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RESEARCH ARTICLE

Conserve the eco-evolutionary dynamic, not the subspecies: phenological divergence and gene flow between temporal cohorts of *Euphilotes ancilla* endemic to southern Nevada

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Abstract

Euphilotes ancilla purpura and *cryptica* (Lycaenidae), butterflies endemic to the Spring Mountains (Clark Co., Nevada), have been described as two univoltine, temporally isolated, sympatric taxa that utilize different early- and late-flowering larval host plant varieties (*Eriogonum umbellatum*). However, our results from field and laboratory indicate that this is not the case. The subspecies overlap in timing of adult reproductive flight (compilation of field records 1977 to 2018) and laboratory emergence of adults from early-season, non-diapause pupae indicate butterflies are not univoltine. Genetic samples collected from putative *E. a. purpura* (Early cohort) and *cryptica* (Late cohort) subpopulations show no evidence of genetic structure indicative of allochronic isolation in phylogenies of 26 mitochondrial DNA COI haplotypes and 18 nuclear ITS1 alleles. Analysis of molecular variance revealed 89% of mitochondrial DNA variation structured within and among subpopulations, with only 11% between the purportedly isolated subspecies. Analysis of isolation and migration indicated gene flow from the Early to Late cohort was 3 × greater than in the opposite direction. We conclude that, rather than two separate subspecies, *Euphilotes ancilla* exists in a network of partially interconnected subpopulations extending from 1750 to 3000 m across much of the Spring Mountains. Gene flow is related to the timing of adult flight and host plant flowering, contributing to the genetic variation in phenology necessary for evolutionary tracking of shifting flowering periods of larval host plants. Maintenance of connectivity and gene flow across the Spring Mountains is therefore essential for population persistence of both cohorts in the face of environmental change.

Keywords Gene flow · Multi-voltine · Phenological divergence · Temporal isolation

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Introduction

Recognition of the importance of evolutionary processes in conservation biology has led to the perspective that managing contemporary eco-evolutionary dynamics and lineages may be more effective than attempting to manage static taxa (Crandall et al. 2000; Stockwell et al. 2003; Höglund 2009; Shaffer 2013). Consideration of the interaction of contemporary natural selection and gene flow is particularly important for conservation and management decisions when genetic exchange among diverging populations has the potential to enhance local adaptation, genetic diversity, and population persistence in the presence of environmental change (Crandall et al. 2000; Garant et al. 2007; Kinnison and Hairston 2007). However, the interaction of gene flow and adaptive divergence also could have the opposite effect, reducing local adaptation and exacerbating conservation threats through the homogenizing influence of gene flow and the introduction of maladaptive phenotypes from other environments (Crandall et al. 2000; Stockwell et al. 2003; Pressey et al. 2007; Kinnison and Hairston 2007; Wadgymar and Weis 2017).

The influence of gene flow on local adaptation of phenology, a critical factor in conservation biology and population responses to climate change (Parmesan 2006; Forrest and Miller-Rushing 2010; Hindle et al. 2015; Morellato et al. 2016), has several potential outcomes with respect to conservation of taxa. Gene flow could enhance adaptive phenological variation and have a net positive influence on population persistence if migrant phenotypes are biased toward those that match local environmental conditions (Kinnison and Hairston 2007; Ravigné et al. 2009), such as would occur with habitat matching (Edelaar et al. 2008) of phenology. Alternatively, migrant phenological phenotypes could exhibit mismatches between life cycle timing and local environmental conditions and reduce or impede local adaptation (Sherry et al. 2007; Wadgymar and Weis 2017), thereby exacerbating conservation threats. Finally, if adaptive divergence in phenology among populations results in non-overlapping reproductive periods, as expected for a multiple effect trait affecting both local resource use and pre-zygotic reproductive isolation (Smadja and Butlin 2011; Servedio et al. 2011), the consequent cessation of gene flow could increase conservation threats by generating reproductively isolated taxa with more restricted geographic distributions, lower total genetic diversity, and potentially higher extinction risk than the network of inter-connected populations that would have existed prior to the divergence and isolation (Stockwell et al. 2003; Servedio and Noor 2003; Taylor et al. 2013; Dixo et al. 2009).

A conservation related example of adaptive divergence in phenology and the timing of adult reproductive activity, a multiple effect trait, can be found in sky-island mountain populations of Rocky Mountain Dotted Blue butterflies (*Euphilotes ancilla;* Lycaenidae) utilizing larval host plants that differ in flowering phenology. Butterflies in the genus *Euphilotes* typically exhibit adult emergence times, and reproduction tied closely to the flowering of one or a few species of *Eriogonum* because their larvae are specialized to feed on flowers or seeds (Pratt and Ballmer 1986; Pratt 1988, 1994). In the Spring Mountains of southern Nevada, USA, two cohorts of *Euphilotes ancilla* (currently recognized as subspecies, *Euphilotes ancilla purpura* and *E. a. cryptica*) occur sympatrically with adult flight seasons that were not believed to overlap (Austin et al. 2008). After feeding for several weeks, larvae pupate below or near the host plant. The timing of adult emergence is determined by the induction of pupal diapause, overwintering, and diapause intensity, the number of days between cessation of diapause and adult eclosion (Pratt and Ballmer 1986; Austin et al. 2008). However, in some *Euphilotes* populations pupae never enter diapause and emerge as adults later in the same year (bi- or multi-voltine; Pratt and Ballmer 1986; Pratt 1994). Such population or subpopulation variation in the incidence of pupal diapause and adult emergence may strongly influence the likelihood of reproductive isolation, genetic exchange between temporal cohorts, and the effects of gene flow on phenological divergence among populations.

Although *E. a. purpura* and *E. a. cryptica* cannot be distinguished by external morphology or genitalia, their taxonomic description was based on larval phenology and use of two host plant varieties that differ in their flowering phenology, *Eriogonum umbellatum* var. *juniporinum* (flowering May to early July) and *E. u.* var. *subaridum* (flowering July to August), respectively (Austin et al. 2008). The butterfly cohorts were described as biologically isolated subspecies because adults were not observed to overlap in the timing of reproductive flights in the Spring Mountains and field collected larvae from *purpura* and *cryptica*, raised under common laboratory conditions, differed in diapause intensity and the timing of adult eclosion. Austin et al. (2008) based their subspecific designation on the assumption that cohorts were univoltine and that early cohort offspring did not contribute to the late cohort despite their observation that 6% of early cohort pupae did not diapause and, therefore, could have emerged with the late cohort in the same year. If the two cohorts are indeed allochronic subspecies as described (Austin et al. 2008), they should exhibit at least partial evolutionary independence, and, based on their assumed use of host plant varieties with non-overlapping flowering times, represent two distinct, adaptively divergent entities for the purposes of conservation (Haig et al. 2006). If instead the cohorts are not phenotypically or

phylogenetically distinct due to overlapping adult emergence times, multi-voltinism, and/or appreciable gene flow relative to selection, subspecific status may not be warranted and the nature and extent of gene flow will determine how the cohort's eco-evolutionary dynamic will impact genetic variation and adaptation, properties critical for conservation decisions.

Here we further investigate the biology and genetic structure of *E. a. purpura* and *E. a. cryptica* (hereafter designated the Early and Late cohort because subspecies differ only in timing of adult emergence and association with larval host plants) using a combination of field and laboratory studies coupled with genetic analyses. Our purpose was to examine existing understandings and assumptions about the cohorts, including the incidence of pupal diapause, temporal overlap of reproductive adults, and potential for phenological mismatch with flowering larval host plants. In addition, we determined whether the phenotypic and phylogeographic structure of the cohorts was consistent with expectations for subspecies (Moritz 2002; Braby et al. 2012). Drawing from reviews and analyses addressing the criteria for defining and delineating subspecies, particularly with reference to conservation biology (Moritz 2002; Patten et al. 2002; Haig et al. 2006; Braby et al. 2012; Funk et al. 2012; Sackett et al. 2014; Patten 2015; Coates 2018), subspecies should have distinct phenotypes, a minimum of one fixed character state (Braby et al. 2012) or breaks in phenotype distributions (e.g., 75% rule; Patten et al. 2002) and should exhibit genetic distinctness indicative of some degree of evolutionary independence. We used two genetic markers, analysis of molecular variance (Peakall and Smouse 2006, 2012), coalescence based estimates of gene flow (Hey and Nielsen 2004, 2007) and evaluation of phylogenetic separation between the Early and Late season cohorts to assess whether they were allochronic subspecies or host races that could serve as appropriate units for conservation or, alternatively, phenologically divergent populations connected by gene flow, for which conservation and management decisions should address the maintenance of interconnections and genetic exchange.

