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Still here after all these years: the persistence of the Uncompahgre fritillary butterfly

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Abstract The US federally endangered Uncompahgre fritillary butterfly (*Boloria acrocnema*) lives in isolated alpine habitats of the San Juan Mountains of Colorado, USA. Its apparent extirpation from the type locality and its low genetic diversity raised concern in the late 1980s, thus monitoring for this species has continued and genetic samples were collected in 2008 and 2009 from all but one known site, each on a separate peak. Data for five new microsatellite markers were collected from 316 total specimens, including 26 from wings preserved from 1987 and 1988 seasons. Only three main colonies had high enough sample sizes for adequate analyses. We estimated levels of genetic variability and structure, and effective population size. Despite low demographic numbers at these sites, the species has maintained relatively high heterozygosity ranging from 0.41 to 0.46 at three sites. Allelic richness corrected by sample size ranged from 5.3 to 5.9. Genetic structure assessed with non-spatially explicit methods indicated that despite separation on high mountain peaks, colonies were fairly well mixed, which is surprising for these weak fliers with very short growing and adult flight seasons. Estimates of effective population sizes were low, reflecting the life history and limited habitat range for the species. Comparisons at the site with historic and modern specimens revealed a consistent pattern in genetic indices. Our data suggest that the three focal butterfly colonies exist as a metapopulation that persists due to low-level migration between sites and "temporal leakage" via flexibility in development time in this biennial species.

Keywords Microsatellite · Genetic diversity · Conservation genetics

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Introduction

As Turlure et al. (2014) recently pointed out, sound management of species of conservation concern that exist as metapopulations of small isolated sub-populations requires detailed estimates of genetically effective population sizes, gene flow, inbreeding rates, and levels of genetic diversity. These genetic parameters are largely made using molecular markers such as co-dominant nuclear microsatellites (e.g., Watt et al. 2007; Saarinen et al. 2009; Turlure et al. 2014). Because of their comparative small size and short lifespans, individual insects are difficult to observe in space and time. For these reasons, conservation genetics techniques are particularly appealing for use in studying insects on isolated patches of habitat. Additionally, knowledge of key population genetic parameters, including effective population size and population connectivity through gene flow, is essential for some management actions such as population augmentations and re-introductions (e.g., Monroe and Britten 2014). We combined a study of current and past microsatellite variability with the results of an intensive, long-term monitoring program for an endangered alpine

butterfly. Here we discuss the persistence of a narrowly endemic alpine butterfly for which conservation genetics and decades of monitoring in the field suggest a metapopulation structure accompanied by two forms of genetic rescue over time. *Boloria acrocnema*, the Uncompahgre fritillary butterfly, was declared federally endangered a mere 16 years after its discovery (Gall and Sperling 1980) in the San Juan Mountains of Colorado, USA. *B. acrocnema* has very limited distribution in alpine habitat patches of snow willow (*Salix nivalis*) growing above 4,000 m on northeastern exposures. Snow willow serves as the larval host plant and larvae require 2 years to develop in the short growing seasons (Scott 1986). Adults are on the wing for about 3 weeks each summer but are weak fliers and are thus mostly philopatric, only moving distances within suitable habitat <50 m (Gall 1984).

Population estimates for the 1987–1988 flight seasons indicated continued demographic decline at two colonies where quantitative sampling was conducted and there was a lack of significant numbers flying at the type locality (Britten et al. 1994). Estimates of population size in 1987, 1990, and 1991 were zero at Uncompahgre Peak, the type locality. Genetic diversity as measured with 20 allozyme loci indicated less genetic diversity relative to sister species and other butterfly taxa (Britten et al. 1994). Furthermore, their results indicated alternate year cohorts were "leaky" enough to prevent divergence between cohorts and these "leakages" could rescue one cohort after a particularly low emergence year, which is important at the Uncompahgre colony that appeared to have a population crash in the early 1990s. Despite extensive searches for additional colonies over the last three decades, the numbers of locations where *B. acrocnema* colonies are known to exist are less than a dozen. Surveys in the late 1980s at 30 additional sites (Britten et al. 1994) resulted in only a few individual sightings at three locations.

