



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

Nicolas G. Benedict¹, S.J. Oyler-McCance^{1,2}, S.E. Taylor¹, C.E. Braun³ & T.W. Quinn^{1,*}

¹Department of Biological Sciences, University of Denver, Denver, Colorado 80208, USA;

²Mid-continent Ecological Science Center, U.S. Geologic Survey, 4512 McMurry Avenue, Fort Collins, Colorado 80525, USA; ³Grouse 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

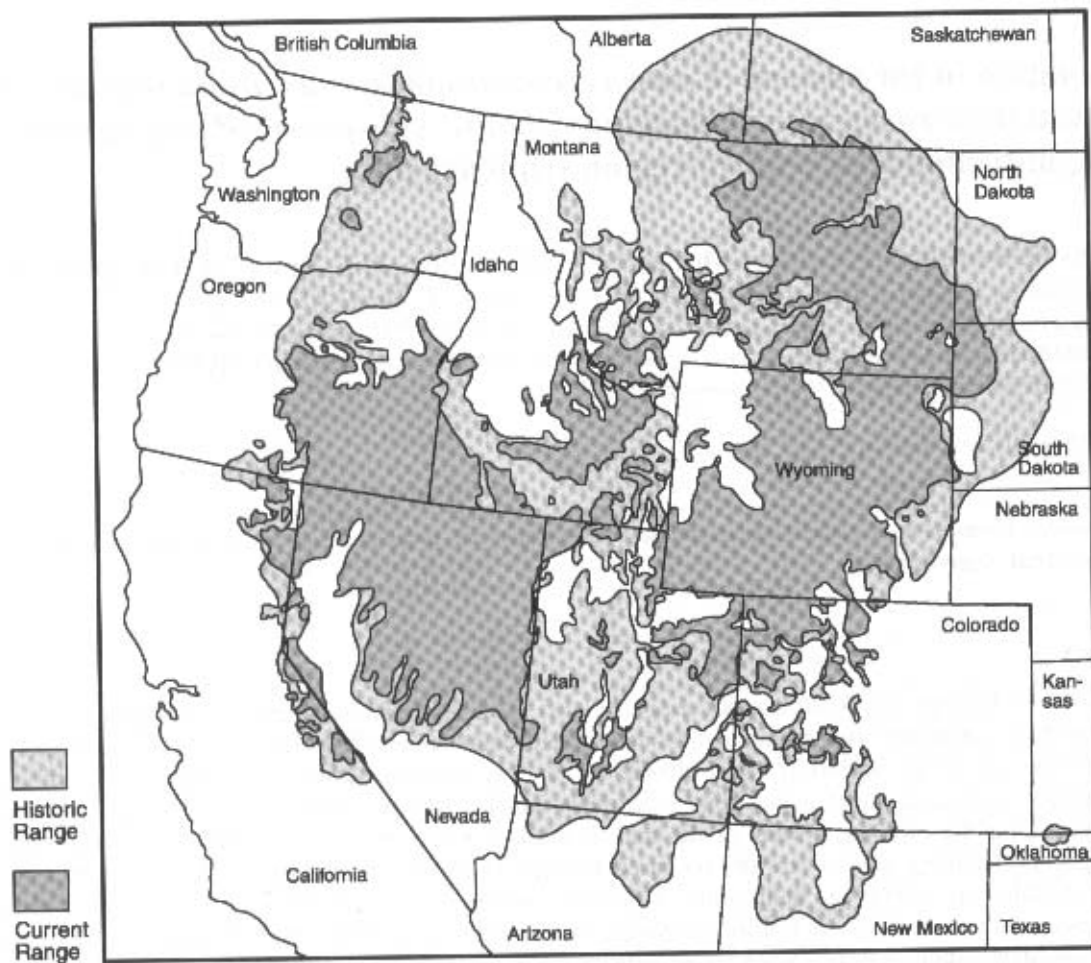


Figure 1. Historic (early 1900s) and current distribution of Sage-grouse in western North America.

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.