Finally, because the known distribution of the Early cohort (*purpura*) was restricted to a small area of conifer woodland in the Spring Mountains (Austin et al. 2008), we conducted surveys throughout the Spring Mountains to determine the geographic ranges of both Early and Late cohorts and to evaluate their population status. This information is important for future management decisions because both subspecies were petitioned for listing as endangered or threatened under the Endangered Species Act and the U. S. Fish and Wildlife Service (USFWS) concluded that the listing may be warranted (USFWS 2012).

Methods

Butterflies and larval host plants

The described subspecies *E. a. purpura* (Austin 1998) and *E. a. cryptica* (Austin et al. 2008) have similar shaped male valvae (*E. ancilla* depicted in Pratt 1988) and do not differ in wing pattern or appearance. Lacking the ability to morphologically identify samples to subspecies, we refer to butterflies as being "Early" cohort, associated with adult or larval use of flowering *E. u.* var. *juniporinum*, from May to June, or "Late" cohort associated with adult or larval use of flowering *E. u.* var. *subaridum*, from June 20 to September 20. Additionally we looked for evidence of the use of other larval host plants and found Late cohort butterflies and larvae associated with flowering *E. u.* var. *versicolor* from June 22 to August 30. Note that when the adult cohorts (putative subspecies) potentially overlap in early June, the designation of Early or Late is determined only by association with or larval occurrence on one of the host plant varieties (consistent with collection of larvae from flowers by Austin et al. 2008).

Geographic distribution, adult flight season, and host plant flowering

Between 2010 and 2018 we conducted surveys in the Spring Mountains to determine the timing of butterfly flight and flowering of larval host plant varieties (*E. u.* var. *juniporinum*, var. *subaridum*, and var. *versicolor*) at 26 known occupied locations following existing survey routes used in past monitoring of butterflies (USFWS 2012), typically a trail through areas with host plants (routes of varying length, 150–1300 m, median 320 m). In surveys, based on a modified Pollard walk (Pollard and Yates 1993), we counted and recorded locations (photographs geo-tagged with a GPS track of the survey) of all butterflies that intersected the volume of space within 5 m to each side and 5 m in front of an observer walking at a slow, deliberate pace along a fixed route. Flowering status and host plants encountered within 5 m of each side of a walked line were also recorded.

The dates and locations of all known records of *E. ancilla* within the Spring Mountains (630 total) were compiled from publications and government reports [1977–2005; 87 Surveys, 121 observations (Austin et al. 2008; Austin and Leary 2008; government reports), Supplementary Table S1) and from our field surveys (2010–2018; 112 Surveys, 499 observations]. The location of *E. u.* var. *juniporinum, E. u.* var. *subaridum*, and *E. u.* var. *versicolor* host plants and their date of flowering

(Fig. 3) were recorded during the 2010 to 2018 butterfly surveys (1,396 records of flowering *Eriogonum umbellatum*, inclusive of the 630 butterflies observed near flowering host plants).

Host plant and diapause experiment

Early season larvae were collected June 21, 2018 from flowers of *E. u.* var. *juniporinum* in a 14 ha area extending south of Willow Creek (Lat 36.417113°; Long − 115.764234°), the location from which larvae were collected in a previous study (Austin et al. 2008). The larvae, individually caged in small plastic cups with mesh top and attached bud vial containing a fresh flower head, were raised in a laboratory with a 14L:10D light cycle and daytime temperature of 29–32 °C and nighttime temperature of 20–24 °C. Larvae were randomly assigned (similar starting mix of larval instars two, three and four) one of three host plant varieties collected from the nearest field locations with flowering *E. u.* var. *juniporinum* (1830–1950 m; source of field caught larvae), *E. u.* var. *subaridum* (2150–2550 m), and *E. u.* var. *versicolor* (2810–2950 m). Larvae were monitored daily and provided with a fresh field-collected flower head as needed (typically every 3 days). A total of 13 larvae were raised on *E. u.* var. *juniporinum*, 13 on *E. u.* var. *subaridum*, and 15 on *E. u.* var. *versicolor* (excluding accidental mortalities). Larvae pupated on the floor of their cup and were maintained under the same light cycle and temperature conditions (above) for five months to determine the timing and incidence of adult emergence, non-diapause early-season pupae, that would emerge during the period of the late-season cohort. Attempts to find and collect a comparable sample of Late cohort larvae in July and August, 2018 were not successful.

Genetic sampling

The majority of the 132 specimens providing genetic data were adults captured by hand net in 2012, with additional individuals added for under-represented geographic areas by opportunistic netting of adults and collection of larvae from flowers from 2013 to 2017 (Supplementary Table S2). Although our initial plan entailed balanced sampling of Early and Late cohorts with a minimum of 15 butterflies from each of eight locations spaced at equal intervals along the roughly northwest to southeast axis of the Spring Mountains, we had difficulty finding Late cohort butterflies and were therefore able to collect Early butterflies from four locations and Late butterflies from only three locations (Fig. 1; locations numbered). We sampled a total of 94 Early cohort butterflies (2012–2015) from Big Timber Spring, Willow Creek North, Willow Creek, and Mud Spring Road in northeast SMNRA where adults were associated with early-flowering *E. u.* var. *juniporinum*. We sampled 42 Late cohort butterflies from locations within the vicinity of Willow Creek, and Harris Mountain where adults were associated with late-flowering *E. u.* var. *subaridum* (Fig. 1). During sampling, we discovered the additional larval host plant variety, *E. u.* var. *versicolor*, and collected Late cohort butterflies from this plant along south Bonanza Trail (Fig. 1). We use the term subpopulation throughout to refer to each of these locations, recognizing the spatial structure of host plant areas as well as the potential stepping-stone patches connecting them.

Genomic DNA was extracted from 132 samples using the Qiagen DNeasy Blood and Tissue Kit following the Supplementary Protocol for Insects (Qiagen, Valencia, CA, USA). We amplified and sequenced the mitochrondrial COI gene using primers and methods described by Ugelvig et al. (2011). We amplified and sequenced the nuclear internal transcribed spacer region 1 (ITS1; Wilson et al. 2013) obtaining primer sequences and guidance from J. Wilson. Outgroup samples used in phylogenetic analyses of COI were derived from several sources: 4 *E. bernardino* specimens collected in 2015 on *Eriogonum fasciculatum* host plants 50 miles south-east of the Spring Mountains; resequencing (1224 bp) mtDNA of 12 *Euphilotes* specimens provided by Matthew Forister, University of Nevada, Reno from a previous study (Wilson et al. 2013, specimen numbers in Supplementary Table S2; 2 *E. ancilla*, 2 *E. battoides*, 2 *E. enoptes*, and 4 *E. pallescens* collected in Nevada, California, Wyoming and Utah); and additional genera from Genbank (https://www.ncbi.nlm.nih.gov/genbank/). To obtain more *E. ancilla, E. enoptes*, and *E. battoides* from locations in California and Nevada, closer to the Spring Mountains, we also used data from 654 bp reads available in a Bar Code of Life (BOLD) project dataset (Ratnasingham and Hebert 2007; samples collected by P. Opler). The datasets generated in the current study are available from the corresponding author on reasonable request.

Genetic analyses

Phylogenetic trees were constructed using maximum-likelihood (MEGA) and Bayesian (MrBayes) methods. We include here the maximum-likelihood trees for COI and ITS1 with posterior values from Bayesian included in each Figure. We ran the function 'modelTest' in the R package 'phangorn' (Schliep 2011) to test for the most likely of 24 evolutionary models for the COI and ITS1 data sets. The General Time Reversible (GTR + G + I) model had the highest AICw (0.99) for both data sets. In MEGA 7.1 (Kumar et al. 2016), we used the Maximum Likelihood method based on the GTR + G + I model (discrete Gamma distribution with 6 categories) with 1100 bootstrap replications. For Bayesian inference of the same COI and ITS1 phylogenies we used MrBayes 3.2.7 (Huelsenbeck and Ronquist 2001).

