FRED FULSTONE, JR.
MARIANNE F. LEINASSAR
Phone: 775-465-2381
Fax: 775-465-1200

F.I.M., CORP.

Farming and Livestock

P.O. BOX 12
SMITH, NEVADA 89430

January 4, 2014

TO: Governor Brian Sandoval and Sagebrush Ecosystem Council

CC: Ted Koch, US Fish and Wildlife Service,
Lyon County Commission,
Mono County Supervisors

SUBJECT:

1. Response to Ted Koch remarks of Dec.3, 1013
2. Response to Sagebrush Ecosystem Council meeting of Dec. 18, 2013

It comes as quite a shock to the whole agriculture and livestock community of Nevada, that the USFWS went against all of their own words and assurances to us regarding the greater sage grouse, and decided to propose a listing of the Bi-State DPS of Sage grouse as “threatened”.

They told all of us what we wanted to hear, and went behind our backs and did what they wanted. The USFWS State director Ted Koch, said that the Bi-State working group plan was the best he had seen. He applauded the Bi-State working group for all their work and for the implementation of programs of the last 10 years on the sage grouse preservation. The programs put in place with the cooperation of NRCS, the over 16,000 acres of re-furbished pinion/juniper land, the conservation easements that have been secured, and all the hard work and sweat put forth to protect the sage grouse. Yet it all seems to be MOOT.

Agency biologists now say that the sage grouse in the Lyon/Mono county region is a separate kind of greater sage grouse than the other sage grouse in the rest of Nevada with a different DNA. The mtDNA [from the female side], is definitely from the Greater Sage Grouse in Nevada and linked to Canada and Washington, but some studies seem to indicate that the nuclear DNA is distinct to this area population.

The genetic evaluation of Bi-State sage grouse does not warrant classification as a subspecies in the following two papers, both attached: (1) S.J. Oyler-McCance, S.E. Taylor, and T.W. Quinn. *“A multilocus population genetic survey of the greater sage-grouse across their range”*. Molecular Ecology (2005)14:1293-1310 and (2) Nicolas G Benedict, SJ Oyler-Mcance, S.E.

Taylor, C.E. Braun, and T.W. Quinn *"Evaluation of the eastern (Centrocercus urophasianus urophasianus) and western (Centrocercus urophasianus phaios) subspecies of Sage Grouse using mitochondrial contol region sequence data"*. Conservation Genetics 4:301-310, 2003

Oyler-McCance et.al. *"A multilocus population genetic survey of the greater sage-grouse across their range"* cites Benedict [et,al] [2003] "it is noted that the Lyon/Mono population represents separation by "ALLOPATRIC FRAGMENTATION".

Allopatric fragmentation means, according to Biology 413{ZOOGEOGRAPHY}, " the separation of a population into two or more geographically isolated populations." Allopatric fragmentation is considered one of the prime, if not major processes, that promotes "evolutionary diversification." This document also states on page [1307] enclosed, "The Bi-State [Lyon/Mono] population is distinct in a way that could be significant in that genetic variation is relevant and necessary to the health and viability of populations, and should be monitored as a MANAGEMENT UNIT [MU]. As reported, the Lyon/Mono population is significant with divergent alleles of nuclear micro DNA but the mtDNA [female], control region types are not reciprocally [present on both sides] monophyletic [developed from a single ancestral type] greater sage grouse despite most newly arisen DNA within this population. Although the Lyon/Mono population could and would be considered a M.U.{ Management Unit] as defined by Moritz [1994], it would NOT be considered an Evolutionary Significant Unit [ESU]. ESU status is necessary for listing under the ESA and the so-called Bi-State sage grouse is just another population of greater sage grouse.

In a lek breeding species such as the greater sage grouse where only a few males do most of the mating, sexual selection can act to influence morphological and behavioral traits at a rate much faster than can be tracked genetically. The nuclear DNA can undergo more of a bottleneck relative to mtDNA [female] inherited in most species. Preliminary comparisons of gross morphology [how they look] and the behavior between the surrounding greater sage grouse populations have revealed little or no differences. S.E. Taylor [unpublished], Young et al [2000].

The distinct population segment is a term used by the USFWS under Endangered Species Act regulations. BLM, FS, and environmental groups whole heartedly endorse the use of DPS in this case to set apart a small group of Greater Sage Grouse, to lock up 1.9 million acres of land for a bird they say hasn't traveled more than a hundred miles in its thousand years history. They base their conclusion on their strongest feelings called professional opinions and not on known facts including the lack of reported sage grouse observations by explorers prior to 1850.

Where did logic and science come from in this case? Not from the "best scientific or commercial data" available. They need to read more and see the WHOLE report, not just the pieces to fit their agenda.

The State director of the USFWS stated at the December 3, 2013 meeting of the Bi-State working group , in Bridgeport, California, that the Governors Sagebrush Ecosystem Council, had NO say in the Bi-State sage grouse issue. The Governor's bill AB461, created a council to oversee ALL the greater sage grouse in Nevada, but according to Mr. Koch, did not apply to or

have anything to do with the decisions, processes or consultations regarding the Bi-State DPS of sage grouse. I hereby challenge all the Governor's AB461 council [Sagebrush Ecosystem Council], to read said bill and all its amendments, and discuss it again. It clearly states on page 4 of the document that "The State of Nevada has authority to manage ALL wildlife belonging to the State that is not listed pursuant to the Endangered Species Act.. That can only mean that it is the duty of the sworn public officials who make up the Sagebrush Ecosystem Council to assemble the best available scientific and commercial data then use that data and the authority of the State to tell the USFWS that any federal data that contradicts the data of Nevada is wrong. USFWS cannot lawfully list the sage grouse until they prove that the data officially held by the State of Nevada is wrong and the federal data is somehow correct.

On July 31, 2012, The Greater Sage Grouse Advisory Committee was created by Executive Order 2012-19, to develop a state specific strategy to conserve the greater sage grouse. It also states on page 4 of Bill AB461, " Whereas, It is in the interest of this State to bring stakeholders and relevant agency experts together on an ongoing basis to guide the implementation of conservation measures sufficient to preclude the need to list the greater sage grouse, the Bi-State sage grouse, and other species that inhabit sagebrush ecosystems within the state."

How can anyone, who knows how to read, not see what this statement says and determine that the council IS required by Nevada law to address the Bi-State Sage grouse issues. They took an OATH of Office to follow and protect the Constitution of Nevada when they oversee the sage hen and all other wildlife species and that includes protection of Nevada and its people from harm regarding federal regulation of the sage hen [all species]. When a law is broken, there are consequences and those consequences may be even more severe for public officials because they have also violated their oath of office. This council can clearly see that the sage grouse in Nevada are ALL Greater Sage Grouse, no matter what part of the state they live in. None are physically separated by geography, even the ones you call a DISTINCT POPULATION SEGMENT. They are still in Nevada, and are still Greater sage grouse, no matter how you decide to look at them. In reality, it does not matter what I believe or you believe, the reality is you are obligated by LAW to do EVERYTHING in your power to protect this bird and the people of Nevada. That starts with the Governor and goes down to the lowest Nevada employee and committee appointee. You are here for Us.

Further the Governor and the respective County Commissioners need to read the "Endangered Species Act of 1973, and see that in Section 4 under the heading of "Determination of Endangered Species and Threatened Species" category, letter C, it states, "The Secretary of the Interior shall by regulation promulgated in accordance with subsection [b] determine whether a species is an endangered or threatened species because of any or all of the following factors …. [c] disease or PREDATION". As for Mr. Koch of the USFWS, and others in the Department of the Interior, who proposed to list the Bi-State population and possibly the entire greater sage grouse population, they clearly understand the predator control is a responsibility of the State. Yet the Governor's council changed the original plan that showed predation as a major threat at the top of the list to a threat at the bottom of the list because you did not want to deal with the environmental groups that oppose killing any species to save another. Right or wrong, you are

obligated under the law to do just that. Federal agencies have a scientist, Dr. Peter Coates, who wrote in his report that over 80% of the loss of nests, eggs, and chicks was due to predation. Yet NDOW and the Governor choose not to address the situation because of political challenges. ESA Section 4 also states under the same heading and under [b] Basis of Determination [1]]a], "The Secretary shall make determination required by subsection [a][1] solely on the basis of the best scientific and commercial data available to him after the review of the status of the species and after taking into accounts those efforts, if any, being made by any State or foreign nation, or any political subdivision of any state or foreign nation, to PROTECT SUCH SPECIES, WHETHER BY PREDATOR CONTROL, PROTECTION OF HABITAT OR FOOD SUPPLY, OR OTHER CONSERVATION PRACTICES, WITHIN ANY AREAS UNDER IT'S JURISDICTION, OR ON THE HIGH SEAS. It is especially important for all parties involved, to read all of Section 4, for it clearly lays out the guidelines which must be followed for a lawful determination of "threatened or endangered species". If you are going use the Endangered Species Act for your ulterior motives, then you must abide by the WHOLE Act, not just pieces to suit your agenda. You ALL must be held accountable to the people of Nevada and to the other 11 states which also face the determination on Greater sage grouse It is not too late for Nevada's Governor to develop a predator control program under the Division of Conservation that would meet the requirements of adequacy and deprive the federal officials of one excuse for listing the sage hen.

Do what is right. Fight for Nevada and against the abusive ESA listing of the Greater Sage Grouse including the Bi-State sage grouse populations.

Please.

(S) Fred Fulstone

Fred Fulstone
FIM Corp
P.O. Box 12
Smith, NV 89430

Conservation Genetics **4**: 301–310, 2003.

Evaluation of the eastern (*Centrocercus urophasianus urophasianus*) and western (*Centrocercus urophasianus phaios*) subspecies of Sage-grouse using mitochondrial control-region sequence data

Nicolas G. Benedict[1], S.J. Oyler-McCance[1,2], S.E. Taylor[1], C.E. Braun[3] & T.W. Quinn[1,*]
[1]*Department of Biological Sciences, University of Denver, Denver, Colorado 80208, USA;*
[2]*Mid-continent Ecological Science Center, U.S. Geologic Survey, 4512 McMurry Avenue, Fort Collins, Colorado 80525, USA;* [3]*Grouse Inc., 5572 N. Ventana Vista Rd., Tucson, Arizona 85750, USA*
*(*Author for correspondence, e-mail: tquinn@du.edu)*

Received 2 January 2002; accepted 15 April 2002

Key words: Centrocercus urophasianus phaios, Centrocercus urophasianus urophasianus, control region, mitochondrial, Sage-grouse

Abstract

The status of Sage-grouse (*Centrocercus urophasianus*) is of increasing concern, as populations throughout its range have contracted as a result of habitat loss and degradation. Historically, Sage-grouse were classified into two subspecies: eastern (*C. u. urophasianus*) and western Sage-grouse (*C. u. phaios*) based on slight differences in coloration noted among eight individuals sampled from Washington, Oregon, and California. We sequenced a rapidly evolving portion of the mitochondrial control region in 332 birds from 16 populations. Although our sampling area covers the proposed boundary between the eastern and western subspecies, no genetic evidence to support the delineation of these subspecies was found. However, a population straddling southwestern Nevada and eastern California was found to contain an unusually high proportion of unique haplotypes, consistent with its genetic isolation from other Sage-grouse populations. Of additional interest was the lack of diversity in the two populations sampled from Washington, one of which contained only a single haplotype. We suggest that multiple lines of evidence are valuable for the formulation of conservation strategies and hence the southwestern Nevada/eastern California population merits further morphological, behavioral, and molecular investigation.

Introduction

The status of Sage-grouse (*Centrocercus urophasianus*) is of increasing concern, as populations throughout its range have been negatively impacted by habitat loss and degradation (Braun 1998). This has resulted in their extirpation from five U.S. states and one Canadian province (Johnsgard 1973; Braun 1998). Remaining populations often become isolated and contain small numbers of individuals (Braun 1995) (Figure 1).

Historically, Sage-grouse were classified into two subspecies: eastern (*C. u. urophasianus*) and western Sage-grouse (*C. u. phaios*) based on slight color differences in eight individuals collected from Washington, Oregon and California (Aldrich 1946). Western Sage-grouse presumably occurred in southern British Columbia, central Washington, east-central Oregon, and northeastern California (Aldrich 1946). Populations in other areas of the range are considered to be eastern Sage-grouse. The validity of this taxonomic distinction has since been questioned (Johnsgard 1983).

While this species has recently been the target of extensive conservation efforts, the taxonomic/genetic relationships between populations/subspecies remain poorly understood. At the southeastern edge of their range, Sage-grouse from southwestern Colorado and

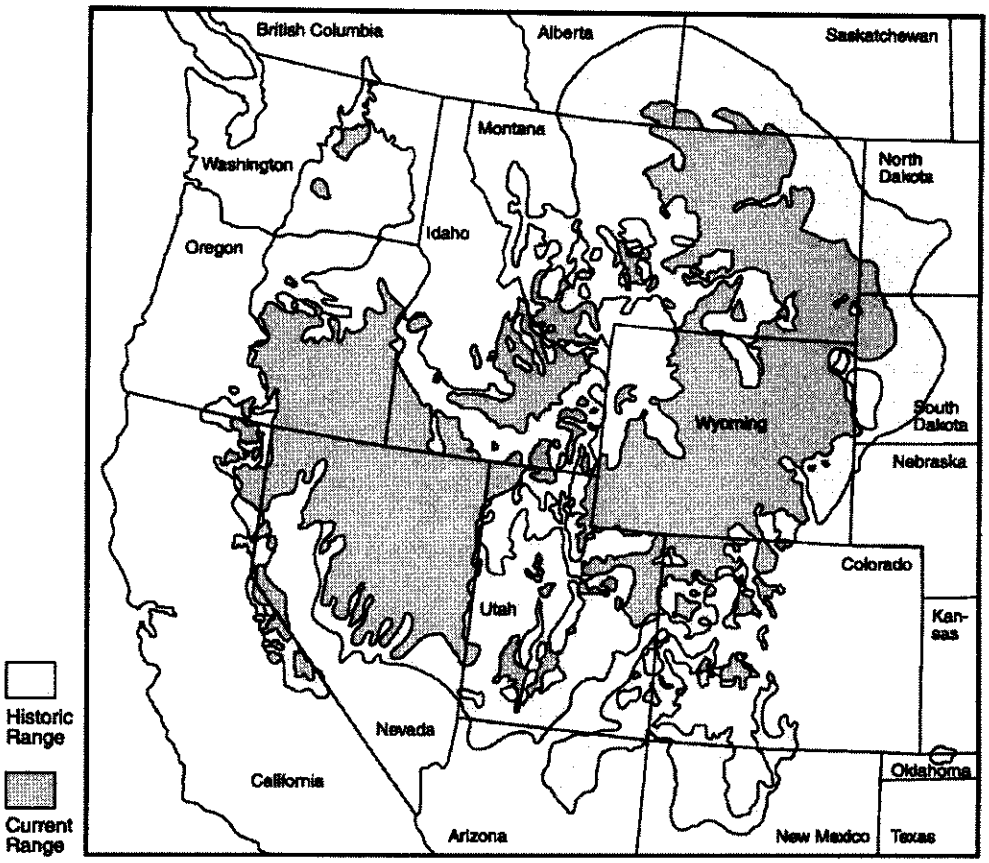

Figure 1. Historic (early 1900s) and current distribution of Sage-grouse in western North America.

southeastern Utah have recently been described as a new species known as Gunnison Sage-grouse (*C. minimus*) (Young et al. 2000), based on morphological (Hupp and Braun 1991), behavioral (Young et al. 1994), and genetic (Kahn et al. 1999; Oyler-McCance et al. 1999) data. For the genetic studies, Oyler-McCance et al. (1999) and Kahn et al. (1999) sequenced a rapidly evolving portion of the control region of mitochondrial DNA (mtDNA) from nine populations of Sage-grouse in Colorado, spanning the boundary between the commonly found Sage-grouse and the Gunnison Sage-grouse. Both these data and additional data from nuclear microsatellites (Oyler-McCance et al. 1999) suggests a lack of gene flow between these groups.

