GENETIC ANALYSIS OF COLUMBIAN SHARP-TAILED GROUSE: A PRELIMINARY STUDY

Final Report

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Dr. Kenneth I. Warheit
Wildlife Research Division
Department of Fish and Wildlife
600 Capitol Way N.
Olympia, WA 98501
360.902.2595
warhekiw@dfw.wa.gov

Dr. Michael A. Schroeder
Wildlife Research Division
Department of Fish and Wildlife
P.O. Box 1077
Bridgeport, WA 98813
509.686.2692
schromas@dfw.wa.gov

INTRODUCTION

The historical range of the Columbian Sharp-tailed Grouse (*Tympanuchus phasianellus columbianus*) extended from the steppe- and shrub-dominated habitats in the inter-mountain regions from British Columbia south to California, Nevada, and Utah, and east to western Montana, Wyoming and Colorado. The subspecies has been extirpated from most of its range and exists now as remnant and isolated populations. The core of Columbian Sharp-tailed Grouse distribution occurs as scattered populations in southeast Idaho and northern Utah where 50-70% of the subspecies' total abundance currently resides (USFWS, 1999). The decline of Sharp-tailed Grouse in Washington has been precipitous and extreme, and it now exist in only six to eight small and fragmented populations in Douglas, Lincoln, and Okanogan Counties (Hays et al., 1998).

As part of Washington Department of Fish and Wildlife's continuing assessment of the status of and recovery efforts for Sharp-tailed Grouse in the state, we have initiated a project to evaluate this subspecies' geographic structure and population genetics in Washington and neighboring states. One of the primary objectives of this study is to determine the genetic relationships of the existing Columbian Sharp-tailed Grouse populations. The purpose of this activity is to determine if cross-transplanting birds from neighboring populations within Washington or moving birds from viable population in Idaho and Utah into Washington is feasible and appropriate. In addition, a genetic analysis of Columbian Sharp-tailed Grouse populations would also permit us to evaluate the degree to which the small and isolated populations in Washington have experienced a loss of genetic diversity either through genetic drift or inbreeding. A loss in genetic diversity may be associated with or foretell a decrease in overall population fitness, similar to that which occurred in the congeneric Greater Prairie Chicken (*Tympanuchus cupido*; Bouzat et al., 1998a,b).

The purpose of this document is to report the results of a preliminary analysis of the population genetics of Columbian Sharp-tailed Grouse, primarily from two general localities in Washington and Idaho. The activities associated with the report were funded by US Department of Interior, Bureau of Land Management (BLM; Contract # HWP000025). The intent of this initial project was to develop laboratory protocols for the amplification and scoring of microsatellite loci, and to provide a preliminary assessment of genetic diversity and geographic structure of Columbian Sharp-tailed Grouse.

METHODS

We did not attempt to develop microsatellite loci for this project. Instead, we screened the literature and Genbank (Benson et al., 2000) for microsatellite loci developed for other Galliform taxa, and evaluated their use in Sharp-tailed Grouse. Our initial literature search provided primer sequences for 16 microsatellite loci, from which we tested appropriate amplification in Sharp-tailed Grouse. We developed polymerase chain reaction (PCR) protocols for these loci, and based on their ease of use and relative variability in a subset of our samples, we used seven of these loci for all subsequent analyses (Table 1).

We obtained a total of 63 blood samples from five general localities (Figure 1; Table 2): Washington (Douglas and Okanogan Counties) [n=20]; Idaho (Power County) [31]; Montana (west of the continental divide, Powell County) [3]; Montana (east of the continental divide, Lewis and Clarke County) [6]; and Alaska (140 km southeast of Fairbanks) [3]. The Washington and Idaho samples are from Columbian Sharp-tailed Grouse, the Alaska samples are from Alaskan Sharp-tailed Grouse (*T. p. caurus*), and the Lewis and Clark County, Montana (eMT) samples are from the Plains Sharp-tailed Grouse (*T. p. jamesi*). The taxonomic identity of the Powell County, Montana (wMT) samples is in doubt (B. Deeble, pers. comm. 2001), but is thought to be from Columbian Sharp-tailed Grouse because the population exists west of the continental divide. Although for this report we treat the Washington and Idaho samples each as a single population, there is some geographic heterogeneity within each of these samples. In Washington, there are two localities from which the samples where drawn: Nespelem, Okanogan County [n=17] and Dyre Hill, Douglas County [3]. The average among-sample distance within each of these localities is 1.0 and 0.0 km, respectively, and the two localities are separated by about 61 km. The Idaho population is divided into roughly four subpopulations, with an additional six individuals scattered between these subpopulations. Although the average among-sample distance within each of these subpopulations is 0.0 km, the four subpopulations are separated by an average of 22 km. Despite this spatial heterogeneity in the Washington and Idaho samples, the results are similar regardless of whether the samples are pooled into two respective populations or are analyzed per subpopulation. As such, we report only the results from the pooled samples.