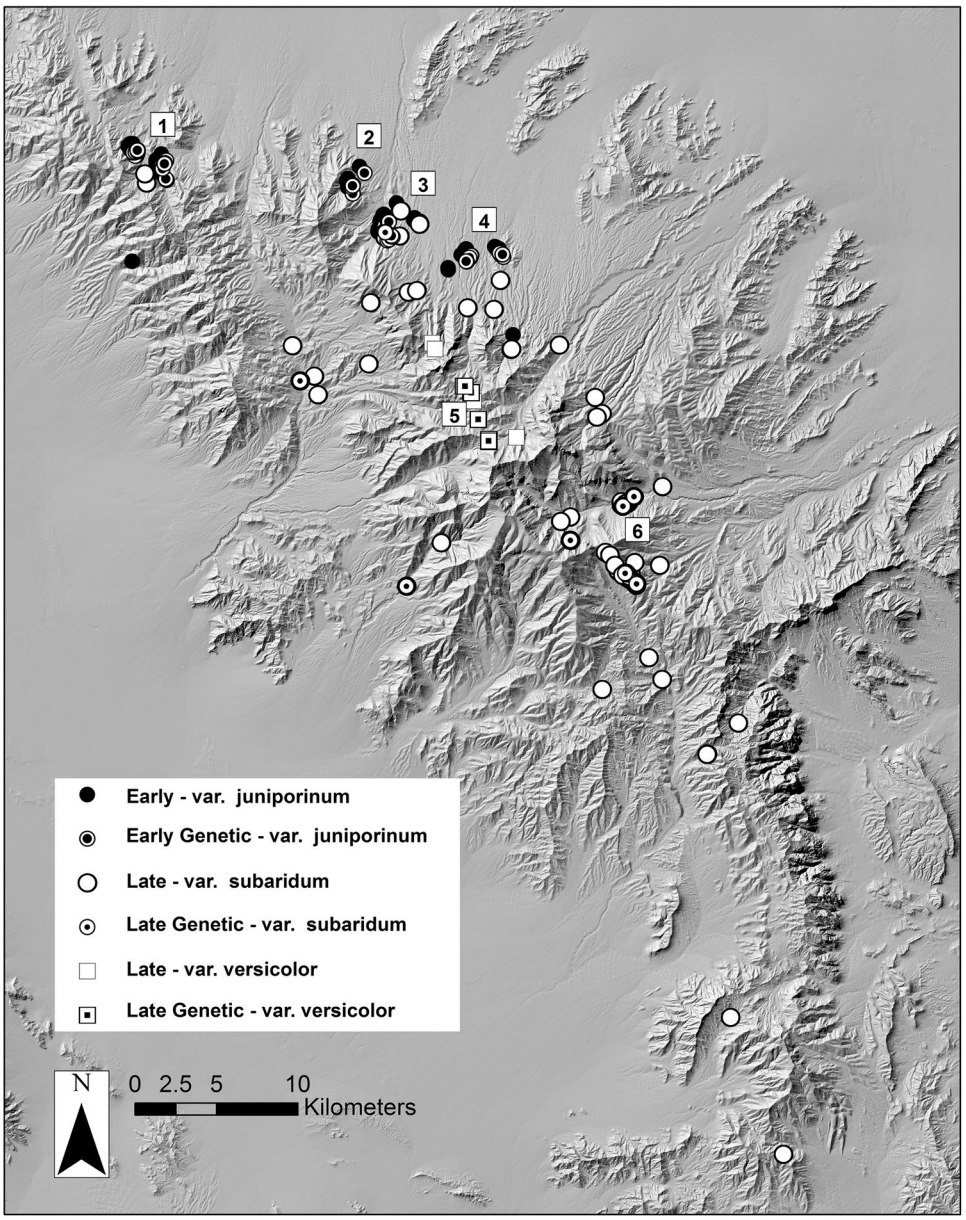

Fig. 1 Locations of 630 *Euphilotes ancilla* within the Spring Mountains, Clark Co., Nevada (records from 1977 to 2018; see "Methods") including Early cohort butterflies (*Euphilotes ancilla purpura*) in association with *Eriogonum umbellatum* var. *juniporinum* larval host plants and Late cohort butterflies (*E. a. cryptica*) in association with *E. u*. var. *subaridum* larval host plants and in association with *E. u*. var. *versicolor* larval host plants (2800 to 3000 m elevation). Individuals collected for genetic sample (2012–2017) from Big Timber Spring (1), Willow Creek North (2), Willow Creek (3), Mud Spring Road (4), south Bonanza Trail (5) and Harris Mountain (6)

With 6 possible rates for substitutions we used default priors for transition rates and nucleotide frequencies, appropriate for a GTR + G + I model, and did not put any constraints on topology. We set chain length to be 125,000,000 generations, with 25% in the burn-in phase, and sampling every 1000 generations.

We constructed a haplotype network for COI using the TCS and median-joining algorithm (Bandelt et al. 1999) in POPART 1.7 (Leigh and Bryant 2015). Analysis of Molecular Variance (AMOVA, GenAlEx 6.5, Peakall and Smouse 2006, 2012) was conducted (9999 permutations) to determine patterns of subspecies (cohort) and subpopulation differentiation in COI haplotype frequencies with 4 subpopulations of Early (Fig. 1, locations 1–4) and 2 subpopulations of Late samples (Fig. 1, locations 5–6). We used a second AMOVA of the same samples to assess whether *E. ancilla* is comprised of three host plant races by estimating genetic divergence among the three larval host plant varieties using a total of 8 subpopulations (samples from the two subpopulations in the Late cohort (Fig. 1, locations 5–6) were subdivided into four smaller subpopulations, two from *E. u*. var. *subaridum* and two from *E. u*. var. *versicolor*).

Gene flow and other demographic parameters were estimated using the Isolation and Migration software IMa (Nielsen and Wakeley 2001; Hey and Nielsen 2004, 2007). The program was implemented with data for both COI and ITS1 from Early (n = 43) and Late (n = 10) samples, setting the inheritance scalar for COI to 0.25 and for ITS1 to 1. Simulations based on 1,000,000 iterations with a burn-in of 10% produced effective sample size (ESS) values indicative

of good mixing during simulations. We report IMa parameter estimates scaled to the per gene mutation rate (unknown for ITS1).

Results

Locations of *E. ancilla* based on observations, publications, and government records from 1977 to 2018 occur from 1710 to 3020 m (Fig. 1; including Austin et al. 2008 locations). In addition to what was known in 2008, we have observed adult or larval Early and Late cohort butterflies at more than 6 new geographic localities within SMNRA in field surveys conducted from 2011 to 2018. The Early season form was found at new localities characterized by widespread occurrence of *E. u.* var. *juniporinum* host plants in open conifer woodlands along bajadas and drainages between 1710 and 2470 m in the northeast of the mountain range (Fig. 1, locations 1 and 2), particularly in areas east and west of Big Timber Spring where only the Late cohort had been observed (Austin et al. 2008). The observation of early-flowering larval host plants in previously unexplored areas extending 17 km to the northwest from Willow Creek and Cold Creek more than triples the known habitat area occupied by the Early cohort.

Late cohort butterflies were observed at relatively low densities utilizing the host plant *E. u.* var. *subaridum* at locations between 1760 and 2250 m as reported in Austin et al. (2008). In addition to these locations, we discovered *E. ancilla* along high elevation ridgelines, from 2800 to 3000 m, in open stands of bristlecone pine *(Pinus longaeva)* utilizing a third host plant variety, *E. u.* var. *versicolor* (Figs. 1, 3, 4). In these ridgeline areas, where flowers bloom between June 20 and the end of August, Late cohort females were observed ovipositing on *E. u.* var. *versicolor* and larvae were collected from host plants in August of 2013, 2015, and 2017. The identification of a new larval host plant variety increases the elevational range of Late cohort *E. ancilla* by 750 m and substantially increases the habitat area of the Late season butterfly along ridges extending for more than 18 km to the north of Lee Canyon at elevations above 2800 m. The larval host plant *E. u.* var. *versicolor* occurs between 2700 and 3050 m and Late cohort *E. ancilla* have been observed between 2800 and 3000 m (Figs. 3, 4). The temporal and spatial overlap of flowering observed between 2011 and 2018 for the three host plant varieties is depicted in Fig. 3.