Because the distinction between the eastern and western subspecies has been questioned (Johnsgard 1983), our objective was to use the methods of Kahn et al. (1999) and Oyler-McCance et al. (1999) to determine whether there was evidence at the genetic level to support designation of the western subspecies. While genetic data alone can only support or not support a subspecies distinction, we believe that, as in Young et al. (2000), morphological, behavioral, and genetic data when used in conjunction, can help clarify such taxonomic questions. In addition, we were interested in providing information relevant to an understanding of gene flow, genetic diversity, and evolutionary history among Sage-grouse populations in Washington, Oregon, Nevada, and California. This type of information can often be used in the development of cohesive management strategies that take genetic distinctiveness into account.

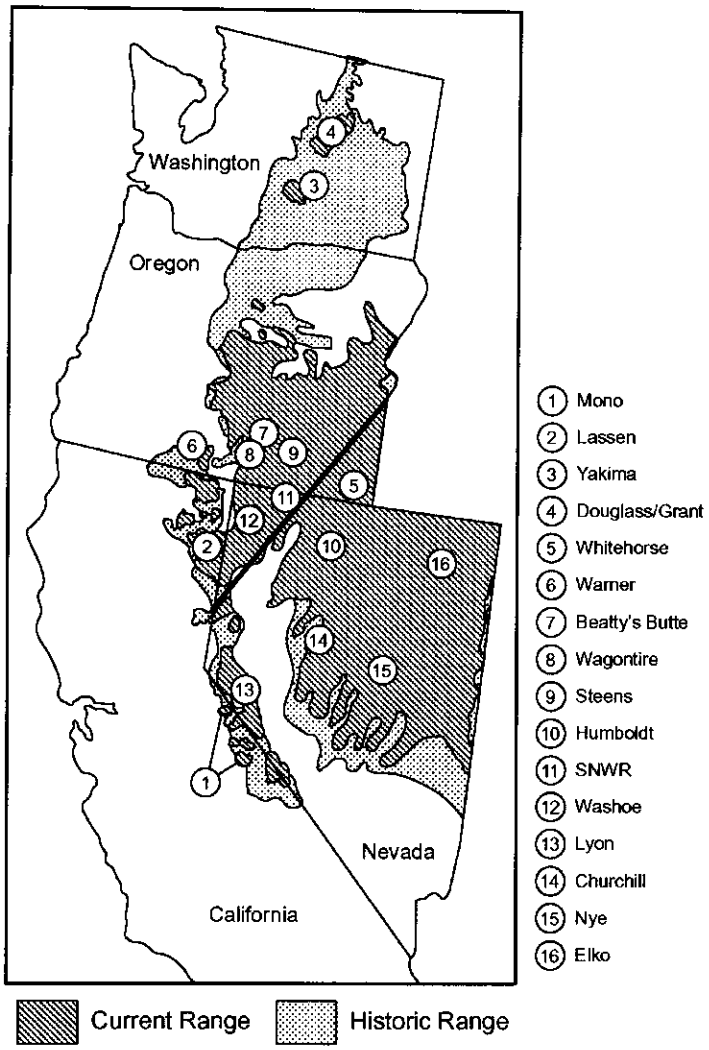

Figure 2. Location of study populations. The solid line denotes the delineation between the eastern and western subspecies as proposed by Aldrich (1946).

Methods

Sage-grouse tissue samples were collected from 16 populations in California, Nevada, Oregon, and Washington (Figure 2), crossing the boundary separating the eastern and western subspecies as described by Aldrich (1946, 1963). Approximately 20 birds were sampled from each population (Table 1). Most tissue samples consisted of muscle obtained from wings of hunter-killed birds. Consequently, these wings were collected by hunt unit, which we are loosely referring to as "populations". These units were delineated by the wildlife professionals most familiar with these birds and the geographic regions in which they reside. These biologists further suggest the Lyon (NV) and Mono (CA) populations are more appropriately considered as a single contiguous population that happens to cross a state boundary (D.S. Blankenship, pers. comm.; S.J. Stiver, pers. comm.; C.E. Braun, pers. comm.; J.R. Young, pers. comm.). To minimize the concern

Table 1. Individuals sampled, by population and haplotype.
Haplotypes underlined and in italics are from clade I, while those in bold are from clade II.
α = represents proportion of haplotypes from clade I.
N* = number of individuals sequenced.
Bottom two rows show the Lyon/Mono population vs. all others

	N*	α	*A*	B	*F*	O	Q	T	U	X	*AG*	*AU*	AV	*AZ*	*BB*	BD	BF	BO	*BX*	*CH*	CJ	CK	CN	CR	*DA*	*DB*	*DC*	*DD*	DH	DL	DM	DS	*DT*	BJ	BK	*BL*	*BM*	BQ	DP	*DQ*
Yakima WA	18	0.00								18																															
Douglass/Grant WA	18	0.11								13																						3	2							
Whitehorse OR	33	0.42	14	1			1	10	4	2											1																		
Steens OR	21	0.29	5	1	1		1	8		4																					1								
Warner OR	19	0.37	4		1		2	2		5													1	2				1					1						
Wagontire OR	19	0.42	6		1		4	2	1	1										1			3																
Beatty's OR	21	0.29	4				1			6			4							2	2	1		1																
Churchill NV	18	0.06		2				5		8				1		1	1																							
Washoe NV	20	0.40	6				3		1	6				1				1	1		1																		
Elko NV	20	0.50	3	3						5	1	4	2												1		1												
Humboldt NV	21	0.67	10	2			1	1		3																4														
Sheldon NWR NV	19	0.16	2			1	2	6	1	6					1																								
Nye NV	20	0.30		4				1		9	1			4	1																									
Lyon NV	20	0.20					1																												5	9	3	1	1		
Mono CA	23	0.65																																	7		4	8		1	3
Lassen CA	22	0.23	5				4			8			1																1	1	2								
			A	B	*F*	O	Q	T	U	X	*AG*	*AU*	AV	*AZ*	*BB*	BD	BF	BO	*BX*	*CH*	CJ	CK	CN	CR	*DA*	*DB*	*DC*	*DD*	DH	DL	DM	DS	*DT*	BJ	BK	*BL*	*BM*	BQ	DP	*DQ*
Others	289	0.31	59	13	3	1	19	35	7	94	2	4	7	6	2	1	1	1	1	3	4	1	4	3	1	4	1	1	1	1	3	3	3							
Lyon/Mono	43	0.44					1																											12	9	7	9	1	1	3

of over-sampling from single broods, primarily adult (86%) females (87%) were sampled after they had already left their lek sites.

The only populations in this study that are no longer hunted are those in Washington. Samples from these birds consisted of either blood or feathers and were provided by M. A. Schroeder of the Washington Department of Fish and Wildlife. These birds were trapped following the methods of Giesen et al. (1982) and blood was collected as described by Oyler-McCance et al. (1999).

In most cases DNA was extracted using a phenol-chloroform based extraction as described by Kahn et al. (1999). All other samples were extracted using either a chelex-based method (Walsh et al. 1991) or the Wizard Genomic DNA Purification System (Promega), following the manufacturer's instructions.

The Polymerase Chain Reaction (PCR) amplification and manual sequencing was performed following the protocol and using the primers outlined by Kahn et al. (1999), in approximately two-thirds of the cases. All reactions were performed using previously described primers, 16775L (Quinn 1992), 521H (Quinn and Wilson 1993), and 418H (Quinn and Mindell 1996). In their study, Kahn et al. (1999) found that 92% of the variation contained in a 380 bp region of the highly variable mitochondrial control region I, was within a 141 bp region. It was this 141 bp hyper-variable region that was sequenced in our study. The remaining one-third of our samples were sequenced using a dye terminator cycle sequencing reaction (Beckman Coulter CEQ2000), using the same primer sets. In these instances, double-stranded PCR products were cleaned using either QIAquick spin columns (Qiagen) or Amicon Microcon-PCR Centrifugal Filter Devices (Millipore), following the manufacturers instructions. The cycle sequencing and subsequent purification of the dye-labeled products was performed using the manufacturer's protocol. These samples were then run on the CEQ2000 automated sequencer (Beckman Coulter).

All sequences were aligned manually and haplotypes were identified using the program MacDNAsis Pro Version 2.0 (Hitachi). Nei's minimum distance (Nei 1972), Roger's distance (Rogers 1972), and Wright's modification of Roger's distance (Wright 1978) were calculated using the software TFPGA (Miller 1997). Neighbor-Joining trees were constructed using the Phylip software package (Felsenstein 1989). A maximum parsimony analysis was performed using the heuristic search algorithm in the software package PAUP*4.0b4a (Swofford 1999), as was done in Kahn et al. (1999). Evaluation of F-statistics was performed using the TFPGA software package (Miller 1997).

To determine whether there was genetic support for the subspecies distinction, we used a randomization test (Manly 1991). In this test, the six populations belonging to the eastern subspecies were pooled as were the nine belonging to the western subspecies. The frequency of each haplotype was calculated for each subspecies, using the following statistic:

$$x = \sum_{i=1}^{38} \frac{(fw_i - fe_i)^2}{\left(\frac{(fw_i + fe_i)}{2}\right)}$$

where fw is the frequency of haplotype i in the western subspecies and fe is the frequency of haplotype i in the eastern subspecies. To compare these frequency differences to those generated with randomized groupings, six populations were randomly assign to the eastern subspecies and nine populations to the western subspecies. The test statistic x was then recalculated. This process was repeated 30,000 times. Our original statistic was then compared to the distribution of the 30,000 randomly generated statistics to determine P values. This procedure was also modified to test whether the Lyon/Mono population and Washington populations were statistically different from all other populations.

Results

Thirty-eight haplotypes were identified among the 332 birds assayed (Table 1). Collectively across all haplotypes, 40 sites were variable. These sites contained 27 transitions, 12 transversions, 7 deletions, 4 insertions, and one site containing both a transition and a transversion. Twenty of these sites were informative for parsimony analysis. All haplotypes fell into one of the two distinct monophyletic clades (Clade I and Clade II) described in Kahn et al. (1999) (Figure 3). Of these 38 haplotypes, 33 had not been described in previous studies by our lab (genbank accession numbers AF543863–AF543895). Labeling of haplotypes by our lab has progressed alphabetically as they have been identified. An evaluation of the distribution of haplotypes revealed that five of the previously identified and widespread haplotypes (A, B, Q, T, and X), were found in at least 6 and as many as 14

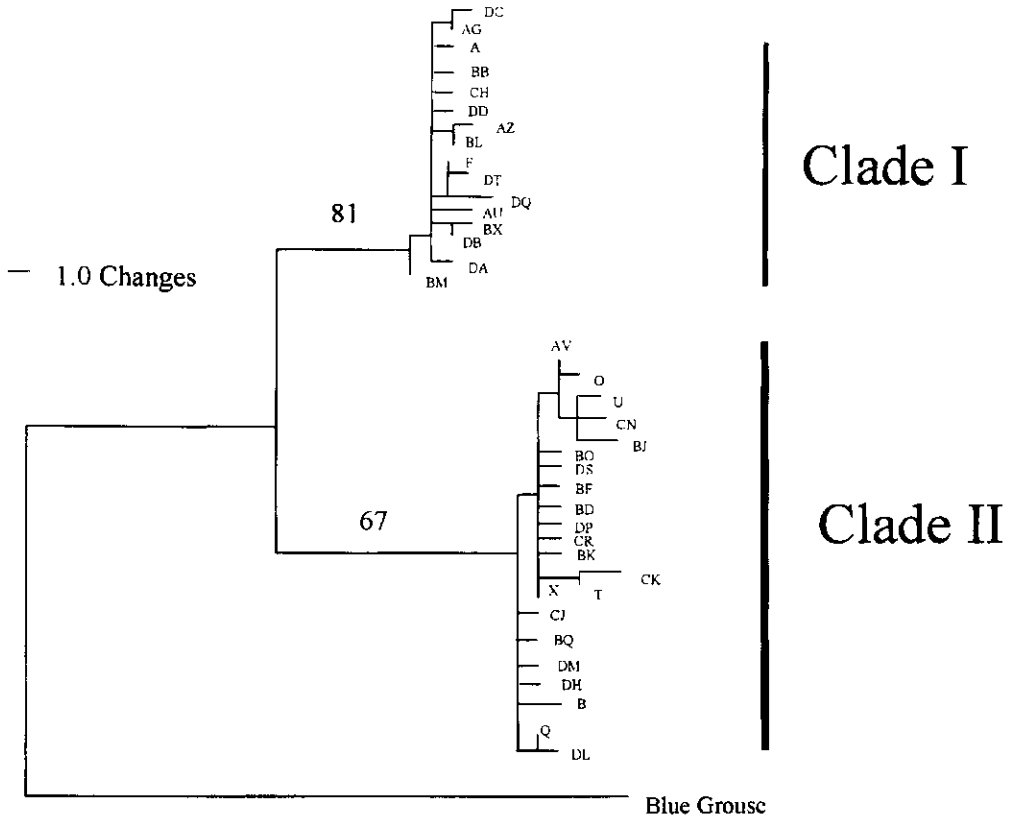

Figure 3. Phylogram of the strict-consensus tree of all haplotypes presented. The tree has a consistency index of 0.882, a retention index of 0.970 and a rescaled consistency index of 0.856. Bootstrap values > 50 are presented on the branches of the tree.

of the populations sampled. Of the birds sampled, 221 (66.6%) had one of these five haplotypes. The X haplotype was found in all populations sampled except the Lyon/Mono population. This widespread haplotype was the only one found in the Yakima (WA) population and constituted the majority of the haplotypes in Douglass/Grant (WA) birds.

Of the 29 newly identified haplotypes, 17 are unique to single populations. Of the remaining 12, only three are present in more than two populations. The most abundant and widespread haplotypes encountered in this study (A through X) are also found in eastern Sage-grouse as far away as Colorado. When these common haplotypes are removed from our data set, only 11 haplotypes that are shared among two or more populations remain.

Since all multiple neighbor-joining trees suggested similar partitioning, a single representative tree is presented (Figure 4). There is no partitioning of the populations representing the eastern and western subspecies. However, the Lyon/Mono and Washington populations do segregate from the other populations.

