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Contrasting Patterns of Population Genetic Structure in Two Great Basin Stoneflies

Kimberly G. Arnaldi
Stetson University

Allyson M. Fenwick
Stetson University

Andrew L. Sheldon
University of Montana

Alicia A. Slater
Stetson University

Introduction

Phylogeography is the study of processes that govern the geographic distribution of genetic variation (Avise 1998). Similar patterns of genetic structure (concordance) in multiple species suggest large, landscape- or climate-level effects. These patterns can help identify hotspots of genetic diversity as well as major drivers of genetic differentiation. Information gathered from genetic analysis can be used in a predictive framework.

In this current study, we examined patterns of genetic differentiation in 'sky island' populations of stream-dwelling insects. Sky islands are mountains isolated by surrounding valleys with different environmental conditions. They are not speciose but are often home to many endemic species (Taubmann et al. 2011; Warshall 1999). Historical or contemporary processes can shape the genetic structure of sky island populations. Historical processes include climatic oscillations that can cause elevational shifts in climate and montane vegetation (Brunsfield et al. 2001). Periods of connectivity and potential gene flow across islands occur when the weather is cooler and wetter and montane vegetation occurs at lower elevations (Waltari and Guralnick 2009). In contrast, periods of isolation occur during warmer and drier periods when vegetation retreats up the mountain; this may restrict gene flow among montane populations and allow for genetic divergence and speciation (Hewitt 2004).

The Great Basin (GB) is an internally drained complex encompassing sky islands and intermontane basins with no outlet to the ocean (Grayson 2011). It lies in western North America and is bordered by the Sierra Nevada to the west and the Rocky Mountains to the east. The GB is well studied, has a well-preserved geological record, and is a hotspot of endemism. Its lake levels have fluctuated, and vegetation has shifted due to climatic oscillations (Grayson 2011). Climatic oscillations and changes in moisture and vegetation patterns through time are expected to have a strong effect on animals that are habitat specialists, particularly those that require a cool and wet habitat.

Stoneflies such as *Hesperoperla pacifica* (Plecoptera:Perlidae) are well suited to the study of population genetic differentiation and species distributions among GB sky islands. Stoneflies have low dispersal tendencies (Nelson 1994), and their life cycle includes an aquatic larval stage and an adult terrestrial stage (Merritt et al. 2008). Adult stoneflies are thought to stay in the same locality even if the environment is favorable for dispersal (Hughes et al. 1999). *Hesperoperla pacifica* is a large, carnivorous stonefly (Allan et al. 1987). Nymphs live in riffles and rocky, boulder-strewn areas with a moderate to fast current. They have a life span of two or three weeks as adults (Nebeker 1971). They are called 'golden stoneflies' because of their yellow-brown color. *Hesperoperla pacifica* is widely distributed from California and New Mexico to Alaska and across the Great Basin. They are not pests and are predators of such other insects in streams as mayflies (Ephemeroptera), midges (Chironomidae), and caddisflies (Trichoptera) (Richardson and Gaufin 1971).

A previous study by Schultheis et al. (2012) found high levels of genetic structure among sky island populations of the stonefly *Doroneuria baumanni* that were particularly pronounced in regions separated by low valleys, even in populations separated by as little as 16 km (Figure 1). They found three clades (Jackson, Western, and Eastern). The groupings shown in Figure 1 represent our a priori hypothesis of genetic structure based on these results from *D. baumanni*. The focus of the current study was to identify whether we find the same geographic pattern in a confamilial species, the stonefly *H. pacifica*, which is known to have a more widespread

distribution. It is still found in cool mountain streams, but it is not restricted to upper elevations like *D. baumanni*.

Because *H. pacifica* is a habitat generalist that occurs at lower elevations, we predicted that it would not be as sensitive to warm climates as *D. baumanni* and would thus experience longer periods of population connectivity. The separation of the sky islands would still lead to genetic differentiation, but we predicted there would be more gene flow among populations of *H. pacifica*, and thus lower levels of genetic differentiation, than were seen in *D. baumanni* (Figure 1). We also predicted that *H. pacifica* populations diverged sometime during the Pleistocene (2.5 million to 12 thousand years ago) but more recently than those of *D. baumanni*. Finding a similar divergence time in a different species would suggest that historical processes shaped the distribution of a number of species in the region.