Adult flight season

The distribution of Julian days for all known records of *E. ancilla* (434 Early, 196 Late, 630 total), pooled across all locations and years from 1977 to 2018 (Fig. 2), revealed one strong peak of adult emergence and flight in late May and a second, relatively broad distribution of emergence times

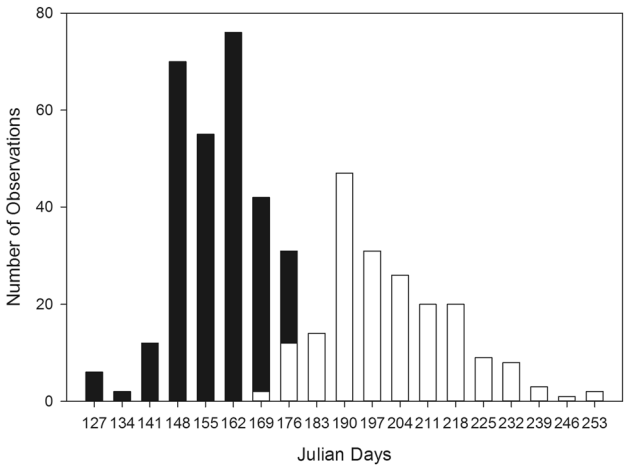

Fig. 2 The distribution of Julian days for all known observations of Early (black) and Late (white) emerging *E. ancilla* from 1977 to 2018 for all locations within the Spring Mountains, Clark Co., Nevada

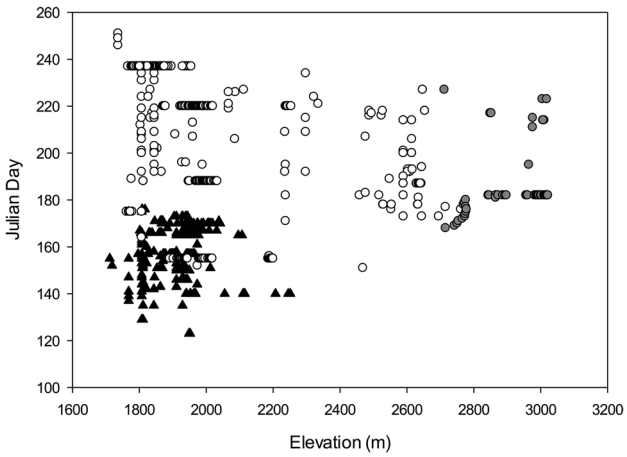

Fig. 3 Julian day for observations of flowering by elevation pooled across all years (2010–2018) at all locations within the Spring Mountains, Clark Co., NV. Symbol color varies by larval host plant with *E. u.* var. *juniporinum* (black filled symbol), *E. u.* var. *subaridum* (open symbol), *E. u.* var. *versicolor* (grey filled symbol). N = 1396 records of flowering *Eriogonum umbellatum*, inclusive of the 630 butterfly observations associated with host plants in Fig. 4

with adult observations relatively equal in occurrence across a 5 to 7 week period from early July through August (Fig. 2). The pronounced peak in early emergence time coincides with a relatively predictable yearly pulse of flowering of *E. u.* var. *juniporinum* in the northern portion of the Spring Mountains (Fig. 2). After this peak, there is a substantially lower level of adult occurrence across all sites in the Spring Mountains matching the drawn out periods of flowering of *E. u.* var. *subaridum* and *versicolor*. The distribution of Julian days for the two emergence groups overlap between day 164 and 176 (Fig. 2). The Early emerging observed during this period of overlap represent 5.7% of all records and

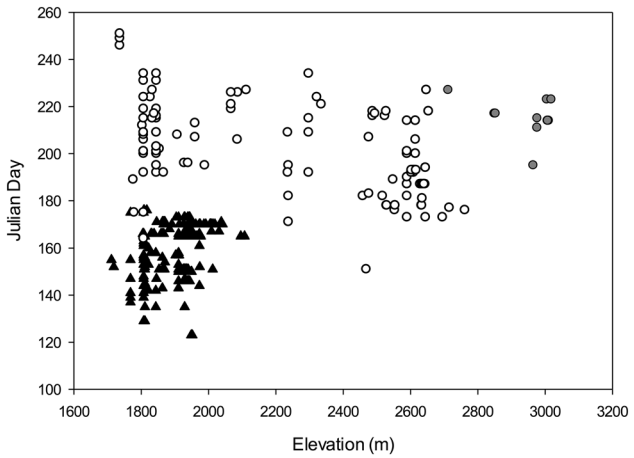

Fig. 4 Julian day for observations of adult *Euphilotes ancilla* by elevation pooled across all years at all locations within the Spring Mountains, Clark Co., NV. Symbol color varies by cohort and larval host plant with early-season cohort utilizing *E. u.* var. *juniporinum* (black filled symbol) and late-season cohort utilizing *E. u.* var. *subaridum* (open symbol) and *E. u.* var. *versicolor* (grey filled symbol). N = 630 records of *Euphilotes ancilla*

the Late emerging butterflies observed during the period of overlap represents 5.1% of the late season records. However, year to year variation in the timing of butterfly emergence and pooling of Julian days for all years causes the total range of dates with adults to exceed the duration of the flight season of Early and Late cohorts within a given year.

The larval host plant *E. u.* var. *juniporinum*, typically flowering in late May, has the lowest elevational range of all the plants utilized by *E. ancilla* butterflies (Fig. 3). Although all of the later flowering plants were found at higher elevations, there was no relationship between the time of Late adult emergence and elevation (Fig. 4). There was temporal and spatial overlap in occurrence of flowering *E. u.* var. *juniporinum* and *subaridum* host plants in the month long interval between 140 and 170 Julian days (e.g, overlap of flowering 20 May to 20 June; Big Timber, Location 1, Fig. 1) and across elevation from 1711 to 2251 m (Fig. 3).

Host plants and larval development

Discovery of temporal overlap in the flowering of all three varieties of *E. umbellatum* (Fig. 3) made it possible to test the development of Early larvae on each of the potential host plant varieties in 2018. Early larvae collected from flowers of *E. u.* var. *juniporinum* on June 20 (Julian day 171) readily fed on flowers of all three varieties, and grew in size, although their survival to pupation varied among the host plant varieties (Chi-square, p = 0.01). The survival to pupation of Early larvae was greatest for larvae feeding on high elevation, late-season *E. u.* var. *versicolor* (85.7%), but not significantly different from larvae feeding on the early-season host plant *E. u.* var. *juniporinum* from which they were collected (72.7%). Survival to pupation was significantly lower for Early larvae feeding on low elevation, late-season *E. u.* var. *subaridum* (38.5%) compared to the other two host plant varieties (Chi-square, p = 0.01).

The incidence of non-diapause pupae for these Early larvae from all of the host plant treatments was more than four times greater than reported by Austin et al. (2008). Within 22–34 days of pupation, 8 of 25 pupae emerged as adults, a non-diapause percentage of 32%. The non-diapause butterflies emerged within the period of flight of the Late cohort, confirming that these populations are not univoltine and a portion of the Early cohort may have descendants contributing to the Late cohort within the same year in the Willow Creek area where the cohorts and host plant varieties are sympatric.

Molecular phylogeography

We obtained 1224 base pairs (bp) of COI sequence data from 126 (87 Early; 39 Late) of the 132 *E. ancilla* specimens collected from the Spring Mountains (Supplementary Table). We obtained 649–660 bp of ITS1 sequence data for 61 specimens but found that several large insertions/deletions caused difficulties in sequencing the remaining 71 specimens. We made several attempts to resequence these samples without success. The nuclear ITS1 sequence data for individuals did not exhibit heteroplasmic sites in the specimens for which we obtained good sequence data. The total sample of adult and larval butterflies from which we acquired sequence data for mitochondrial cytochrome oxidase I (COI, 1224 bp) or mitochondrial COI and internal transcribed spacer region 1 (ITS1, 649–660 bp) was 126 Spring Mountains *Euphilotes ancilla*, with 87 Early and 39 Late samples.

The sample of 126 *Euphilotes ancilla* from the Spring Mountains yielded 26 unique haplotypes differing by 1 to 15 bp changes (A–Z; Table 1). All of the 26 haplotypes identified in these samples were relatively similar to each other (maximum sequence divergence = 1.2%, average divergence = 0.6%) with 34 segregating sites and overall nucleotide diversity of 0.0051. A large portion (n = 68) of samples were comprised of two common haplotypes (B and E; 36 and 32 samples respectively). Four haplotypes were found in both cohorts (Table 1). Haplotype E was present in all subpopulations with roughly similar frequency in both cohorts. Haplotype B was the most common overall, present in all Early subpopulations and found in one Late butterfly. Fifteen haplotypes were represented by only one sample each.