The distribution of novel haplotypes was evaluated, as was the proportion of novel haplotypes among groups. The frequency with which these novel haplotypes are found in their respective groups ranged from 0 (Whitehorse, Wagontire, Beattys, Steens, Sheldon NWR, and Nye), to a high of 97.7% (Lyon/Mono) (Figure 5). With the exception of Lyon/Mono, no population had more than 30% of its individuals comprised of these novel haplotypes. The F-statistics provided no support for the subspecies distinction (Fst = 0.0356, $p > 0.05$).

The randomization test showed no genetic support for the subspecies distinction ($x = 1.49$, $P > 0.05$). In contrast, the distribution of haplotypes in

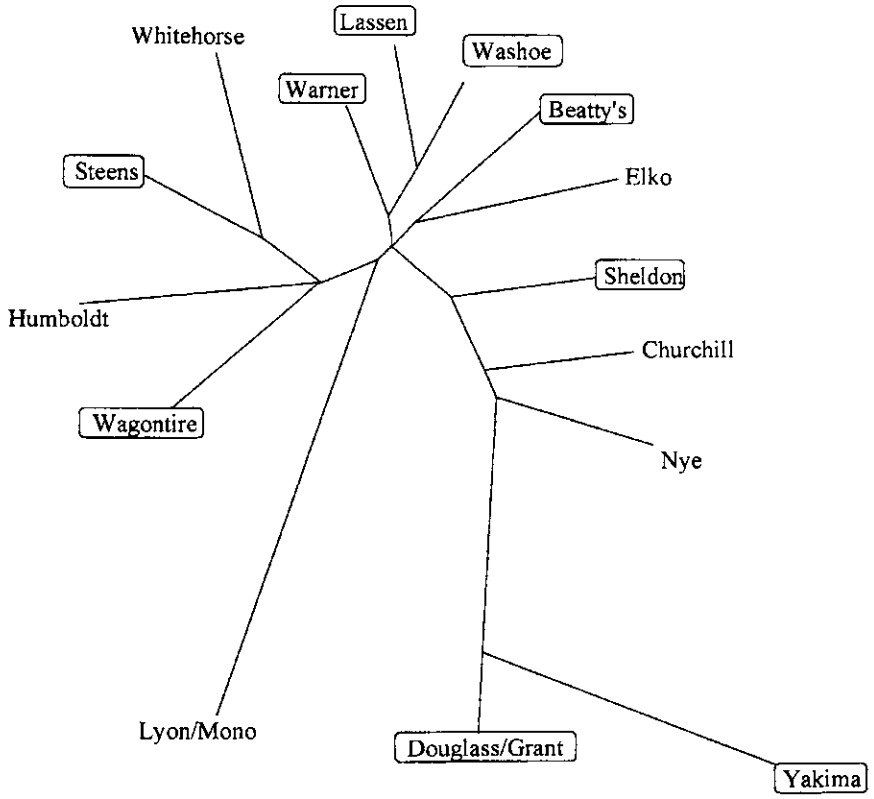

Figure 4. Neighbor-Joining tree constructed using Wright's (1978) modification of Roger's genetic distance (Boxed populations represent the western subspecies, while unboxed populations represent the eastern subspecies).

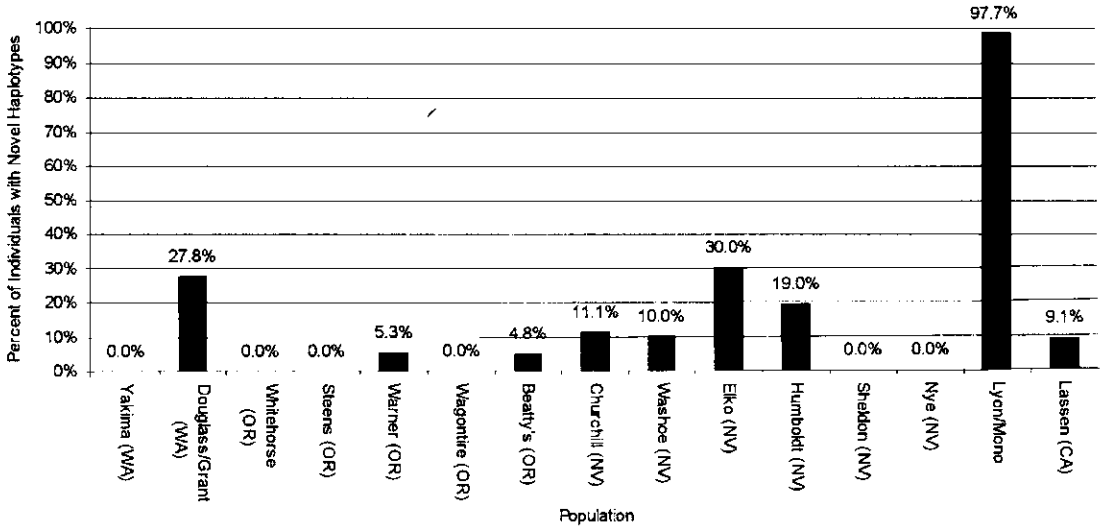

Figure 5. Proportion of individuals per population with novel haplotypes.

Lyon/Mono was statistically different from all other populations ($x = 3.86$, $P < 0.001$). The Washington populations were also statistically different from all other populations ($x = 2.61$, $P < 0.05$).

Discussion

Fossil records from the Pleistocene document Sage-grouse in Colorado, Nevada, New Mexico, Utah, Wyoming, and Idaho (Shufeldt 1913; Howard and Miller 1933; Howard 1952; Miller 1963; Miller 1965; McDonald and Anderson 1975; Grayson 1976; Emslie 1985; Emslie and Heaton 1987; Emslie 2001). By 6,000 years ago Sage-grouse were also documented in northern California (Miller 1963; Grayson 1976). Pollen records suggest that the requisite sagebrush (*Artemisia* spp.) habitat was patchily distributed throughout the southwestern United States during the Pleistocene (Van Devender and King 1971; Wright et al. 1973; Madsen and Currey 1979; Emslie 1986; Nowak et al. 1994; Hall and Valastro 1995; Koehler and Anderson 1995). It would follow that Sage-grouse were limited to these patchily distributed refugia during this Epoch. This may explain the two distinct monophyletic haplotype clades described by Kahn et al. (1999). These two clades are thought to have begun diverging approximately 850,000 years ago in two geographically isolated populations of Sage-grouse. Under this hypothesis the two clades subsequently intermixed as these populations re-converged.

Analysis of Molecular Variance (AMOVA) is often used for the analysis of sequence based population data in part because it can weight allelic/haplotypic differences according to the number of base substitutions between them. However, in this case, the largest differences between haplotypes come in comparisons between members of Clade I and Clade II. It is the considerable sequence divergence between these two haplotype clades that pose unique difficulties in performing conventional molecular analyses (Figure 3). These differences actually relate to biogeographic conditions that no longer exist (see above) and hence weighting haplotypes according to those differences adds more noise than signal to the analysis. The subtle molecular differences among the modern populations that we have sampled are found in the relatively shallow branches of the respective clades and become obscured when haplotypes of its divergent sister clade are included. All populations, except Yakima (WA), contain multiple haplotypes from both clades. Furthermore, since neither clade is predominant in all populations, neither can be independently evaluated in our molecular analyses, as we would thus encounter unacceptably low sample sizes. Consequently, our analyses focused primarily on the distribution of haplotypes among our populations, rather than on haplotype distances. It is specifically because of these difficulties that statistical tests such as AMOVA were forsaken for the frequency based randomization test previously described.

The number of haplotypes per population ranged from one (Yakima, WA) to nine (Warner, OR), with an average of 6.4. Most populations had a combination of common, rare, and novel haplotypes. The distribution of widespread, common haplotypes showed there was no obvious genetic subdivision between the eastern and western subspecies. In addition, 42% of birds in this study share five haplotypes (A, B, F, X, AG) with populations from Colorado and Utah (Kahn et al. 1999). The Washington populations and the Lyon/Mono population are obvious exceptions to this overall pattern.

Ten of sixteen populations sampled contain novel haplotypes that, to date, are unique to those populations. Typically, these haplotypes vary from those previously described by a single base change (Figure 3). They occur in low frequency in most populations, typically fewer than 10% of the individuals. In stark contrast, 87.5% of the haplotypes found in the Lyon/Mono population are novel, constituting 97.7% of the birds sampled (Figure 5). The only shared haplotype is from a single individual possessing the widespread Q haplotype. Further, the Lyon/Mono population does not contain the ubiquitous X haplotype that has been found in every other population sampled in this study. This high proportion of novel haplotypes coupled with the lack of the X haplotype suggest the Lyon/Mono population has been isolated from neighboring populations for a considerable amount of time. Further, since novel haplotypes closely related to both of the divergent Sage-grouse mitochondrial clades can be found, it is likely that the isolation of this population occurred after the intermixing of historic populations representing the two major haplotype clades. Over thousands and perhaps tens of thousands of years, factors such as mutation, genetic drift, and the fixation of rare haplotypes have resulted in the significant divergence of the Lyon/Mono population from other Sage-grouse populations.

The Washington populations contain the lowest level of haplotype diversity observed. Although two

haplotypes are unique to the Douglass/Grant population, a single haplotype (X) is found in the majority of individuals (86.1%). Low allelic diversity is expected in populations that have recently experienced severe bottlenecks (Hoelzel et al. 1993; Zink 1994; Bouzat et al. 1998; Le Page et al. 2000). Given that these populations now occupy between 8 and 10% of their original range (Friedman and Carlton 1999), such a bottleneck is plausible. Nonetheless, these results could also be explained by the founder effect as the species' range expanded into its northwestern edge during relatively recent postglacial periods.

The neighbor-joining tree shows a lack of dichotomy between the populations representing the eastern and western subspecies (Figure 4). The long branch length of the Lyon/Mono population is attributable to the unique allelic composition of these birds, as evidenced by both their high proportion of novel haplotypes as well as the lack of the widespread X haplotype. Conversely, the long branch representing the Washington populations can be explained by their relative low level of haplotype diversity. This lack of genetic diversity, rather than their unique allelic composition, sets the Washington birds apart.

Using mtDNA sequence data, we found no evidence to support the subspecies delineation proposed by Aldrich (1946). These data, however, did uncover the distinctiveness of the Washington and Lyon/Mono populations. The low genetic diversity in the Washington populations is likely a reflection of population declines (Schroeder et al. 2000). The probable loss of genetic variation caused by this bottleneck and its potentially long-term adverse impact (Bouzat et al. 1998; Le Page et al. 2000) should be addressed as management strategies are developed for these populations. Active management, such as translocation of birds, may be justified to ensure their continued persistence. Preservation of genetic diversity represented by the unique allelic composition of the Lyon/Mono population is also of particular importance for conservation. Given the likelihood that the distinctiveness of neutral genetic markers extends to genes under adaptive selection, this population should be managed independently to avoid the translocation of other Sage-grouse into this area.

Studies in our lab are ongoing to further evaluate populations of Sage-grouse throughout their range, using nuclear microsatellite markers. Meanwhile, it will be critical that additional morphological and behavioral studies of the Lyon/Mono population be undertaken to address taxonomic questions. Sound conservation strategies require that multiple and mutually supportive lines of evidence be used to make prudent delineations at the species and subspecies level.

Acknowledgements

We thank the Colorado Division of Wildlife, Nevada Division of Wildlife, Western Association of Fish and Wildlife Agencies, National Fish and Wildlife Foundation, USDA/Forest Service, and USDI/Bureau of Land Management for funding in support of this and ongoing research. We thank D. S. Blankenship of the California Department of Fish and Game, K. R. Durbin and E. V. Rickerson of the Oregon Department of Fish and Wildlife, S. J. Stiver of the Nevada Department of Wildlife, and D. L. Mitchell of the Utah Division of Wildlife Resources for providing Sage-grouse wings and location information. We also thank the many other wildlife managers who coordinated sample collection and hunters who cooperated with those managers. C. A. Wassell and C. Marander of Colorado State University edited the distribution map which was provided to us by M. A. Schroeder of the Washington Department of Wildlife. Statistical review and comments were provided by K. P. Burnham and J. C. Fogleman. Supporting instrumentation was available through the National Science Foundation grant 9977691.

References

Aldrich JW (1946) New subspecies of birds from western North America. *Proceedings of the Biological Society of Washington*, **59**, 129–136.

Aldrich JW (1963) Geographic orientation of American tetraonidae. *Journal of Wildlife Management*, **27**, 529–544.

Bouzat JL, Cheng HH, Lewin HA, Westemeier RL, Brawn JD, Paige KN (1998) Genetic evaluation of a demographic bottleneck in the Greater Prairie Chicken. *Conservation Biology*, **12**, 836–843.

Braun CE (1995) Status and distribution of Sage-grouse in Colorado. *Prairie Naturalist*, **27**, 1–9.

Braun CE (1998) Sage-grouse declines in western North America: what are the problems? *Proceedings of the Western Association of State Fish and Wildlife Agencies*, **78**, 139–156.

Emslie SD (1985) The late Pleistocene (Rancholabrean) avifauna of Little Box Elder Cave, Wyoming. University of Wyoming. *Contributions in Geology*, **23**, 63–82.

Emslie SD (1986) Late Pleistocene vertebrates from Gunnison County, Colorado. *Journal of Paleontology*, **60**, 170–176.

Emslie SD (2001) The early and middle Pleistocene avifauna from Porcupine Cave, Colorado. In: *Early and Middle Pleistocene Biodiversity and Environmental Change: The Porcupine Cave*

Fauna from Colorado (ed. Barnowsky A). Univ. California Press, Berkeley, in press.

Emslie SD, Heaton TH (1987) The late Pleistocene avifauna of Crystal Ball Cave, Utah. *Journal of Arizona-Nevada Academy of Science*, **21**, 53–60.

Friedman M, Carlton DC (1999) Petition for a rule to list the Washington population of western Sage-grouse, *Centrocercus urophasianus phaios*, as "threatened" or "endangered" under the Endangered Species Act, 16 U.S.C. Sec. 1531 et seq (1973) as amended. United States department of the Interior – Fish and Wildlife Service, Spokane, WA.

Felsenstein J (1989) *PHYLIP – Phylogeny Inference Package*. Version 3.2. University of Washington – Department of Genetics, Seattle, WA.

Giesen KM, Schoenberg TJ, Braun CE (1982) Methods for trapping Sage-grouse in Colorado. *Wildlife Society Bulletin*, **10**, 224–231.

Grayson DK (1976) A note on the prehistoric avifauna of the Lower Klamath Basin. *Auk*, **93**, 830–833.

Hall SA, Valastro S Jr (1995) Grassland vegetation in the southern Great Plains during the last glacial maximum. *Quaternary Res.*, **44**, 237–245.

Hoelzel RA, Halley J, O'Brien SJ, Campagna C, Arnbom T, Le Boef B, Ralls K, Dover GA (1993) Elephant Seal genetic variation and the use of simulation models to investigate historical population bottlenecks. *Journal of Heredity*, **84**, 443–449.

