

Hierarchical spatial genetic structure in a distinct population segment of greater sage-grouse

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Abstract Greater sage-grouse (*Centrocercus urophasianus*) within the Bi-State Management Zone (area along the border between Nevada and California) are geographically isolated on the southwestern edge of the species' range. Previous research demonstrated that this population is genetically unique, with a high proportion of unique mitochondrial DNA (mtDNA) haplotypes and with significant differences in microsatellite allele frequencies compared to populations across the species' range. As a result, this population was considered a distinct population segment (DPS) and was recently proposed for listing as threatened under the U.S. Endangered Species Act. A more comprehensive understanding of the boundaries of this genetically unique population (where the Bi-State population begins) and an examination of genetic structure within the Bi-State is needed to help guide effective management decisions. We collected DNA from eight sampling locales within the Bi-State ($N = 181$) and compared those samples to previously collected DNA from the two most proximal populations outside of the Bi-State DPS, generating mtDNA sequence data and amplifying 15 nuclear microsatellites. Both mtDNA and microsatellite analyses support the idea that the Bi-State DPS represents a genetically unique population, which has likely been separated for thousands of years. Seven mtDNA haplotypes were found exclusively in the Bi-State population and represented 73 % of individuals, while three haplotypes were shared

with neighboring populations. In the microsatellite analyses both STRUCTURE and FCA separate the Bi-State from the neighboring populations. We also found genetic structure within the Bi-State as both types of data revealed differences between the northern and southern part of the Bi-State and there was evidence of isolation-by-distance. STRUCTURE revealed three subpopulations within the Bi-State consisting of the northern Pine Nut Mountains (PNa), mid Bi-State, and White Mountains (WM) following a north–south gradient. This genetic subdivision within the Bi-State is likely the result of habitat loss and fragmentation that has been exacerbated by recent human activities and the encroachment of singleleaf pinyon (*Pinus monophylla*) and juniper (*Juniperus* spp.) trees. While genetic concerns may be only one of many priorities for the conservation and management of the Bi-State greater sage-grouse, we believe that they warrant attention along with other issues (e.g., quality of sagebrush habitat, preventing future loss of habitat). Management actions that promote genetic connectivity, especially with respect to WM and PNa, may be critical to the long-term viability of the Bi-State DPS.

Keywords Bi-State · Distinct population segment · Gene flow · Genetic diversity · Greater sage-grouse · Microsatellites · Mitochondrial DNA

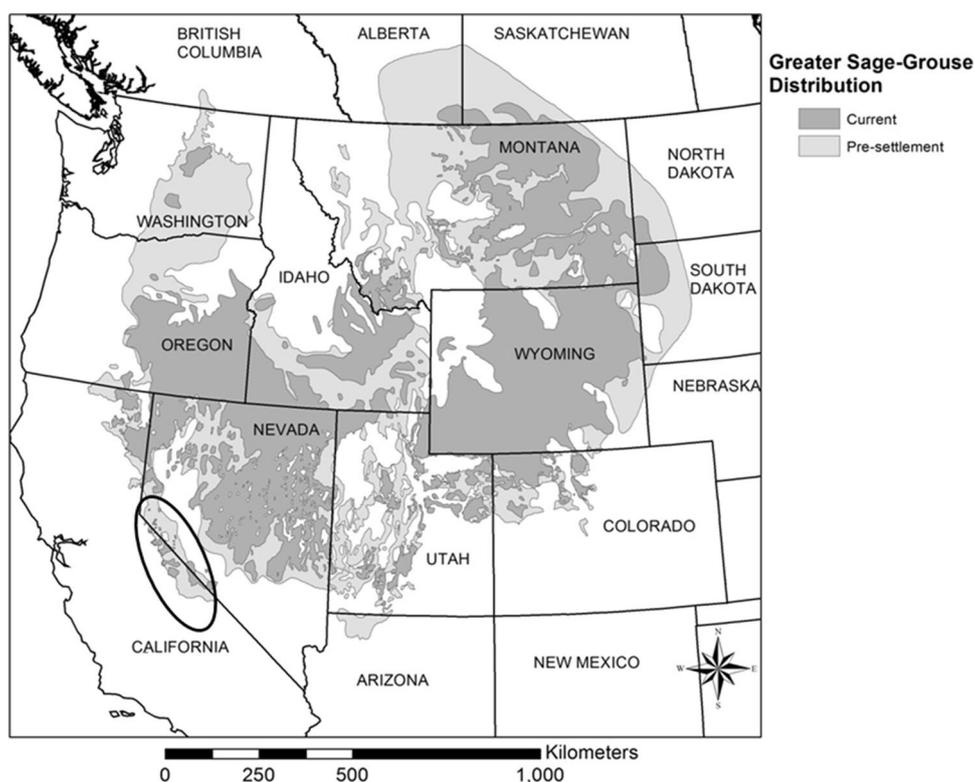
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Introduction

Greater sage-grouse (*Centrocercus urophasianus*) are ground-dwelling birds that occur only in western North America within sagebrush (*Artemisia tridentata*) steppe ecosystems (Johnsgard 1983). Greater sage-grouse populations have declined dramatically because of the loss,

Fig. 1 Current and pre-settlement distribution of greater sage-grouse (from Schroeder et al. 2004). The Bi-State (referred to as Lyon/Mono in previous genetic studies) population is circled



fragmentation, and degradation of sagebrush habitat (Braun 1998; Connelly et al. 2011), which has resulted in the isolation of small populations from larger populations existing in more contiguous habitat (Fig. 1). One potential exception to this overall pattern of isolation due to habitat loss is the population that exists along the border between Nevada and California identified as the Bi-State Management Zone (Bi-State Local Planning Group, unpublished conservation plan), hereafter Bi-State (Fig. 1). Historically, this population was connected to populations north of the Bi-State (near Lassen, CA) only through a narrow peninsula of sagebrush on the northern edge of the region, surrounded on all other sides by mountain ranges and large expanses of non-sagebrush vegetation. This peninsular connectivity has led to a greater degree of geographic isolation and, thus, genetic distinctiveness of the Bi-State compared to other populations (Fig. 1). The sagebrush peninsula north of the Bi-State no longer exists, largely due to urbanization and increased human land-use practices. Additionally, expansion of singleleaf pinyon (*Pinus monophylla*) and juniper (*Juniperus* spp.) woodlands has replaced sagebrush habitat resulting in small, isolated patches of sagebrush within the Bi-State.