Maximum likelihood phylogenetic trees of the COI haplotypes were constructed with two different sets of congeners. The first utilized the full 1224 bp sequence from all Spring Mountains *Euphilotes ancilla* in combination with: nearby sample of *E. bernardino* haplotype, 12 samples of *E. ancilla*,

Table 1 Number of samples (126 total) in two seasonal cohorts representing each of 26 COI haplotypes for butterflies associated with larval host plant varieties *E. u.* var. *juniporinum*, var. *subaridum*, and var. *versicolor*

	A	B*	C	D	E*	F	G	H*	I	J	K	L	M*	N	O	P	Q	R	S	T	U	V	W	X	Y	Z
Early Cohort *juniporinum*	**1**	**39**	1		**26**	**2**		8	2			**1**	**1**	**2**						1	1				1	2
Late Cohort *subaridum*		**1**		1	**9**		2	1		1	1					1			1			1	**1**	**1**		
Late Cohort *versicolor*					9			1					1		1	2	1	1	1							
Total	1	40	1	1	44	2	2	10	2	1	1	1	2	2	1	3	1	1	2	1	1	1	1	1	1	2

Four haplotypes with letters marked (*) are found in both Early (*E. a. purpura*) and Late cohorts (*E. a. cryptica*). Haplotypes in bold cells are found within areas of sympatry of the Early and Late cohorts and their larval host plants *E. u.* var. *juniporinum* and var. *subaridum*

E. battoides, E. enoptes, and *E. pallescens* used in a previous study of *E. pallescens* in Nevada (Wilson et al. 2013). The tree based on 34 variable sites depicts a monophyletic genus *Euphilotes* and a monophyletic group including both cohorts from the Spring Mountains (Fig. 5). Within the Spring Mountains, the 26 lettered haplotypes of *E. ancilla* exhibit relatively minor topological structure, with only 3 or 4 supported branches, none of which correspond to phylogenetic differences between Early and Late samples. However, Early and Late samples are mixed with representatives of several other congeners including *E. pallescens* (Wilson et al. 2013).

The cohorts of *E. ancilla* from southern Nevada (Fig. 5) formed a well-supported clade with representatives of *E. pallescens, E. enoptes*, and *E. bernardino* (clade 1 from Wilson et al. 2013) that was phylogenetically distinct from the clade with other *E. ancilla* samples from Utah, Wyoming, and Montana (clade 2 from Wilson et al. 2013), polyphyletic with respect to COI. Because we did not have 1224 bp COI sequence data from *E. ancilla* elsewhere in Nevada or California, we constructed a second tree with our Spring Mountains *E. ancilla* samples and representatives of conspecifics and congeners collected from Nevada and California using a 654 bp subset of the COI data (BOLD; P. Opler unpublished data). In this second maximum likelihood phylogeny with six *Euphilotes* species (Supplementary Fig. S1), there was strong support for two mtDNA clades within the genus. The samples of *E. ancilla* from southern Nevada were structured within clade 1 with samples of *E. ancilla* from Donner Pass, California, whereas *E. ancilla* samples from northern Nevada, California, Utah, and Montana were in clade 2. The broader geographic taxonomic coverage also revealed that three of the six species, *E. ancilla, E. battoides* and *E. pallescens*, were each polyphyletic with respect to mtDNA with representatives in clade 1 and clade 2. Of the other three species, *E. enoptes* and *E. bernardino* were in clade 1 whereas *E. glaucon* was in clade 2. (Supplementary Fig. S1).

A minimum spanning network of all *E. ancilla* Early and Late haplotypes illustrates the relationships within the Spring Mountains (Fig. 6). The haplotype diversity in the 38 Late samples was quite high with a total of 14 haplotypes. However, there is no clear network structure indicating that Late sample haplotypes share similar origins indicative of the long term isolation expected of chronological subspecies. The Late sample haplotypes share origins with haplotypes that are common in both Early and Late samples; haplotypes are intermingled throughout the total genetic space occupied by the Early group rather than clustered within a single group. Importantly, both Early and Late samples share, at relatively comparable frequencies, the common haplotype, E in every subpopulation. Moreover both groups contain unique low frequency haplotypes associated with haplotype E (Fig. 6). Although the common haplotype B is present in all Early

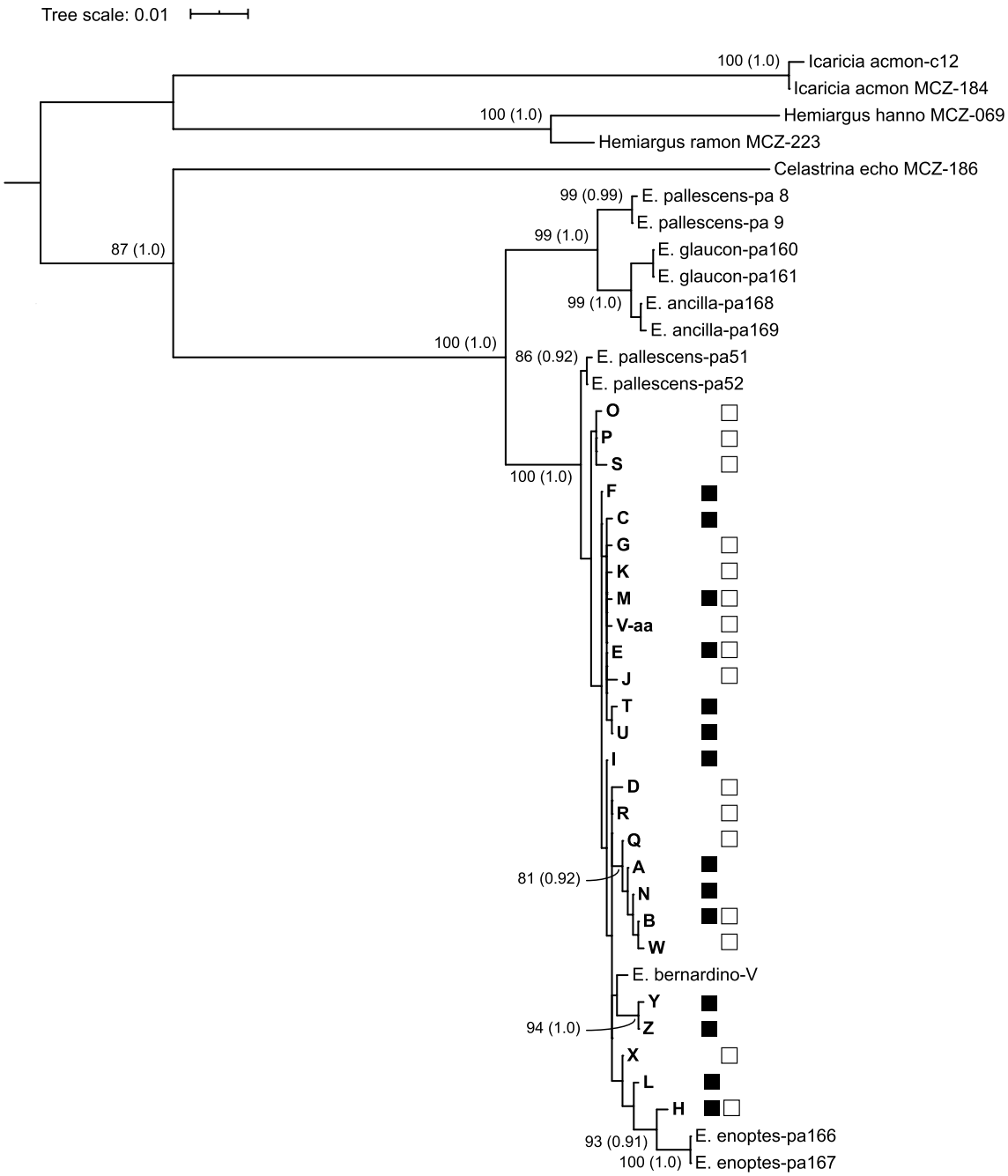

Fig. 5 Maximum Likelihood tree of 26 COI haplotypes (A–Z; 1224 bp) sampled from two seasonal cohorts of *Euphilotes ancilla* (Early cohort—black; Late cohort—white; see "Methods") in the Spring Mountains, Clark Co., Nevada. Outgroup taxa include *E. bernardino*, *E. pallescens*, *E. enoptes*, and *E glaucon* (Wilson et al. 2013), in addition to specimens of three genera from Genbank (see Supplementary Table S2). Haplotypes B and E are common (each greater than 25% of all samples). Branch length is scaled to the number of substitutions and bootstrap support and posterior probabilities (MrBayes) in parentheses are labeled on each branch (all values greater than 75%)

subpopulations, B is in particularly high frequency, along with a set of closely related haplotypes in the samples from the Willow Creek sympatric area. Importantly, one of the Late samples from Willow Creek also had the haplotype B and another individual had the haplotype W, one mutational step away from B. Haplotype Q (3 steps from B) was also found in the Late cohort Bonanza trail subpopulation (Fig. 6).