Continued concern for the status of this species has resulted in implementation of its recovery plan that includes extensive annual population monitoring at three sites, each associated with a unique mountain peak (Uncompahgre Peak, Redcloud Peak and Site 3), colony persistence surveys at eight additional known sites, and a reassessment of genetic diversity with newer microsatellite markers. The goals of this study were to: (1) estimate the genetic structure and variability of the species within and among the three largest colonies, (2) to investigate historic changes in variability at one of these sites using samples collected in 1987 and 1988, and (3) to test for temporal structure between broods of this biennial species. Because *B. acrocnema* exists near the upper limit of vegetative growth in the San Juan and other nearby mountain ranges, it is particularly exposed to extirpations and potential extinction due to stochastic or deterministic events associated global climate change. Strong population genetic structuring and loss of genetic diversity are expected to precede losses of colonies at isolated sites that support the butterfly.

Materials and methods

Genetic sample collection and DNA extraction

Exact sample locations for previously unknown locations will not be provided for this study due to the threat collectors pose to an already endangered species, but samples were taken in southwestern Colorado, USA (thus we cannot include a map). At the end of field observations in 2008 and 2009 flight seasons, small (about 2 × 2 mm) wing clips were taken in the field from adult male butterflies from all known sites except one, placed into waxed envelopes, and shipped to USD where they were stored in a −20 °C freezer until extraction. Wing clips were collected from 12 alpine sites in the San Juan Mountains that included sub-sites at three largest colonies, Redcloud (2 sub-sites), Uncompaghre (4 sub-sites), and Site 3 (2 sub-sites) where there were disjunct patches of snow willow no more than 1800 m from neighboring occupied patches. We also had entire sets of wings collected at the same two sub-sites at Redcloud during the 1987 and 1988 flight seasons that had been stored in plastic laminate at room temperature.

To extract DNA from the 1987–1988 preserved samples, small pieces of wing were cut from the laminate and forceps were first used to pull apart the laminate and then to scrape wing tissue and glue off of the plastic. This resulted in a tiny sticky ball made of wing bits and glue, which were processed the same as the fresh wing clips. Samples were placed into individual 0.2-µl tubes and soaked in 35 µl of sterile deionized and demineralized water overnight at 4 °C. The next day, genomic DNA was isolated with the Zygem (Hamilton New Zealand) PrepGEM insect extraction kit following the manufacturer's instructions, except we doubled the incubation time. After extraction, the samples were spun down, and isolates were placed into a 96-well plate.

Microsatellite development and optimization

Lepidopteran taxa are known to be difficult to design primers for microsatellites (Meglecz et al. 2004), however, we were able to develop primers for five microsatellite loci from pooled DNA extracted from ten *B. acrocnema* specimens following the slightly modified subtractive hybridization procedures of Hamilton (1999) and Glenn and Schable (2005). We sequenced 192 clones and were

careful to choose repeat regions that were not shared among the markers and carefully checked flanking regions for similarity (Meglecz et al. 2004) before designing primers in Primer 3 software (Rozen and Skaletsky, 2000). Suggestions for primers by Primer 3 were then tweaked by hand and checked for melting temperatures and self-binding on IDT's oligo-analyzer website (http://www.idtdna.com/analyzer/applications/oligoanalyzer/) to maximize performance. Twelve potential markers were screened which resulted in the five markers we optimized for genotyping *B. acrocnema* with fluorescently labeled primers. Loci were amplified in 10-µl volumes of 5 µl of Qiagen (Valencia, CA) multiplex master mix, 1 µl of water, 2 µl of forward (4 µM labeled) and reverse (1 µM) primer mix, and 2 µl of DNA template. Reaction conditions were as follows: 15 min hot start at 95 °C followed by 30 cycles of 94 °C for 30 s, primer-specific annealing temperatures (Table 1) for 45 s, 72 °C for 45 s followed by 8 cycles of 94 °C for 30 s, second primer-specific annealing temperature (Table 1) for 45 s, and 72 °C for 45 s with a final extension at 72 °C for 10 min. PCR products were multiplexed and separated with high-resolution gel capillary electrophoresis on an Applied Biosystems 3500 genetic analyzer and scored in GeneMarker (SoftGenetics; State College, PA). Each plate contained PCR positive and negative control samples.