Experimental Methods and Procedures

Sampling

Hesperoperla pacifica larvae were collected by kickscreen in June 2002 (SR, PF, JK, HM, TY, SC, JB) and June 2009 (DC, RM, DM, SO, and SS) (Range names are associated with initialisms in Table 1; Figure 1). All larvae were preserved in 95% ethanol at -80°C.

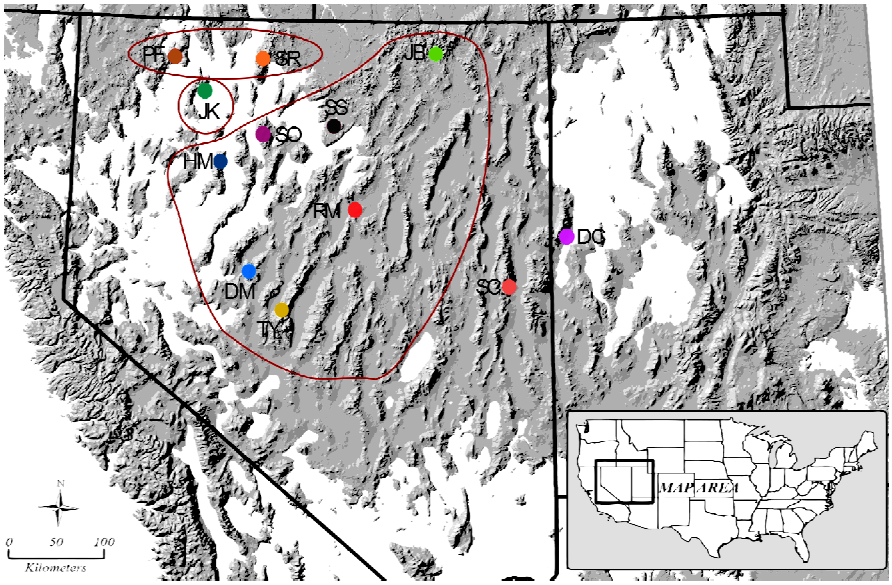

Figure 1: Great Basin region. White indicates elevations less than 1500 m above sea level. Labels are as in Table 1. Groupings represent our a priori hypothesis of genetic structure based on the results from *D. baumanni* (adapted from Figure 1 in Schultheis et al. 2012, reprinted by permission).

Molecular methods

Genomic DNA was isolated using the PureGene kit (Gentra Systems). The primers cyt-*b* 728F (5'- GGA CGA GGG ATG TAT TAC GG-3') and cyt-*b* 745R (5'- AGG GGT CTT CAA CAG GTC GT-3') from Kauwe et al. (2004) amplified an 867 bp fragment of the mitochondrial

cytochrome *b* gene. Gene fragments were amplified in 12.5 μL reactions, which contained 2.5 μL 5X PCR buffer (Promega), 0.875 μL 25 mM $MgCl_2$ (Promega), 0.5 μL 8 mM dNTPs (Promega), 0.5 μL 10 mM primers, and 0.4 μL *Taq* Polymerase (Promega). The temperature regime for amplification of the cyt-*b* gene was an initial denaturation of 94°C for 3 min, followed by 34 cycles of 1 min at 94°C, 1 min annealing temperature of 54°C and 1.5 min at 72°C. A final extension step of 7 min at 72°C ended the regime. PCR products were checked on a 1% agarose gel stained with ethidium bromide and visualized with a UV light source. PCR products were purified using the ExoSAP procedure (Pandey et al. 2013) and were sent to Operon Technologies in Huntsville, Alabama for sequencing. All PCR products were sequenced in both directions. Sequences were edited and aligned in Geneious 6.1.5 which gave us a consensus sequence for each individual sequence after having checked for errors. Sequences were trimmed in Geneious so that all individuals were represented, yielding a final data matrix of 568 bp.

Phylogeny and Divergence time estimates

Evolutionary models and partitioning strategies were evaluated with Kakusan4 (vers. 4.0.2012.12.14; Tanabe 2011), which chose a single partition and the GTR model for the data under the AICc4 criterion. We estimated phylogenetic relationships using MrBayes (vers. 3.2.2; Ronquist et al. 2012), with 1 million generations, discarding the first 100,000 generations as burnin. We used Tracer (vers. 1.5; Rambaut et al. 2013) to verify that the Markov chain had reached stationarity before the burnin period and that the posterior distribution was appropriately sampled.