A previous rangewide genetic study of greater sage-grouse (Oyler-McCance et al. 2005a) revealed that the distribution of genetic variation showed a gradual shift in both mitochondrial and nuclear markers across the species'

range, suggesting that movements were typically among neighboring populations and not across the species' range. The Bi-State population (called Lyon/Mono by Benedict et al. 2003; Oyler-McCance et al. 2005a) was an exception to this pattern as it was found to be genetically distinct because it was characterized by a high proportion of unique mitochondrial DNA (mtDNA) haplotypes (Benedict et al. 2003; Oyler-McCance et al. 2005a), and significant population differentiation as measured using microsatellite analysis (Oyler-McCance et al. 2005a). As a result, the Bi-State area was considered by the U.S. Fish and Wildlife Service to be a distinct population segment (DPS) and was recently proposed for listing as Threatened under the Endangered Species Act. The rationale for protecting this population was that maintaining the unique genetic diversity in the Bi-State could protect the evolutionary potential of greater sage-grouse in this area (Benedict et al. 2003; Oyler-McCance et al. 2005a). Concern for the survival of this small, isolated population and how it may be affected by environmental and demographic stochasticity has created a need for closer examination of this population.

Previous genetic research on this population included only limited samples from a single location in Lyon County, Nevada, and two locations in Mono County, California, which represented only a portion of the geographically isolated population (Fig. 1). Further, samples were obtained in the fall, such that the breeding location of

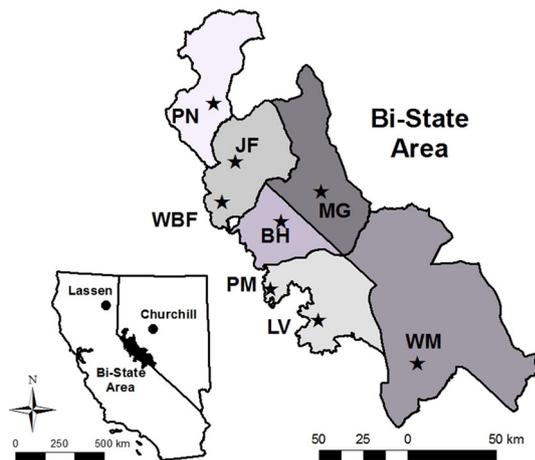


Fig. 2 Map of the Bi-State area showing sampling locations of all sampling locales within the Bi-State and the two neighboring populations (Churchill, NV and Lassen, CA) that are outside the Bi-State area

those grouse was unknown. The extent of movement among discrete areas of sagebrush within the Bi-State (Fig. 1) remains unknown as do the specific boundaries of the DPS. To address these issues, eight distinct breeding locations within the Bi-State were sampled (sampling locales) with the goal of analyzing samples with the same techniques used in previous studies (Benedict et al. 2003; Oyler-McCance et al. 2005a). Our specific objectives were to (1) compare data within the Bi-State to the nearest neighboring populations, (2) determine whether all sampling locales within the Bi-State carried the unique genetic signature defined previously, and (3) examine genetic structure among the eight sampling locales within the Bi-State. These findings will provide a better understanding of genetic variation at a finer scale within the Bi-State, which will help guide management and conservation actions for this isolated, distinct population. This study will also provide a more thorough comparison of this population to neighboring populations, which can help further refine the boundary of the Bi-State DPS.

Methods

Study area

The Bi-State straddles the border between Nevada and California and is separated from other continuous sagebrush habitat in California and Nevada (Fig. 1). This area is comprised of smaller, discrete patches of sagebrush intermixed with habitat unsuitable for sage-grouse, which may separate groups of birds from one another. The sampling locales in this study included Jackass Flat (JF, $N = 16$), Wheeler Burcham Flat (WBF, $N = 8$), Bodie

Hills (BH, $N = 31$), Parker Meadows (PM, $N = 13$), Long Valley (LV, $N = 17$), Pine Nut Mountains (PN, $N = 49$), Mount Grant (MG, $N = 15$), and White Mountains (WM, $N = 32$) (Fig. 2). Several mountain ranges separated the northern and southern ends of the study area with elevations ranging from 1,660–3,770 m. Vegetation types across sites consisted of big sagebrush interspersed with little sagebrush (*A. arbuscula*). Native bunchgrasses and perennial forbs dominated the understory vegetation. Cheatgrass (*Bromus tectorum*) was present but uncommon and pinyon pine and juniper woodlands were relatively common at elevations of 1,850–3,000 m.

To compare the Bi-State to those outside the Bi-State we used DNA from samples collected previously (Benedict et al. 2003; Oyler-McCance et al. 2005a) from the two populations most proximal to the Bi-State (Lassen and Churchill; Fig. 2). Importantly, the genetic make-up of both Churchill and Lassen was typical of sage-grouse in the western portion of their range, while the genetic make-up of birds from the Bi-State was unique (Benedict et al. 2003; Oyler-McCance et al. 2005a).

Field and laboratory procedures

Using a spotlight trapping method (Giesen et al. 1982) we trapped and collected blood from 181 birds at eight sampling locales within the Bi-State (Fig. 2). DNA was extracted using the GenomicPrep Blood DNA Isolation Kit (General Electric) with modifications following Oyler-McCance et al. (2005b). In addition, we included 19 samples from Churchill and 55 samples from Lassen which were described in a previous analysis (Benedict et al. 2003; Oyler-McCance et al. 2005a). Only a subset of the Lassen samples ($N = 22$) were sequenced previously and are used in this study.

Mitochondrial DNA analysis

We used the primers 16775L (Quinn 1992) and H21 (Quinn and Wilson 1993) to amplify a portion of the mitochondrial control region sequenced in previous studies (Kahn et al. 1999; Benedict et al. 2003; Oyler-McCance et al. 2005a). Amplifications were performed in 25 μ L reactions consisting of ~ 20 ng of template DNA, 0.2 mM of each dNTP, 0.5 μ M forward primer, 0.5 μ M reverse primer, 1.25 U GoTaq Flexi DNA polymerase (Promega), 1.5 mM $MgCl_2$ and 1 \times GoTaq Flexi Buffer (Promega). Amplification conditions were as follows: 94 $^{\circ}C$ for 2 min, then 94 $^{\circ}C$ for 1 min, 55 for 1 min 30 s, 72 $^{\circ}C$ for 2 min for 40 cycles, followed by 72 $^{\circ}C$ for 10 min. The PCR products were purified for sequencing by the addition of 5 U exonuclease I (USB) and 0.5 U shrimp alkaline phosphate (USB) and a subsequent 37 $^{\circ}C$ incubation for

30–45 min. These two enzymes were denatured by a 15 min 80 °C incubation. Sequencing was performed in 10 µL reactions consisting of 2 µL prepared template, 0.5 µM of either forward or reverse primer, and 0.5 µL BigDye v3.1 in 1× sequencing buffer (Applied Biosystems). Sequencing reactions were cleaned following the manufacturer's protocol for ethanol/EDTA/sodium acetate precipitation. Purified sequenced products were run on an AB3500 Genetic Analyzer (Applied Biosystems).