Genetic analyses

Genotypes were analyzed for the presence of null alleles (MICROCHECKER; van Oosterhout et al. 2006). Linkage disequilibrium and Hardy–Weinberg equilibrium (HWE; Guo and Thompson 1992) were checked with Arlequin 3.11 (Excoffier and Lischer 2010). The 2-year development for this species may result in genetically isolated cohorts in odd- and even-numbered years. To test for cohort differences between 2008 and 2009 in the three largest colonies at Redcloud, Uncompahgre and Site 3, we used analysis of molecular variance (AMOVA; Excoffier et al. 1992) and Nei's unbiased genetic identity (Nei 1972), both calculated in GenAlEx 6.4 (Peakall and Smouse 2006). Finally, we analyzed genetic structure for the three largest colonies with both cohorts combined where we used ten independent runs of 300,000 iterations after a 100,000 burn-in period in STRUCTURE, testing K from one to ten, using both the correlated and uncorrelated allele options. The delta-K value (Evanno et al. 2005) and STRUCTURE bar plots were used to infer the most biologically relevant number of populations.

Further analyses were conducted on colonies per their respective mountain peaks. Observed and expected heterozygosity values were calculated in GenAlEx (Peakall and Smouse 2006), we tested Hardy–Weinberg Equilibrium (HWE) with the method of Guo and Thompson (1992) in Arlequin 3.11, and allelic richness was calculated by rarefaction (Petit et al. 1998) in FSTAT (Goudet 1995). A hierarchical pattern of genetic structure for populations combined over both cohorts at the three main colonies was quantified with 1000 permutations in AMOVA using Arlequin 3.11. Bottlenecks, defined as zero observed adults over a flight season at a site, were directly observed at the Uncompahgre site in 1987, 1990, and 1991, so BOTTLENECK (Cornuet and Luikart 1996) was used to see if these population crashes could be detected with microsatellites. We advise caution in interpreting these results due to low power given our small sample sizes. Evidence for a

Table 1 Locus name, primer sequences, annealing temperatures (Ta), allele size range (basepairs) and repeat motif of five isolated microsatellite loci from *Boloria acrocnema*

Locus	Primer sequences (5′–3′)	Ta (°C)	Allele size range	Repeat motif[a]	Number of Alleles[b]
Ba122	F: ATTTGAGTTCATAACCCCC R: AGAACAATCCCGACTAATTCACC	54.5; 50.5	164–208	$(CT)_{10}(GT)_{21}$	15
Ba190	F: AACTCAGAGGTTCAATGTATGC R: GGTTCATCCGTTCGGGAGC	57.0; 53.0	168–190	$(GT)_{14}$	6
Ba133	F: TATCAGATTGTCAAAGG R: CCGCATTCGTCTCCATCAC	57.0; 53.0	192–206	$(AAG)_{10}$	5
Ba134a	F: AGGTAAAATAGCTTCATCGACTCC R: ACCAGTAGTTCCTGAGATTAACAC	60.0; 56.0	208–352	$(GTT)_{33}$	31
Ba134b	F: CCCCTGGTGAGTGGTCATC R: TACCCACGAAGGAACGGAG	60.0; 56.0	189–193	$(CAAA)_4(CA)_7$	3

Annealing temperatures are for the first set and second set of cycles in each thermal cycling protocol

[a] Repeat motif identified in sequenced clone

[b] Number of alleles for all genotyped (N = 316) *B. acrocnema*

bottleneck was tested using the stepwise and two-phase (TPM) mutation models in 100,000 replications, with parameters for the TPM estimation set at variance = 30 and probability = 10 %. Effective population size (Ne) for colonies in 2008 and 2009 on Redcloud, Uncompahgre, and the Site 3 peaks was estimated with the one-sample method of Tallmon et al. (2004a) in the online program ONeSAMP (Tallmon et al. 2008) using a maximum effective population size of 100. Ne estimates for 1987 and 1988 populations on Redcloud were also estimated in ONeSAMP. Other sampling sites did not yield enough individuals to employ this estimator with reasonable robustness, and our results should be interpreted with caution since we only have data for five loci, and at least ten are highly recommended (Tallmon et al. 2008).

