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Lack of evidence for reproductive isolation among ecologically specialised lycaenid butterflies

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Abstract. 1. The evolution of reproductive isolation between recently diverged or incipient species is a critical component of speciation and a major focus of speciation models. In phytophagous insects, host plant fidelity (the habit of mating and ovipositing on a single host species) can contribute to assortative mating and reproductive isolation between populations adapting to alternative hosts. The potential role of host plant fidelity in the evolution of reproductive isolation was examined in a pair of North American blue butterfly species, *Lycaeides idas* and *L. melissa*.

2. These species are morphologically distinct and populations of each species utilise different host plants; however they share 410 bp haplotypes of the mitochondrial cytochrome oxidase subunit I (COI) gene, indicating recent divergence.

3. Some populations using native hosts exhibited strong fidelity for their natal host plant over the hosts used by nearby populations. Because these butterflies mate on or near the host plant, the development of strong host fidelity may create reproductive isolation among populations on different hosts and restrict gene flow.

4. Tests of population differentiation using allozyme allele frequency data did not provide convincing evidence of restricted gene flow among populations. Based on morphological differences, observed ecological specialisation, and the sharing of genetic markers, these butterflies appear to be undergoing adaptive radiation driven at least partially by host shifts. Neutral genetic markers may fail to detect the effects of very recent host shifts in these populations due to gene flow and/or the recency of divergence and shared ancestral polymorphism.

Key words. Butterfly conservation, host plant fidelity, *Lycaeides*, Lycaenidae, mitochondrial DNA, reproductive isolation, specialisation.

Introduction

The origin of biodiversity is one of the oldest, least understood, and most important issues in evolution and ecology. Recent studies have argued either that rapid niche shifts may be important during (ecological) speciation (Orr & Smith, 1998; Hatfield & Schluter, 1999; Rundle *et al.*, 2000) or that niches are conserved over (vicariant) speciation events (Peterson *et al.*, 1999). The role that niche shifts play in divergence of butterflies in the family Lycaenidae was examined. Lycaenid butterflies are a species-rich group comprising as many as 6000 species, more than 47% of butterfly (Papilinoidea) species (Robbins, 1982). The possibility that strong host plant fidelity evolves rapidly after host shifts in these philopatric butterflies and that reproductive isolation develops as an epiphenomenon of this host plant adaptation was examined.

Feder (1998) defined host fidelity as the tendency of phytophagous insects to mate and oviposit on the same host plant species. Host fidelity is a measure of host specificity and is relevant in discussions of speciation because it can create reproductive isolation in the form of positive

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assortative mating (Maynard Smith, 1966; Diehl & Bush, 1989; Rice & Hostert, 1993; Wood *et al*., 1999). Speciation is defined here as a process that leads to reproductive isolation among populations that may eventually lead to reciprocal monophyly.

Ecological factors relating to host preference have been shown to drive natural selection on phytophagous insects. For example, local adaptation to host plant populations and individual plants has been demonstrated in sedentary (Edmunds & Alstad, 1978; Alstad & Edmunds, 1989; Karban, 1989; Hanks & Denno, 1994) and relatively mobile (Via, 1989; Mopper *et al*., 1995; Traxler & Joern, 1999) insects. Rapid changes in host preference during host shifts have also been documented. For example, Singer *et al*. (1993) documented rapid host shifts in the butterfly *Euphydryas editha* (Boisduval) (Nymphalidae) resulting from changes in human land use practices. Little is known, however, about the role that host shifts play in speciation of butterflies.

Ecological factors, including those associated with host or habitat shifts, have been implicated in sympatric speciation (Bush, 1969; Tauber & Tauber, 1977, 1989; Rausher, 1984; Rosenzweig, 1987; Craig *et al*., 1993; Feder *et al*., 1998; Itami *et al*., 1998; Rundle *et al*., 2000; Emilianov *et al*., 2001), although host shifts also occur without reproductive isolation (Thompson, 1994; Radtkey & Singer, 1995). Furthermore, while local genetic differentiation has been found in insects, sometimes at very fine geographical scales (McCauley & Eanes, 1987; Alstad & Corbin, 1989; Rank, 1992), it is not always clear whether this variation is adaptive. It is also not always clear whether differentiation arises as a result of a host shift or existed previously (but see Groman & Pellmyr, 2000). The analysis of the role of ecological factors in speciation has been confounded somewhat with arguments about the possibility and frequency of sympatric speciation. That is, ecological speciation (Schluter, 1998) and sympatric speciation are often equated even though ecological factors, including host-related selection, may certainly act in allopatric situations (Funk, 1998). Some of the difficulty arises due to the apparent rarity of observable speciation events and/or the difficulty of recognising them, especially non-sympatric events. Cases of very recent or on-going speciation offer opportunities to examine the role that ecological factors play in speciation (Kondrashov & Kondrashov, 1999).