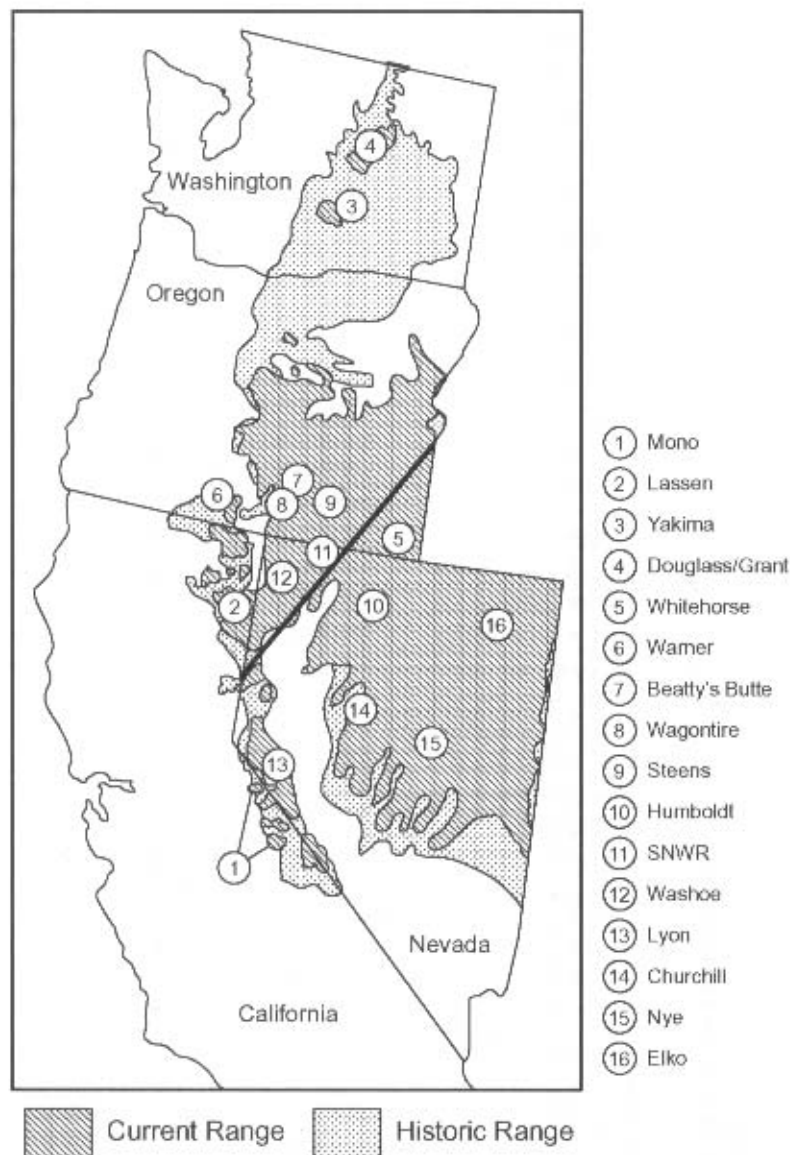


Figure 2. Location of study populations. The solid line denotes the delineation between the eastern and western subspecies as proposed by Aldrich (1946).

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

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 manufacturer's 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 TFGPA (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 TFGPA 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