Results

A total of 316 specimens from 12 alpine sites was genotyped at five microsatellite loci, only 15 individuals failed to amplify at only one locus each. These five loci varied in the total number of alleles discovered in all 316 specimens, ranging from 3 to 31 alleles (Table 1). Loci Ba122 and Ba134a were highly variable with 15 and 31 alleles in contrast to the other three loci with low numbers of alleles (Table 1). Of all the individuals that amplified, 26 were from the 1987 and 1988 cohorts and the rest were from recent 2008 and 2009 cohorts (Table 2). Only locus Ba133 in the Uncompahgre colony was diagnosed to have null alleles due to homozygous excess, there was no evidence for large allele drop-out, and because it is likely that our small and endangered populations could have homozygous excess, we used all five loci in further analyses. Linkage disequilibrium was significant in only ten out of 120 possible location-by-locus combinations but there was no consistent pattern across location-by-locus pairs and only three pairs of loci were linked in two of the 12 locations. Of these, one pair (Ba134a and Ba134b) was linked at two of the smaller colonies which were excluded from all further analyses, another pair (Ba133 and Ba134b) was linked at Uncompaghre and Site a, which is a small colony site that was excluded from further analyses and the third pair (Ba122 and Ba133) was linked at Uncompaghre and Site 3. Furthermore, Excoffier and Slatkin (1998) and Excoffier and Lischer (2010) note that *P* values of likelihood ratio tests for linkage disequilibrium are determined by the level of linkage between two loci and departure from Hardy–Weinberg equilibrium at either, or both, of the two loci estimated to be in linkage disequilibrium. Locus Ba134b showed significant deficiencies in heterozygotes in all three colonies (see below) suggesting that low heterozygosity, not linkage equilibrium alone, was responsible for this result. Given this, all loci were used in further analyses.

For all further analyses, only the three largest colonies with sample sizes ≥20 samples total from all sub-sites in either year were included, thus nine sites were excluded due to a lack of power (Table 2). These three largest colonies were sampled for both odd- and even-year cohorts, AMOVA results within and among cohorts were significant (all three colonies $p = 0.01$) but variation was partitioned mostly within cohorts (96 % Redcloud, 96 % Uncompahgre, and 97 % Site 3) rather than among cohorts and Nei's mean unbiased genetic identity (Nei 1972) over both cohorts were high (0.91 Redcloud, 0.98 Uncompahgre, and 1.02 Site 3). Allelic richness and heterozygosity levels were also very similar between cohorts (Table 3). Given this, we pooled data from 2008 and 2009 for further analyses in order to maximize sample sizes.

Overall cohorts and largest colonies, STRUCTURE analyses resulted in a delta K value of two (Fig. 1), and the assignment of individuals into one colony at Redcloud and Site 3 and a second at Uncompahgre. However, individuals from all three sites were assigned with a minimum of 20 % membership in one of the groups relative to the other, indicating a lack of structure corresponding to populations.

Thus, the rest of the analyses will be based on three colonies at each physical mountain peak, Redcloud, Uncompahgre, and Site 3. Hierarchical structure from AMOVA analyses was significant ($p = 0.00001$) and a majority of the variation was within sub-sites (95.7 %), with 2.5 % among sub-sites within a colony and only 1.8 % among main colonies. Pairwise Fst estimates among the three sites were: Redcloud/Uncompahgre = 0.017, Redcloud/Site 3 = 0.01, Uncompahgre/Site 3 = 0.016 and Fst among the three sites combined was 0.04. Allelic richness ranged from 2.0 to 20.8 alleles per locus, but in general it was similar at all three sites, except for locus Ba133 which was monomorphic at Redcloud (Tables 4, 5). The fixed allele at locus Ba133 at Redcloud was at high frequency at Uncompahgre (0.974) and Site 3 (0.890). Per locus, observed heterozygosity was fairly close to expected heterozygosity at all three sites, and mean values were also very similar, but observed means was always greater than expected means (Table 4). In 14 tests (one locus was monomorphic at one site) for conformity to HWE, only seven locus-by-location combinations were out of HWE, and this dropped to three locus-by-location combinations when significance was adjusted (Bonferroni, Rice 1989) for experiment-wide error rates. Locus Ba134b was out of HWE at all three locations.

There was no evidence in our microsatellite data of a bottleneck, using the sign test or Wilcoxon test for any of the three colonies, which is not surprising since we only

Table 2 Site names of *B. acrocnema* colonies in the San Juan Mountains, CO, USA where males were sampled for wing clips during even- and odd-year cohorts

Sampled colony name (sub-sites)	Even year sample size	Odd year sample size	H_E (± SE) for total sample
Uncompahgre (total)	71	25	0.43 (0.15)
Lower	18	14	na
Middle	30	5	na
Upper	15	5	na
Upper 6	8	1	na
Redcloud (total)	37	36	0.42 (0.16)
Upper	13	18	na
Lower	24	18	na
1987/88 Redcloud (upper)	7	19	0.40 (0.10)
Site 3	27	19	0.47 (0.14)
Site a	2	10	0.53 (0.11)
Site b	3	9	0.46(0.14)
Site c	9	6	0.51 (0.13)
Site d	6	6	0.48 (0.12)
Site e	4	9	0.55 (0.11)
Site f	2	0	na
Site g	2	0	0.27 (0.17)
Site h	3	0	na
Site i	4	0	na