Microsatellite analysis

We amplified 15 microsatellite loci for all samples (MSP11, MSP18, SGMS06.4, SGMS06.6, SGMS06.8; Oyler-McCance and St. John 2010, SGCA5, SGCA11, SGCTAT1; Taylor et al. 2003, TUT3, TUT4, TUD3; Segelbacher et al. 2000, BG6, BG16; Piertney and Höglund 2001, TTT3; Caizergues et al. 2003, TTD6; Caizergues et al. 2001). Amplifications were performed in 10 µL PCRs consisting of 20 ng of template DNA, 0.2 mM of each dNTP, 0.25 µM dye-labeled forward primer, 0.25 µM reverse primer, 0.625 U GoTaq Flexi DNA polymerase (Promega), 2.25 mM MgCl₂ and 1× GoTaq Flexi Buffer (Promega). The amplification conditions for all loci except TTD6 were as follows: 94 °C for 2 min, then 94 °C for 30 s, annealing temperature (52 °C: MSP11, SGMS06.8; 54 °C: BG16; 55 °C: MSP18, SGCA5; 57 °C: BG6; 58 °C: SGMS06.4, SGMS06.6, SGCA11, TTT3; 60 °C: SGCTAT1, TUT3, TUT4, TUD3) for 30 s, 72 °C for 30 s for 40 cycles, then 72 °C for 10 min and a final extension at 60 °C for 45 min. The locus TTD6 was amplified using a 'Touchdown' protocol: 94 °C for 3 min, 10 cycles of 30 s denaturation at 94 °C, 30 s of annealing starting at 65 °C and dropping by 1 °C per cycle, and 30 s of extension at 72 °C, followed by a further 20 cycles consisting of 30 s denaturation at 94 °C, 30 s of annealing at 55 °C, 30 s of extension at 72 °C, with a 10-min period of extension at 72 °C following the last annealing step. PCR products were multi-loaded based on product size and primer label, combined with GeneScan LIZ 600 (Applied Biosystems) and electrophoresed through a capillary gel matrix using an AB3500 Automated DNA Sequencer (Applied Biosystems). We determined allele sizes for each locus using GeneMapper v4.1 software (Applied Biosystems).

Data analysis

Mitochondrial DNA sequences were edited using Sequencher Version 4.1.4 (Gene Codes, Ann Arbor, MI) and assigned to the appropriate haplotype by comparing it to published sequences (Kahn et al. 1999; Benedict et al. 2003; Oyler-McCance et al. 2005a). Sequences were aligned using MEGA ver. 5 (Tamura et al. 2011) using the

default parameters. We used the program jModelTest (Guindon and Gascuel 2003; Posada 2008) to determine the best evolutionary model (using Akaike's Information Criterion) given our sequence alignment. Phylogenetic analyses were performed using Bayesian inference within MrBayes, version 3.12; (Ronquist and Huelsenbeck 2003), using the best model of evolution determined by jModelTest. We ran two independent analyses in MrBayes for 1 million generations each using four chains and default values for all parameters, sampling from the posterior every 100 generations. We discarded the first 25 % of the stored trees as burn-in and summarized the remaining trees as a 50 % majority consensus tree. To determine whether different analytical methods gave congruent results, phylogenetic analyses of these data were also conducted using a maximum likelihood analyses in MEGA ver. 5 (Tamura et al. 2011) incorporating the most appropriate model of evolution (as determined using jModelTest) and using the close-neighbor interchange algorithm with 1000 initial trees and 1000 bootstrap replicates. Phylogenetic trees were viewed in FigTree (Rambaut 2008).

We calculated gene diversity in Arlequin 2.001 (Schneider et al. 2001) within each sampling locale in the Bi-State DPS and also within the two neighboring populations. We also conducted pairwise F_{ST} tests among all pairs of sampling locales in Arlequin 2.001 (Schneider et al. 2001) that examined differentiation among sampling locales within the Bi-State population and between the Bi-State sampling locales and each of the neighboring populations. Similarly, we combined all the Bi-State samples into one population and calculated pairwise population F_{ST} tests among the Bi-State and the two neighboring populations. For these tests we calculated F_{ST} using haplotype frequencies rather than incorporating the mutational differences between the haplotypes since our data has two deeply divergent clades. Sampling locales were deemed to be significantly different using a Bonferroni correction ($\alpha = 0.002$), while differences among populations were identified using a greater alpha level ($\alpha = 0.02$).

The mean number of microsatellite alleles for each sampling locale was calculated and the observed and expected levels of heterozygosity were estimated using Genalex (Peakall and Smouse 2012). Allelic richness, which adjusts for discrepancies in sample size by incorporating a rarefaction method, and F_{IS} was estimated in FSTAT 2.9.3.2 (Goudet 1995). To examine the reliability of the microsatellite loci, each locus was tested (by sampling locale) for departures from Hardy–Weinberg equilibrium (HWE; Guo and Thompson 1992) in Arlequin 2.001 (Schneider et al. 2001) using a Bonferroni correction ($\alpha = 0.0003$). We then tested for deviations from HWE for panmixia among the three populations in the study: Lassen, Churchill, and the Bi-State (all sampling locales combined)

using GenePop for the web (Raymond and Rousset 1995). Finding a significantly positive F_{IS} value within the Bi-State, we tested for departures from HWE for panmixia among each sampling locale within the Bi-State as well.

Similar to the mtDNA analysis, for the microsatellite analysis, we conducted pairwise F_{ST} tests among pairs of sampling locales examining differentiation among sampling locales within the Bi-State population and between the Bi-State sampling locales and each of the neighboring populations. Similarly, we combined all the Bi-State samples into one population and calculated pairwise population F_{ST} tests among the Bi-State and the two neighboring populations. Sampling locales were deemed to be significantly different using a Bonferroni correction ($\alpha = 0.002$), while differences among populations were identified using a greater alpha level ($\alpha = 0.02$).