We used Qiagen DNeasy Spin Tubes for extracting DNA from each of the blood samples following the standard nucleated blood protocols provided with the kit. PCR microsatellite protocol development and locus amplification were conducted on MJ Research PTC-200 thermocyclers and the DNA fragments were visualized using florescently labeled primers and an Applied Biosystems ABI Prism 377 sequencer. Each sample was run with an internal size standard (Applied Biosystems GeneScan 500) and sized using GeneScan 3.1 and Genotyper 2.5 software. We conducted statistical analyses using Genepop 3.3 (Raymond and Rousset, 1995), MEGA 2.0 (Kumar et al., 2001), and GDA 1d16c (Lewis and Zaykin, 2001), or using macros and programs developed by us and implemented on Microsoft Excel 2000.

RESULTS and DISCUSSION

Genetic Diversity within Population

Samples sizes are small for each of the populations, especially for the Alaska and two Montana populations where sample sizes per locus range from two to six individuals (Table 2). As such, no definitive conclusions should be made from these data concerning levels of genetic diversity within each of these populations, or the genetic divergence among the populations. The sample sizes from the Washington and Idaho populations are larger than those from the other localities and do provide sufficient power to discern significant differences where they occur (i.e., minimize the probability of making a Type II statistical error).

Genetic diversity can be used as a measure of population health and evolutionary potential. That is, it is assumed that a population, as a whole, has a greater potential to cope with a variety of environmental effects with a diverse array of genotypes than a population with reduced genetic diversity (see Hedrick, 1996 for caution in using this assumption). We provide several measures

of genetic diversity for each of the populations, although the sizes for Alaska and Montana samples are insufficient to describe the true molecular variance for each population. LLSD4 is the most variable locus within each population, with as many as 14 alleles in the Idaho samples (Table 2). Overall, all loci are polymorphic in the Washington and Idaho samples, and both populations show similar levels of allelic diversity (Table 2) and expected heterozygosity (Table 3). This indicates that in terms of the number of alleles per locus, the Washington and Idaho samples show the same level of variability (Table 4). As expected, there is less allelic diversity in the wMT and Alaska populations where we sampled only three individuals from each locality. Although our eMT sample consisted of only six individuals, the expected heterozygosity in this population was comparable to both the Washington and Idaho samples (Table 3).

Although the degree of allelic diversity in the Washington and Idaho samples are the same, the Washington population has a deficit in the number of observed heterozygotes, and as such, the population is not in Hardy-Weinberg equilibrium at two to four of the seven loci (Table 3). None of the other populations show a heterozygote deficit and all other populations are in Hardy-Weinberg equilibrium at all loci. What this means is that although both the Washington and Idaho samples have roughly the same number of alleles, those alleles are represented significantly more as homozygotes in Washington than in Idaho. In other words, the Idaho samples show significantly greater within-individual genetic diversity than the Washington samples.

A reduction in the number of heterozygotes and deviations from Hardy-Weinberg equilibrium can result from several different factors, including inbreeding, selection, mutation, and immigration, or can be an artifact of null or non-amplifying alleles. Since it is only the Washington population that has heterozygote deficiencies, and it would be unlikely that selection or altered mutation rates would be affecting only this population. Furthermore, as we show below, there appears to be little or no gene flow and therefore effectively no migration among all populations in this analysis. Therefore, the most plausible hypotheses for the deficiencies of heterozygotes in Washington are either null alleles or inbreeding. Null alleles can be in either a heterozygote or homozygote form. An individual with a heterozygote null allele has one non-amplifying allele (the null allele) and one amplifying allele. Since the one amplifying allele is the only allele that is visualized electrophoretically, the sample appears as a homozygote for that single amplifying allele. A homozygote null allele has two non-amplifying alleles and since no alleles are visualized electrophoretically, the sample appears as a blank, thereby reducing the population's sample size for that locus. The per locus samples sizes for Washington population are considerably more variable than those for the other localities (Table 2) suggesting that null alleles may be present. An alternative hypothesis for variable number of amplifying samples per locus is poor quality DNA. If a reduction in sample size at a particular locus is a function of homozygote null alleles and if there is a direct relationship between the number of homozygote and heterozygote null alleles (i.e., if a null allele is present it should occur in both homozygote and heterozygote states), there also should be a direct relationship across all loci between a population's sample size and the probability of heterozygote deficit. That is, loci with small sample sizes should also have low p-values for heterozygote deficit (low p-value indicates that the null hypothesis of no heterozygote deficit has been rejected – i.e., there is indeed a heterozygote deficit). However, as shown Figure 2, there appears to be no relationship between p-values and sample size. In fact, except for one locus (LLSD7), those loci with the highest sample sizes seem to be the loci with a deficit in heterozygotes. This suggests that the reduction in heterozygotes in the Washington population is not due to null alleles.