Location names, other than two already well-known sites, are coded to protect *B. acrocnema* from poachers. Sample sizes reflect successful amplification at five microsatellite loci (25 specimens failed to amplify at one locus). H_E is unbiased heterozygosity averaged over all loci (calculated in GenAlEx 6.4.1), not reported for sites with less than ten individuals over both years. Minor sites designated by letters were not included in analyses due to small sample sizes

Table 3 Mean allelic richness and mean unbiased expected heterozygosity of five loci over all sub-populations at three main colonies for each cohort

	Redcloud	Uncompahgre	Site 3
2008 Allelic richness (±SE)	5.7 (2.7)	5.3 (2.8)	5.9 (2.2)
2009 Allelic richness (±SE)	5.8 (3.3)	5.8 (2.8)	5.6 (2.4)
2008 Heterozygosity (±SE)	0.41 (0.16)	0.42 (0.16)	0.45 (0.14)
2009 Heterozygosity (±SE)	0.41 (0.16)	0.46 (0.14)	0.46 (0.13)

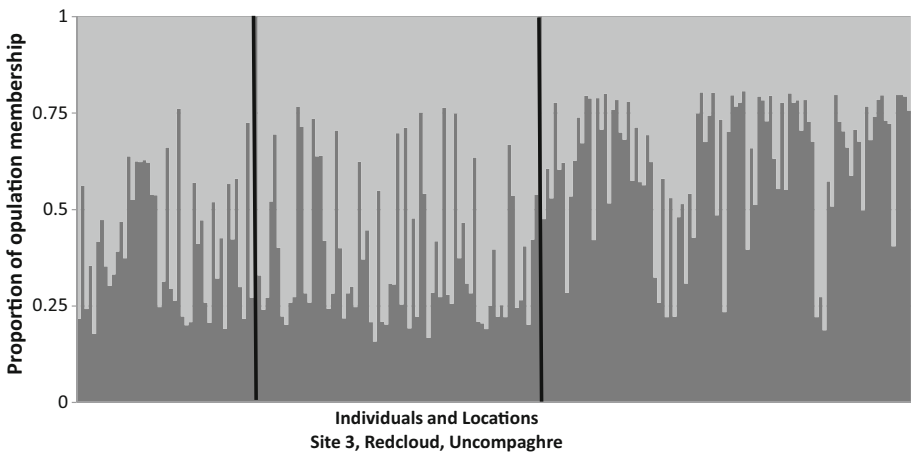

Fig. 1 Population probability of membership in K = 2 colonies analyzed over both years of *Boloria acrocnema* from the three largest colonies at Site 3, Redcloud and Uncompahgre peaks. *Vertical black lines* separate individuals from each corresponding sample site. Samples were analyzed as in text in STRUCTURE 2.2.3 (Pritchard et al. 2000)

Table 4 Sample size (N), allelic richness (AR), observed heterozygosity (Ho), unbiased expected heterozygosity (He) for all loci calculated over 2008–2009 cohorts in colonies of *Boloria acrocnema*

Colony	Locus	N	AR	Ho	He	HWE
Uncompahgre	Ba122	96	5.1	0.25	0.25	0.16
	Ba190	93	2.0	0.41	0.35	0.14
	Ba133	96	2.6	0.03	0.05	0.02*
	Ba134a	94	19.8	0.97	0.94	0.56
	Ba134b	96	3.0	0.93	0.59	0.00**
Mean (±SE)		95	6.5 (3.4)	0.52 (0.19)	0.44 (0.15)	na
Redcloud	Ba122	73	6.1	0.36	0.35	0.17
	Ba190	73	2.0	0.21	0.21	1.00
	Ba133	73	1.0	0.00	0.00	monomorphic
	Ba134a	69	18.0	0.87	0.91	0.05*
	Ba134b	72	3.0	0.96	0.62	0.00**
Mean (±SE)		72	6.0 (3.1)	0.48 (0.19)	0.42 (0.16)	na
Site 3	Ba122	46	8.9	0.39	0.40	0.03*
	Ba190	45	3.0	0.13	0.22	0.01*
	Ba133	44	5.0	0.18	0.19	0.18
	Ba134a	46	20.8	0.96	0.93	0.63
	Ba134b	44	3.0	0.82	0.58	0.00**
Mean (±SE)		45	8.1 (3.3)	0.50 (0.17)	0.46 (0.14)	na
Grand mean (±SE)		71	7.6 (3.9)	0.50 (0.10)	0.44 (0.08)	na