To examine genetic structure within and around the Bi-State (Churchill, Lassen, and all sampling locales within the Bi-State), we used STRUCTURE 2.00 (Pritchard et al. 2000). In this program, individuals were grouped into clusters without regard to the assigned population or sampling locale using a model-based clustering analysis. The number of unique genetic clusters (K) was initially estimated by conducting five independent runs each of $K = 1-10$ with 100,000 Markov Chain Monte Carlo (MCMC) repetitions and used 100,000 initial iterations as the burn-in period using the model with admixture, correlated allele frequencies, and no prior location information. An additional set of five independent runs was then conducted with $K = 1-7$ with 500,000 MCMC repetitions and a 250,000 burn-in period using the above model. We chose the optimal value of K using the ΔK method described by Evanno et al. (2005) in STRUCTURE HARVESTER (Earl and vonHolt 2011). The use of multiple runs to evaluate K in STRUCTURE can produce several distinct solutions due to label switching across replicates. To alleviate this issue, we used CLUMPP (Jakobsson and Rosenberg 2007) to produce our final output. Results from CLUMPP were visualized using the software DISTRUCT (Rosenberg 2002). We also examined genetic structure in the program Genetix (Belkhir et al. 2004) by conducting a factorial components analysis using the 3D for populations setting. We tested for isolation by distance using a Mantel (1967) test, which looks for a correlation between genetic distance (F_{ST}) and geographic distance. This test was conducted using the software ZT (Bonnet and Van de Peer 2002).

Results

Within the region that we sequenced, 44 sites were variable and 23 were parsimony informative. Of the 209 individuals that were sequenced, 20 haplotypes were identified, two

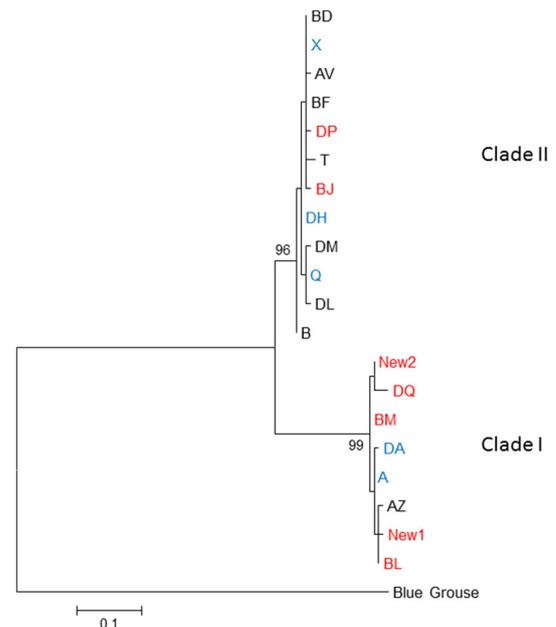


Fig. 3 Phylogenetic tree of the control region haplotypes based on maximum likelihood analysis. Haplotypes in *black* were found in Churchill and Lassen (and other greater sage-grouse populations) and not in the Bi-State. Haplotypes in *red* are found only in the Bi-State. Haplotypes in *blue* are found in both the Bi-State and at least one of the neighboring populations. Haplotype Q is shown here in *blue* because it was found in the Bi-State in previous studies (Benedict et al. 2003; Oyler-McCance et al. 2005a, b) but was not found in the Bi-State samples sequenced in this study. Similarly, haplotype DA is shown in *blue* as it was found in Elko, NV previously but was not found in Churchill or Lassen. (Color figure online)

(New1 and New2) had not yet been described for this area or anywhere else across the range (Benedict et al. 2003; Oyler-McCance et al. 2005a). Those sequences have been deposited in GenBank (accession numbers KF956707 and KF956708). The HKY +I model provided the best fit to the mtDNA data. The relationship among haplotypes was consistent with previous studies (Kahn et al. 1999; Benedict et al. 2003; Oyler-McCance et al. 2005a) with all haplotypes belonging to one of two divergent clades. Haplotypes that are unique to the Bi-State occurred in both of the divergent clades (Fig. 3). Seven haplotypes (DP, BJ, DQ, BM, BL, New1, and New2) were found only in the Bi-State population representing 73 % of the individuals (Table 1; Fig. 3). Three haplotypes (A, X, and DH) were found both within the Bi-State and in the neighboring populations (Table 1; Fig. 3). Haplotype Q was not found in any of the Bi-State samples sequenced here but was found previously in the Bi-State, and haplotype DA was found in the Bi-State and also in Elko, NV yet not in Churchill or Lassen (Benedict et al. 2003; Oyler-McCance et al. 2005a).

Gene diversity values were similar across all sampling locales with the exception of PM which had much lower

Table 1 mtDNA haplotypes and genetic diversity for 8 sampling locales within the Bi-State and two neighboring populations

	N	Haplotypes																Gene diversity	Number of haplotypes			
		Haplotypes																				
		A	B	Q	T	X	AZ	AV	BD	BF	BJ	BL	BM	DA	DH	DL	DM			DP	DQ	New1
LV	15				1				1	7	1						4			1	0.7429	6
BH	30								13	5	5			3				4			0.7540	5
WBF	8								1		4			2				1			0.7500	4
PM	13								11		2										0.2821	2
WM	29				6						12						8		3		0.7241	4
JF	15	1							6		3	1						4			0.7714	5
PN	44	12							2	7	2			18			1	2			0.7431	7
MG	15	1							3	2	3						4	2			0.8667	6
Lassen	22	5		4										1							0.8052	7
Churchill	18		2		5	8	1	1						1	1	2					0.7451	6

gene diversity (Table 1). The gene diversity of each sampling locale, with the exception of PM, was also similar to what was found in Churchill and Lassen, the two neighboring populations (Table 1). Parker Meadows had only two haplotypes present, which was fewer than other areas. Wheeler Burcham Flat and WM also had lower numbers of haplotypes with only four compared to 5–7 found in other sampling locales and in Churchill and Lassen. Pairwise F_{ST} tests among all the sampling locales revealed that there were significant differences among some of the sampling locales (Table 2). White Mountain and PN were significantly different from 5 and 6 other sampling locales respectively (Table 2), making them the most highly differentiated of all the Bi-State samples. All Bi-State sampling locales were significantly different from Lassen and Churchill. Lassen and Churchill were not significantly different from each other (Table 2). When all sampling locales were combined into one population, the Bi-State population was significantly different from both Churchill and Lassen ($P < 0.005$ for both tests), and Churchill and Lassen were not significantly different from each other ($P = 0.03$).