The role of ecological factors has been studied in a case of recent or on-going speciation in the butterfly genus *Lycaeides* (Lycaenidae) in North America. Two nominal species, *L. idas* (Linnaeus) and *L. melissa* (W. H. Edwards), are morphologically distinct but are indistinguishable at the molecular genetic level and are clearly of recent origin (Nice & Shapiro, 1999). If ecological factors relating to host shifts are important during differentiation in these butterflies, high levels of host fidelity for natal host plants and evidence of restricted gene flow among populations using alternative hosts are expected. Host fidelity need not be heritable. Any form of host fidelity could reduce gene flow among the habitat and host plant islands occupied by these butterflies significantly. Alternatively, if these butterflies have not developed differences in preference for their natal hosts and exhibit broad host preferences, low levels of host fidelity are expected. This alternative may arise if host use varies geographically and is determined by local host species abundance (Wiklund, 1982; Thompson, 1993) or if significant gene flow occurs between populations preventing the fixation of heritable host preference differences. Niche overlap was quantified and the hypothesis that strong host fidelity develops rapidly during host shifts was tested by measuring oviposition preference in populations of *L. idas* and *L. melissa* from California and Nevada, U.S.A. (Fig. 1). Additionally, the hypothesis that host fidelity can restrict gene flow was tested by measuring population differentiation using data from genetic markers.

This group of butterflies includes several endangered taxa. The Lotis Blue *L. i. lotis* is now extinct and the Karner Blue *L. m. samuelis* is protected under the U.S. Endangered

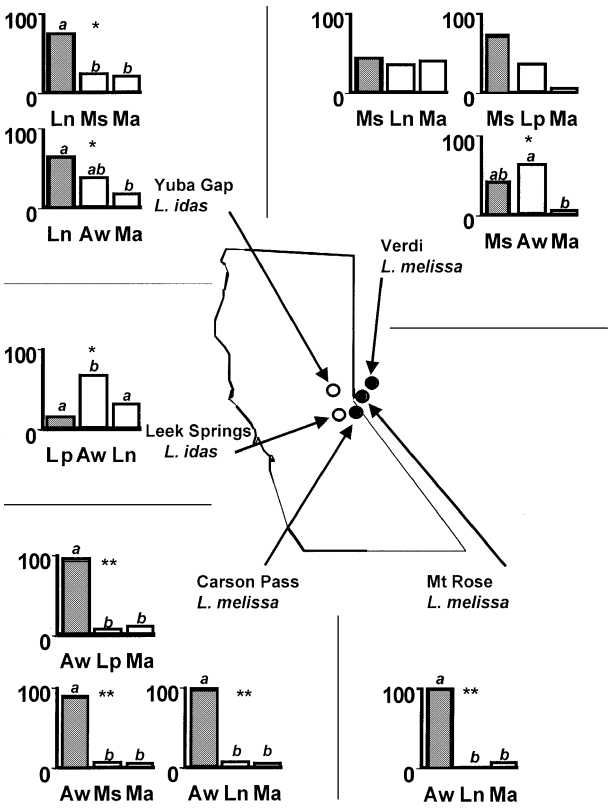

Fig. 1. The locations of the five focal populations of the *Lycaeides melissa*/*L. idas* complex are indicated on a truncated map of northern California, U.S.A. Bar graphs indicate the per cent of eggs laid on each available plant (*y* axis on bar graphs) in oviposition experiments for each population and each experiment. Shaded bars designate natal host for each population. Plant abbreviations: Aw, *Astragalus whitneyi*; Lp, *Lupinus polyphyllus*; Ms, *Medicago sativa*; Ln, *Lotus nevadensis*; Ma, *Melilotus alba*. Different letters indicate significantly different proportion of eggs laid on each plant. Asterisks indicate significance of multiple comparisons (Conover, 1999): $^{**}P < 0.01$, $^{*}P < 0.05$.

Species Act and the subject of a major recovery effort (Arnold, 1993; Andow *et al.*, 1994). An understanding of the factors that drive divergence in this group of butterflies is not only of theoretical interest; elucidation of the factors causing evolutionary differentiation may lead to greater understanding of the basis of endangerment and inform management decisions.

Materials and methods

Butterfly biology

The ranges of the nominal species *Lycaeides idas* and *L. melissa* are broadly overlapping in western North America, especially in California where their ranges are interdigitated and the butterflies occur in sympatry in at least one location (A. M. Shapiro and C. C. Nice, pers. obs.). These butterflies feed on papilionaceous legumes (Fabaceae) in California and Nevada. There is considerable overlap in the recorded use of hosts for each species, however local populations of each species are apparently monophagous. For example, *L. melissa* populations using introduced alfalfa *Medicago sativa* L. apparently ignore readily available native hosts. Where *L. idas* and *L. melissa* occur in close proximity, for instance in the Sierra Nevada of California, they are always found using different host plants. *Lycaeides melissa* at Carson Pass, California use *Astragalus whitneyi* A. Gray as their host and apparently ignore *Lupinus polyphyllus* Lindley, which is the host for the nearby population of *L. idas* at Leek Springs, California. Indeed, conspecific populations of *L. idas* in close proximity can be found using different host plants. In other words, the polyphagy at the species level appears to be the result of localised specialisation and is not the product of a generalist strategy (Fox & Morrow, 1981). This apparent localised specialisation is coupled with the mating behaviour of these butterflies. Mating occurs on or very near the host plant (Pellmyr, 1982; Scott, 1986; J. A. Fordyce and C. C. Nice, pers. obs.). Females perch on the host plant, males fly near the plants searching for females, and the courtship behaviour and mating usually occur on the host.

