0 (Schneider et al. 2000). Additionally, the structure of COII variation between *P. communis* and *P. albescens* was explored using spatial analysis of molecular variation (SAMOVA) with the program SAMOVA 1.0 (Dupanloup et al. 2002). SAMOVA is based on a simulated annealing procedure that partitions genetic variation into geographically continuous groups of populations that maximize ϕ_{CT} (Excoffier et al. 1992). The number of groups (k) was determined by analyzing values of k between 2 and 10 and choosing the value that maximizes ϕ_{CT} or the value of ϕ_{CT} when it plateaus. We chose $k = 3$ because it is the number of groups that corresponded to where ϕ_{CT} values plateau.

Results

Alignment of 450 bp of COII sequences from *P. communis* and *P. albescens* revealed 10 unique haplotypes. Phylogenetic analyses failed to capture *P. communis* and *P. albescens* as separate, reciprocally monophyletic groups (Fig. 2a). In fact, haplotypes C, D, and H are shared between the two morphologically defined species (Table 1). The statistical parsimony network of the COII sequences illustrates little divergence (Fig. 2b); maximum sequence divergence was 0.895%.

The nuclear gene, *wingless*, exhibited an exceedingly high level of variation with no two sequences identical. Most of this variation was in the form of heterozygotes, inferred by peaks observed on the chromatogram of similar amplitude occurring at the same location in both sequencing directions. Only nine of the 44 genotypes did not include at least one heterozygous site (median number of heterozygous sites = 3). We entertained the possibility that such surprisingly high variation might be a consequence of editing errors, or errors occurring in the sequencing reaction. Sequences were edited twice independently, without using a reference sequence or knowledge of codon position, and both times we arrived at the same conclusion. In total, we detected 54 variable sites out of 404 bp, 43 of which were transitions. Despite the high level of variation detected at the nucleotide level, there were no amino acid substitutions. All but one of these synonymous substitutions occurred at the third position. This level of variation for *wingless* in the Lepidoptera is unprecedented, based on an examina-

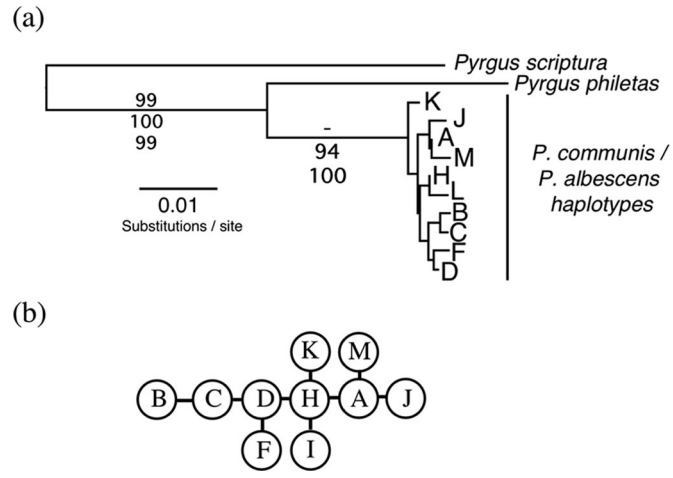

Fig. 2. (a) Neighbor-joining phylogram of *P. communis* and *P. albescens*. The outgroups used during the analysis (*Polites sabuleti* and *Hesperia juba*) are excluded from the illustration. Number above node is maximum likelihood bootstrap support (GTR+Γ) (100 pseudoreplicates), below is maximum parsimony and neighbor-joining (1,000 pseudoreplicates for each). Letters correspond to unique haplotypes detected in *P. communis* and *P. albescens*. (b) Statistical parsimony haplotype network for *P. communis*/*P. albescens*. Locales for haplotypes provided in Table 1.

tion of variation within species and between closely related species from sequences available on GenBank (also see Forister et al. 2004). If the observed variation were due to errors in editing and/or amplification, and we assume that this error should be random with respect to codon position, the probability that we would observe such high variation confined only to synonymous mutations is extremely low ($P < 1.0^{-24}$). The number of heterozygous sites within most sequences was too large, and samples within populations too low, to confidently infer fully phased genotypes. Phylogenetic analyses of *wingless* genotypes was not informative; these analyses did not distinguish between *P. communis* and *P. albescens* (data not shown).