Nuclear microsatellite analysis

Only one of the 15 loci (TUD3) consistently departed from HWE across most sampling locales/populations, and thus we removed that locus from our analysis. Of the remaining 14 loci, we found only four locus/sampling locale combinations that were out of HWE and they occurred in four different loci and three sampling locales. Since none of those loci were consistently out of HWE across sampling locales, we used the remaining 14 loci for our analysis. In our HWE test for panmixia among the three populations, we found that the Bi-State population was significantly out of HWE ($F_{IS} = 0.114$, $P < 0.0001$). When we examined HWE among sampling locales, only one sampling locale (PN) continued to be out of HWE ($F_{IS} = 0.0768$, $P = 0.003$; Table 3). The mean number of alleles per sampling locale ranged from 4.00 to 8.21. Within the Bi-State observed heterozygosity values were similar (Table 3) with the highest value in JF and the lowest in BH and MG. Allelic richness, which accounts for differences in sample size, was also similar among sampling locales with the highest in MG and lowest in PM. Pairwise F_{ST} tests among all the sampling locales revealed that there were significant differences among almost all of the sampling locales (Table 2). Only WBF and JF were not significantly different. All sampling locales were significantly different from Churchill and Lassen (Table 2). When all the sampling locales were combined into one population, all populations (Bi-State, Churchill, and Lassen) were significantly different from each other ($P < 0.02$).

Table 2 Pairwise F_{ST} values for 8 sampling locales within the Bi-State population and two neighboring populations using mtDNA control region data (below diagonal) and microsatellite data (above diagonal)

	LV	BH	WBF	PM	WM	JF	PN	MG	Lassen	Churchill
LV	0.00000	0.10081	0.08168	0.13830	0.12151	0.10486	0.11966	0.12376	0.13343	0.10523
BH	0.15113	0.00000	0.07502	0.09585	0.10615	0.07621	0.08113	0.02739	0.11256	0.12909
WBF	0.22157	0.08351	0.00000	0.12364	0.14356	0.05016	0.12609	0.07315	0.13825	0.14608
PM	0.44110	0.11384	0.40794	0.00000	0.15011	0.11753	0.13301	0.10775	0.13727	0.14048
WM	0.17214	0.20610	0.07277	0.41731	0.00000	0.12793	0.12589	0.12138	0.14198	0.13917
JF	0.21131	-0.00616	0.06747	0.15676	0.18705	0.00000	0.10852	0.05585	0.10599	0.12783
PN	0.18675	0.16780	0.13631	0.40049	0.24690	0.19851	0.00000	0.10960	0.11356	0.11367
MG	0.04195	0.03840	0.05312	0.27158	0.06157	0.02494	0.14215	0.00000	0.12550	0.15415
Lassen	0.20488	0.21798	0.20873	0.42141	0.17462	0.19858	0.16051	0.15274	0.00000	0.06210
Churchill	0.23327	0.25007	0.25284	0.46512	0.19173	0.24216	0.25602	0.19583	0.07480	0.00000

Significant differences are noted in bold

In the STRUCTURE analysis, the optimal number of genetic clusters revealed a K of four given the data (Fig. 4a). The Churchill and Lassen populations represented one unique cluster (green) and there were three additional clusters within the Bi-State (Fig. 4a). The PN sampling location was split into two clusters (Fig. 4a), one shown in blue (PNa) and one shown in yellow (PNb). Six other sampling locales in the mid Bi-State (JF, WBF, MG, BH, PM, and LV) also were part of the yellow genetic cluster (Fig. 4a). Long Valley was the only sampling locale that appeared to be slightly admixed with individuals largely yellow, but with some mixture of red. The WM sampling locale consisted largely of a single cluster shown in red (Fig. 4a). We calculated F_{IS} and conducted pairwise population F_{ST} tests among the three clusters within the Bi-State and all were significantly different (Table 4). Because we found one cluster within the Bi-state that was made up of individuals from multiple sampling locales geographically separated and spread across the middle of the Bi-State region (PNb, JF, WBF, MG, BH, PM and LV), we reran STRUCTURE within this group using the same methods and parameters described for the initial analysis. This allowed us to examine hierarchical genetic structure as is recommended by Evanno et al. (2005). We found that the mid Bi-State group consisted of two sub clusters, with PNb, JF, WBF, and MG aligned in one sub cluster (yellow) and PM and LV aligned in a second sub cluster (orange; Fig. 4b).

The FCA analysis (Fig. 5) showed (1) the neighboring populations outside the Bi-State clustered together (circled in red), (2) most of the Bi-State individuals clustered together (circled in green), and (3) a subset of the PN sampling locale (PNa) clustered together (circled in blue). Within the green polygon, it is evident that the WM sampling locale shown in pink clustered together at the top of the green polygon and was most closely aligned with the

LV sampling locale, which is shown in yellow (Fig. 5). The Mantel test revealed that there was a positive correlation between genetic distance and geographic distance ($r = 0.912369$, $P = 0.0001$).

Discussion

We found 20 mtDNA haplotypes, each of which fell into one of the two divergent monophyletic clades (Fig. 3) in sage-grouse described by Kahn et al. (1999), Benedict et al. (2003), and Oyler-McCance et al. (2005a). Kahn et al. (1999) and Benedict et al. (2003) argued that the two divergent clades in sage-grouse are likely the result of allopatric groups of sage-grouse separated during the Pleistocene Epoch that have since rejoined. The genetically unique Bi-State population includes haplotypes from both divergent clades, which suggests that this population became isolated from other sage-grouse populations (and began its divergence) after the two clades were re-joined less than 850,000 years ago (Kahn et al. 1999). We found two haplotypes that have not been previously described in greater sage-grouse. Both of these haplotypes aligned with clade I and were rare, yet were only single substitutions from existing Bi-State haplotypes BL and BM, suggesting that these haplotypes may be recent mutations from more common Bi-State haplotypes.