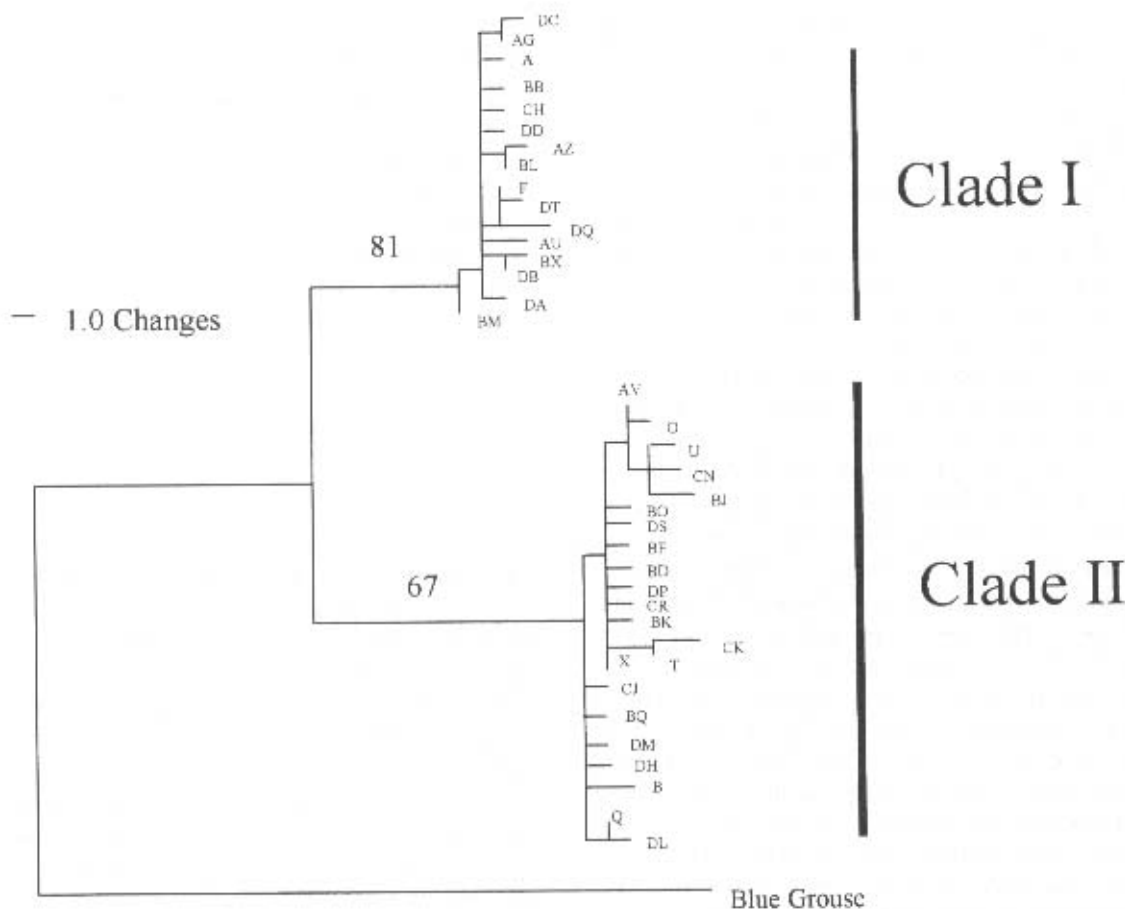


Figure 3. Phylogram of the strict-consensus tree of all haplotypes presented. The tree has a consistency index of 0.882, a retention index of 0.970 and a rescaled consistency index of 0.856. Bootstrap values > 50 are presented on the branches of the tree.

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 ($F_{st} = 0.0356$, $p > 0.05$).