Vladimir Nabokov delineated these species by differences in wing pattern and the form of the male genitalia (Nabokov, 1949). A discriminant function analysis of variation in male genitalic characters clearly distinguished the two species (Nice & Shapiro, 1999). Two male genitalic measures (falx length and uncus length) accounted for 83% of the morphological variation between species. The discriminant function analysis based on the genitalic measures misclassified only 2.5% of males that were identified to species by wing phenotype. These species are indistinguishable using both nuclear markers (allozymes) and mitochondrial markers (mtDNA) (Nice & Shapiro, 1999), indicating recent divergence (Neigel & Avise, 1986). Genetic distances calculated from allozyme allele frequencies from 14 populations of *L. idas* and *L. melissa* from California and Nevada were extremely small (Nei's $D = 0.002$–0.078) and the two species were not monophyletic (Nice & Shapiro, 1999). Analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) of mtDNA cytochrome oxidase subunit I (COI) gene sequences revealed that $< 3\%$ of the total molecular variance was partitioned among species (Nice & Shapiro, 1999). This incongruence between morphological and molecular data indicates that divergence has either occurred recently or is ongoing and that selective factors may be important components of diversification. An alternative is that the morphological characters used to distinguish the species exhibit phenotypic plasticity or that the phenotype is host dependent (or host induced). This appears unlikely as individuals of both species reared to adulthood in the laboratory on the same hosts exhibit normal *L. idas* and *L. melissa* phenotypes (C. C. Nice, unpublished).

The lack of significant differences at the molecular level would be expected if the genomes of these species evolve in a mosaic fashion (Ting *et al.*, 2000). That is, molecular evolution may proceed at different rates for different loci or groups of loci within a genome. Rates of evolution may be significantly higher for loci that contribute to reproductive isolation or are relevant in the context of ecological speciation compared with rates for presumed neutral loci (Butlin & Tregenza, 1998; Ting *et al.*, 2000). In fact, such differences between quantitative trait loci and neutral markers may be considered evidence for divergent natural selection (Whitlock, 1999). The absence of intermediate morphologies in Sierran populations of *Lycaeides* indicates that divergent selection on morphological characters is quite strong and/or that gene flow among populations is low. Strong host fidelity in *Lycaeides* is one mechanism by which reproductive isolation may evolve and restrict gene flow. This hypothesis was tested by measuring host fidelity in populations of *Lycaeides* that use different larval host plants. The allozyme data of Nice and Shapiro (1999) were also re-examined to test specifically for population differentiation that may result from the evolution of host fidelity. Do females prefer the hosts they use in the field? If so, is gene flow restricted among populations using alternative hosts?

Oviposition experiments

Geographic specialisation in oviposition preference by a phytophagous insect may occur in two ways (Thompson, 1993): phytophagous insects evolve heritable differences in preference for a particular host species or their host choice may be affected by ecological variables such as relative host species abundance, host phenology, or larval conditioning. The phenomenon of larval conditioning, also known as *Hopkins host selection principle* (Hopkins, 1917), posits that females prefer to lay eggs on the plant species that they ate as larvae; however there is little or no experimental evidence that supports this hypothesis (Szentesi & Jermy, 1990), especially in the Lepidoptera (Wiklund, 1974;

Stanton, 1979; Tabashnik *et al*., 1981; Thompson & Pellmyr, 1991; Bossart & Scriber, 1995; Kuussaari *et al*., 2000).

If *L. idas* and *L. melissa* populations have developed differences in female oviposition preference, high levels of host fidelity and differences in preference hierarchies (i.e. the ranking of potential host species) are expected. If, on the other hand, host use is determined by some other mechanism(s), broader host ranges with low levels of fidelity for the natal host are expected (Thompson, 1993). To assess host fidelity in these butterflies, oviposition preference tests were conducted with wild-collected females from four populations (Fig. 1). Populations using either native host plants (Carson Pass, Mt Rose, Leek Springs, Yuba Gap) or a recently introduced host plant (Verdi) were chosen to test the hypotheses that females specifically prefer their natal host and that host fidelity is lower for butterflies with a shorter evolutionary history with a host plant than for those with a longer association.

For 10 experiments, three wild-caught females (individual caged females do not lay eggs; three females interact and stimulate oviposition) from the same population were enclosed in an arena ($\approx 2000\,cm^3$) with three plants: their natal host plant, a negative control, *Melilotus alba* Medikus, which is a legume that is not recorded as a host of either *L. melissa* or *L. idas*, and a test plant (the natal host of an adjacent population). Each experiment consisted of nine to 11 replicates (number of replicates was dependent on capture success and mortality of females in transit). Sample sizes are shown in Table 1. Females were collected from Carson Pass, California (38°58′N, 119°83′W), Mt Rose, Nevada (39°32′N, 119°96′W), Yuba Gap, California (39°31′N, 120°63′W), and Verdi, Nevada (39°51′N, 119°99′W). The Carson Pass and Mt Rose (*L. melissa*) sites are above the treeline in the Sierra Nevada. The Yuba Gap (*L. idas*) site is at mid-elevation on the west slope of the Sierra Nevada. The Verdi (*L. melissa*) site is located at relatively low elevation, east of the Sierra Nevada. Natal hosts are: Carson Pass, *Astragalus whitneyi*; Mt Rose, *A. whitneyi*; Yuba Gap, *Lotus nevadensis* (S. Watson) E. Greene; Verdi, *M. sativa*. *Medicago sativa* was introduced into North America less than 150 years ago (Bolton *et al*., 1972; Michaud *et al*., 1988). Populations and test plants were: Carson Pass (three experiments), *L. polyphyllus*, *M. sativa*, *L. nevadensis*; Mt Rose (one experiment), *L. nevadensis*; Yuba Gap (two experiments), *M. sativa*, *A. whitneyi*; Verdi (three experiments), *L. nevadensis*, *A. whitneyi*, *L. polyphyllus*. The Mt Rose population was included for comparison with the ecologically and morphologically similar Carson Pass population.