The AMOVA results showed that partitioning the molecular variance between the two nominal taxa accounted for nearly 50% of the COII variation but <10% of the variation in *wingless* (Table 2). SAMOVA was used as an exploratory tool to examine geographic structure of mitochondrial variation. Three geographically continuous regions were defined (Fig. 3; Table 1). Region I consisted entirely of locales with the *P. communis* form (Fig. 3; Table 1). Regions II and III consisted primarily of the *P. albescens* form; yet, region III also included *P. communis* in the Sierra Nevada. Based upon these groupings, ≈ 63% of COII variation and ≈ 12% of *wingless* variation was explained.

Table 2. Analysis of molecular variance results using two grouping schemes

Data	Source	Variance	% total	*P*	Φ_{ST}
Grouping by morphologically defined species					
COII	Between	0.346	49.9	<0.001	0.499
	Within	0.348	50.1		
wingless	Between	0.026	1.6	0.210	0.016
	Within	1.640	98.4		
Grouping by three regions identified by SAMOVA					
COII	Among	0.441	63.6	<0.001	0.636
	Within	0.252	36.4		
wingless	Among	0.111	6.57	<0.001	0.065
	Within	1.578	93.43		

Discussion

Our phylogenetic analyses of mitochondrial and nuclear genetic data failed to provide evidence that *P. communis* and *P. albescens* are "good" phylogenetic species. It is unlikely that additional sequence information from the loci examined here would reveal important phylogenetic structure relevant to the current nominal designations, especially given that the two entities share mitochondrial haplotypes in the Sierra Nevada and southern California based upon our current data. However, additional sequence data from multiple loci might be informative in regard to past demographic history and patterns of gene flow between the entities if examined in a coalescent framework (Rosenberg and Nordborg 2002, Hein et al.

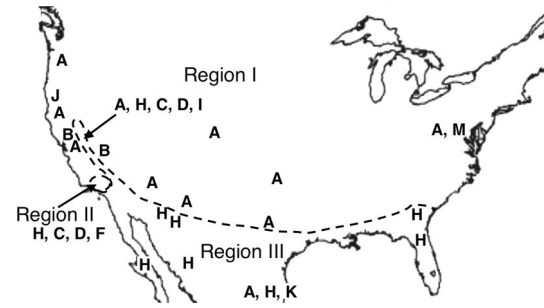

Fig. 3. Distribution of COII haplotypes. Dashed lines indicate approximate boundaries of geographic regions indicated by SAMOVA. See Table 1 for haplotype frequency information.

2005). Despite the lack of reciprocal monophyly in our analyses, *P. communis* and *P. albescens* are, nevertheless, distinct entities that can be distinguished by geographic distribution, male genitalic morphology, and average size.

There was a clear indication of geographic structuring in genetic variation that was largely congruent with taxonomic designation. Although a substantial amount of mitochondrial variation was explained by taxonomy, this is confounded with the role of geography because *P. communis* is largely a northern entity, with a range that extends into central Canada, and *P. albescens* southern (Burns 2000). Spatial analysis of molecular variance defined three distinct regions that, with the exception of the Sierra Nevada *P. communis,* which was grouped with *P. albescens* in region III, were exclusive for either taxon (region I for *P. communis* and regions II and III for *P. albescens*). Interestingly, the analysis separated *P. albescens* from southern California, south of the Transverse Ranges, from the remainder of *P. albescens.* This region contained a distinctly different composition of mitochondrial haplotypes, some of which occurred in the Sierra Nevada samples. In fact, nearly all of the haplotype diversity occurs in southern California and the Sierra Nevada, whereas throughout the remainder of our samples *P. communis* and *P. albescens* nearly always possess their respective haplotypes (haplotype A for *P. communis* and haplotype H for *P. albescens*). The matching of particular haplotypes with male genitalic morphology in the southeast is consistent with historical evidence for recent eastward range expansion by *P. albescens* (Burns 2000, Calhoun 2002). The Sierra Nevada *P. communis* were the only samples to contain haplotypes characteristic of both entities; although haplotypes characteristic of *P. albescens* occur at a higher frequency, these populations are clearly *P. communis* based on morphology.