We estimated within and among clade divergence times in BEAST (vers. 1.5.4; Drummond & Rambaut 2007). For each population, we used the HKY+ Γ model with an uncorrelated relaxed lognormal clock to estimate divergence times among populations. This model was selected because confidence intervals in preliminary analyses were wide. We were able to narrow them somewhat with this model, because it has the fewest states to estimate parameters. Divergence times were estimated using two independent runs that sampled every 10,000th generation for 100 million generations. We used a lognormal prior distribution with a calibration point based on estimated time of speciation between *H. pacifica* and *D. theodora* of 2–5 million years before present (ybp), following Carstens et al. (2005). Thus, priors for the time to most recent common ancestor (tMRCA) for *H. pacifica* and *D. theodora* were set at a median of 3.5 and standard deviation of 0.17; this yielded a 95% credible interval between 2 and 5 million ybp. The tree prior used for divergence dating was the coalescent / constant size. For the ucld.mean parameter, the following priors were used: mean = 0.125, SD = 1.1, and offset = 0; this yielded a rate range of 0.5–5.4 substitutions per site per million years, which represented a wide range of known substitution rates for insect mtDNA genes. The outgroup used to root the tree was *Doroneuria theodora*. LOGCOMBINER (vers. 1.5.4) was used to combine the trace and tree files, and estimates of median time to most recent common ancestor were obtained from the combined file. For comparison of divergence time estimates with *D. baumanni*, populations were grouped into the Eastern (HM, TY, JB, DC, DM, RM, SC, SO, SS), Western (PF, SR), or Jackson (JK) group.

Population genetic analysis

Levels of genetic differentiation within and among mountain ranges (*F* and *Φ* statistics) were determined by an AMOVA analysis implemented in ARLEQUIN (vers. 3.11; Excoffier et al. 2005) using 16,000 permutations in the Tamura-Nei model of nucleotide substitution. The AMOVA yielded F_{ST}, Φ_{ST}, number of migrants (*Nm*), and exact test of sample differentiation estimates. F_{ST} values were calculated using haplotype frequency only, whereas the Φ_{ST} calculations included both haplotype frequency and sequence divergence (a measure of how different two sequences are). Due to the low level of support for groupings in the Bayesian tree (a grouping that includes a common ancestor and all the descendants of that ancestor; see Figure 2), we used the geographic populations (mountain ranges = populations) for genetic structure testing in ARLEQUIN. A haplotype network was created in HAPSTAR (vers. 0.5), using minimum spanning network data generated in ARLEQUIN. The network shows how the haplotypes (unique DNA sequences) are connected to each other.

Table 1: Collection locations, codes, sample sizes, and haplotypes for *H. pacifica* from the Great Basin, Nevada, and Utah. Numbered haplotypes represent 568 base pairs of cyt *b.*

Location	Latitude, Longitude	N	Haplotype (s)	*D.baumanni* clade
Pine Forest Range (PF)				
Big Creek, Humboldt Co., NV	41.67, -118.67	10	2	W
Santa Rosa Range (SR)				
Rebel Creek, Humboldt Co., NV	41.60, -117.75	4	2, 7	W
Jackson Mountains (JK)				
Bottle Creek, Humboldt Co., NV	41.32, -118.32	3	1	JK
Humboldt Range (HM)				
Star Creek, Humboldt Co., NV	40.55, -118.11	1	1	E
Toiyabe Range (TY)				
South Twin River, Nye Co., NV	38.53, -117.15	2	5, 7	E
Jarbidge Mountains (JB)				
Jarbidge River, Elko Co., NV	41.81, -115.41	9	5, 7	E
Desatoya Mountains (DM)				
Big Den Creek,Churchill Co., NV	39.43, -117.67	4	5	E
Roberts Mountains (RM)				
Dry Creek, Eureka Co., NV	39.92, -116.29	5	3,4	E
Sonoma Range (SO)				
Sonoma Creek, Pershing Co., NV	40.85, -117.66	3	1, 8, 9	E
Snowstorm Mountain (SS)				
Frazier Creek, Elko Co., NV	41.33, -116.97	3	5, 6	E
Deep Creek Mountains (DC)				
Trout Creek, Juab Co., UT	39.85, -113.91	5	1, 5, 10, 11	-
Schell Creek Range (SC)				
Cleve Creek, White Pine Co., NV	39.33, -114.72	8	5	-

Results

Phylogeny and Divergence time estimates

The tree yielded a single clade that was not geographically distinct, with the exception of Roberts Mountains and Snowstorm Mountain (Figure 2), both of which showed strong support (posterior probability 0.97).