In another experiment of similar design, *L. idas* females were collected from Leek Springs, California (38°71′N, 120°25′W) and given three choices that did not include the negative control. The Leek Springs site is a wet meadow at mid-elevation on the west slope of the Sierra Nevada (Fig. 1). For this experiment, the choices included *Lupinus polyphyllus* (the natal host of the Leek Springs population), *L. nevadensis* (host of the Yuba Gap *L. idas* population), and *A. whitneyi* (host of the alpine *L. melissa* populations at Mt Rose and Carson Pass). This experiment consisted of 13 replicates.

All host plants used were collected on the same day as the butterflies and from localities where the test populations were present (except *M. alba*, which does not occur in sympatry with all *Lycaeides* populations). Each arena consisted of an air-spun polyester mesh bag (Kleen Test Products, Brown Deer, Wisconsin) attached to a circular cardboard base, with the stems of each plant projecting through the base into a common water reservoir. Females remained enclosed in the arena and were watered and fed three times a day with a saturated sucrose solution sprayed directly onto the bag. Each experiment lasted 48 h, after which the females were removed and the total number of eggs on each plant was counted. Each arena was considered a block and the number of eggs laid on each available plant

Table 1. Results of natal host oviposition preference experiments. Results of multiple comparisons are given in Fig. 1.

Species/population/ natal host	Test plants	*n*	T_3	*P*
L. idas				
Yuba Gap, California	*M. alba*, *M. sativa*	10	7.95	< 0.005
L. nevadensis	*M. alba*, *A. whitneyi*	10	9.12	< 0.005
L. melissa	*M. alba*, *L. nevadensis*	8	0.28	> 0.250
Verdi, Nevada	*M. alba*, *A. whitneyi*	9	5.49	< 0.025
M. sativa	*M. alba*, *L. polyphyllus*	9	3.23	< 0.100
L. melissa	*M. alba*, *L. polyphyllus*	11	22.83	< 0.001
Carson Pass, California	*M. alba*, *L. nevadensis*	11	19.41	< 0.001
A. whitneyi	*M. alba*, *M. sativa*	10	17.05	< 0.001
L. melissa				
Mt. Rose, Nevada				
A. whitneyi	*M. alba*, *L. nevadensis*	10	31.42	< 0.001
L. idas				
Leek Springs, California				
L. polyphyllus	*A. whitneyi*, *L. nevadensis*	13	9.68	< 0.005

per arena was analysed using the Quade test, a rank-based randomised blocked ANOVA (Potvin & Roff, 1993; Conover, 1999). Thus each plant was ranked one, two, or three in each arena. All host-preference experiments were carried out in a greenhouse at the University of California, Davis. These tests of oviposition preference occurred in experimental arenas of small volume where available plants were in very close proximity and often interdigitated. This arrangement only allowed an assessment of short-distance cues, not long-distance cues, such as plant architecture. Females in these experiments were using short-range cues, probably relying on leaf chemistry for making their oviposition decisions. In the field as well as in the experiments, females are observed walking on the plants after alighting, apparently inspecting the plant before laying eggs (J. A. Fordyce and C. C. Nice, pers. obs.). Electrophysiological investigations have shown that lepidopteran species commonly use sensillae located on the tarsi for chemoreception (Blaney & Simmonds, 1990; Roessingh *et al.*, 1991) and that these cues are often important in oviposition choice (Stanton, 1979; Wiklund, 1982; Papaj, 1986).

Morphometric analysis of male genitalia

Variation in the form of the genitalia from individual males from the focal populations (see Nice & Shapiro, 1999) was assessed using principal component analysis. Three measurements were made for each male following the methods of Nabokov (1949). The three measurements were: falx length, humerulus length, and uncus length (Fig. 2). Differences between the genital morphology of the two species were determined using MANOVA and univariate ANOVAs on the principal component scores of each male. All statistical analysis for the genitalia study was carried out using the JMP® IN software version 4.03 (Sall *et al.*, 2001). Principal component scores were then used to plot the location of each male in morphological space to illustrate the differences between populations and species. Numbers of males for each population were: Carson Pass (36), Mt Rose (24), Yuba Gap (25), Leek Springs (21), Trap Creek (9), Verdi (10).