Howard H (1952) The prehistoric avifauna of Smith Creek Cave, Nevada, with a description of a gigantic raptor. *Bulletin of Southern California Academy of Sciences*, **51**, 50–54.

Howard H, Miller AH (1933) Bird remains from cave deposits in New Mexico. *Condor*, **35**, 15–18.

Hupp JW, Braun CE (1991) Geographical variation among Sage-grouse populations in Colorado. *Wilson Bulletin*, **103**, 255–261.

Johnsgard PA (1973) *Grouse and Quails of North America*. University of Nebraska Press, Lincoln, NE.

Johnsgard PA (1983) *Grouse of the World*. University of Nebraska Press, Lincoln, NE.

Kahn NW, Braun CE, Young JR, Wood S, Mata DR, Quinn TW (1999) Molecular analysis of genetic variation among large- and small-bodied Sage-grouse using mitochondrial control-region sequences. *Auk*, **116**, 819–824.

Koehler PA, Anderson RS (1995) Thirty thousand years of vegetation changes in the Alabama Hills, Owens Valley, California. *Quaternary Research*, **43**, 238–248.

Le Page SL, Livermore RA, Cooper DW, Taylor AC (2000) Genetic analysis of a documented population bottleneck: introduced Bennett's wallabies (*Macropus rufogriseus rufogriseus*) in New Zealand. *Molecular Ecology*, **9**, 753–763.

Madsen DB, Currey DR (1979) Late Quaternary glacial and vegetation changes, Little Cottonwood Canyon area, Wasatch Mountains, Utah. *Quaternary Research*, **12**, 254–270.

Manly B (1991) *Randomization and Monte Carlo Methods in Biology*. Chapman and Hall, New York, NY.

McDonald HG, Anderson E (1975) A late Pleistocene vertebrate fauna from southeastern Idaho. *Tebiwa*, **18**, 19–37.

Miller L (1963) Birds and Indians in the Western Bulletin of the Southern California Academy of Sciences, **62**, 178–191.

Miller L (1965) Bird remains from an archaeological site in the Beaverhead Mountains of southeastern Idaho. *Tebiwa*, **8**, 17–20.

Miller MP (1997) Tools For Population Genetic Analyses (TFPGA) version 1.3. A Windows program for the analysis of allozyme and molecular population genetic data. Arizona State University, Tempe, AZ.

Nei M (1972) Genetic distance between populations. *American Naturalist* **106**, 283–292.

Nowak CL, Nowak RS, Tausch RJ, Wigand PE (1994) Tree and shrub dynamics in northwestern Great Basin woodland and shrub steppe during the late-Pleistocene and Holocene. *American Journal of Botany*, **81**, 265–277.

Oyler-McCance SJ, Kahn NW, Burnham KP, Braun CE, Quinn TW (1999) A population genetic comparison of large- and small-bodied Sage-grouse in Colorado using microsatellite and mitochondrial DNA markers. *Molecular Ecology*, **8**, 1457–1465.

Quinn TW (1992) The genetic legacy of Mother Goose: phylogeographic patterns of Lesser Snow Goose (*Chen caerulescens caerulescens*) maternal lineages. *Molecular Ecology*, **1**, 105–117.

Quinn TW, Mindell DP (1996) Mitochondrial gene order adjacent to the control region in crocodile, turtle and tuatara. *Molecular Phylogenetics and Evolution*, **5**, 344–351.

Quinn TW, Wilson AC (1993) Sequence evolution in and around the control region in birds. *Journal of Molecular Evolution*, **37**, 417–425.

Rogers JS (1972) Measures of genetic similarity and genetic distance. In: *Studies in genetics VII*, pp. 145–154. University of Texas, Austin, TX.

Schroeder MA, Hays DW, Livingston MF, Stream LE, Jacobson JE, Pierce DJ (2000) Changes in the distribution and abundance of Sage-grouse in Washington. *Northwestern Naturalist*, **81**, 104–112.

Shufeldt RW (1913) Review of the fossil fauna of the desert region of Oregon, with a description of additional material collected there. *Bulletin of the American Museum of Natural History*, **32**, 123–178.

Slatkin M (1985) Rare alleles as indicators of gene flow. *Evolution*, **39**, 53–65.

Swofford DL (1999) PAUP: Phylogenetic Analysis Using Parsimony, Version 4.0 beta 4a. Sinauer Associates inc. Sunderland, MA.

Van Devender TR, King JE (1971) Late Pleistocene vegetational records in western Arizona. *Journal of the Arizona Academy of Science*, **6**, 240–244.

Walsh PS, Metxger DA, Higuchi R (1991) Chelex 100 as a medium for single extraction of DNA for PCR-based typing from forensic material. *BioTechniques*, **10**, 506–513.

Wright HE Jr., Bent AM, Hansen BS, Maher LJ Jr (1973) Present and past vegetation of the Chuska Mountains, northwestern New Mexico. *Geological Society of America Bulletin*, **84**, 1155–1180.

Wright S (1978) *Variability Within and Among Natural Populations. Evolution and Genetics of Populations*. University of Chicago Press, Chicago, IL.

Young JR, Hupp JW, Bradbury JW, Braun CE (1994) Phenotypic divergence of secondary sexual traits among Sage-grouse (*Centrocercus urophasianus*) populations. *Animal Behavior*, **47**, 1353–1362.

Young JE, Braun CE, Oyler-McCance SJ, Hupp JW, Quinn TW (2000) A new species of Sage-grouse (*Phasianidae: Centrocercus*) from southwestern Colorado. *Wilson Bulletin*, **112**, 445–453.

Zink RM (1994) The geography of mitochondrial DNA variation, population structure, and species limits in the Fox Sparrow (*Passerella iliaca*). *Evolution*, **48**, 96–111.

Molecular Ecology (2005) 14, 1293–1310

doi: 10.1111/j.1365-294X.2005.02491.x

A multilocus population genetic survey of the greater sage-grouse across their range

S. J. OYLER-MCCANCE,*† S. E. TAYLOR† and T. W. QUINN†

*U.S. Geological Survey, Fort Collins Science Center, 2150 Centre Ave, Building C, Fort Collins, CO 80526 USA, †Rocky Mountain Center for Conservation Genetics and Systematics, Department of Biological Sciences, University of Denver, Denver, CO 80208 USA

Abstract

The distribution and abundance of the greater sage-grouse (*Centrocercus urophasianus*) have declined dramatically, and as a result the species has become the focus of conservation efforts. We conducted a range-wide genetic survey of the species which included 46 populations and over 1000 individuals using both mitochondrial sequence data and data from seven nuclear microsatellites. Nested clade and STRUCTURE analyses revealed that, in general, the greater sage-grouse populations follow an isolation-by-distance model of restricted gene flow. This suggests that movements of the greater sage-grouse are typically among neighbouring populations and not across the species, range. This may have important implications if management is considering translocations as they should involve neighbouring rather than distant populations to preserve any effects of local adaptation. We identified two populations in Washington with low levels of genetic variation that reflect severe habitat loss and dramatic population decline. Managers of these populations may consider augmentation from geographically close populations. One population (Lyon/Mono) on the southwestern edge of the species' range appears to have been isolated from all other greater sage-grouse populations. This population is sufficiently genetically distinct that it warrants protection and management as a separate unit. The genetic data presented here, in conjunction with large-scale demographic and habitat data, will provide an integrated approach to conservation efforts for the greater sage-grouse.

Keywords: gene flow, genetic diversity, greater sage-grouse, microsatellites, mtDNA, nested clade analysis

Received 20 September 2004; revision received 29 October 2004; accepted 12 January 2005

Introduction

The range of the greater sage-grouse (*Centrocercus urophasianus*) historically spanned 12 western US states and three Canadian provinces (Schroeder *et al.* 2004), yet this species currently occupies only 56% of its historic (pre-European period) range (Fig. 1) with extirpations in at least one state and one province (Connelly & Braun 1997; Schroeder *et al.* 2004). Regional population declines have been dramatic, ranging from 17% to 47% (Connelly & Braun 1997). These declines are likely linked to the loss, fragmentation, and degradation of sagebrush (*Artemisia* spp.) habitat (Braun 1998), resulting in the isolation of small populations from larger populations existing in more contiguous habitat (Fig. 1). Consequently, the greater sage-grouse have become a species of conservation concern and petitions have been filed to list them for protection under the US Endangered Species Act.

Management of the greater sage-grouse has previously been based on information from studies of demographic rates and habitat requirements that have focused on local populations (reviewed in Connelly *et al.* 2000). The distribution of genetic variation among populations across the entire range of the greater sage-grouse has been unknown despite increasing pressure on managers to make difficult decisions about which populations may be more 'important' than others. The identification of any genetically discrete groups of the greater sage-grouse is paramount to the development of greater sage-grouse management plans. In addition, faced with an increasingly fragmented distribution with small and isolated populations, it is important to determine the relative amount of genetic diversity contained in each population. Populations with relatively low

Correspondence: Sara J. Oyler-McCance, Fax: 303-871-3471; E-mail: sara_oyler-mccance@usgs.gov

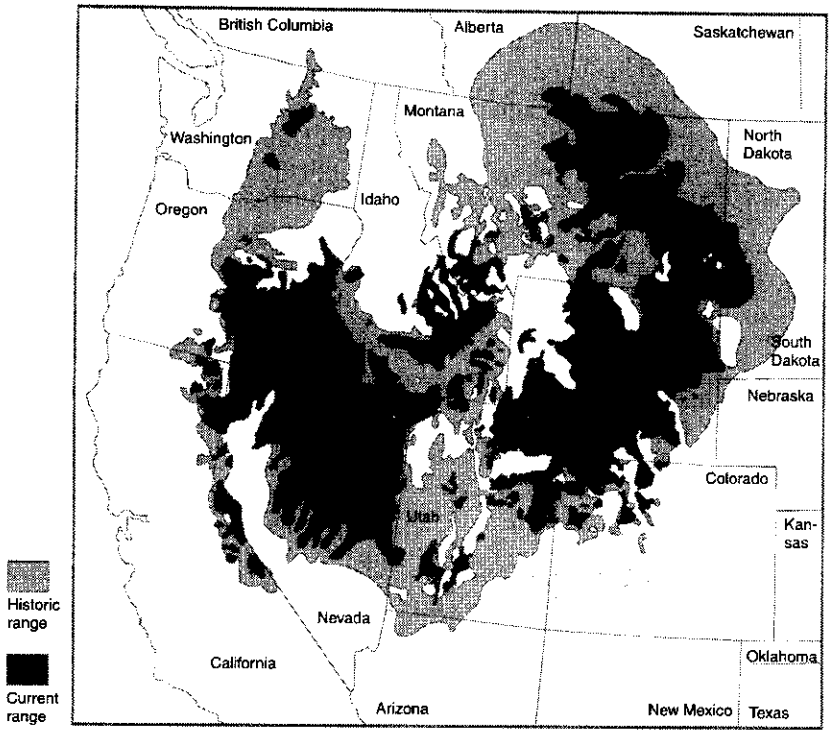

Fig. 1 Historic and current distribution of the greater sage-grouse (from Schroeder *et al.* 2004).

levels of genetic diversity can suffer from inbreeding effects and can be more susceptible to parasitic agents and disease. Genetic data can provide information relevant to an understanding of gene flow, isolation, genetic diversity, and the evolutionary history of a species. Further, it can facilitate a cohesive management strategy that takes genetic distinctiveness into account, based in part on a clear picture of the entire 'genetic landscape' of a species. This increases the efficiency of management decisions and adds to their scientific foundation.

Previous population genetic studies of sage-grouse have focused on assessing taxonomic status. Kahn *et al.* (1999) and Oyler-McCance *et al.* (1999) used mitochondrial and nuclear markers to document the genetic distinctiveness of sage-grouse in southwestern Colorado. This, combined with morphological (Hupp & Braun 1991) and behavioural (Young *et al.* 1994) information led to the recognition of a new species of sage-grouse (Young *et al.* 2000), the Gunnison sage-grouse (*Centrocercus minimus*). Benedict *et al.* (2003) investigated whether or not genetic data supported a subspecific taxonomic delineation in the western part of the greater sage-grouse range that had long been questioned. These studies provided useful taxonomic information and knowledge of the distribution of genetic variation locally, yet they lacked the range-wide perspective necessary to make management decisions regarding the greater sage-grouse at the species level. Here we greatly extend the sampling range and density of previous studies to provide a comprehensive examination of the distribution of genetic variation across the entire range of the greater sage-grouse using both mitochondrial DNA (mtDNA) sequence data and data from nuclear microsatellites.

Materials and methods

Tissue collection and DNA extraction

Forty-six populations from all US states with populations of the greater sage-grouse (11) and one Canadian province (Alberta) were included in this study. The Owyhee, Oregon population was included solely in the microsatellite analysis and the Converse, Wyoming population was only included in the mtDNA analysis. We collected approximately 20 samples per population. Blood samples were collected from the Alberta, Lyon/Mono, South Dakota, Strawberry Valley, and Yakima populations. Feather samples were collected from the Douglass/Grant population. For all other populations, including most samples from Lyon/Mono and South Dakota, muscle tissue was obtained from the wings of hunter-killed birds. As in Benedict *et al.* (2003), most population names correspond to hunt units. DNA was extracted from most samples using either a phenol–chloroform method (Kahn *et al.* 1999) or the Wizard Genomic DNA Purification System (Promega) following the manufacturer's instructions. Some blood samples were later re-extracted using the GenomicPrep Blood DNA Isolation Kit (Amersham Biosciences) using the modifications of Oyler-McCance *et al.* (in press).

Mitochondrial sequencing

A 146-base pair portion of hypervariable control region I was amplified using polymerase chain reaction (PCR) and sequenced using a dye terminator cycle sequencing reaction (Beckman Coulter CEQ8000) as described by Benedict *et al.* (2003). This region was used because it was known to contain approximately 92% of the variable sites in a larger 380-base pair region spanning control region I (Kahn *et al.* 1999).

Microsatellite fragment analysis

Seven nuclear microsatellite loci (*LLST1*, *SGCA5*, *SGCA9*, *SGCA11*, *LLSD3*, *LLSD8*, and *ADL0230*) were screened using the methods described in Oyler-McCance *et al.* (in press). Briefly, PCRs were performed using a dye-labelled forward primer and amplified products were then run on the CEQ 8000 Genetic Analysis System (Beckman Coulter).