HWE is the *p* value of the exact test using Markov chain with 1000 dememorization steps (Guo and Thompson 1992)

* is significant at $\alpha = 0.05$

** still significant after Bonferroni adjustment for multiple comparisons (0.0035)

used 5 loci, and a minimum of 20 is recommended (Cornuet and Luikart 1996). Effective population size estimates are very low, ranging from 12 to 52, with 95 % CI 10-116 in 2008–2009 (Table 6). Estimates for the 1987–1988 cohorts at Redcloud are within the same range at 33 and 52 respectively (Table 5).

Discussion

Despite predictions made in an earlier population genetics study of the Uncompahgre fritillary (Britten et al. 1994), the butterfly remains extant in the San Juan Mountains. The microsatellite data, though based on a small number of loci, suggest that the species has maintained moderate levels of genetic variability over time and that the three sites where it is most abundant are somewhat genetically connected to each other.

Current population genetic variability and ecology

Britten et al. (1994) concluded that while the biannual life cycle of the Uncompahgre fritillary may be an important demographic buffer against extirpation at any given site, even- and odd-year broods experience enough "genetic leakage" between them (e.g., even-year larvae taking 1 or

Table 5 Sample size (N), allelic richness (AR), observed heterozygosity (Ho), and unbiased expected heterozygosity (He) for all loci calculated over the 1987–1988 cohorts in colonies of *Boloria acrocnema* at Redcloud

Colony	Locus	N	AR	Ho	He	HWE
Redcloud	Ba122	26	6.0	0.31	0.31	0.99
	Ba190	27	3.0	0.74	0.14	0.01*
	Ba133	27	1.0	0.00	0.00	monomorphic
	Ba134a	27	13.8	0.74	0.86	0.50
	Ba134b	26	3.0	0.73	0.52	0.04*
Mean (±SE)			5.4 (2.27)	0.37 (0.16)	0.37 (0.15)	na

HWE is the *p* value of the exact test using Markov chain with 1000 dememorization steps (Guo and Thompson 1992)

* is significant at $\alpha = 0.05$, none of them were still significant after Bonferroni adjustment for multiple comparisons (0.01)

Table 6 Effective population (N_e) estimates for cohorts at the three largest colonies as estimated by ONeSAMP (Tallmon et al. 2004a, 2008)

Colony	Cohort	Sample size	Ne estimate	Lower 95 % CI	Upper 95 % CI
Uncompahgre	2008	71	51	34	93
	2009	25	31	23	41
Redcloud	1987	20	33	20	69
	1988	26	52	32	116
	2008	39	49	33	85
	2009	36	31	23	45
Site 3	2008	27	20	15	28
	2009	19	12	10	16

3 years to develop and emerging as adults in the odd year) to eliminate any temporal genetic structure at each site. Our microsatellite data corroborate this conclusion. There may be an optimum rate of genetic leakage between broods that mitigates loss of genetic diversity due to genetic drift in each brood while allowing enough demographic independence between them to allow the extirpation of one brood, which could be re-established later, while not resulting in the loss of the entire colony at the site. This plasticity in development time is probably a large factor in the ability of the species to maintain even a modest level of genetic diversity over time and to persist in its alpine habitat. Monroe and Britten (2014) detected a similar phenomenon in another endangered insect, the Hine's emerald dragonfly (*Somatochlora hineana*), that can take up to 5 years to develop from egg to adult. Temporal rescue in the form of leakage and "genetic rescue" (Tallmon et al. 2004b) through migration from other locations are likely what have allowed the Uncompahgre colony to persist from 1988 to 2009 despite not being detected in 1987, 1990, and 1991. However, flight season has since been determined to be highly variable between years ranging from early June to mid-August (K. Alexander, unpublished data), which means that emergence could have been missed in 1987, 90, and 91, since there was no genetic bottleneck detected with our analyses.

Genetically effective population sizes (N_e) for the 2008 and 2009 cohorts at our three focal sites are cause for concern for the persistence of the species and are on the low end of the range of N_es reported for other endangered insects (see Monroe and Britten 2015, Turlure et al. 2014). However, the trend for the Uncompahgre colony is encouraging; estimates of both genetic diversity and N_e suggest that genetic diversity at this site rebounded after at least three seasons where there were no observed adults 1987–2009. At all three of our sites the even-year brood has a larger N_e than the odd-year although there is broad overlap in the confidence intervals for the N_e estimates.