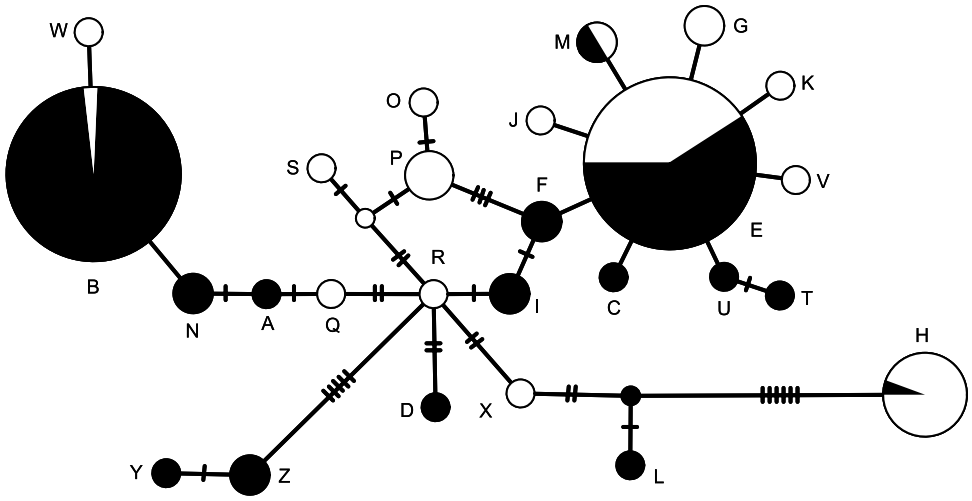

Fig. 6 Minimum spanning (TCS) network of *Euphilotes ancilla* haplotypes from the Spring Mountains, Nevada. The circle size reflects the number of individuals exhibiting a haplotype and the filled region reflects the proportion of haplotype present in the Early (black) versus Late (white) samples

Analysis of molecular variance

Based on six subpopulation samples, four from Early and two from Late cohorts, AMOVA (GenAlEx 6.5, Peakall and Smouse 2006, 2012) of all mtDNA haplotype frequencies indicated significant structure for all sources of variation with 76.2% of all genetic variation within populations, 13.1% of variation among populations, and only 10.7% of haplotype differences attributed to Early and Late cohorts (Table 2). The Phi value for genetic structure between the cohorts was less than that attributed to differences among subpopulations. A second AMOVA testing for genetic divergence among the three larval host plant varieties using a total of 8 subpopulations produced less structure among the three host plants than had been accounted for between the cohorts (samples from the two subpopulations in the Late cohort were subdivided into four subpopulations, two from *E. u.* var. *subaridum* and two from var. *versicolor*). All sources of variation were significant with 77.7% (Phi = 0.23) of all genetic variation within populations, 15.3% (Phi = 0.16) of variation among populations, and only 7.9% (Phi = 0.08) of haplotype differences attributed to divergence among the three larval host plants (Table 1). These results do not support consideration of *E. ancilla* as three separate larval host plant races in the Spring Mountains.

ITS1 analyses

Of the 126 butterflies with COI sequence we were able to get complete ITS1 sequence data from 49 Early and 10 Late cohort samples. Of the 59 individuals there were 18 different alleles, all distinct from ITS1 alleles of three congeners. Despite the small sample size of 10 Late cohort with ITS1 sequences, we found 6 different ITS1 alleles, one-third of the 18 ITS1 alleles in the total sample. The phylogeny of ITS1 (Fig. 7) is monophyletic for all 18 alleles with no phylogeographic pattern and no pattern of divergence related to Early or Late cohorts.

With respect to the subset of butterflies for which we had both COI haplotypes and ITS1 sequences, there was evidence of shared haplotypes and ITS1 alleles among sympatric Early and Late butterflies. Of the 6 ITS1 alleles (I-1, I-4, I-11, I-17, I-19, and I-20) in Late samples, two were shared with Early samples. Six of 10 Late and 23 of 49 Early samples had one of the two common shared alleles, ITS1-17 and − 20. Examining the two common COI haplotypes (B and E) relative to ITS1 haplotypes, 10 of the 23 Early samples that have shared ITS1 also have the shared, common COI-E and 2 of 10 Late butterflies have shared common COI and ITS1. The overlap in individuals carrying the 2 ITS1 markers that are common and shared between the cohorts

Table 2 Analysis of molecular variance (AMOVA) of 126 COI haplotype samples among six sample locations (see "Methods") with four subpopulations of Early cohort (Fig. 1, 1–4) and two subpopulations of Late cohort (Fig. 1, 5–6) *E. ancilla*

Source of variation	d.f.	SS	Estimated variance	Percentage of variance	*Phi-pt* Value	p
Between cohorts	1	29.28	0.34	10.7	0.107	0.001
Among subpopulations	4	43.15	0.41	13.1	0.145	0.001
Within subpopulations	120	290.84	2.42	76.2	0.237	0.001

AMOVA Degrees of freedom, Sum of Squares, Estimated genetic variance, Percentage of genetic variation (%), and Significance level are based on 9999 permutations

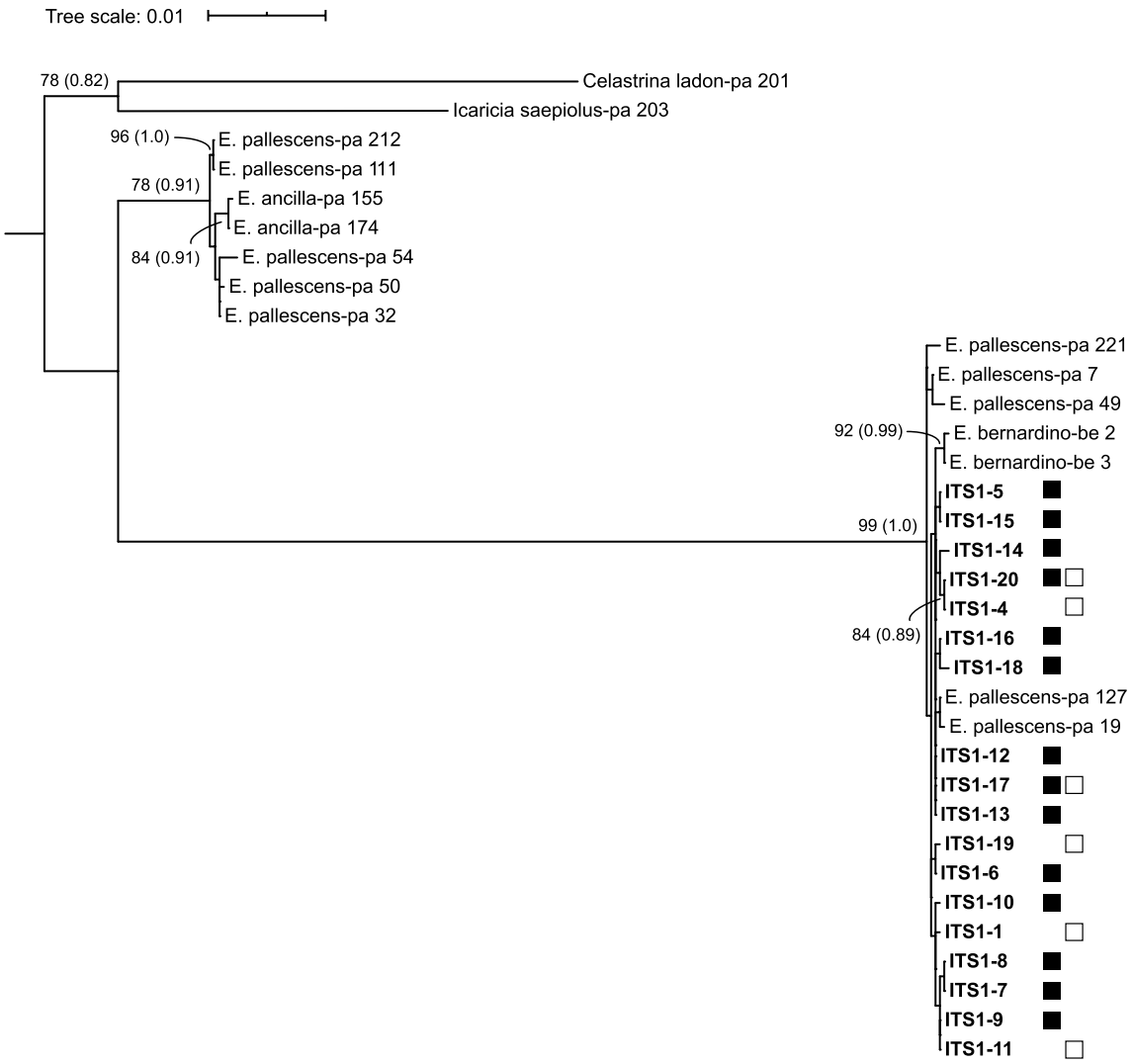

Fig. 7 Maximum Likelihood tree of 18 ITS1 alleles (649 bp) sampled from two seasonal cohorts of *Euphilotes ancilla* (Early cohort—black; Late cohort—white; see "Methods") in the Spring Mountains, Clark Co., Nevada. Outgroup taxa include *E. Bernardino* and *E. pallescens* (Wilson et al. 2013), in addition to specimens of two genera from Genbank (see Supplementary Table S2). Branch length is scaled to the number of substitutions and bootstrap support and posterior probabilities (MrBayes) in parentheses are labeled on each branch (all values greater than 75%)

provides evidence of mixing that is similar to the pattern observed for mtDNA haplotypes.