Data analysis

All mtDNA sequences were edited and aligned using SEQUENCHER version 4.1.4 and haplotypes were identified using programs MACDNASIS PRO version 2.0 (Hitachi) and GENETOOL. Maximum-parsimony analysis of all haplotypes was conducted using PAUP* version 4.1 (Swofford 2003). Blue grouse (*Dendragapus obscurus*) was used as an outgroup because it has been confirmed by molecular work (Ellsworth *et al.* 1996; Lucchini *et al.* 2001) to be the closest extant relative to sage-grouse. An heuristic analysis was conducted keeping best trees only, with maxtrees set at 100. The starting tree was obtained by stepwise addition with swapping on the best tree when multiple starting trees exist. The addition sequence was simple, with the outgroup used as the reference taxon. Five hundred trees were held at each step. Branch swapping was carried out with the tree-bisection–reconnection (TBR) algorithm, saving multiple trees and swapping on the best trees only. This analysis was followed by an heuristic bootstrap analyis using the default settings but with 1000 replicates. We used nested clade analysis (NCA) to differentiate patterns of population history and gene flow. This was performed by generating an unrooted haplotype cladogram using the statistical parsimony software TCS version 1.13 (Clement *et al.* 2000). The cladogram was constructed following the algorithm of Templeton *et al.* (1992) with ambiguities resolved following Crandall & Templeton (1993) and Crandall *et al.* (1994). The resulting cladogram was then nested using procedures from Templeton *et al.* (1987) and input along with geographical coordinates of all populations in the software program GEODIS version 2.2 (Posada *et al.* 2000). The program GEODIS calculates the clade distance (D_c), nested clade distance (D_n), and the average interior distances minus the average tip distances $(I\text{-}T)_c$ and $(I\text{-}T)_n$. These four statistics were used in conjunction with the key provided by Templeton (1998) and subsequently updated in Templeton (2004) to examine if the observed clade structure provided information about biological processes such as restricted gene flow, allopatric fragmentation, or long-distance migration events.

We calculated the total number of microsatellite alleles per locus and the mean number of alleles for each population. Microsatellite loci were tested (by population) for departures from Hardy–Weinberg equilibrium (HWE) (Guo & Thompson 1992) using the computer program ARLEQUIN 2.001 (Schneider *et al.* 2001). A test for linkage disequilibrium (LD) among pairs of loci within each population was performed using GENEPOP (http://wbiomed.curtin.edu.au/genepop/) on the Web (Markov chain parameters: 5000 dememorization steps, 500 batches, 5000 iterations per batch) (Raymond & Rousset 1995).

Pairwise population genetic distances (R_{ST}, Slatkin 1995) were calculated in ARLEQUIN (Schneider *et al.* 2001). The R_{ST} values were used to construct a neighbour-joining (NJ) tree using PHYLIP 3.57 (Felsenstein 1989) that was viewed using TREEVIEW 1.6.6 (Page 1996).

R_{ST} values were used to perform an analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) in ARLEQUIN. AMOVA partitions the molecular variance (microsatellite allele size) into three categories: between groups, among populations, and among individuals within populations. We tested for population bottlenecks using the software BOTTLENECK (Cornuet & Luikart 1997) and the Wilcoxon test under the TPM model with 1000 replications. Population structure was also examined using STRUCTURE 2.00 software (Pritchard *et al.* 2000). In this program, individuals were grouped into clusters without regard to the assigned population using a model-based clustering analysis. The number of 'populations' (K) was initially estimated by conducting five independent runs each of $K = 1$–45 with 100 000 Markov chain Monte Carlo (MCMC) repetitions and a 100 000 burn-in period using the model with admixture, correlated allele frequencies, and no prior information. An additional set of five independent runs was then conducted with $K = 5$–15 with 500 000 MCMC repetitions and a 500 000 burn-in period using the above model. A Mantel (1967) test was used to look for a correlation between genetic distance and geographical distance using the software ZT (Bonnet & Van de Peer 2002).

Results

Mitochondrial analysis

We sequenced a portion of the mitochondrial control region I in 614 individuals, adding to the 466 individuals that had been sequenced previously (Kahn *et al.* 1999; Benedict *et al.* 2003). Of the 1080 total individuals sequenced over the course of this study and our previous work, 80 unique

Table 1 Haplotype frequencies for all populations. Haplotypes in clade I are represented first in normal text. Haplotypes in clade II are shown in italics

(a) Haplotypes in clade I

Population	*N*	Proportion in clade I	Proportion in clade II	Number of haplotypes	Haplotypes																															
					A	DT	D	DR	AA	AG	BM	BL	AC	AZ	AU	EF	EL	DB	E	F	CH	DQ	EC	ED	EM	ET	EV	L	BB	BX	DA	DC	DD	FA	FB
Blue Mt., Colorado	21	0.36	0.64	11	1		1		1				1																							
Cold Springs, Colorado	25	0.57	0.43	7	3		1						1														2								
Eagle, Colorado	26	0.40	0.60	5	2		4																													
Middle Park, Colorado	21	0.33	0.67	6			2												1																	
North Park, Colorado	23	0.38	0.63	8	4		3												2																	
Box Elder, Utah	28	0.71	0.29	7	10	2			1	1													1													
Wayne, Utah	25	0.50	0.50	8	6	4			1				1																							
Rich, Utah	26	0.64	0.36	9	3	5			1				1										1												1	1
Diamond, Utah	26	0.56	0.44	9	9	1			1				2			1																				
Blue Mt. Utah	18	0.60	0.40	5					1				2			1																				
Strawberry Valley, Utah	23	0.25	0.75	4				15																												
Kemmerer, Wyoming	18	0.43	0.57	7		2			2							1																				
Farson, Wyoming	25	0.40	0.60	5	2		1																													
Rawlins, Wyoming	20	0.40	0.60	5									1													2									
Bighorn, Wyoming	20	0.00	1.00	4																																
Weston, Wyoming	20	0.10	0.90	10	4																															
Converse, Wyoming	13	0.08	0.92	6	1																															
Rosebud, Montana	23	0.00	1.00	4																																
Beaverhead, Montana	22	0.29	0.71	7	1																						2								
Valley, Montana	26	0.17	0.83	6			2																													
Phillips, Montana	18	0.22	0.78	9	1																				1										
Fergus, Montana	23	0.00	1.00	4																																
Harding, South Dakota	21	0.17	0.83	6			1																													
Slope, North Dakota	36	0.20	0.80	5													3																			
Bowman, North Dakota	22	0.17	0.83	6													1																			
Alberta	29	0.25	0.75	8	1																				1										

Table 1 *Continued*

(a)

Population	*N*	Proportion in clade I	Proportion in clade II	Number of haplotypes	Haplotypes																															
					A	DT	D	DR	AA	AG	BM	BL	AC	AZ	AU	EF	EL	DB	E	F	CH	DQ	EC	ED	EM	ET	EV	L	BB	BX	DA	DC	DD	FA	FB
Riddle, Idaho	44	0.36	0.64	11	17		1		2	2																										
Curlew Valley, Idaho	19	0.50	0.50	8	1	3			1															1												
Medicine Lodge, Idaho	20	0.20	0.80	5	7																															
Magic Valley, Idaho	49	0.54	0.46	13	8	2			2	7						1							1	1												
Whitehorse, Oregon	33	0.14	0.86	7	14																															
Steens, Oregon	21	0.29	0.71	7	5															1																
Warner, Oregon	19	0.38	0.63	8	4	1														1													1			
Wagontire, Oregon	19	0.38	0.63	8	6															1	1															
Beattys Butte, Oregon	21	0.25	0.75	8	4																2															
Churchill, Nevada	18	0.17	0.83	6										1																						
Washoe, Nevada	20	0.38	0.63	8	6									1															1							
Elko, Nevada	20	0.63	0.38	8	3					1					4																1	1				
Humboldt, Nevada	21	0.33	0.67	6	10													4																		
Sheldon, Nevada	19	0.29	0.71	7	2																								1							
Nye, Nevada	20	0.50	0.50	6						1				4															1							
Lassen, California	22	0.14	0.86	7	5																															
Lyon/Mono, NV/CA	54	0.40	0.60	10	1						10	9										3														
Yakima, Washington	25	0.00	1.00	1																																
Douglass, Washington	18	0.33	0.67	3		3																														
Total – Clade I	1080				141	23	16	15	13	12	10	9	9	6	4	4	4	4	3	3	3	3	3	2	2	2	2	2	2	1	1	1	1	1	1

Table 1 *Continued*

(b) Haplotypes in clade II

Population	Haplotypes																																														
	B	*C*	*X*	*EJ*	*T*	*EH*	*Q*	*EP*	*BJ*	*BK*	*W*	*S*	*AL*	*AV*	*U*	*AD*	*H*	*Z*	*CJ*	*CN*	*EB*	*DH*	*CR*	*DM*	*DS*	*EI*	*ES*	*AE*	*AF*	*EE*	*EK*	*EN*	*EQ*	*EU*	*EX*	*O*	*AM*	*BD*	*BF*	*BO*	*CK*	*DL*	*BQ*	*DP*	*EA*	*EO*	*ER*	*EW*	*EZ*
Blue Mt., Colorado	*8*	*1*										*1*				*1*		3										2	1																					
Cold Springs, Colorado	*7*	*10*																*1*																															
Eagle, Colorado	2	*15*															3																															
Middle Park, Colorado	*7*	*9*											*1*				*1*																															
North Park, Colorado	*5*	*6*	*1*														*1*																				*1*											
Box Elder, Utah	12																																		1												
Wayne, Utah	*7*										4	1																							1												
Rich, Utah	*6*	*4*									4	1																																			
Diamond, Utah	*6*	*2*			1							3																																			
Blue Mt. Utah	11	*3*																																													
Strawberry Valley, Utah	*3*	*4*									1																																					
Kemnerer, Wyoming	*9*	*1*										2		1																																	
Farson, Wyoming	13	*8*										1																																			
Rawlins, Wyoming	*9*	*6*																																2													
Bighorn, Wyoming	2	11		5									2																																	
Weston, Wyoming	2	*5*		1		1										1										3		1		1															
Converse, Wyoming	4	*3*				2	2																							1																
Rosebud, Montana		*9*		12				1																									1													
Beaverhead, Montana	13	2	1	1									2																																	
Valley, Montana		13		7		1		2																									1													
Phillips, Montana		4		4		2		1													2									2															
Fergus, Montana		10		8				4					1																																	
Harding, South Dakota		3		9		6																				1																				1
Slope,		7		21		3																				2																			

Table 1 *Continued*

(b)

Population	Haplotypes																																																
	B	*C*	*X*	*EJ*	*T*	*EH*	*Q*	*EP*	*BJ*	*BK*	*W*	*S*	*AL*	*AV*	*U*	*AD*	*H*	*Z*	*CJ*	*CN*	*EB*	*DH*	*CR*	*DM*	*DS*	*EI*	*ES*	*AE*	*AF*	*EE*	*EK*	*EN*	*EQ*	*EU*	*EX*	*O*	*AM*	*BD*	*BF*	*BO*	*CK*	*DL*	*BQ*	*DP*	*EA*	*EO*	*ER*	*EW*	*EZ*
North Dakota																																																	
Bowman, North Dakota	1	5		9		5																									1																		
Alberta		5		6		5		9					1																																			1	
Riddle, Idaho	5	2	8		4		1						1																																1				
Curlew Valley, Idaho	8	1	1													3																																	
Medicine Lodge, Idaho	8	1	1																		3																												
Magic Valley, Idaho	9	6	7		1											1					1									2																			
Whitehorse, Oregon	1		2		10		1								4				1																														
Steens, Oregon	1		4		8		1																	1																									
Warner, Oregon			5		2		2													1			2																										
Wagontire, Oregon			1		2		4								1					3																													
Beattys Butte, Oregon			6				1							4					2				1																		1								
Churchill, Nevada	2		8		5																																	1	1										
Washoe, Nevada			6				3								1				1																					1									
Elko, Nevada	3		5											2																																			
Humboldt, Nevada	2		3		1		1																																										
Sheldon, Nevada			6		6		2								1																					1													
Nye, Nevada	4		9		1																																												
Lassen, California			8				4							1								1		2																		1							
Lyon/ Mono, NV/ CA			2				1		16	10																																	1	1					
Yakima, Washington			25																																														
Douglass, Washington			12																						3																								
Total-Clade II	170	156	121	83	41	25	23	17	16	10	9	9	8	8	7	6	5	4	4	4	4	3	3	3	3	3	3	2	2	2	3	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1

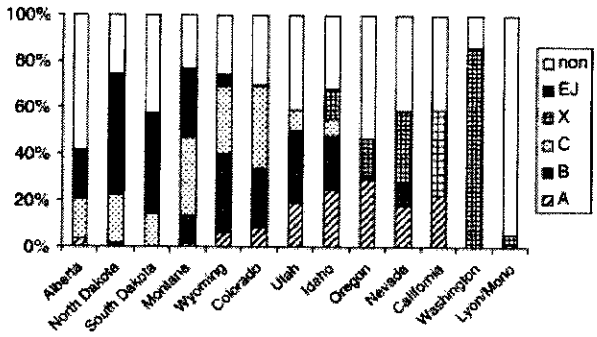

Fig. 2 Proportion of individuals in each state with common haplotypes (non represents haplotypes that are not common). The haplotypes EJ, X, C, B, and A were the most common haplotypes found in the study. Each bar represents the proportion of each of these common haplotypes for every state.

mtDNA haplotypes were identified (Table 1). Of these 80 haplotypes, 28 are newly described here (Accession nos AY850036–AY850062, and AY846747). Parsimony analysis distributed all haplotypes into one of two distinct monophyletic clades (31 in clade I, 49 in clade II). Of the 100 trees of shortest length (124 steps) that were retained, all maintained monophyly of those two clades. Bootstrap support was 91% for clade I and 88% for clade II. The maximum DNA sequence difference between the two clades was 18.4% and the minimum difference between any greater sage-grouse haplotype and the outgroup sequence was 23.4%. Along the 146-base pair sequence, 60 sites were variable with 39 transitions, 18 transversions, and 8 insertions/deletions. Five of those sites were both transitions and transversions.

The average number of haplotypes per population was 6.9 with a high of 13 haplotypes in Magic Valley and a low of one in Yakima (Table 1). Five haplotypes (A, B, C, X, and EJ) were common and widespread representing 62% of all individuals sequenced. Haplotype A was found virtually everywhere with the exception of Washington, North and South Dakota, and parts of Wyoming and Montana. (Fig. 2). Haplotype B was present in most populations except in areas of Montana, South Dakota, Oregon, California, and Washington while haplotype C was widespread except in Oregon, Nevada, California, and Washington (Fig. 2). Haplotype X was more localized spanning Idaho, Oregon, Nevada, California, and Washington as was haplotype EJ, which is found primarily in Wyoming, Montana, North and South Dakota, and Alberta (Fig. 2). The Lyon/Mono population (Fig. 2) has an extremely low percentage of individuals with common haplotypes (5%). Of the 54 individuals from the Lyon/Mono population, 50 are characterized by haplotypes unique to that population.

In the NCA, statistical parsimony revealed five separate networks, three that were composed of only one haplotype (haplotypes CJ, BX, or DC). The two networks that represented the remaining 77 haplotypes corresponded to the two distinct clades described previously (Kahn *et al.* 1999; Benedict *et al.* 2003). The 95% plausible set of both networks was comprised of many haplotypes and each contained several ambiguous connections that were resolved using the frequency and topology criterion. The two networks were nested resulting in a final network (Fig. 3). Because the three other networks contained only one haplotype per network, they were not used in subsequent analyses.