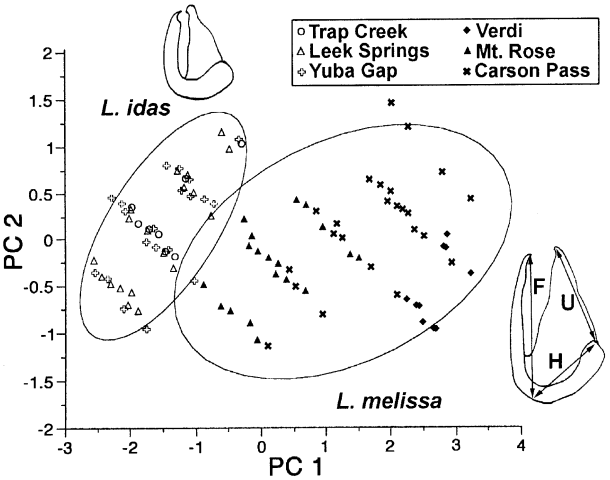

Fig. 2. Principal component scores for *Lycaeides idas* and *L. melissa* males from analysis of genitalic morphology following the methods of Nice and Shapiro (1999) and Nabokov (1949). Ellipses indicate the 95% confidence interval for each species. Individual males are plotted and identified by population (symbols) and by morphological species (shaded symbols = *L. melissa*, unshaded symbols = *L. idas*). Examples of the form of the male genitalia of each species are also provided. The illustration of *L. melissa* genitalia includes a depiction of the three measurements used: falx length (F), humerulus length (H), and uncus length (U).

Tests of population differentiation using allozymes

To test the hypothesis that strong host fidelity may restrict gene flow, the data from 10 allozyme loci reported by Nice and Shapiro (1999) were re-examined. The GENEPOP version 3.2a software (Raymond & Rousset, 1995a) was used to test for population differentiation among the focal populations in this study using the test of Raymond and Rousset (1995b). This procedure uses a Markov chain method to obtain an unbiased estimation of the exact test of Fisher (1935). The Genetic Data Analysis version 1.0 software (Lewis & Zaykin, 1997) was also used to calculate pairwise F_{ST} estimators (θ_{ST} values) according to the formulae of Weir and Cockerham (1984) and Weir (1996). Statistical significance of θ_{ST} values was assessed by 1000 bootstrap replicates.

One of the focal populations, the Yuba Gap population, was not included in the original allozyme survey because it was discovered only after that investigation was completed. The Trap Creek population (39°36′N, 120°64′W) examined in the initial allozyme survey could not be assessed for oviposition preference due to logistical constraints, however the Trap Creek and Yuba Gap populations of *L. idas* are similar morphologically (see Results, Fig. 2) and share identical mtDNA haplotypes (Fig. 3). Both populations use *L. nevadensis* as the larval host in the field and these populations are located within 7 km of each other. For these reasons, it was assumed that allozyme allele frequencies in the Trap Creek and Yuba Gap samples were similar and the allozyme data from Trap Creek were used as an approximation of the allozyme allele frequencies that would be found in the Yuba Gap location.

Mitochondrial DNA methods

The Leek Springs and Yuba Gap populations of *L. idas* examined in the oviposition experiments were not included in the original mtDNA survey reported by Nice and Shapiro (1999). Therefore, 440 bp of the mitochondrial cytochrome oxidase subunit I (COI) gene were sequenced from two individuals from each of these populations. Additional COI sequences were also obtained from two individuals from the Carson Pass population of *L. melissa* and two

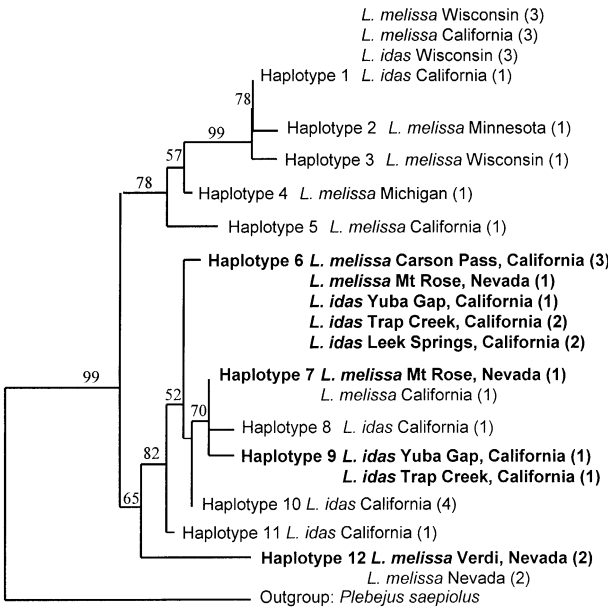

Fig. 3. Neighbour-joining tree of mtDNA cytochrome oxidase sub-unit I haplotypes. The topology of the maximum-likelihood tree was the same as that presented in this figure. Haplotypes present in the four populations measured for oviposition preference are indicated in bold. Bootstrap values are indicated above branches.

individuals from the Trap Creek *L. idas* population. Extraction, amplification, and sequencing methods were described by Nice and Shapiro (1999). From these sequences, 410 continuous nucleotides could be read reliably for all specimens. This portion of the COI region of the mtDNA genome corresponds approximately to positions 1781–2191 of the *Drosophila yakuba* reference sequence (Simon *et al.*, 1994). These eight sequences were combined with 29 other COI sequences that included individuals from the other populations in this study (Nice & Shapiro, 1999). Neighbour-joining using Kimura two-parameter corrected distances and Jukes–Cantor corrected distances, and a maximum likelihood analysis were performed with the PAUP* software version 4.0b3a (Swofford, 1993).