Benedict et al. (2003) argued that the Bi-State population has been isolated from other populations for thousands or perhaps tens of thousands of years. This idea was based on the fact that most individuals within the Bi-State carry mtDNA haplotypes that are not found elsewhere across the range of greater sage-grouse. In this study, seven of the eleven haplotypes found in the Bi-State are not found elsewhere across the range, representing 73 % of the individuals. This percentage is lower than was reported by

Table 3 Genetic diversity of 8 sampling locales within the Bi-State and the neighboring populations across 14 microsatellite loci

Sampling locale	Number of samples	Mean Ho	Mean He	Mean F_{IS}	Mean number of alleles per sampling locale	Allelic richness
LV	17	0.661	0.608	-0.0532	4.78	4.05
BH	31	0.613	0.616	0.0200	6.00	4.28
WBF	7	0.653	0.609	0.0039	4.21	4.21
PM	13	0.654	0.607	-0.0374	4.00	3.35
WM	32	0.613	0.595	-0.0138	5.00	3.80
JF	16	0.677	0.634	-0.0333	4.71	4.02
MG	15	0.615	0.622	0.0459	5.36	4.42
PN	49	0.618	0.662	0.0768	6.79	4.37
Lassen	55	0.755	0.757	0.0106	8.21	5.40
Churchill	19	0.710	0.710	0.0321	6.50	5.11

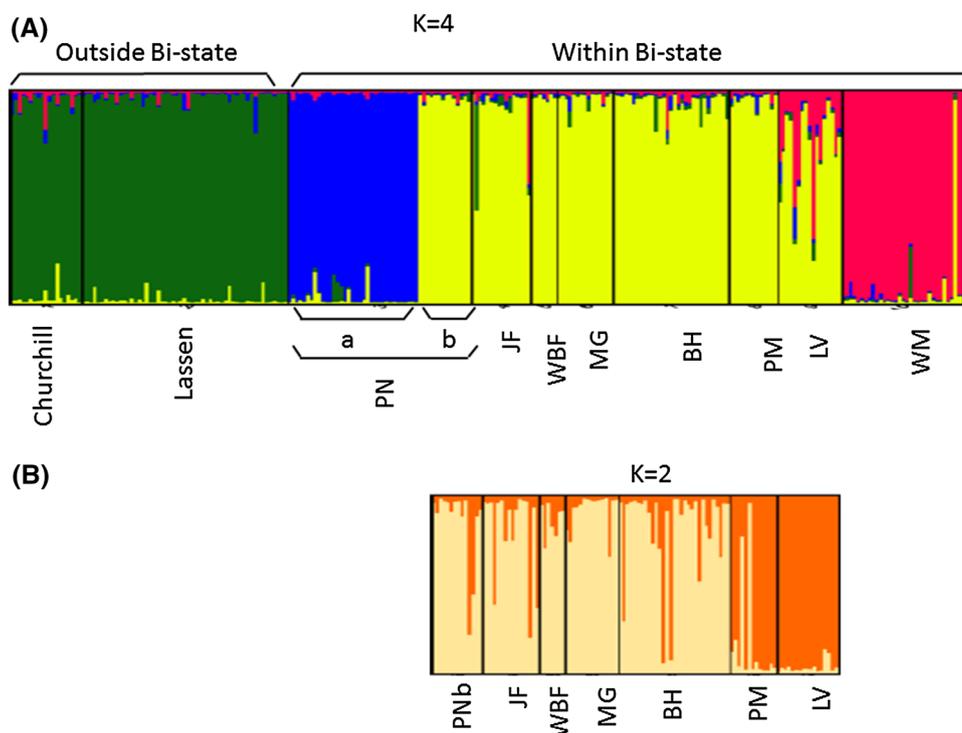


Fig. 4 Estimated population genetic structure at two hierarchical levels (A and B) based on allele frequency variation from 14 microsatellite loci as calculated in STRUCTURE. Populations and sampling locales are ordered in a north to south direction in both A and B. **a** Genetic structure around and within the Bi-State with the optimal number of distinct genetic clusters (K) of four. Each distinct cluster is represented by a unique color. Each vertical bar represents

an individual greater sage-grouse. The colors on each vertical bar represent the individual's estimated membership in each of the four unique genetic clusters. **b** STRUCTURE analysis within the mid Bi-State cluster investigating further genetic substructure. The optimal number of genetic sub clusters within the middle Bi-State cluster was two. (Color figure online)

Benedict et al. (2003) and is likely due to the inclusion of birds from the Pine Nut Mountains area, which represents the closest link to the Lassen birds.

The mitochondrial and nuclear markers revealed a consistent pattern of genetic differentiation between the Bi-State and the neighboring populations of greater sage-grouse. The mtDNA data set showed that all newly

sampled locales within the Bi-State contained haplotypes that were consistent with previous findings from studies with limited samples from this region (Benedict et al. 2003; Oyler-McCance et al. 2005a). Specifically, all sampling locales had the BM and BJ haplotypes that are characteristic of the Bi-State, with the exception of one sampling locale where the haplotype BJ was not represented.

Table 4 Mean F_{IS} values and pairwise F_{ST} values for the three groups (subpopulations) identified in STRUCTURE within the Bi-State

Subpopulation	Mean F_{IS}	Mid Bi-State	WM
Mid Bi-State	0.067		
WM	-0.014	0.08699	
PNa	-0.003	0.13272	0.17357

All pairwise F_{ST} tests were significantly different (shown in bold)

Additionally, the Bi-State population, and all sampling locales therein, were found to be significantly different from the Churchill and Lassen populations when comparing mtDNA haplotype frequencies and microsatellite allele frequencies (pairwise F_{ST}). The microsatellite F_{ST} values between the Bi-State sampling locales and Churchill and Lassen may, in fact, be underestimated (Balloux and Lugin-Moulin 2002). Similarly, the microsatellite data set showed that Lassen and Churchill represented one genetic cluster, separate from the Bi-State greater sage-grouse (Figs. 4a, 5). These results corroborate Benedict et al. (2003) and Oyler-McCance et al. (2005a) who found that greater sage-grouse in the Bi-State area were significantly different from greater sage-grouse throughout the rest of the range.

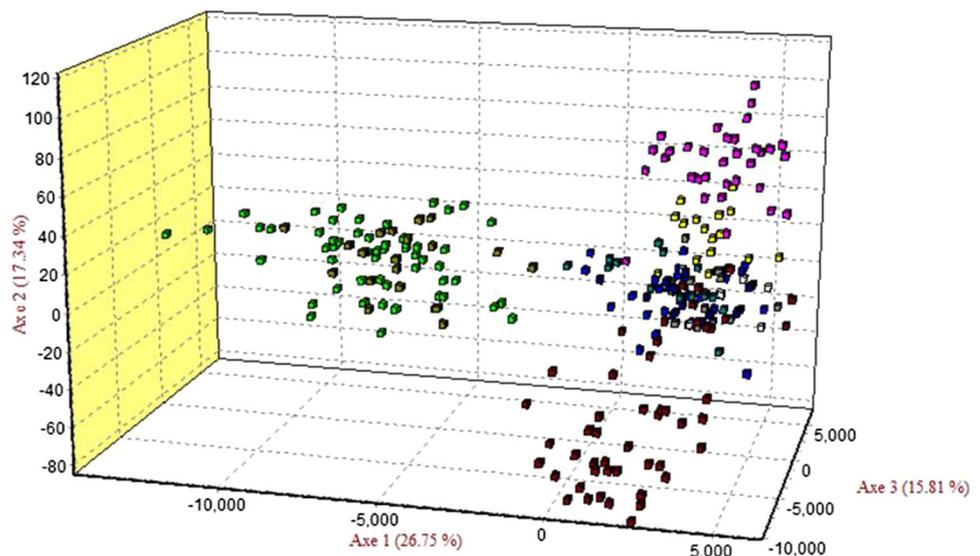
The amount of differentiation between the Bi-State and other range-wide sage-grouse populations is relatively large compared to differentiation among populations outside of the Bi-State. Oyler-McCance et al. (2005a) suggested that the amount of differentiation between the Bi-State and other greater sage-grouse was comparable to the amount of differentiation between greater sage-grouse and its sister species Gunnison sage-grouse. They surmised that