We rejected the null hypothesis of no relationship between the mitochondrial haplotype genealogy and the geographical distribution of haplotypes for 29 of the 39 clades in the analysis (Table 2). Eighteen of those 29 clades were uninformative, categorized variously as inconclusive, insufficient genetic resolution, or inadequate genetic sampling (Table 2) using the updated key by Templeton (2004). Eleven clades,

Table 2 Characteristics of each clade described using nested clade analysis

Continuous range expansion	Allopatric fragmentation	Restricted gene flow with isolation by distance	Inadequate geographic sampling	Insufficient genetic resolution	Inconclusive	No relationship
2-3	1-3	1-5	1-1	1-9	1-18	1-2
2-4	1-8	1-13	1-4	1-22	1-19	1-11
		1-20		1-32	1-30	1-14
		2-1			1-31	1-15
		2-8			2-6	1-25
		3-4			2-7	1-26
		3-5			2-9	1-27
					2-11	2-2
					2-13	2-10
					3-1	3-3
					3-2	
					4-1	
					4-2	

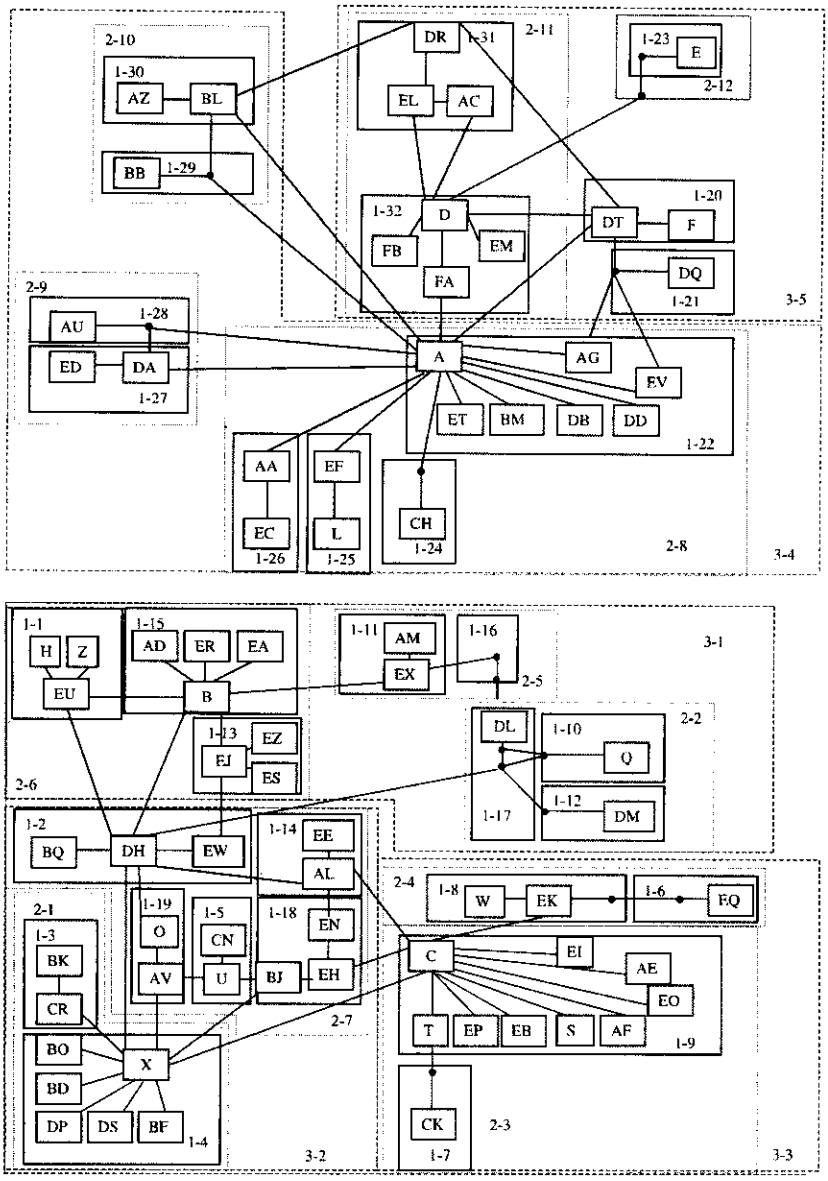

Fig. 3 Unrooted estimated 95% parsimony cladogram of 80 haplotyopes detected in the greater sage-grouse. Haplotypes are represented by letters. Lines represent single mutational events, dots represent intermediate haplotypes not found in our sample but necessary to link haplotypes that were found. Numbers represent the level of nesting in the analysis. Most haplotypes fell into one of two distinct clades (previously described by Kahn *et al.* 1999 and Benedict *et al.* 2003). The placement for connection of these two clades could not be determined so they are represented separately as clade I (top) and clade II (bottom). Three haplotypes could not be connected with confidence to either clade or each other and thus are not included here.

however, did provide insight into the biogeographical history of the greater sage-grouse. Clades 2-3 and 2-4 were characterized as continuous range expansion and two clades (1-3 and 1-8) represented patterns associated with allopatric fragmentation. The pattern of restricted gene flow with isolation by distance was the most prominent being characterized by seven clades (1-5, 1-13, 1-20, 2-1, 2-8, 3-4, and 3-5).

Microsatellite analysis

The number of microsatellite alleles per locus across all populations ranged from five (*LLST1*) to 31 (*SGCA9*). The mean number of alleles per population across all seven loci ranged from 3.1 alleles in Douglass/Grant to 7.1 alleles in Alberta (Table 3). One population, Strawberry Valley, was shown to have undergone a recent population bottleneck ($P = 0.0078$). There were 27 significant departures from HWE ($P < 0.05$) among the 315 possible combinations of population and loci. Because of the large number of combinations (multiple tests), it is possible that some departures were caused by chance. To correct for multiple tests, the P value was lowered to 0.00016 (Bonferroni method) and only one population/locus comparison was significant ($P < 0.00016$). The significant departure was in the Eagle population at the *SGCA9* locus. The test for LD examined each pair of loci in each population for a total of 945 possible comparisons. Using the Bonferroni correction, the P value was

Table 3 Sample population names, locations, sample size, expected heterozygosity (H_E) and allelic richness for each locus, mean number of alleles and assigned cluster (identified by STRUCTURE analysis) for each population

			LLST1		*SGCA5*		*SGCA9*		*SGCA11*		*LLSD3*		*LLSD8*		*ADL0230*			
Population	State/ Province	*N*	H_E	Allelic richness	H_E	Allelic richness	H_E	Allelic richness	H_E	Allelic richness	H_E	Allelic richness	H_E	Allelic richness	H_E	Allelic richness	Mean no. of alleles	Assigned cluster
Blue Mountain-CO	Colorado	25	0.26	2	0.83	8	0.84	7	0.82	7	0.63	5	0.65	5	0.8	6	5.71	3
Cold Springs	Colorado	30	0.36	2	0.84	9	0.75	7	0.84	8	0.6	5	0.69	4	0.77	8	6.14	3
Eagle	Colorado	26	0.39	2	0.8	9	0.84	8	0.8	8	0.71	4	0.64	3	0.77	6	5.71	5
Middle Park	Colorado	21	0.52	4	0.87	9	0.85	8	0.83	7	0.65	5	0.57	3	0.71	4	5.71	5
North Park	Colorado	22	0.42	3	0.79	8	0.77	11	0.89	10	0.58	6	0.64	3	0.61	4	6.43	5
Box Elder	Utah	31	0.3	2	0.82	8	0.81	15	0.74	5	0.68	6	0.6	6	0.75	6	6.86	8
Wayne	Utah	27	0.14	2	0.59	5	0.83	10	0.68	4	0.53	4	0.49	4	0.7	6	5	7
Rich	Utah	31	0.48	3	0.82	9	0.82	11	0.81	9	0.64	5	0.67	6	0.61	4	6.71	3
Diamond	Utah	27	0.42	3	0.79	8	0.87	11	0.81	7	0.66	5	0.58	3	0.7	5	6	3
Blue Mountain-UT	Utah	18	0.43	2	0.55	7	0.72	8	0.69	4	0.45	5	0.57	3	0.62	5	4.86	3
Strawberry Valley	Utah	23	—	1	0.77	6	0.77	7	0.77	5	0.29	2	0.57	3	0.58	3	3.86	7
Kemmerer	Wyoming	21	0.52	3	0.84	8	0.8	8	0.86	6	0.5	4	0.7	5	0.7	6	5.71	3
Farson	Wyoming	25	0.41	2	0.87	9	0.8	9	0.81	9	0.67	4	0.64	3	0.81	6	6	3
Rawlins	Wyoming	20	0.56	2	0.85	10	0.84	10	0.85	8	0.67	5	0.73	6	0.74	6	6.71	3
Bighorn	Wyoming	20	0.41	2	0.77	7	0.61	6	0.81	8	0.23	3	0.68	4	0.83	6	5.14	8
Weston	Wyoming	20	0.35	2	0.7	7	0.84	15	0.78	7	0.44	3	0.78	5	0.78	5	6.29	9
Rosebud	Montana	25	0.43	2	0.78	8	0.9	12	0.7	9	0.48	5	0.73	6	0.71	5	6.71	1
Beaverhead	Montana	19	0.26	3	0.88	8	0.87	10	0.81	8	0.46	4	0.75	5	0.73	4	6	4
Valley	Montana	29	0.33	2	0.66	6	0.91	17	0.76	9	0.53	5	0.76	5	0.72	4	6.86	1
Phillips	Montana	19	0.37	2	0.8	7	0.93	14	0.73	7	0.45	4	0.73	5	0.74	4	6.14	1
Fergus	Montana	30	0.38	2	0.76	8	0.88	13	0.77	8	0.53	3	0.78	6	0.72	4	6.29	1
Harding	South Dakota	26	0.43	2	0.54	4	0.88	15	0.64	6	0.12	3	0.78	5	0.69	4	5.57	9
Slope	North Dakota	36	0.49	2	0.66	5	0.88	11	0.61	5	0.26	3	0.71	4	0.69	4	4.86	9
Bowman	North Dakota	24	0.5	2	0.69	5	0.87	12	0.57	5	0.32	3	0.79	5	0.75	6	5.43	9
Alberta	Alberta	36	0.38	2	0.77	8	0.91	13	0.85	12	0.51	5	0.67	5	0.69	5	7.14	1
Riddle	Idaho	25	0.5	4	0.78	6	0.72	9	0.77	5	0.65	4	0.69	5	0.76	5	5.43	2
Curlew Valley	Idaho	19	0.46	3	0.87	7	0.78	10	0.84	7	0.64	5	0.75	7	0.7	5	6.29	8
Medicine Lodge	Idaho	36	0.43	4	0.85	9	0.86	17	0.84	10	0.62	4	0.73	6	0.72	6	8	4
Magic Valley	Idaho	31	0.46	3	0.76	7	0.76	13	0.77	7	0.61	6	0.71	6	0.78	7	7	8

Table 3 *continued*

Population	State/ Province	*N*	*LLST1*		*SGCA5*		*SGCA9*		*SGCA11*		*LLSD3*		*LLSD8*		*ADL0230*		Mean no. of alleles	Assigned cluster
			H_E	Allelic richness	H_E	Allelic richness	H_E	Allelic richness	H_E	Allelic richness	H_E	Allelic richness	H_E	Allelic richness	H_E	Allelic richness		
Whitehorse	Oregon	18	0.26	4	0.81	7	0.74	7	0.8	7	0.69	5	0.75	7	0.74	5	6	8
Steens	Oregon	22	0.6	3	0.79	7	0.73	10	0.81	7	0.73	4	0.78	5	0.8	6	6	2
Warner	Oregon	22	0.44	3	0.83	7	0.28	4	0.79	7	0.71	4	0.77	5	0.83	7	5.29	2
Wagontire	Oregon	22	0.52	3	0.85	8	0.49	6	0.84	8	0.72	3	0.76	6	0.78	5	5.57	2
Beattys Butte	Oregon	24	0.46	3	0.75	6	0.74	7	0.84	7	0.69	4	0.77	7	0.79	6	5.71	2
Owyhee	Oregon	25	0.5	3	0.78	6	0.69	9	0.78	7	0.73	6	0.67	6	0.84	8	6.43	8
Churchill	Nevada	19	0.45	4	0.79	6	0.63	7	0.75	6	0.6	6	0.65	5	0.69	5	5.57	8
Washoe	Nevada	22	0.42	3	0.81	6	0.64	7	0.69	7	0.74	5	0.75	7	0.7	5	5.71	2
Elko	Nevada	22	0.56	4	0.85	8	0.85	12	0.85	7	0.61	4	0.75	7	0.81	7	7	8
Humboldt	Nevada	24	0.41	4	0.8	8	0.73	10	0.77	7	0.7	5	0.71	6	0.79	5	6.43	8
Sheldon	Nevada	23	0.41	3	0.81	6	0.72	7	0.84	7	0.65	4	0.68	4	0.81	6	5.29	2
Nye	Nevada	23	0.4	4	0.79	6	0.81	10	0.83	7	0.66	4	0.71	7	0.67	6	6.29	8
Lyon/Mono	Nevada/ California	68	0.51	3	0.78	6	0.42	5	0.32	7	0.71	7	0.69	6	0.68	6	5.71	10
Lassen	California	55	0.51	3	0.74	5	0.64	11	0.67	8	0.66	6	0.79	7	0.74	5	6.43	2
Yakima	Washington	29	0.43	2	0.07	2	0.62	4	0.4	4	0.61	3	0.41	4	0.58	4	3.29	6
Douglass/ Grant	Washington	21	0.29	2	0.07	2	0.58	4	0.74	5	0.7	3	0.09	2	0.73	4	3.14	6

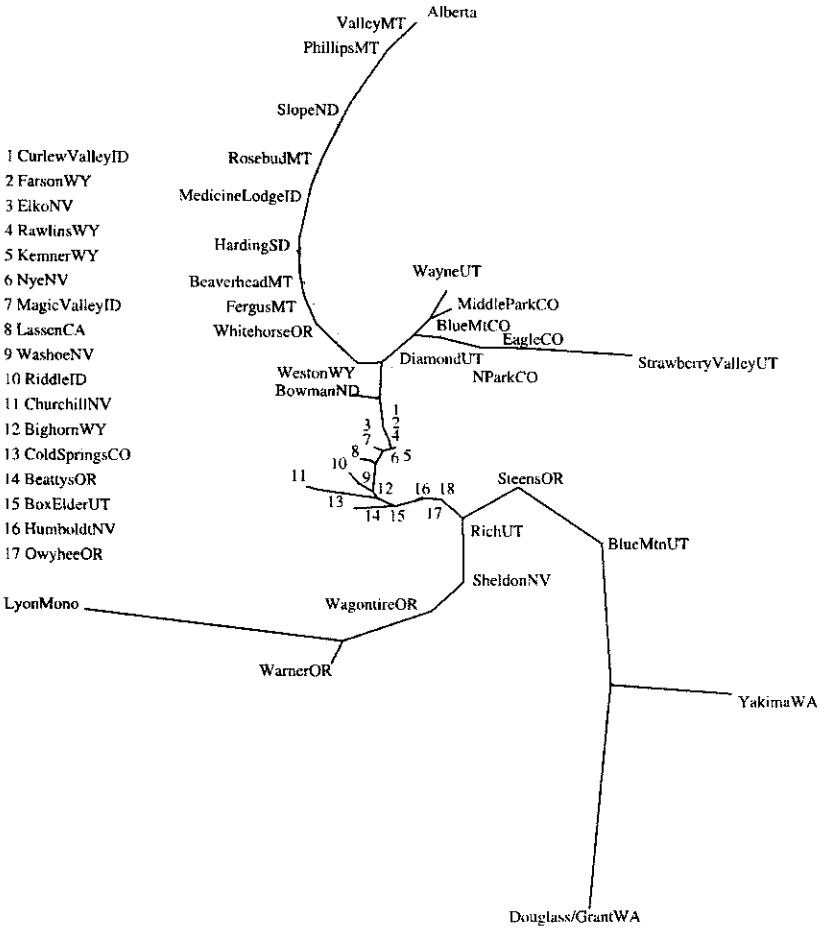

Fig. 4 Neighbour-join tree constructed using the genetic distance R_{ST} for 45 populations of the greater sage-grouse. Population names are represented followed by a two-letter abbreviation of the corresponding state. Samples from the Canadian province Alberta are labelled Alberta. The Lyon/Mono population, which spans the border of Nevada and California, is labelled LyonMono.

lowered to 0.00005. There was only one significant comparison, the *SGCA9* and *SGCA11* loci in the Eagle population.