Results

Oviposition experiments

The mean ($\pm$ SE) number of eggs laid per arena over all oviposition experiments was 20.8 ± 2.1. Females of *L. melissa* from Carson Pass and Mt Rose preferred their natal host, *A. whitneyi*, overwhelmingly over the natal hosts of nearby populations (Table 1; Fig. 1). Similarly, females of *L. idas* from Yuba Gap showed preference for their natal host plant, *L. nevadensis*. Females of the Verdi population of *L. melissa* that used non-native *M. sativa* showed no preference for their natal host. Likewise, females of the Leek Springs *L. idas* population did not exhibit preference for their natal host *L. polyphyllus*. Interestingly, both of these populations showed some preference for *A. whitneyi* (Table 1, Fig. 1). The only experiment with Verdi females that indicated any preference was the one in which *A. whitneyi* was present, however multiple comparisons failed to distinguish a preference between *A. whitneyi* and *M. sativa* and only showed preference for *A. whitneyi* over *M. alba*. Females from Leek Springs clearly preferred *A. whitneyi* over their natal host and the natal host of the Yuba Gap population. This lack of fidelity to the natal hosts in the Verdi and Leek Springs populations could reflect an ancestral relationship with an *Astragalus* species in addition to relatively recent association with *M. sativa* or *L. polyphyllus* respectively.

The results from these oviposition trials with females from Leek Springs and Verdi also indicate that larval conditioning is not an important determinant of oviposition preference. Females from both populations prefer to lay on plants that do not occur at those locations. Females from the Verdi population, from a relatively low elevation site in the western Great Basin, laid more eggs on *A. whitneyi*, which occurs above the tree-line in the Sierra Nevada, than on *M. sativa*, though the difference between the relative number of eggs laid on each was not significant. The Leek Springs females also prefer *A. whitneyi*, which does not occur at that mid-elevation site. If larval conditioning is important, these females would be expected to choose the host that is most abundant at their natal location, or at least they should prefer to oviposit on a host that actually occurs there. This rejection of the Hopkins host selection principle agrees with other studies of Lepidoptera (Wiklund, 1974; Stanton, 1979; Tabashnik *et al.*, 1981; Thompson & Pellmyr, 1991; Bossart & Scriber, 1995; Kuussaari *et al.*, 2000).

Morphometric analysis of male genitalia

The means of the three male genitalic measures were larger for *L. melissa* [means (10^{-5} m) $\pm$ SE: falx length $= 54.1 \pm 0.6$, humerulus length $= 38.2 \pm 0.2$, uncus length $= 37.1 \pm 0.3$; $n = 70$] compared with *L. idas* [means (10^{-5} m) $\pm$ SE: falx length $= 39.7 \pm 0.2$, humerulus length $= 34.6 \pm 0.2$, uncus length $= 28.9 \pm 0.2$; $n = 55$]. This was in contrast to wing size measurements, which were on average >10% larger for *L. idas* (J. A. Fordyce *et al.*, unpublished). The first two principal components accounted for >97% of the observed variation in male genitalia. PC1 was largely an index of overall size of the genitalia and accounted for 88% of the total variation being equally loaded by each measurement [measurement and eigenvector coefficient: falx length (0.60), humerulus length (0.55), uncus length (0.58)]. PC2 accounted for 10% of the variation and was loaded mostly by humerulus length (eigenvector coefficient, 0.81) and to a lesser extent by the lengths of the uncus and falx (eigenvector coefficients -0.53 and -0.23 respectively). The contrast between the sign associated with humerulus length and the length of the uncus and falx indicates that PC2 represents

a difference in shape between *L. idas* and *L. melissa* male genitalia.

Comparison of the principle component scores between *L. idas* and *L. melissa* showed a significant difference in male genitalic morphology between these two species (MANOVA, $F_{3,147} = 186.52$, $P < 0.001$). Subsequent univariate ANOVAs revealed that only PC1 and PC2 differed significantly between the two species ($F_{1,149} = 386.94$, $P < 0.001$, $F_{1,149} = 10.77$, $P < 0.01$ respectively). The morphological separation of *L. idas* and *L. melissa* is shown in Fig. 2.

Tests of population differentiation using allozymes

The two tests of population differentiation did not provide congruent results. The exact test of population differentiation of Raymond and Rousset (1995b) demonstrated that there are significant differences in allele frequencies among some populations (Table 2). The Verdi population of alfalfa-feeding *L. melissa* was differentiated significantly from all other populations in accordance with the mtDNA data (Fig. 3). Within the group of very closely related Sierran populations, the Carson Pass *L. melissa* and Yuba Gap *L. idas* populations showing fidelity for alternative native hosts showed significant divergence in allozyme allele frequencies, however the Leek Springs *L. idas* population, in which females did not exhibit strong fidelity for their natal host, was also differentiated from the Carson Pass population.

Tests of population differentiation using the ANOVA approach of Weir and Cockerham (1984) and Weir (1996) also indicated that the Verdi population was significantly different from all other populations, however none of the pairwise θ_{ST} values among Sierran populations was significantly different from zero. Despite apparently strong host fidelity observed at Carson Pass and Yuba Gap, there was no clear evidence of restricted gene flow. The incongruence in the results of the two tests of differentiation may result from the failure of the θ_{ST} approach to estimate confidence intervals correctly when the number of polymorphic loci is small (Raymond & Rousset, 1995b).