Gene flow

Implementation of the HKY mutation model with Isolation and Migration software (IMa) generated good estimates of *theta* for the Early cohort, migration from Early to Late, and migration from Late to Early cohort butterflies. The best solution for IMa produced sharp peaks for marginal posterior probabilities and estimates of *theta-1* for Early samples and gene flow in both directions with *m-1* for Late into Early and *m-2* for Early into Late cohorts (Table 3, Fig. 8) but did not yield good estimates of the effective population size associated with Late populations and the ancestral population (estimates of *theta* for the Late cohort and ancestral population were identical although ESS values indicated good mixing that led to convergence). Despite the uncertainty in the estimates of *theta* for the Late cohort and ancestral population, these values were consistently much greater than *theta* associated with Early samples (Table 3), reflecting the greater genetic diversity of the Late cohort. Importantly, the estimate of *m-2* for gene flow from the Early to Late cohort was nearly three times greater than in the opposite direction, as would be expected from the asymmetrical influence of non-diapause pupae from the Early cohort emerging and contributing to reproduction in the Late cohort.

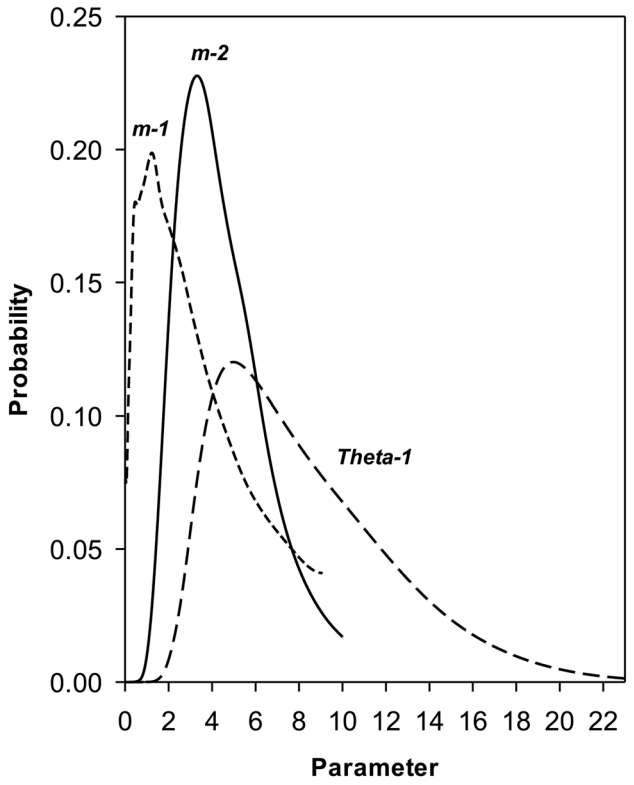

Fig. 8 Marginal posterior probabilities of population genetic parameters from isolation with migration analysis (IMa) of *Theta-1* ($4N_1u$) for the Early cohort (*E. a*. purpura) with *m-1* the rate for genes coming into Early from Late and *m-2* the rate for genes coming into Late from Early (see text)

Discussion

Overall, our results are not consistent with the division of Spring Mountains *E. ancilla* into two univoltine, temporally isolated subspecies or three host plant races. The distributions of Early and Late cohort emergence times, pooled across years, partially overlap by at least 12 Julian days for butterflies associated with the three varieties of flowering *E. umbellatum*. Although the use of pooled dates overestimates the within-year duration of adult flight and the extent of overlap in Early and Late emergence times, there is the potential for butterflies in the tails of these two temporal distributions in some years to hybridize. Regardless of overlap, directional gene flow will occur if some Early cohort progeny do not diapause as pupae and emerge during the period of the Late cohort flight season, as observed in laboratory rearing of field-caught larvae. Although our conclusions differ from Austin et al. (2008) with respect to voltinism, experimental results from both studies demonstrate the potential for Early cohort progeny to contribute to the Late cohort. Indeed, our finding of 30% non-diapause pupae compared to 6% non-diapause pupae found by Austin et al. (2008) for the same source population is consistent with the

Table 3 Parameter estimates of population genetic parameters from IMa program isolation with migration analysis and simulation of divergence between Early (n = 43) and Late (n = 10) using all samples for which we had both COI and ITS1 sequence data

Parameter[a]	*Theta-1* Early *purpura*	p	*Theta-2* Late *cryptica*	p	*Theta-a* ancestral	p	*m-1* Late to Early	p	*m-2* Early to Late	p
Estimate[b]	4.98		0.12	83.73	0.03	83.73		0.13	1.23	0.19	3.31	0.23

p is the height of the marginal posterior probability peak.

[a]Parameter estimates include the per gene mutation rate, *u* (e.g. *Theta-1* = $4N_1u$, and *m-1* = m_1/u, where m_1 is the rate for genes coming into population 1 from population 2).

[b]Based on 1,000,000 iterations with a burn-in of 10% with one mitochondrial and one nuclear gene sampled.

differences in experimental rearing temperature, 30 °C compared to ambient (21 °C) temperature, respectively. Pratt and Ballmer (1986) reported that higher developmental temperatures (28–35 °C) were more likely to inhibit pupal diapause than lower temperatures in *E. enoptes*, several populations of which had variable pupal diapause and multi-voltinism. As field temperatures encountered by *E. ancilla* exceed 35 °C during June and July at Willow Creek, the 30% emergence from non-diapause pupae we observed is indicative of pupae in the field that would emerge within the Late cohort butterflies, causing gene flow in areas of sympatry. These results are not consistent with current assumptions about univoltine *E. a. purpura* and *E. a cryptica*.

Phenological divergence and gene flow

A coalescent analysis of COI and ITS1 DNA sequences in a model of isolation and migration (IMa) provided estimates of gene flow between the cohorts while accounting for ancestral polymorphism (Nielsen and Wakeley 2001; Hey and Nielsen 2007; Marko and Hart 2011). Importantly, the estimate of Early to Late cohort gene flow was three times greater than in the opposite direction, as expected given the directionality due to emergence of non-diapause pupae. Even the smaller, more robust estimate of population effective migration rate for the Early cohort (*theta-1* × *m-1* = 6.13) was high and well above values hypothesized to offset neutral divergence. There was no detectable phylogeographic structure of haplotypes or alleles indicative of two putatively isolated subspecies *purpura* and *cryptica* (Austin et al. 2008), although with only two genetic markers this result does not preclude the existence of significant genetic structure in other parts of the genome (Forister et al. 2008; Funk et al. 2012; Coates et al. 2018). Multiple alleles of COI and ITS1 were shared between Early and Late cohorts including four of the 26 mitochondrial haplotypes and two of the 18 ITS1 alleles. Networks based on DNA site differences among mitochondrial haplotypes and among ITS1 alleles also did not exhibit the structure expected of sympatric subspecies. With 61% of COI haplotypes and 67% of ITS1 alleles private, present in only one individual or population, most of the genetic variance is structure within and among subpopulations, as evidenced in the AMOVA of mitochondrial haplotypes. The Early and Late cohorts contribute a relatively modest influence on phylogeographic structure, less than would be expected for univoltine allochronic subspecies utilizing different host plant varieties (Forister et al. 2011; Santos et al. 2011). Gradish et al. (2019) also reported minimal mtDNA structure among sympatric cohorts of the biennial butterfly (*Oeneis macounii*).

Gene flow between cohorts has the potential to enhance adaptive variation in phenology if adult emergence coincides with flowering of host plants suitable for larval development. In an investigation of gene flow and population structure using allozymes in a congener, *E. enoptes*, Peterson (1995) found no evidence of reduced genetic exchange with respect to differences in adult emergence along an elevational gradient. Our observations of relatively continuous flowering of host plants from June to August, across a wide range of locations and elevations, indicate that variable butterfly emergence times are not likely to produce mismatch with flower phenology. The spatial and temporal continuity of available flowering host plants and the butterflies' flights to the nearest flowering host plants (observed in this study and Peterson (1997) for a congener) generate matching habitat choice (Edelaar et al. 2008; Nicolaus and Edelaar 2018) that facilitates maintenance of a broad range of butterfly emergence times and reduces nonadaptive or constraining influences of gene flow on the evolution of phenology.