both groups (Bi-State and Gunnison sage-grouse) may have been isolated for similar amounts of time. Isolation of the Bi-State population is likely due to geographic and landscape features (for example, lack of sagebrush habitat) that have been in place since before English settlement (Schroeder et al. 2004). Unlike Gunnison sage-grouse, however, Bi-State greater sage-grouse have not developed the distinct differences in plumage, morphology (Schroeder 2008), and behavior (Taylor and Young 2006) that have led to the reproductive isolation and speciation in Gunnison sage-grouse.

The levels of genetic differentiation among greater, Bi-State, and Gunnison sage-grouse have all been measured using a small number of neutral genetic markers (Benedict et al. 2003; Oyler-McCance et al. 2005a, b). While these types of markers are appropriate for measuring time since isolation, such neutral markers cannot measure genes associated with traits potentially under selection and relevant for speciation. New comparisons of greater, Bi-State, and Gunnison sage-grouse using genomic methods (> 13,000 single nucleotide polymorphisms), however, have shown that by including many more markers, some of which are potentially under selection, Gunnison sage-grouse are much more highly differentiated (U. S. Geological Survey, unpublished data), even though they may have been separated for a similar amount of time. Such a phenomenon is not uncommon in organisms with highly skewed mating systems where sexual selection is a driving force in speciation (Spaulding 2007; Oyler-McCance et al. 2010). This is important as it relates to refining taxonomic delineations within this species complex.

Informed management of a unique population like the Bi-State DPS requires an understanding of the genetic

Fig. 5 Factorial components analysis of Bi-State and neighboring populations of greater sage-grouse using 14 microsatellite loci. Samples in the red polygon represent the individuals in the neighboring populations of Churchill and Lassen. Samples in the blue polygon represent a subset of individuals from the PN sampling locale (PNa). Samples in the green polygon represent the remaining samples in the Bi-State. The individuals within the green polygon that are pink are from the WM sampling locale. (Color figure online)



structure within it. All analyses of genetic structure within the Bi-State suggest some level of genetic subdivision, particularly separating part of PN (northernmost Bi-State) and WM (southernmost Bi-State), with a group of sampling locales in the middle of the Bi-State (Fig. 4a). The STRUCTURE analysis defined three subpopulations (PNa, mid Bi-State, and WM) within the Bi-State (Fig. 4a) and all were significantly different from one another in pairwise F_{ST} tests using microsatellite data (Table 4). Interestingly, the subset of individuals within PN (PNa) that appear to have a different assignment in STRUCTURE (Fig. 4a) all have mtDNA haplotypes that are shared with Lassen (A and DH). Further evidence using FCA analysis also revealed that the PNa subpopulation formed a group different from the rest of the Bi-State birds (blue polygon in Fig. 5). Like the PNa, the WM subpopulation shows differentiation from the rest of the sampling locales within the Bi-State. The WM subpopulation had typical unique Bi-State haplotypes (BM and DQ) yet lacked the common BJ. When examining the microsatellite data in STRUCTURE, this differentiation was also evident (Fig. 4a). These patterns of genetic structure appear to fit a north–south gradient (Figs. 4a, 5) that is further supported by a positive isolation-by-distance that was found using the Mantel test. The northernmost (PNa) and southernmost (WM) subpopulations are differentiated with some amount of mixing from WM into the LV sampling locale just north of WM (Fig. 4a). The FCA analysis offered similar evidence but more subtly revealed that the WM individuals were separated from other sage-grouse within the Bi-State yet aligned most closely with the neighboring sampling locale LV. Additional STRUCTURE analyses at lower hierarchical levels, within the mid Bi-State (Fig. 4b), revealed patterns of relatedness among individuals from sampling locales that were geographically close, aligning the northern sampling locales (PNb, JF, WBF, and BH) and the southern sampling locales (PM and LV).

Pine Nut Mountains is the most northerly sampling locale in the Bi-State DPS and was likely loosely connected to the Lassen population historically through a somewhat contiguous band of sagebrush-steppe along the foothills of the Sierra Nevada Mountains. Today, connectivity that may have allowed a limited amount of gene flow between PN and Lassen has been lost largely as a result of loss of sagebrush habitat and severe fragmentation caused by continued expansion of urban development (e.g., population centers of Reno, Carson City, Gardnerville, etc.). Although evidence suggests that genetic differentiation among sampling locales of sage-grouse along the Sierra Nevada Mountains in a north to south gradient has been taking place for thousands of years, perhaps only recently has connectivity been completely lost with increased urbanization. Interestingly, data from an ongoing telemetry

study of the sage-grouse sampled for this study suggested that the birds in PN that share haplotypes with the Lassen population (PNa) used a different area during the spring months (northern end of the PN) than those sage-grouse (PNb) that consisted of haplotypes similar to the rest of the Bi-State DPS (southern end of the PN; U.S. Geological Survey, unpublished data). These telemetry data also indicated that PNb birds that were followed using radio- and GPS-telemetry were tracked to other sampling locales within the mid Bi-State subpopulation, which is consistent with the second level of the hierarchical STRUCTURE analysis (Fig. 4b) that aligns PNb birds with other sampling locales in the northern part of the mid Bi-State. However, sample sizes were limited and these phenomena warrant further investigation.

The sharing of haplotypes of sage-grouse in the PNa with those found in Lassen suggests that this is an important subpopulation within the Bi-State DPS largely because it represents the last known link to greater sage-grouse outside the Bi-State and maintaining this subpopulation may help preserve the overall genetic variation across this region. The population management unit (PMU) for PN largely consists of pinyon–juniper woodlands, which has been identified as the most important threat to greater sage-grouse in this area (Bi-State Technical Advisory Committee Nevada and California 2012). These woodlands likely fragment sagebrush habitat by forming barriers to movement, as sage-grouse are known to avoid conifers (Commons et al. 1999; Casazza et al. 2011).