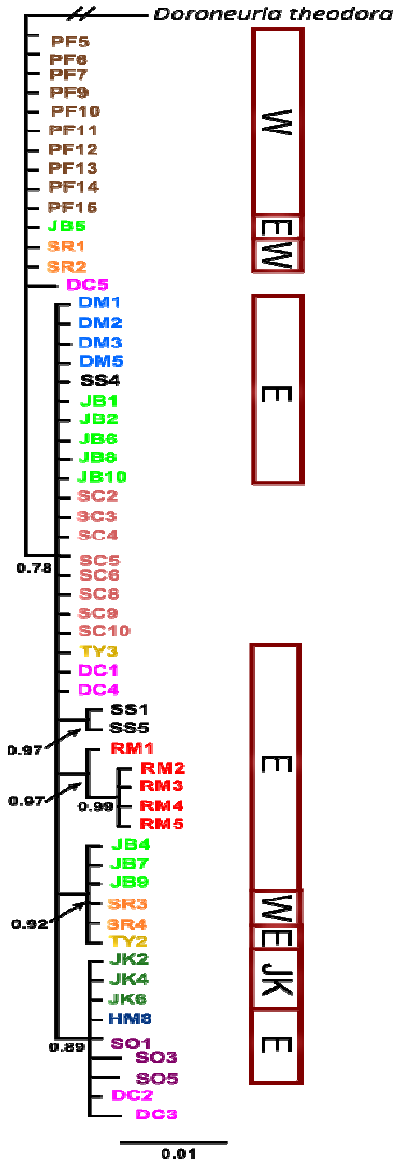

Figure 2: Gene tree for Great Basin *H. pacifica* populations inferred using 568 bp of cyt *b* mtDNA and Bayesian inference. Numbers below nodes indicate Bayesian posterior probabilities greater than 0.5. Location codes are as in Table 1. Colors indicate the population of origin as in Figure 1. Clade groupings of populations correspond to clades identified for *D. baumanni*. These

served as our a priori hypotheses for clades in *H. pacifica*: W = western, E = eastern, and JK = Jackson. Populations that do not have a corresponding clade were not sampled for *D. baumanni*.

The divergence times among ranges in *H. pacifica* populations are more recent than previously found in *D. baumanni* (Table 2b). This can be seen in more detail through within-clade estimates: the Western clade is represented by Pine Forest and Santa Rosa populations, and all appear to have diverged more recently in *H. pacifica*. The Jackson clade diverged most recently in *H. pacifica*, a pattern similar to that of *D. baumanni* (Table 2a). In addition, the divergence time ranges for both species are overlapping.

Table 2: **(a)** Mean within and overall clade (based on *D. baumanni* clades) divergence time estimates in years before present calculated in BEAST vers. 1.8.0. Parentheses indicate 95% confidence intervals. The Eastern clade for *H. pacifica* includes Jarbidge, Humboldt, Snowstorm, Roberts Mountains, Sonoma, Toiyabe, Deep Creek, Schell Creek, and Desatoya Mountains; The Western clade includes Pine Forest and Santa Rosa. Numbers reported are for *D. baumanni* from Table 3 in Schultheis et al. (2012). **(b)** Overall estimates.

(a) within-clade			
	E	W	JK
D. baumanni	183,000 (63,655-325,800)	144,000 (36,546-289,300)	59,830 (7,761-139,700)
H. pacifica	67,525 (1,147-231,800)	73,150 (30,000-197,700)	37,300 (1,019-56,200)
(b) overall			
D. baumanni	642,200 (107,300-1,815,000)		
H. pacifica	547,400 (52,900-1,400,000)		

Population genetic analysis

We collected data for a total of 57 individuals from 12 populations. The final alignment was 568 bp; eleven unique haplotypes (DNA sequences) were found (Figure 3). Snowstorm, Roberts, Deep Creek, and Sonoma all had some unique haplotypes. Four haplotypes were shared across multiple populations, including some that were very far geographically, such as JK and DC that are 525 km apart.