Despite this lack of significance in pairwise θ_{ST} values among the Sierran populations, examination of the actual values revealed that Carson Pass and Trap Creek (Yuba Gap) were the most differentiated and that lower θ_{ST} values were detected between both of these populations and Leek Springs. In other words, the two Sierran populations with the greatest observed host fidelity exhibited the highest level of genetic divergence. This pattern, though not statistically significant at $\alpha = 0.05$, is congruent with the prediction that host-associated assortative mating may reduce gene flow between populations exhibiting strong host fidelity

Mitochondrial DNA

The addition of sequences, including a new haplotype (haplotype 9), to the COI phylogeny of *Lycaeides* did not alter the topology or clarify the relationship between *L. idas* and *L. melissa* significantly. Neighbour-joining and maximum-likelihood analyses yielded the same tree topology (Fig. 3). Neither species forms a monophyletic group, however two distinct clades are evident. One clade (haplotypes 6–12) is confined to the Sierra Nevada and north Coast Ranges of California and an adjacent area of western Nevada. The other clade covers a large geographic area including southern California and Oregon and east to Michigan. These clades are separated by substantial genetic distance (sequence divergence between haplotypes 4 and 11 is 1.5%) (Nice & Shapiro, 1999) and both contain populations of *L. melissa* and *L. idas*. All of the Sierran populations included in the oviposition experiments belonged to a single clade (Fig. 3). This phylogeographic pattern has recently been confirmed by a much larger survey of sequence variation in the mitochondrial AT-rich region (C. C. Nice *et al.*, unpublished). That survey included sample sizes of at least 20 individuals from each of the Sierran populations sampled in this study.

Discussion

The most striking aspect of the results reported here is the sharing of genetic markers between morphologically and ecologically differentiated populations and nominal species. Given the low vagility of these butterflies and the patchy distribution of their host plants, a relatively high level of genetic differentiation is expected (Peterson & Denno,

Table 2. Results of tests for population differentiation. Below diagonal: The probability method of Raymond and Rousset (1995b) provides *P*-values for pairwise population comparisons of allele frequencies. *P*-values < 0.05 indicate significant differences between populations. Above diagonal: Pairwise θ_{ST} values. Values were calculated using the formulae of Weir and Cockerham (1984) and Weir (1996). Significance was estimated from 1000 bootstrap replicates. θ_{ST} values that are significantly different from zero are indicated in bold. The Trap Creek population data were used as an approximation for the Yuba Gap population (see text). Comparisons involving the Verdi population are outlined to indicate that this population contains a mtDNA haplotype that is distinctly different from the haplotypes found in samples from populations 1–3 (Fig. 3).

Population	1	2	3	4
Trap Creek (Yuba Gap) *L. idas*		0.019	0.084	**0.194**
Leek Springs *L. idas*	0.640		0.048	**0.143**
Carson Pass *L. melissa*	**0.005**	**0.028**		**0.078**
Verdi *L. melissa*	**0.000**	**0.000**	**0.001**	

1998). The results from tests of population differentiation do not indicate clearly that the host fidelity observed in some populations has resulted in allele frequency divergence between those populations, as would be expected if host fidelity restricted gene flow. The exact test did indicate some differentiation, however it is not clear whether this is a product of isolation via host fidelity. There are at least four explanations for these results: (1) host fidelity simply does not restrict gene flow in *Lycaeides*; (2) while females exhibit host plant fidelity and, consequently, habitat fidelity, males do not and male-mediated gene flow is common, though this does not explain shared mtDNA haplotypes; (3) gene flow may be occurring through populations with low levels of host fidelity or preference for hosts other than their natal hosts (i.e. Leek Springs), linking populations that would otherwise be isolated; or (4) host shifts and the evolution of host fidelity have occurred extremely recently and there has been insufficient time for detectable genetic divergence and lineage sorting to occur. The genetic variation observed in these populations may represent ancestral polymorphism. If this is the case, the mtDNA and allozyme markers used in this study cannot provide information about gene flow because little or no genetic differentiation has occurred since divergence. This lack of monophyly due to ancestral polymorphism has even been observed in reproductively isolated and allopatric species of *Drosophila* (Kliman *et al*., 2000). Consequently, the possibility of barriers to gene flow between populations and species is not precluded by the data from the markers used in this study.

Given the patchy distribution of their host plants, the sedentary nature of the butterflies, the level of host preference observed in some populations such as Carson Pass and Mt Rose, and the morphological differences that are maintained in the Sierra Nevada (Fig. 2), host fidelity appears to be a likely mechanism for the evolution of reproductive isolation in *Lycaeides*, however little genetic evidence supporting this view was found with the markers used in this study. *Lycaeides idas* and *L. melissa* in the Sierra Nevada are indistinguishable genetically with the markers used here. It is possible that these butterflies are not nearly as sedentary as they appear to observers in the field or that *accidental* movements caused by meteorological phenomena occur frequently and result in considerable gene flow. King (1998) conducted mark–release–recapture studies with the endangered Karner Blue *L. m. samuelis* and observed surprisingly long flights by males of up to 1 km or more. It is possible that males do not exhibit the levels of host fidelity observed for females in the experiments. Male-mediated gene flow may explain the lack of differentiation in allozyme allele frequencies. This explanation is unsatisfying because males in the field appear to locate host plants as a first step in locating mates. Furthermore, male-mediated gene flow does not explain the observed shared mtDNA haplotypes, which are maternally inherited. It would seem that host fidelity in males would be selectively advantageous in the competition for mates. Furthermore, this explanation requires fairly strong selection in the face of this gene flow to maintain the morphological differences observed between species.