Of the 990 population pairwise (R_{ST}) genetic distances, 194 were significant ($P = 0.00005$, Bonferroni corrected). Most notably, the Lyon/Mono population was significantly different from all other populations except Steens, Wagontire, Warner, Sheldon, and Box Elder. The Douglass/Grant, Yakima, and Alberta populations differed significantly from 27, 32, and 25 other populations, respectively.

The R_{ST} genetic distance tree also indicated that the Douglass/Grant and Yakima populations and the Lyon/Mono population were genetically distant from each other and from all other populations (Fig. 4). When the population groups suggested by the R_{ST} values (Douglass/Grant and Yakima, Lyon/Mono, Alberta) were tested against all other populations and each other (four total groups), the AMOVA based on the R_{ST} distances revealed that most of the variation in the two categories of interest was explained by the among groups (9.93%) category, rather than the among populations within groups category (6.71%) (Table 4a).

STRUCTURE assigned each individual a probability of belonging to each of 10 clusters. Each population was assigned to the appropriate cluster based on the largest number of individuals with a certain cluster assignment (Table 3, Fig. 5). The number of populations assigned to clusters ranged from 1 (Lyon/Mono, cluster 10) to 10 (various populations from Nevada, Idaho, Wyoming, Utah, and Oregon, cluster 8). R_{ST} genetic distances were recalculated based on the STRUCTURE clusters. An AMOVA based on the 10 clusters indicated that, relative to the AMOVA based on four groups (Table 4a), the proportion of among-group variation remained nearly the same (8.91%) while the among-populations–within-groups variation was reduced (1.86%) (Table 4b). The Mantel test revealed that there was a positive correlation between genetic distance and geographical distance ($r = 0.4312$, $P = 0.00001$) (Fig. 6).

Discussion

The 80 mtDNA haplotypes fell into one of two monophyletic clades as described by Kahn *et al.* (1999) and Benedict *et al.* (2003). The two clades are not separated geographically. In fact, all but four populations contain individuals with haplotypes from both clades. Kahn *et al.* (1999) and Benedict *et al.* (2003) have previously argued that these two clades may have resulted from the

Table 4 Analysis of molecular variance using seven microsatellite loci

(a) Forty-five populations, four groups. Group 1, Lyon/Mono; group 2, Alberta; group 3, Douglass/Grant, Yakima; group 4, all other populations

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	3	5712	7.53	9.93
Among populations within groups	41	13024.15	5.06	6.71
Within populations	2317	146534.18	63.24	83.36

(b) Forty-five populations, 10 groups. Groups are the 10 clusters identified in the STRUCTURE analysis (see Table 3)

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	9	14229.92	6.32	8.91
Among populations within groups	35	4506.23	1.32	1.86
Within populations	2317	146534.18	63.24	89.23

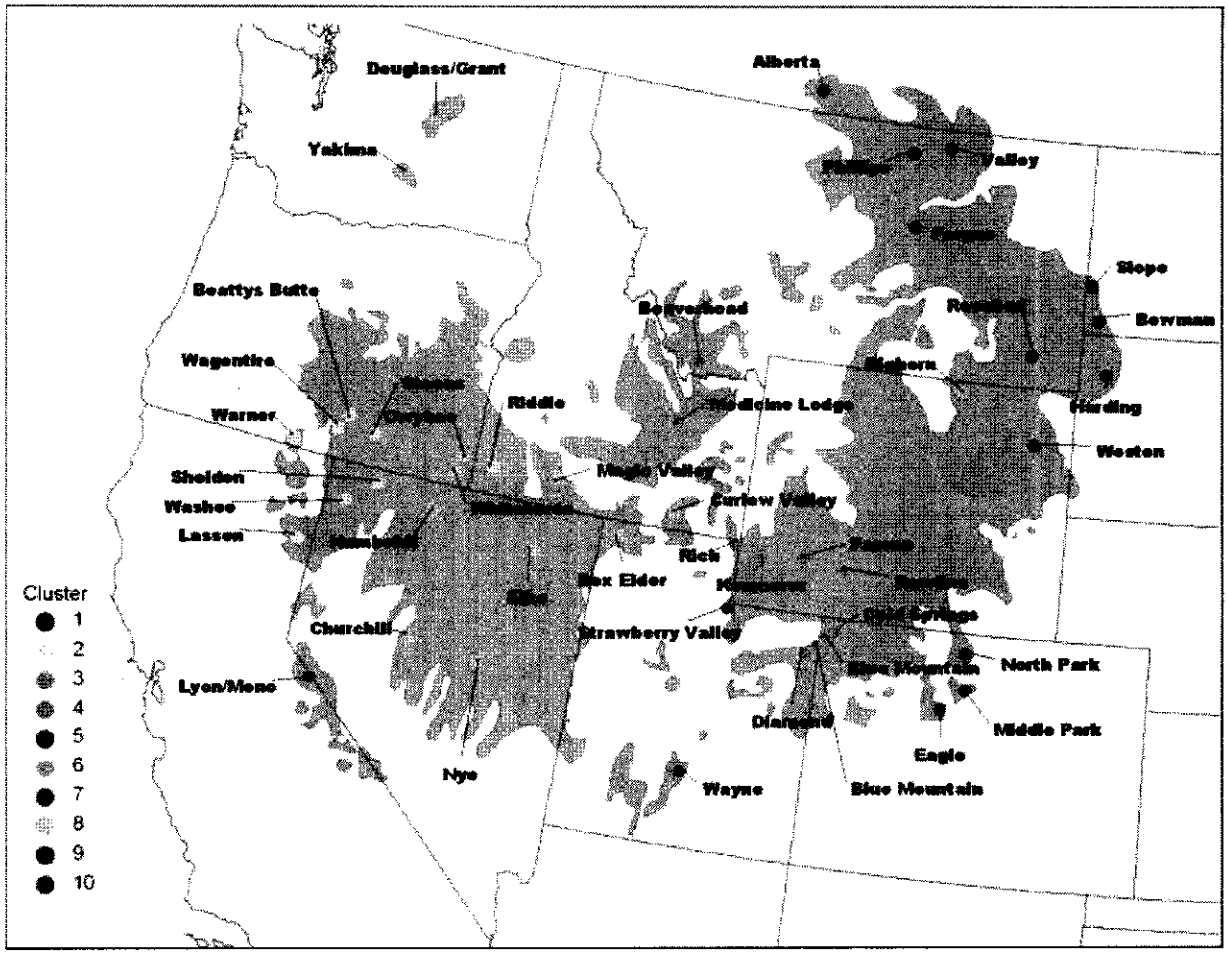

Fig. 5 Map of sampling sites for the microsatellite analysis colour coded by the cluster each population has been assigned to using STRUCTURE analysis.

separation of sage-grouse into two allopatric groups approximately 850 000 BP, perhaps in association with the patchy distribution of sagebrush habitat during the Pleistocene epoch.

In each population the percentage of individuals in each clade shifted across the range with many populations in the north (particularly the northeast) containing few or no haplotypes from clade I (Table 1). This may suggest a

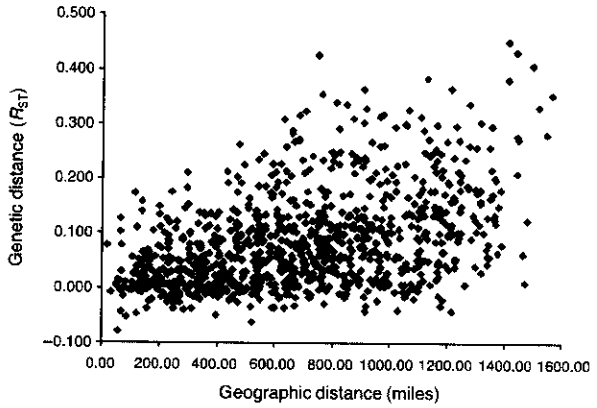

Fig. 6 Relationship between the genetic distance R_{ST} and geographical distance for all pairs of populations of the greater sage-grouse.

range expansion to the north and northeast following the Pleistocene epoch. Fossil records have documented sage-grouse during the Pleistocene in the south-central and southeastern part of their current range (Shufeldt 1913; Howard & Miller 1933; Howard 1952; Miller 1963, 1965; McDonald & Anderson 1975; Grayson 1976; Emslie 1985, 2004; Emslie & Heaton 1987) and more recently (6000 BP) in western portions of the range (Miller 1963; Grayson 1976), yet sage-grouse have not been recorded during this period in the northern part of their current range.

Results from our NCA suggest continuous range expansion in two of our nested clades (2-3 and 2-4). Populations in clade 2-3 are found throughout most of the range, yet populations in clade 2-4 occur only in the central and northeastern part of the range, in Utah, Wyoming, Montana, and North Dakota. More recent evidence suggests that the range expansion, particularly in the northeast, has continued to present day. Schroeder *et al.* (2004) provided a pre-European period distribution of the greater sage-grouse that they developed by examining early written observations of sage-grouse. Although some ambiguities exist, they propose that the distribution of sage-grouse was following a northward and eastward transition into areas not originally occupied in the early 1800s (Schroeder *et al.* 2004). Our data are consistent with this observation and provide support for the idea that shifts in sagebrush habitat distribution may have provided the greater sage-grouse an opportunity for range expansion, particularly in the northeastern part of their range.

The distribution of genetic variation shows a gradual shift across the range in both mitochondrial and nuclear data sets. An examination of the distribution of the most common mtDNA haplotypes demonstrates this phenomenon (Fig. 2). Haplotype A is the most widespread occurring in all but North Dakota, South Dakota, and Washington. Haplotype X is found primarily in the western part of the range, while haplotypes B and C are found in the central and eastern part of the range. Haplotype EJ is found only in the northeastern part of the range in Alberta, Montana, North and South Dakota, and Wyoming. This pattern suggests localized gene flow with isolation by distance (i.e. movement among neighbouring populations yet not across the range).

Results from the NCA confirm this finding with seven clades characterized by restricted gene flow with isolation by distance (1-5, 1-13, 1-20, 2-1, 2-8, 3-4, and 3-5). The lower order (more localized) clades (1-5, 1-13, 1-20) represented smaller portions of the range, yet the higher order (regional) clades (2-1, 2-8, 3-4, 3-5) represented most of the range. This suggests that restricted gene flow with isolation by distance is a range-wide phenomenon.

Analysis of our microsatellite data showed a similar pattern. The Mantel test showed a positive correlation between genetic distance and geographical distance suggesting an isolation-by-distance phenomenon (Fig. 6). In addition, the STRUCTURE analysis best grouped our data into 10 clusters (Fig. 5). All clusters were made up of populations geographically adjacent suggesting again patterns of localized gene flow and isolation by distance. The smaller, more fragmented populations on the periphery of the range (North Park, Middle Park, and Eagle in Colorado, Strawberry Valley and Wayne in Utah, Lyon/Mono in Nevada/California, and Douglass/Grant and Yakima in Washington) made up their own clusters suggesting lower amounts of gene flow in these areas.

Direct knowledge of the dispersal distances of the greater sage-grouse is limited. In one Colorado study, the respective median natal dispersal distances for 12 males and 12 females was 7.4 km and 8.8 km, respectively (Dunn & Braun 1985), distances more apt to be between neighbouring leks than between non-neighbouring populations. Some greater sage-grouse have been documented to move seasonally between summer and winter ranges. One study in Idaho estimated the average distance of these movements to be 13.1 km (Connelly *et al.* 1988). Our data are consistent with these studies suggesting that gene flow is likely limited to the movement of individuals between neighbouring populations and not likely the result of long-distance movements of individuals (across large portions of the range). This information is important because conservation efforts often consider translocations and augmentation of existing populations using animals from outside populations. Our data suggest linkages among neighbouring populations and differences among distant populations, raising the possibility that local adaptations may exist and that translocations should involve neighbouring populations rather than geographically distant populations.

Levels of genetic variation differed among populations (Tables 1 and 3). The highest level was found in Magic

(17)

Valley in the mtDNA data set with 13 haplotypes per population and in Alberta in the microsatellite data set with an average of 7.14 alleles. In both mtDNA and micrsosatellite data sets, the least amount of genetic diversity (Tables 1 and 3) was in the two Washington populations, Yakima and Douglass/Grant, with one and three mtDNA haplotypes per population and an average of 3.29 and 3.14 microsatellite alleles per population, respectively.

Pairwise population R_{ST} tests also showed that Douglass/Grant and Yakima were significantly different from most populations (27, 32). Our NJ tree constructed using R_{ST} genetic distances (Fig. 4) showed that the two Washington populations were among the populations with the longest branches. The significant results of R_{ST} genetic distance comparisons are largely a reflection of the small number of alleles found in both populations.

Interestingly, the two Washington populations did not show signs of a recent population bottleneck as was found in Strawberry Valley, which had been documented to have had a severe population decline because of predation problems within the last 10 years (Utah Division of Wildlife, unpublished). The test for population bottlenecks, however, only detects recent bottlenecks on the order of 0.2–4.0 generations (Luikart & Cornuet 1998). Population declines in Washington have been estimated to be at least 77% between 1960 and 1999 (Schroeder *et al.* 2000) suggesting that declines have been ongoing and significant for 40 years. The lack of genetic diversity in the Washington populations is not surprising given their small population size and isolation (Fig. 1) and the fact that they currently occupy only 8% of their historic range (Schroeder *et al.* 2000).