Recent population genetic structure

Our results indicate that the three largest colonies of the Uncompahgre fritillary, each existing as two to four nearby sub-populations, are somewhat connected by gene flow. STRUCTURE assignment probabilities suggest high admixture between the colonies. These results are not consistent with the insular distribution of the butterfly's habitat and the ecological diversity caused by the strong topographic relief between occupied sites. But they are consistent with our AMOVA results and pairwise Fst estimates which both suggest considerable gene flow among the three sites. Turlure et al. (2014) found a similar pattern when comparing estimated population differentiation with STRUCTURE to F-statistics for the threatened cranberry fritillary (*Boloria aquilonaris*). It is conceivable that between-site gene flow could occur given that the occupied sites are 20–25 km straight line distance apart and that suitable intermediate sites could act as stepping-stone habitat patches. The Uncompahgre fritillary is a weak flier but may be capable of occasional movements of this magnitude. Accidental colonization may also account for some of the small numbers of butterflies that occur at other sites in the area (Table 2). Survey results for more than a decade in the area suggest that these may be transient occurrences that do not establish persistent populations (K. Alexander, unpubl data). In addition to the genetic and demographic rescue afforded by temporal leakage between broods, we suggest that low-level gene flow among populations may contribute to long-term persistence of the species. In their review of the concept of genetic rescue, Tallmon et al. (2004b), conclude that genetic rescue through gene flow in space and the resulting maintenance of heterosis are enhanced in small, somewhat genetically isolated subpopulations within a metapopulation that does not experience strong selection. These appear to be the circumstances under which *B. acrocnema* has persisted over decades. It appears likely that the Uncompahgre

colony has persisted despite documented extirpations due to both forms of rescue: leakage from off-year broods and migration into the site. Similar processes are likely occurring at our other two focal sites, but have gone undetected because our long-term monitoring has not documented colony extirpations in either. Similarly, the minor sites listed in Table 2 may receive occasional migrants, but these are unable to establish self-sustaining colonies.

Historic genetic data

Comparison between the 1987/88 and the 2008/09 Redcloud population data are instructive regarding the capacity of the Uncompahgre fritillary for maintaining its genetic diversity over time. Our genetically effective population size estimates are similar for both time periods suggesting that the species can maintain its genetic diversity over time. Our genetic variability estimates also indicate that this colony can maintain its genetic variability on the order of decades. Interestingly, the Redcloud colony remained monomorphic for the same allele at one of our five loci (Ba133) over the intervening 22 years between samples. The Uncompahgre and Site 3 colonies had three and five alleles, respectively, segregating at this locus in the 2008/2009 samples. Both the Uncompahgre and Site 3 colonies maintained the shared alleles at Ba133 with the Redcloud population at high frequencies, somewhat mitigating the monomorphy at this locus on Redcloud as evidence for a complete lack of gene flow among the three colonies. That is, because it was at high frequency in all three colonies, the common allele had the highest probability of flowing into the Redcloud population from the other two where it would not be detected as an allele brought in by immigrants to Redcloud.

Conservation implications

Our historic and current microsatellite and long-term monitoring data suggest that the Uncompahgre fritillary has persisted because of two forms of rescue: temporal rescue via developmental leakage between broods and more traditional spatial rescue through immigration (Brown and Kodric-Brown 1977; Tallmon et al. 2004b). These processes would allow *B. acrocnema* to re-establish after extirpation at the Uncompahgre site and to maintain genetic diversity for $\sim$20 years at Redcloud. The level of genetic diversity at Site 3 is also explained by these mechanisms. Occasional detection without persistence at other sites is consistent with this hypothesis. This interpretation leads to three important implications for the management of Uncompahgre fritillary colonies and their habitat. First, it appears to be imperative that all three colonies remain extant. The flow of individuals and genes between sites, even if at low levels, is critical to the persistence of this small metapopulation. Second, the Uncompahgre Peak colony may at times function as a population sink while at other times, it may be self-sustaining for some period. The Uncompahgre site is the only one of the three that is impacted by an annual sheep drive in the area which perhaps accounts for impacts to the population dynamics and genetics of this population. Finally, the role of butterflies on intermittently occupied sites in terms of metapopulation dynamics is unknown. It would be prudent; however, to protect these sites from significant human impacts at least until the potential role of the individuals at the occasionally occupied sites is better understood. While direct anthropogenic threats to the species (e.g., collecting, trampling, and livestock grazing) have been mitigated to some extent, the potential effects of global climate change have not been directly addressed. Continuation of the current monitoring program and additional population genetics surveys are recommended for this species.