The idea that the Leek Springs population may be acting as a genetic *bridge* between Sierran populations is intriguing. The Leek Springs *L. idas* population has low host fidelity and females actually preferred the host plant used by *L. melissa* at Carson Pass. The low level of fidelity at Leek Springs may result in less assortative mating that allows gene flow with nearby populations, including nearby *L. melissa* populations. Most models of host- or habitat-associated differentiation focus on two hosts and bifurcating evolutionary lineages (e.g. Rice, 1987; Johnson *et al*., 1996; Dieckmann & Doebeli, 1999; Kondrashov & Kondrashov, 1999). If host preference or fidelity arises in more complicated ways with more than two alternative hosts, however, the process of differentiation might be slowed or subverted. Host shifts may occur in non-synchronised or non-simultaneous ways, and differing levels of host fidelity or differences in oviposition preference between populations may facilitate gene exchange.

The lower observed host fidelity in the Leek Springs and Verdi populations may reflect the recency of the shift onto new hosts relative to other populations. Both may currently be undergoing evolution of host fidelity. Although it might be suggested that these individuals are actually polyphagous and employing a distinctly different host plant strategy, this is unlikely as the use of alternative hosts in most of these populations has not been observed in over 10 years of study. This is a realistic possibility for the Verdi population, however, because the switch to alfalfa can only have occurred in the 150 years since the introduction of alfalfa into North America (Bolton *et al*., 1972; Michaud *et al*., 1988). If the Leek Springs population has shifted to *L. polyphyllus* recently, it may serve as a source of migrants to other (older) populations such as Carson Pass or Yuba Gap. Such gene flow could presumably prevent differentiation among Sierran populations. The obvious objection to this explanation comes from the Verdi population. Strong preference in this population was not observed, yet it is differentiated in terms of allozymes and mtDNA haplotypes; however this population may belong to a lineage that is clearly historically distinct from the Sierran populations (Nice & Shapiro, 1999; C. C. Nice *et al*., unpublished; Fig. 3).

In general, evidence of genetic differentiation among populations using different host plants is equivocal. Several studies of insect populations that are adapted to different host species or habitats have detected genetic differentiation (e.g. Craig *et al*., 1993; Singer & Thomas, 1996; Feder, 1998; Dobler & Farrell, 1999) while other investigators have found little or no host-associated genetic differentiation (Tong & Shapiro, 1989; Sperling & Harrison, 1994; Sperling & Hickey, 1994; Downie, 2000). One investigation of hairstreak butterflies found evidence of both situations in different areas (Nice & Shapiro, 2001) and Radtkey and Singer (1995) reported genetic variation in oviposition preference among populations using different hosts, but this variation was not associated with mtDNA sequence variation. This situation may result from the timing of

investigations relative to the host shift event. If the evolution of host fidelity occurs very rapidly after a host shift, investigations may fail to detect genetic differentiation due to the recency of divergence. This may be especially true if adaptive differences involve changes at a small number of loci (Tong & Shapiro, 1989; Sperling, 1994; Sperling & Harrison, 1994). The genomes of these species may evolve as a mosaic, with neutral markers failing to track differentiation in the short term (Ting *et al*., 2000). In other words, there is some time directly after a host shift during which genetic differentiation at neutral markers will not be detectable (Neigel & Avise, 1986). Genetic markers that show clear differentiation are required for an effective analysis of gene flow. The low levels of differentiation observed in allozymes and mtDNA indicate the recency of divergence but cannot provide reliable estimates of rates of gene flow.

The oviposition data reported here together with the data from molecular markers and male genital morphology indicate that the evolution of host plant fidelity and concurrent morphological differentiation may occur very rapidly in *Lycaeides*, more rapidly than mtDNA sequence divergence or lineage sorting and more rapidly than changes in allozyme allele frequencies. As a result, there are substantial oviposition preference differences among populations of morphologically defined butterfly species that share mtDNA haplotypes (Figs 1, 2, and 3).

Evolution in these butterflies appears to be proceeding via a complicated temporal and geographical mosaic (Thompson, 1999) of host plant shifts and morphological differentiation across a large portion of western North America. In the case of *Lycaeides* and perhaps also for other lycaenids, the locally specialised taxa that are products of adaptive radiation involving multiple host shifts may be particularly susceptible to habitat disruption that has had an adverse impact on their hosts. These results suggest that this may be a mechanism by which these butterflies are prone to endangerment.

These results also illustrate the difficulty of identifying conservation units for special attention when evolution proceeds rapidly. Multiple data sets (morphological, ecological, behavioural, genetic) may be required for interpreting the evolutionary history and genetic structure of threatened taxa in such situations. Examination of genetic structure at neutral markers alone may fail to detect evolutionarily significant units because unlinked, ecologically important loci may diverge under selection more rapidly than neutral markers diverge via drift and/or mutation.

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