The AMOVA analysis suggested that there was significant genetic structure among populations ($\Phi_{ST} = 0.63$, $P < 0.01$ and $F_{ST} = 0.39$, $P < 0.01$). In *H. pacifica,* five out of 66 (8%) pairwise Φ_{ST} values were significantly different from 0 (with a Bonferroni correction of $P = 0.05/66 = 0.007$); these ranged from 0.59 to 1 (Table 3). Significant comparisons included the PF, DC, JB, RM, and SC populations.

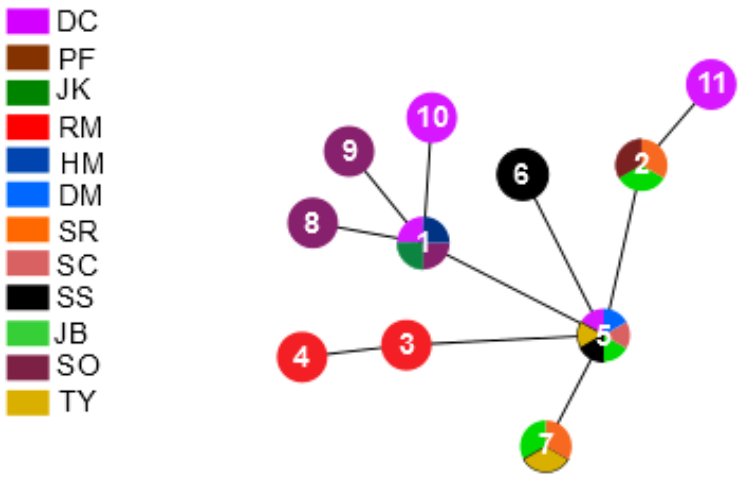

Figure 3: *H. pacifica* haplotype network of 11 haplotypes of 568 bp of the cyt *b* gene created in HAPSTAR (vers. 0.5.) n = 57. Numbers indicate the haplotype number. Connections between circles indicate a single base pair change between sequences.

Table 3: Above diagonal, pairwise Φ_{ST} values (16,000 permutations, Tamura and Nei distance method). Below diagonal, Slatkin's linearized N_m values. Bold numbers indicate a significant result in the permutation test ($P < 0.007$). Asterisks indicate significant results from the exact test ($P < 0.007$).

	DC	DM	HM	JB	JK	PF	RM	SC	SO	SR	SS	TY
DC	-	0.04	-0.50	0.15	0.12	**0.59***	0.61	0.21	0.14	0.12	0.20	-0.03
DM	10.88	-	1.00	0.05	1.00	1.00	0.87	0.00	0.67	0.33	0.58	0.38
HM	∞	0.00	-	0.50	0.00	1.00	0.86	1.00	-1.00	0.33	0.60	0.33
JB	2.83	9.23	0.50	-	0.63	**0.72***	**0.73**	0.17	0.58	0.02	0.37	-0.34
JK	3.78	0.00	∞	0.29	-	1.00	0.91	1.00	0.00	0.61	0.80	0.77
PF	0.35	0.00	0.00	0.20	0.00	-	**0.96***	**1.00***	0.90	0.57	0.92	0.92
RM	0.32	0.07	0.08	0.18	0.05	0.02	-	0.92	0.79	0.71	0.80	0.77
SC	1.90	∞	0.00	2.48	0.00	0.00	0.04	-	0.80	0.51	0.73	0.63
SO	3.10	0.25	∞	0.36	∞	0.06	0.13	0.13	-	0.50	0.57	0.44
SR	3.64	1.00	1.00	24.48	0.32	0.38	0.20	0.47	0.50	-	0.37	-0.21
SS	2.06	0.36	0.33	0.87	0.12	0.04	0.12	0.18	0.37	0.84	-	0.32
TY	∞	0.80	1.00	∞	0.15	0.04	0.15	0.30	0.63	∞	1.05	-

Discussion

Our results support the hypothesis that historical and contemporary processes have helped shape the genetic structure of *H. pacifica* populations in the Great Basin. Our hypothesis predicting lower levels of genetic differentiation of *H. pacifica* populations was supported. The Snowstorm, Roberts, Sonoma, and Deep Creek populations were genetically differentiated populations. The other populations had low Φ_{st} values, which suggests there is some dispersal and contemporary gene flow among them. In contrast, *D. baumanni* populations had much

higher overall and pairwise F_{ST} and Φst values; this suggests genetic isolation with little dispersal among populations.