As for host plant suitability, early cohort larvae were able to complete development and pupate on flowers of three varieties of *Eriogonum umbellatum*, with equal survival of larvae feeding on early-season *E. u.* var. *juniporinum* and late-season *E. u.* var. *versicolor* but lower survival on *E. u.* var. *subaridum*. The high survival of Early larvae on *E. u.* var. *versicolor* flowers, indicates that gene flow between low and high elevation populations would not cause mismatch in larval–host plant compatibility. Early to Late cohort gene flow in low elevation areas with late-season *E. u.* var. *subaridum* could be opposed by natural selection associated with the reduced suitability of this host plant variety for Early larvae. However, the current lack of information about the performance of Late larvae on the same plant precludes determination of any maladaptive effect of gene flow or its magnitude. Overall, the broad distribution of emergence times, availability of flowering host plants, and the butterfly's ability to find even solitary flowering host plants contribute to an eco-evolutionary scenario in which low to moderate gene flow, due in part to variation in pupal diapause, has the potential to contribute to adaptive variation in phenological traits and voltinism. Given the potential for climate driven shifts in the timing of host plant flowering and the strong influence of flowering on *E. ancilla* population dynamics, the persistence of these endemic butterflies is likely to be strongly influenced by the ongoing eco-evolutionary interplay of gene flow and selection across the host plant varieties of the Spring Mountains sky-island (Crandall et al. 2000; Kinnison and Hairston 2007).

Subspecies and conservation

The criteria defining subspecies and the evolutionary and conservation implications of taxonomy below the species level have been the subject of many articles across a wide array of taxa (e.g., Patten et al. 2002; Zink 2004; Phillimore and Owens 2006; Haig et al. 2006; Mallet 2008; Braby

et al. 2012; Sackett et al. 2014; Mee et al. 2015). Although there is a long history of debate about the use and value of subspecific taxonomy, many authors agree that application of standardized criteria to define subspecies is warranted and useful in conservation decisions if taxa reflect both distinct adaptive phenotypes and distinct genetic differences due to historical isolation and evolutionary independence (Moritz 2002; Haig et al. 2006; Braby et al. 2012; Patten 2015; Coates et al. 2018). Criteria sufficient to provide diagnosable phenotypic differences among subspecies include at least one fixed morphological or behavioral character state (Braby et al. 2012) or, for continuous traits, a break in phenotypic distribution such that 75% of individuals in one taxon can be distinguished from more than 99% of individuals in another (Patten et al. 2002; Patten 2015). To address whether subspecies exhibit population genetic structure indicative of evolutionary independence relative to other units, researchers typically use neutral genetic markers to quantify phylogeographic structure or to estimate gene flow (Crandall et al. 2000; Moritz 2002; Haig et al. 2006; Braby et al. 2012; Sackett et al. 2014). With the expectation of reduced gene flow, reduced sharing of haplotypes or alleles, and greater disparities in genetic frequencies among subspecies in comparison to among populations within subspecies, hierarchical analyses such as AMOVA and analyses of gene trees can be used to determine if sets of genetically divergent populations align with phenotypically defined subspecies (e.g. Braby et al. 2012; Sackett et al. 2014, Mee et al. 2015; Prentice et al. 2019; Wilson et al. 2013; Taylor et al. 2013, Pavlova et al. 2014).

The phenotypic differences proposed to distinguish *Euphilotes ancilla purpura* and *cryptica*, intensity of pupal diapause, adult emergence time, and larval host plant variety (Austin et al. 2008) are all related to phenological divergence and, as multiple effect traits (Smadja and Butlin 2011), have the potential to cause temporal isolation. Therefore, adaptive divergence in adult emergence time that generates phenologically distinct subspecific groups, would be expected to generate a corresponding signature of genetic divergence indicative of reduced gene flow and evolutionary independence between the taxa. In this manner, divergence of multiple effect traits in general, can be predicted to simultaneously generate phenotypically distinct and genetically distinct taxa on a continuum from subspecies to species, similar to what has been hypothesized for allochronic subspecies and species of Lepidoptera (Yamamoto and Sota 2009; Santos et al. 2011; Gradish et al. 2019). However, *E. a. purpura* and *cryptica*, do not meet this expectation. The phenotypic divergence in adult emergence (Fig. 2) and intensity of pupal diapause (Austin et al. 2008) appear to satisfy the 75% rule, however, there is no corresponding genetic structure between the subspecies for COI and ITS1 relative to the divergence and structure observed among subpopulations within subspecies. The weak genetic structure and relatively high gene flow between the allochronic Early and Late cohorts (putative subspecies) can be explained by our observation of mixed voltinism and emergence of non-diapause pupae into the late cohort. In studies of biennial insects, two butterfly species with sympatric, allochronic cohorts also did not exhibit evidence of genetic structure between alternate year cohorts (Kankare et al. 2002; Gradish et al. 2019), consistent with gene flow due to variability in emergence during off-years.

The known distribution and abundance of endemic *Euphilotes ancilla* in the Spring Mountains of southern Nevada has been increased substantially by field surveys conducted from 2010 to 2018. The geographic extent and total abundance of early-season *E. ancilla* is more than three times greater than previously known (compare to Fig. 1, Austin et al. 2008). As for late-season *E. ancilla*, we have discovered females ovipositing on, and larvae developing in, flowers of *E. u.* var. *versicolor* in relatively open bristlecone forest (*Pinus longaeva*) between 2800 and 3000 m. These observations increase the breadth of larval host plants utilized by *E. ancilla*, expand the elevational range by 800 m, and increase the habitat area along more than 10 km of mountain ridges above 2800 m. This high elevation habitat is particularly important in terms of butterfly movement, dispersal, and conservation due to the stepping stone habitat connectivity it may provide along the north–south axis of the Spring Mountains as well as between the east-and west-side conifer woodland habitat areas known to harbor diffuse subpopulations of Late cohort butterflies.

In contrast to the shallow relatively unstructured topology in the clade that contains *E. ancilla* Early and Late cohorts endemic to the Spring Mountains, other populations and species of *Euphilotes* harbor two distinct clades in phylogenies of COI (Fig. 5, Supplementary Fig. S1; Clade 1 and 2 in Wilson et al. 2013) and ITS1 (Fig. 7). The monophyletic group including our Early and Late cohorts, *E. ancilla* from Donner Pass, California, and four other congeners is the same as the mitochondrial clade 1 identified by Wilson et al. (2013) within *E. pallescens* in central and northern Nevada. Unlike the Spring Mountains cohorts, morphologically distinct subspecies of *E. pallescens* and several restricted subspecies of conservation concern (Wilson et al. 2013) mainly contain haplotypes of mitochondrial clade 2, although some also have haplotypes of clade 1. For populations or subspecies of *Euphilotes* that contain both mtDNA clades, it will be important to determine if this genetic structure aligns with adaptive phenotypic differences that could delineate separate conservation units. From a broader evolutionary perspective, in depth multi-locus or genomic analyses will be required to elucidate what combination of hybridization, introgression, and/or rapid diversification with incomplete lineage sorting (Wilson et al. 2013; Zhang et al. 2019) underlies the

persistence of the same mitochondrial clades in *E. ancilla*, *E. battoides*, and *E. pallescens*.

Conclusion

We now have a fairly complete picture of how *E. ancilla* populations interact across the Spring Mountains. Early season emergence produces high densities of butterflies in relatively restricted areas. These populations produce non-diapause pupae allowing genetic mixing into later emerging populations that occur at much lower densities but across a wide range of elevations and areas; high elevation populations utilizing a different variety of larval host plant facilitate gene flow across the range. Austin et al. (2008) envisioned the conservation of a putative *E. a. purpura* subspecies as being strongly associated with protection of a few low elevation sites. With these new understandings we recognize that maintenance of *E. ancilla* into an uncertain future requires conservation of populations utilizing three host species across a wide range of elevations and across the Spring Mountains. We need to conserve the eco-evolutionary dynamic, not the subspecies.

Our results indicate that conservation and management plans should focus on maintenance of both cohorts of *E. ancilla* as a partially connected, evolving set of subpopulations harboring a broad range of phenotypic and genetic variants, particularly with reference to life history phenology and adult eclosion. As stated by Crandall et al. (2000), an important goal of this conservation should be the preservation of the evolutionary dynamic, the ongoing processes of chronological divergence and gene flow as subpopulations track the flowering of three host plant varieties across elevational gradients in the Spring Mountains. Given their specialization on a single floral resource, and the ability of adults to find small clusters of flowering plants in a diffuse population of plants stretching from 1750 to 3000 m, habitat matching or biased gene flow is likely to enhance the genetic diversity and persistence of these sky-island endemics in the face of future climatic fluctuation if conservation is focused on preservation of the evolutionary dynamic not the taxonomic units.

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