The high elevation WM subpopulation is located at the southwestern edge of the species' range east of the Sierra Nevada Mountains. Previously collected radio-telemetry data indicated that average elevation for these sage-grouse was $3,200 \pm 220$ m with some individuals commonly located over 3,600 m (U.S. Geological Survey, unpublished data). Sagebrush-dominated vegetation communities are primarily found at the sub-alpine elevation zone between 2,900–3,660 m (Mooney et al. 1962). Vegetation on lower elevation slopes (1,980–2,900 m) is dominated by pinyon and juniper woodland. Similar to the PN area, this band of woodlands likely substantially reduces movement to and from nearby sampling locales. The closest known breeding location to the WM is LV, separated by a distance of about 60 km, and our genetic data suggests that LV is the only likely sampling locale that maintains a connection to WM (Figs. 4a, 5). A large majority of the area between WM and LV consists of fragmented habitat and is unsuitable for greater sage-grouse based on a recent habitat suitability index (U.S. Geological Survey, unpublished data).

Habitat management actions that promote connectivity between the outer subpopulations (PNa and WM) to other areas within the Bi-State DPS may be crucial to increase

gene flow and maintain genetic diversity within subpopulations. For example, both subpopulations would benefit from reduction in conifers to promote sagebrush-dominated corridors linking the subpopulations. Further, conservation measures that reduce habitat fragmentation within the PNa and WM subpopulations, such as targeted removal of trees within areas of recent conifer expansion, may help preserve connectivity of seasonal habitat within the subpopulations.

The level of genetic diversity in the Bi-State DPS as a whole is comparable to levels of diversity found elsewhere across the species range (Oyler-McCance et al. 2005a). This is important and points to the fact that the differences documented in the Bi-State as a whole were not likely caused by a genetic bottleneck or a founder event. Among sampling locales within the Bi-State area, PM and WM had the lowest levels of genetic diversity in both number of mtDNA haplotypes (two and four respectively) and in microsatellite allelic richness. Parker Meadows represents a small, isolated group of greater sage-grouse in the Bi-State. This sampling locale has consistently lower lek counts than most other sampling locales in the Bi-State (California Department of Fish and Wildlife, unpublished data). Parker Meadows is surrounded by areas that are unsuitable for sage-grouse based on a recent habitat suitability analysis (U.S. Geological Survey, unpublished data). Greater sage-grouse within PM are confined to a relatively small area and the low level of genetic diversity may indicate genetic drift and/or a genetic bottleneck. Recent reductions in levels of gene flow from neighboring sampling locales may be exacerbating these phenomena. It is possible that the relatively low genetic variation we observed in PM was a function of limited sample size. However, during trapping efforts we observed fewer sage-grouse compared to other sampling locales and lek counts were among the lowest within the Bi-State suggesting that PM is simply a small sampling locale. Thus, while the sample size was lower compared to others in the data set, we believe that these samples were of sufficient size to capture the total genetic variation within this sampling locale.

Reduced genetic variation within PM is important information for wildlife managers and conservationists. Although the importance of maintaining substantial genetic variation in small populations is debated, most biologists agree that genetic variation is relevant to maintaining population viability and should be addressed in management plans (O'Brien and Evermann 1988; Quattro and Vrijenhoek 1989). Populations with low genetic variation may face enhanced susceptibility to parasitic agents or infectious disease such as West Nile Virus, which is a known threat for greater sage-grouse (Naugle et al. 2004). Further, reproductive success of an isolated population of a close relative, greater prairie-chicken (*Tympanuchus*

cupido) was shown to be reduced due to a bottleneck caused by habitat loss (Bouzat et al. 1998; Westemeier et al. 1998). Recent observations have indicated that nest abandonment rates are higher at PM and eggs from abandoned nests are generally not fertile (U.S. Geological Survey, unpublished data; Scott Gardner, California Department of Fish and Wildlife, personal observation). Translocations augmenting the PM are a potential conservation strategy based on results from this study. Our original STRUCTURE analysis suggests that any of the sampling locales within the mid Bi-State subpopulation would be appropriate sources for translocation into PM, yet BH and LV are closest geographically and show some admixture with PM (Fig. 4b). Nevertheless, continued monitoring of genetic diversity in PM may be warranted and characteristics of fitness as they relate to genetic diversity should be more closely examined in this sampling locale.

Conclusion

As reported previously, (Benedict et al. 2003; Oyler-McCance et al. 2005a) the Bi-State population is genetically unique and may have been isolated from other greater sage-grouse for thousands or tens of thousands of years. We document a similar pattern here with seven of the eleven mtDNA haplotypes unique to the Bi-state representing 73 % of the individuals. While our percentage of individuals with novel haplotypes is lower than was reported by Benedict et al. (2003) and Oyler-McCance et al. (2005a) (97.7 and 88.89 % respectively), it is still far higher than the average of unique haplotypes across the species range (8.37 %, Oyler-McCance et al. 2005a) and is likely due to the inclusion of birds from the Pine Nut Mountains area, which represents the closest link to the Lassen birds. Within the Bi-State population we found finer scale genetic structure that has likely been exacerbated by recent human activities and the encroachment of pinyon and juniper trees. The findings from this hierarchical structure-based approach allowed us to further delineate the genetic structure within the DPS and identify three subpopulations (PNa, mid Bi-State, and WM) that would benefit from conservation actions. The WM subpopulation, at higher elevation and at the southernmost edge of the Bi-State, is genetically isolated, such that maintaining or even improving connectivity with the LV sampling locale may be beneficial. Additionally, the PM sampling locale is small, with low genetic diversity and should be monitored. Managing WM, LV, and PM to maintain connectivity with neighboring sampling locales may be important for their long-term viability. Further, PN contains individuals carrying the unique Bi-State genetic signature (PNb, part of

the mid Bi-State subpopulation) and also a group of individuals that are a mix of Lassen and Bi-State (PNa subpopulation). Pine Nut Mountains, at the northernmost edge, represents the boundary of the Bi-State. All other sampling locales carry the unique Bi-State signature. While genetic concerns may be only one of many priorities for the conservation and management of the Bi-State, we believe that they warrant attention along with other issues. Conservation strategies for the Bi-State should include preventing future habitat loss and fragmentation, enhancing existing habitat, protecting and maintain connectivity among and within subpopulations, and monitoring genetic diversity.

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