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

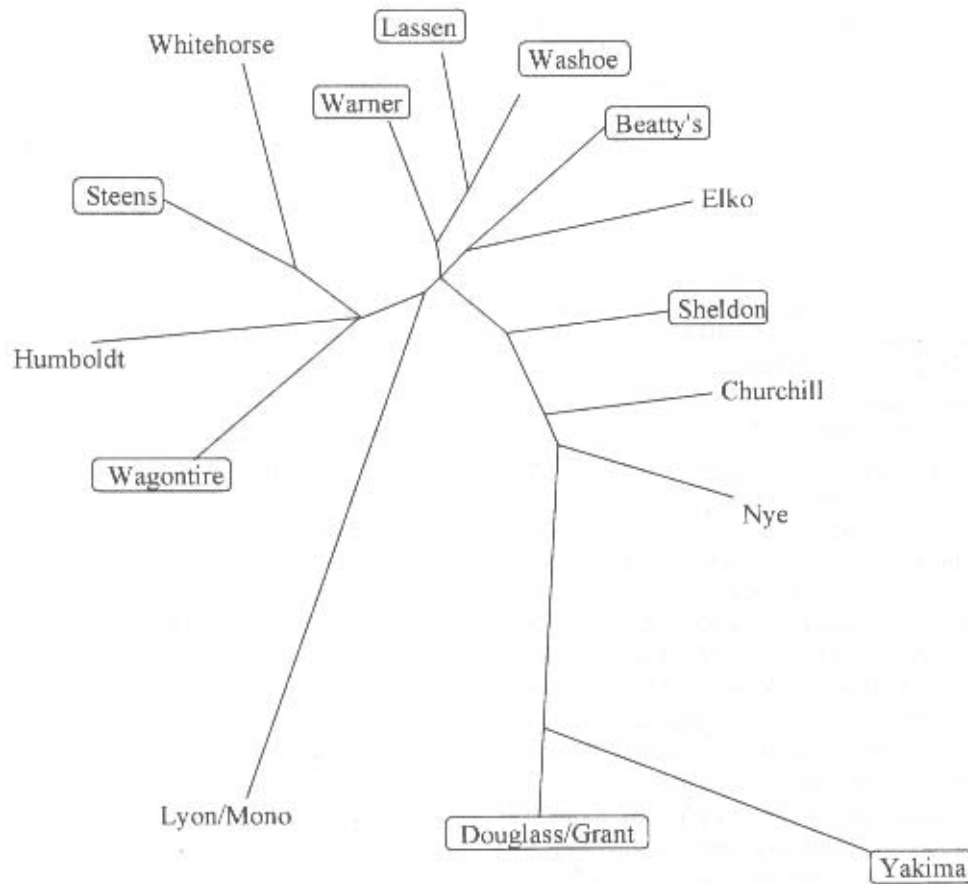


Figure 4. Neighbor-Joining tree constructed using Wright's (1978) modification of Roger's genetic distance (Boxed populations represent the western subspecies, while unboxed populations represent the eastern subspecies).

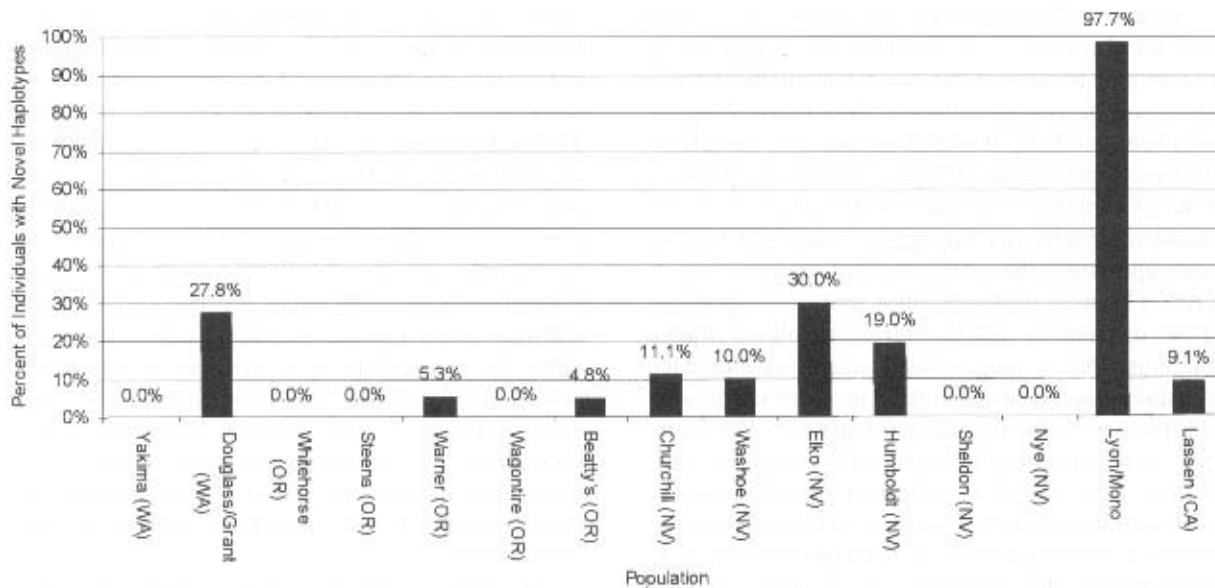


Figure 5. Proportion of individuals per population with novel haplotypes.

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. Further-

more, 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.

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