There are at least two explanations for the incongruence between morphology and mitochondrial genetic variation in the Sierra Nevada. First, *P. communis* may simply be a polytypic species exhibiting two distinct, yet variable, male genitalic morphologies. This would be surprising given that, with the exception of the *P. communis* found in the Sierra Nevada (region III), particular mitochondrial haplotypes are consistently associated with particular male genitalic morphologies. Furthermore, variation observed in male genitalic morphology of this group is apparently bimodal, with no intermediate *P. communis*/*P. albescens* morphology (Burns 2000). A second explanation is that the occurrence of mostly *P. albescens* associated haplotypes in the Sierra Nevada is a consequence of introgression due to past, or ongoing, gene flow. This region is known to be an important area of contact between formerly isolated lineages (e.g., Gompert et al. 2006a), corresponding to Remington's Pacific-Rocky Mountain suture zone VI and described by Austin and Murphy (1987) as a "center of differentiation." Recently, Swenson and Howard (2005) identified this region as a "hybrid zone hot spot." The Sierra Nevada and other western mountain ranges have been shown to be important geographic features that limit gene flow in other butterfly species (Nice and Shapiro 2001, Forister et al. 2004, Nice et al. 2005, Oliver and Shapiro 2007). In the context of possible hybrid origin of the Sierra Nevada *P. communis*, it is important to note that they differ from adjacent *P. communis* and *P. albescens* by being univoltine, undoubtedly adaptive in the short season associated with high elevation. They are also unique compared with neighboring populations in feeding (apparently) exclusively on native malvaceous host plants (*Sidalcea*). Almost all multivoltine populations now feed opportunistically on naturalized weedy Malvaceae, especially the genus *Malva;* this is even true of the Sierraville, CA, population (1,500 m), which is only ≈12 km from the nearest univoltine (2,000 m) population (A.M.S., unpublished data). The relatively high diversity of mitochondrial haplotypes associated with our Sierra Nevada samples supports current, or recent, gene flow. Sequences of the nuclear gene *wingless* are no help in distinguishing between these scenarios, because they show no geographic pattern in the distribution of variation. This is presumably due to ancestral polymorphisms, suggesting a relatively recent origin of these species. The fact that our sample from Mendocino Pass (Glenn Co.) in the high north Coast Ranges of northwest California (≈2,000 m) is morphologically *P. communis* and possesses the common *P. communis* haplotype (haplotype A) is worth noting. This is a montane–meadow population using a native *Sidalcea* host plant, but it is bivoltine and geographically removed from the Sierra Nevada; faunistically, the high North Coast Range is more similar to the Klamath Mountains than to the Sierra Nevada (Shapiro 1992).

It also should be noted that our sampling is biased toward California, and increased sampling there increases the probability of detecting more genetic diversity. Acknowledging this, other observations are worth considering. Our largest single sample was from a morphologically *P. communis* population in south-central Texas ($n = 16$); this sample was fixed for a single mitochondrial haplotype (A), which is associated exclusively with the *P. communis* phenotype. Our single samples from Florida and Georgia contained only haplotype H, which is usually associated with *P. albescens;* and each of these samples was morphologically *P. albescens.* The single occurrence of haplotype A in Mexico was associated with the *P. communis* morphology (Table 1). Thus, the Sierra Nevada was the only region where haplotype could not predict morphology, or vice versa.

The primary motivation for this study was to assess the phylogenetic status of *P. communis* and *P. albescens.* It is clear from our data that the two entities are not now (or have recently not been) completely reproductively isolated species, at least in parts of California. However, the distribution of mitochondrial variation suggests the two entities have distinct histories and may have relatively recently come into secondary contact. The substantial variation and lack of structure associated with the nuclear sequence information, by comparison, is consistent with a recent

origin of the two entities. Mitochondrial variation that is incongruent with the morphological and ecological discontinuities used to describe lepidopteran taxa has been recorded in other systems, and is likely a consequence of recent divergence (ancestral polymorphism), introgression, or both (Gompert et al. 2006b; Forister et al. 2008). Further work, with more rigorous application of finer grain molecular markers, is required to understand current or recent patterns of gene flow and the mechanisms that maintain the observed discontinuities in morphology and geographic distribution. The pattern of variation observed for *P. communis* and *P. albescens* is an additional example of discordance associated with the Sierra Nevada and nearby mountain ranges.

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