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References

Britten HB, Brussard PF, Murphy DD (1994) The pending extinction of the Uncompahgre fritillary butterfly. Conserv Biol 8:86–94

Brown JH, Kodric-Brown A (1977) Turnover rates in insular biogeography: effect of immigration on extinction. Ecology 58:445–449

Cornuet JM, Luikart G (1996) Description and power analysis of two test for detecting recent population bottlenecks from allele frequency data. Genetics 144:2001–2014

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14:2611–2620

Excoffier L, Lischer HE (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Res 10:564–567

Excoffier L, Slatkin M (1998) Incorporating genotypes of relatives into a test of linkage disequilibrium. Am J Hum Genet 62:171–180

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491

Gall, LF, Sperling FAH (1980) A new high altitude species of *Boloria* from southwestern Colorado (Nyphalidae), with a discussion of phenetics and hierarchical decisions. J Lepid Soc 34:230–252

Gall LF (1984) Population structure and recommendations for conservation of the narrowly endemic alpine butterfly, *Boloria acrocnema* (Lepidoptera:Nymphalidae). Biol Conserv 28:1–28

Glenn TC, Schable NA (2005) Isolating microsatellite DNA loci. Method Enzymol 395:202–222

Goudet J (1995) FSTAT (Version 1.2): a computer program to calculate F-statistics. J Hered 86:485–486

Guo SW, Thompson EA (1992) Performing the exact test of Hardy–Weinberg proportion for multiple alleles. Biometrics 48:361–372

Hamilton MB (1999) Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. Mol Ecol 8:521–523

Meglecz E, Petenian F, Danchin E, D'Acier AC, Rasplus JY, Faure E (2004) High similarity between flanking regions of different microsatellites detected within each of two species of Lepidoptera*: Parnassius apollo* and *Euphydryas aurinia*. Mol Ecol 13:1693–1700

Monroe EM, Britten HB (2014) Conservation in Hine's sight: the conservation genetics of the federally endangered Hine's emerald dragonfly, *Somatochlora hineana*. J Insect Conserv 18:353–363

Monroe EM, Britten HB (2015) Single-sample estimation of effective population size in several populations of the endangered Hine's emerald dragonfly. J Freshwater Sci 34:1058–1064

Nei M (1972) Genetic distance between populations. Am Nat 106:283–291

Peakall R, Smouse PE (2006) GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6:288–295

Petit RJ, Mousadik EL, Pons O (1998) Identifying populations for conservation on the basis of genetic markers. Conserv Biol 12:844–855

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155:945–959

Rice WR (1989) Analyzing tables of statistical tests. Evol 43:223–225

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, pp 365–386. http://fokker.wi.mit.edu/primer3/

Saarinen EV, Austin JD, Daniels JC (2009) Genetic estimates of contemporary effective population size in an endangered butterfly indicate a possible role for genetic compensation. Evol Apps 3:28–39

Scott JA (1986) The butterflies of North America. Stanford University Press, Stanford p 583

Tallmon DA, Luikart G, Beaumont MA (2004a) A comparative evaluation of a new effective population size estimator based on approximate Bayesian computation. Genetics 167:977–988

Tallmon DA, Luikart G, Waples RS (2004b) The alluring simplicity and complex reality of genetic rescue. TREE 19:489–496

Tallmon DA, Koyuk A, Luikart G, Beaumont MA (2008) ONeSAMP: a program to estimate effective population size using approximate Bayesian computation. Mol Ecol Res 8:299–301

Turlure C, Vandewoestijne S, Baquette M (2014) Conservation genetics of a threatened butterfly: comparison of allozyme, RAPDs and microsatellites. BMC Gen 15:114–124

van Oosterhout C, Weetman D, Hutchinson WF (2006) Estimation and adjustment of microsatellite null alleles in non-equilibrium populations. Mol Ecol Notes 6:255–256

Watt PC, Saccheri IS, SJ Kemp, Thompson DJ (2007) Effective population sizes and migration rates in fragmented populations of an endangered insect (*Coenagrion mercuraile*: Odonata). J An Ecol 76:790–800