While the importance of maintaining substantial levels of genetic variation in a population has been the topic of considerable debate, most agree that genetic variation is relevant to the health and viability of populations and that it must be addressed and monitored in management plans (O'Brien & Evermann 1988; Quattro & Vrijenhoek 1989). Bouzat *et al.* (1998) and Westemeier *et al.* (1998) showed that fertility and hatching success of greater prairie chickens *(Tympanuchus cupido)* were reduced because of a bottleneck caused by habitat loss. The Washington populations of the greater sage-grouse, a close relative of the greater prairie chicken (both are members of Tetraoninae), have experienced similar isolation and reduction in population size resulting from loss of habitat and likely have the same potential for inbreeding effects. Further, genetically depauperate populations face enhanced susceptibility to parasitic agents or infectious disease such as West Nile virus, which has been shown to be a significant threat in the greater sage-grouse (Naugle *et al.* 2004). Management strategies for these populations have included the consideration of translocations from other populations since natural gene flow appears unlikely given the geographical isolation of these populations. Our genetic data suggest that any translocations or augmentations of the Washington populations should involve populations that are geographically close.

Using mtDNA sequence data, Benedict *et al.* (2003) previously noted that the Lyon/Mono population was genetically unique compared to other populations in California, Nevada, Oregon, and Washington. Our study substantiates their findings. While an additional 24 populations were added by our data set, the observation remains that Lyon/Mono contains mostly novel haplotypes not found elsewhere across the range (Table 1). In fact, 93% of individuals from Lyon/Mono had novel haplotypes, while the average percentage of novel haplotypes among all other populations was 8.37. The genetic diversity present in Lyon/Mono is comparable to (if not higher than) most other populations (11 haplotypes) suggesting that the differences are not caused by a genetic bottleneck or founder event.

This pattern was found as well in the nuclear data set. Pairwise population R_{ST} tests revealed that although there were many population pairs (194 of 990) that were significantly different, Lyon/Mono were significantly different from almost all other populations, reinforcing its genetic distinctiveness. Further, in the STRUCTURE analysis, the Lyon/Mono population was the only population forming its own cluster, which again supports the idea that this population is genetically distinct.

Benedict *et al.* (2003) suggested that the Lyon/Mono population has been isolated from other greater sage-grouse populations for thousands or perhaps tens of thousands of years, noting that most members of the population carry mitochondrial haplotypes that are not found elsewhere across the species range. In total, there are seven novel haplotypes of 10 found in the population, and 48 of the 54 individuals from Lyon/Mono carry one of those seven. The results of our NCA support the theory of Benedict *et al.* (2003) as one of our clades (1-3) representing the Lyon/Mono separation was characterized by allopatric fragmentation.

The concept of evolutionary significant units (ESUs) is increasingly used to set management goals for populations or groups of populations below the species level (e.g. Parker *et al.* 1999). Although the most appropriate definition of an ESU is currently being debated, the general concept is that a population that has diverged a significant amount genetically is evolutionarily independent from other populations. The debate involves the question of how much genetic differentiation is significant and the strictest definition incorporates the phylogenetic species concept. According to Moritz (1994), an ESU should 'be reciprocally monophyletic for mtDNA alleles and show significant divergence of allele frequencies at nuclear loci', whereas a management unit (MU) would require 'significant divergence of alleles at nuclear or mitochondrial loci'.

We have demonstrated that Lyon/Mono has significant divergent allele frequencies of nuclear microsatellite loci,

but the mtDNA control region haplotypes are not reciprocally monophyletic despite most being newly arisen within this population. Although the Lyon/Mono population would be considered an MU as defined by Moritz (1994), it would not be considered an ESU. We believe, however, that Moritz's (1994) restrictive definition of ESU should not be applied without careful consideration of several aspects of the breeding biology of the species under consideration. In some cases, reciprocal monophyly may appear long after complete and irreversible isolating mechanisms are in place. Further, the time it takes to reach reciprocal monophyly in mitochondria is dependent upon such factors as effective population size of females, and population dynamics related to expanding vs. contracting populations. In a lek-breeding species such as the greater sage-grouse where a few males do most of the mating, sexual selection can act to influence morphological and behavioural traits at a rate much faster than can be tracked genetically. Also, as a consequence of that breeding biology, the nuclear genome may undergo more of a bottleneck relative to the maternally inherited mitochondrial genome than it would in most species. In essence, this would delay the time that it takes the mitochondrial genome to reach reciprocal monophyly relative to the amount of differentiation that is simultaneously occurring in the nuclear genome.

Surprisingly, the Lyon/Mono population is at least as divergent from other populations of the greater sage-grouse as Gunnison sage-grouse are from the greater sage-grouse by virtue of the large number of new haplotypes unique to that population. Gunnison sage-grouse were recognized as a new species of sage-grouse based on morphological, behavioural, and genetic data (Young *et al.* 2000). Preliminary comparisons of gross morphology and behaviour between Lyon/Mono and surrounding greater sage-grouse populations, however, have revealed little or no differences (S. E. Taylor, unpublished). This suggests that while Lyon/Mono may have been isolated for an amount of time similar to the isolation of Gunnison sage-grouse, they have not experienced a significant divergence in morphology or behavioural characteristics as has been documented in Gunnison sage-grouse (Young *et al.* 2000), which ultimately led to their reproductive isolation.

Because Lyon/Mono is so genetically different, however, they deserve special attention. They certainly qualify as a distinct population segment from a genetic standpoint and may even warrant consideration as a new subspecies based on our genetic data. However, more comprehensive morphological and behavioural comparisons should be performed before a change in taxonomic status should be considered. Regardless of the label placed on this population, it should be managed separately and protected because of its genetic distinctiveness as it may contain genetic variation that may be important to the survival of the species over large timescales.

Our study documented the distribution of genetic variation across the entire range of the greater sage-grouse, determining that the Lyon/Mono population has a unique history of isolation distinct from all other populations and that two populations in Washington have low levels of genetic diversity. Further, we found that isolation by distance has left in imprint on greater sage-grouse gene pools, and that local adaptation is a realistic possibility for the species and should be considered in decisions involving translocations. This genetic data used in conjunction with large-scale demographic and habitat data will provide an integrated approach to conservation efforts for the greater sage-grouse.

Acknowledgements

We are grateful to the Western Association of Fish and Wildlife Agencies for providing major funding and coordinating collection of sample materials. We also thank the Colorado Division of Wildlife, the Nevada Division of Wildlife, and the Oregon Department of Fish and Wildlife, particularly C. E. Braun and S. Stiver, for providing the initial impetus and support for earlier sage-grouse genetic studies that led to this wider survey. Biologists in state wildlife agencies in California, Idaho, Montana, Nevada, North Dakota, South Dakota, Utah, Washington, and Wyoming were also helpful in selecting sample areas and facilitating tissue collection. C. L. Aldridge provided material from Alberta. We thank J. R. Young, N. W. Kahn, N. G. Benedict, and S. M. Pearson for their earlier work on this or related projects. We also thank H.-P. Liu, J. St. John, and three anonymous reviewers for helpful comments on earlier versions of this paper.

References

Benedict NG, Oyler-McCance SJ, Taylor SE, Braun CE, Quinn TW (2003) Evaluation of the eastern (*Centrocercus urophasianus urophasianus*) and western (*Centrocercus urophasianus phaois*) subspecies of sage-grouse using mitochondrial control-region sequence data. *Conservation Genetics*, **4**, 301–310.

Bonnet E, Van de Peer Y (2002) zt: a software tool for simple and partial Mantel tests. *Journal of Statistical Software*, **7**, 1–12.

Bouzat JL, Cheng HH, Lewin HA *et al.* (1998) Genetic evaluation of a demographic bottleneck in the greater prairie chicken. *Conservation Biology*, **12**, 836–843.

Braun CE (1998) Sage-grouse declines in western North America: what are the problems? *Proceedings of the Western Association of State Fish and Wildlife Agencies*, **78**, 139–156.

Clement M, Posada D, Crandall K (2000) tcs: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1660.

Connelly JW, Braun CE (1997) Long-term changes in sage grouse *Centrocercus urophasianus* populations in western North America. *Wildlife Biology*, **3**, 229–234.

Connelly JW, Browers HW, Gates RJ (1988) Seasonal movements of sage grouse in southeastern Idaho. *Journal of Wildlife Management*, **52**, 116–122.

Connelly JW, Schroeder MA, Sands AR, Braun CE (2000) Guidelines to manage sage grouse populations and their habits. *Wildlife Society Bulletin*, **28**, 967–985.

(16)

Cornuet JM, Luikart G (1997) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics*, **144**, 2001–2014.

Crandall KA, Templeton AR (1993) Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. *Genetics*, **134**, 959–969.

Crandall KA, Templeton AR, Sing CF (1994) Intraspecific phylogenetics, problems and solutions. In: *Phylogeny Reconstruction* (eds Scotland RWL, Siebert DJ, Williams DM), pp. 273–297. Clarendon, Oxford.

Dunn PO, Braun CE (1985) Natal dispersal and lek fidelity of sage grouse. *Auk*, **102**, 621–627.

Ellsworth DL, Honeycutt RL, Silvy NJ (1996) Systematics of grouse and ptarmigan determined by nucleotide sequences of the mitochondrial cytochrome-*B* gene. *Auk*, **113**, 811–822.

Emslie SD (1985) The late Pleistocene (Rancholabrean) avifauna of Little Box Elder Cave, Wyoming. *University of Wyoming, Contributions in Geology*, **23**, 63–82.

Emslie SD (2004) The early and middle Pleistocene avifauna from Porcupine Cave, Colorado. In: *Early and Middle Pleistocene Biodiversity and Environmental Change: The Porcupine Cave Fauna from Colorado* (ed. Barnowsky A), pp. 127–140. University of California Press, Berkeley.

Emslie SD, Heaton TH (1987) The late Pleistocene avifauna of Crystal Ball Cave, Utah. *Journal of Arizona-Nevada Academy of Science*, **21**, 53–60.

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.

Felsenstein J (1989) PHYLIP *(Phylogeny Inference Package), Version 3.2.* Department of Genetics. University of Washington, Seattle.

Grayson DK (1976) A note on the prehistoric avifauna of the Lower Klamath basin. *Auk*, **93**, 830–833.

Guo S, Thompson E (1992) Performing the exact test of Hardy–Weinberg proportions for multiple alleles. *Biometrics*, **48**, 361–372.

Howard H (1952) The prehistoric avifauna of Smith Creek Cave, Nevada, with a description of a gigantic raptor. *Bulletin of Southern California Academy of Sciences*, **51**, 50–54.

Howard H, Miller AH (1933) Bird remains from cave deposits in New Mexico. *Condor*, **35**, 15–18.

Hupp JW, Braun CE (1991) Geographical variation among sage-grouse populations in Colorado. *Wilson Bulletin*, **103**, 255–261.

Kahn NW, Braun CE, Young JR, Wood S, Mata DR, Quinn TW (1999) Molecular analysis of genetic variation among large- and small-bodied sage-grouse using mitochondrial control-region sequences. *Auk*, **116**, 819–824.

Lucchini V, Hoglund J, Klaus S, Swenson J, Randi E (2001) Historical biogeography and a mitochondrial DNA phylogeny of grouse and ptarmigan. *Molecular Phylogenetics and Evolution*, **20**, 149–162.

Luikart G, Cornuet JM (1998) Empirical evaluation of a test for identifying recently bottlenecked populations from allele frequency data. *Conservation Biology*, **12**, 228–237.

Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research*, **27**, 209–220.

McDonald HG, Anderson E (1975) A late Pleistocene vertebrate fauna from southeastern Idaho. *Tebiwa*, **18**, 19–37.

Miller L (1963) Birds and Indians in the West. *Bulletin of the Southern California Academy of Sciences*, **62**, 178–191.

Miller L (1965) Bird remains from an archaeological site in the Beaverhead Mountains of southeastern Idaho. *Tebiwa*, **8**, 17–20.

Moritz C (1994) Defining 'evolutionary significant units' for conservation. *Trends in Ecology and Evolution*, **9**, 373–375.

Naugle DE, Aldridge CL, Walker BL *et al.* (2004) West Nile virus: pending crisis for greater sage-grouse. *Ecology Letters*, **7**, 704–713.

O'Brien SJ, Evermann JF (1988) Interactive influence of infectious disease on genetic diversity of natural populations. *Trends in Ecology and Evolution*, **3**, 254–259.

Oyler-McCance SJ, St. John J, Taylor SE, Quinn TW (2005) Population genetics of Gunnison sage-grouse: implications for management. *Journal of Wildlife Management*, in press.

Oyler-McCance SJ, Kahn NW, Burnham KP, Braun CE, Quinn TW (1999) A population genetic comparison of large- and small-bodied sage-grouse in Colorado using microsatellite and mitochondrial DNA markers. *Molecular Ecology*, **8**, 1457–1465.

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences*, **12**, 357–358.

Parker KM, Sheffer RJ, Hedrick PW (1999) Molecular variation and evolutionarily significant units in the endangered Gila topminnow. *Conservation Biology*, **13**, 108–116.

Posada D, Crandall KA, Templeton AR (2000) GEODIS: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology*, **9**, 487–488.

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

Quattro JM, Vrijenhoek RC (1989) Fitness differences among remnant populations of the endangered Sonoran topminnow. *Science*, **245**, 976–978.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Schneider S, Roessli D, Excoffier L (2001) ARLEQUIN: *a software for population genetics data analysis Version 2.001.* Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Schroeder MJ, Hays DW, Livingston MF *et al.* (2000) Changes in the distribution of sage-grouse in North America. *Northwestern Naturalist*, **81**, 104–112.

Schroeder MJ, Aldridge CA, Apa AD *et al.* (2004) Distribution of sage-grouse in North America. *Condor*, **106**, 363–376.

Shufeldt RW (1913) Review of the fossil fauna of the desert region of Oregon, with a description of additional material collected there. *Bulletin of the American Museum of Natural History*, **32**, 123–178.

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics*, **139**, 457–462.

Swofford DL (2003) PAUP*. *Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4.* Sinauer Associates, Sunderland, Massachusetts.

Templeton AR (1998) Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology*, **7**, 381–397.

Templeton AR (2004) Statistical phylogeography: methods of evaluating and minimizing inference errors. *Molecular Ecology*, **13**, 789–809.

Templeton AR, Boerwinkle E, Sing CXF (1987) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. I. Basic theory and an analysis

(17)

of alcohol dehydrogenase activity in *Drosophila*. *Genetics*, **117**, 343–361.

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred form restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics*, **132**, 619–633.

Westemeier RL, Brawn JD, Simpson SA *et al.* (1998) Tracking the long-term decline and recovery of an isolated population. *Science*, **282**, 1695–1698.

Young JW, Braun CE, Oyler-McCance SJ, Hupp JW, Quinn TW (2000) A new species of sage-grouse (Phasianidae: *Centrocercus*) from southwestern Colorado. *Wilson Bulletin*, **112**, 445–453.

Young JW, Hupp JW, Bradbury JW, Braun CE (1994) Phenotypic divergence of secondary sexual traits among sage-grouse (*Centrocercus urophasianus*) populations. *Animal Behaviour*, **47**, 135D–1362.

Portions of the data collected for this study comprised Sonja Taylor's MS thesis; she subsequently collected additional microsatellite data as a research associate. Sara Oyler-McCance (Conservation Geneticist with the US Geological Survey) and Tom Quinn (Associate Professor at the University of Denver) have been collaborating on genetic studies of sage-grouse for a number of years. They are co-directors of the Rocky Mountain Center for Molecular Conservation Genetics and Systematics at the University of Denver.