For *D. baumanni*, there were approximately 30 different haplotypes found in a similar geographic area with slightly more samples (n = 90) (Figure 3 in Schultheis et al. 2012). The haplotypes grouped into three major clades that were separated by many mutational steps (Figure 5 in Schultheis et al. 2012). *Hesperoperla pacifica* shows a much different pattern; several haplotypes were shared across a broad region.Populations that were quite distinct genetically in *D. baumanni* shared haplotypes in *H. pacifica* (e.g., PF, SR, and TY) (Figure 5 in Schultheis et al. 2012). Furthermore, the Jackson Range was considered its own clade in *D. baumanni* because it had the largest number of mutational steps from other clades (Figures 3 and 5 in Schultheis et al. 2012), whereas in *H. pacifica,* the haplotype from the Jackson range (Hap 1) is shared with three other mountain ranges (Table 1, Figure 2). Haplotype sharing is also reflected in the gene tree for *H. pacifica* which was not as resolved as that of *D. baumanni.*

Overall Φ_{ST} values for *H. pacifica* indicated genetic structure but this number was much lower than the Φ_{ST} value for *D. baumanni* ($\Phi_{ST} = 0.90$, $P < 0.01$), and the results of the AMOVA were consistent with those of the phylogenetic analysis. There were high levels of genetic differentiation among certain populations but low levels among others. Pairwise Φ_{ST} values for all populations indicate that contemporary dispersal among sky islands by both aquatic larvae, which can disperse through streams, and aerial adults, is restricted among some populations but not others.

The Bayesian analysis and the haplotype network suggest that Snowstorm, Roberts, Sonoma, and Deep Creek populations are genetically isolated in *H. pacifica*. This is a different result than for *D. baumanni* populations, where the populations that were very different from others were at the Jackson Range, Pine Forest, Santa Rosa, and the Sierra Nevada sites.

The contrasting patterns of genetic differentiation in *H. pacifica* and *D. baumanni* are not surprising given the differences in the geographic distribution of the haplotypes in the two species and may be a result of different habitat tolerances. *Doroneuria baumanni* is restricted to upper elevations and headwater streams, whereas *H. pacifica* has less stringent habitat preferences and is more of a habitat generalist, occurring in rivers, creeks, and springs at lower elevations which may favor gene flow among populations. *H. pacifica* is therefore not as sensitive to climate change as *D. baumanni*.

Our analysis in BEAST dated the divergence of all *H. pacifica* clades to 547,400 ybp ($CI_{95\%}$ 52,900-1,400,000), which is during the Pleistocene. While *D. baumanni* divergence times were older (suggesting longer isolation), the timing of divergence was similar, with all estimates well within the Pleistocene and the Jackson clade being most recent. We did not detect any recent effects on genetic isolation, but the tools we were using may be unable to detect very recent influences. However because our sample sizes are small and not equally distributed, the results should be interpreted with caution.

Kauwe et al. (2004) also performed a phylogeographic study of stoneflies in the western United States. They used cyt *b* to investigate the patterns of genetic differentiation in *Pteronarcys californica* populations. Their results showed a pattern of restricted gene flow with isolation by distance, which they suggested may be a result of dispersal via connected streams and rivers. Their study also implied that the observed pattern of genetic variation was directed by long distance, overland dispersal. These results mirror those in our study–we also found gene flow across high valleys–and confirm why it is important to study multiple species to infer broad scale historical influences such as climate changes.

Mitochondrial DNA has some limitations such as being prone to selective sweeps which wipe out genetic diversity and only reflecting dispersal of females because it is maternally inherited (Hurst and Jiggins 2005). However, mtDNA is still a useful marker to use because its high levels of variation allow detection of recent genetic divergence, as found in our study. Other advantages of mitochondrial DNA is that its fast evolutionary rates give us high information content per base pair sequenced. It is useful for studying recent changes, it is easy to amplify, and it has a faster mutation rate because of its inefficient mutation repair mechanism. It can also be used to determine phylogenetic relationships of closely related taxa. It is still best to use it in combination with an estimate from other markers, such as nuclear loci, as it will lead to more confident population parameter estimates and a better test for phylogenetic hypotheses (Schultheis et al. 2014).

The results of our study underscore the benefits of a comparative approach to understanding the influences of combined historical and contemporary processes on population isolation in the Great Basin.

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