Table 4 shows the distribution of alleles for each locus in each of the five populations. In addition, we also provide an inbreeding coefficient (*f* or F_{IS}) for each locus and as a single measure of inbreeding averaged over all loci. This inbreeding coefficient measures the probability that alleles at a single locus from an individual are identical by descent. Alleles that are identical by descent are those from related individuals, and as such, this coefficient measures the degree to which the parents of an individual are related. From a heterozygosity perspective, this inbreeding coefficient measure the extent to which the observed heterozygosity in a population is lower than the expected heterozygosity, and it is assumed that this reduction is the result of inbreeding. An inbreeding coefficient greater than zero suggests here that inbreeding is occurring in that population. In the Washington population, six of the seven loci show inbreeding coefficients greater than zero, while in the Idaho population, none of the loci show inbreeding coefficients greater than zero (Table 4). Overall, all populations, except Washington, show an inbreeding coefficient not significantly different from zero, while the coefficient in Washington is 0.26 (Table 4). The upper and lower bounds of a bootstrap provide a means by which each of the coefficients can be tested for similarities. The range of inbreeding coefficients for Idaho, wMT, eMT, and Alaska samples are all overlapping and cross zero indicating that the coefficients in these populations are the same and are equal to zero. However, the lower bound of the coefficient in Washington is 0.17 (Table 4), indicating that the coefficient is significantly positive and different from all other populations. These data suggest that the reduction in heterozygotes in Washington is a result of inbreeding.

Genetic Differentiation Among Populations

Because the Washington and Idaho samples were drawn from the same subspecies, while the Alaska and eMT samples represent two additional subspecies, our initial hypothesis was that the Idaho and Washington samples would be most similar to each other and significantly different from both Alaska and eMT. That hypothesis proved to be false. Since the identity of the wMT samples was unknown, but though to be from the Columbian Sharp-tailed Grouse, we also assumed that if indeed the wMT samples were from this subspecies, they would be genetically more similar to Washington and Idaho than the neighboring eMT samples (Plains Sharp-tailed Grouse). We provide two measures of population differentiation (Table 5). In calculating the coancestry coefficient (θ), we did not assume Hardy-Weinberg equilibrium, and as such, θ measures to degree to which the genotypes for these seven microsatellite loci have differentiated between each pair of populations. Coancestry coefficients equal to zero indicate no differentiation. Each of the coancestry coefficients in Table 5 are significantly greater than zero, indicating that the genotypes in each of these populations are significantly different from each other. Furthermore, the largest coefficients (indicating greater differentiation) for each the Washington, Idaho, and wMT populations were with the Alaska and eMT populations, which is not surprising considering the fact that the Alaska and eMT populations are from different subspecies. However, the coefficients for the Idaho samples appear constant through all pairwise comparisons suggesting an equal distance between these samples and the other four populations (Table 5). This is inexplicable, but may be related to the fact that the sample sizes are grossly unbalanced in each of these calculations (e.g., n= 31 for Idaho and n=3 for Alaska).

Figure 3 is a minimum evolution tree based on the coancestry coefficient in Table 5. A minimum evolution tree is the one tree whose topology out of all possible topologies has the smallest sum of all branch lengths (Nei and Kumar, 2000). The plot is essentially a graphical representation of the coancestry coefficient matrix, and should not be construed as a phylogenetic tree. The tree is unrooted because no outgroup was designated in this analysis. However, from the perspective of the Columbian Sharp-tailed Grouse, the root would be placed

at either the AK (Alaska subspecies) or the eMT (Plains subspecies) node. This tree shows the close association between the Washington and wMT samples, and this association is maintained regardless of whether the root for the tree is placed at the Alaska or eMT node.

In the lower part of the matrix in Table 5 we provide probability values testing the null hypothesis that the genotypic distribution between each pair of populations is identical. That is, small values indicate significant differentiation between the pair of populations. All pairwise comparisons show highly significant population differentiation, except those involving wMT, whose sample size and degree of differentiation provided little power to adequately test for differences (except in wMT's comparison with Idaho, where Idaho large sample size provided sufficient power to test for differences). What this means is that although there may be a significant genetic differentiation between wMT and Washington, Alaska, or eMT, the sample sizes in the analyses are insufficient to test for that differentiation. Nevertheless, the data from Table 5 suggest that there is little or no migration of individuals among all sample localities.

CONCLUSIONS

Based on the analyses presented in this report, we reached the following two conclusions. However, we emphasize that although the sample sizes for both the Washington and Idaho samples appear adequate for the types of analyses presented here, they are still relatively small (especially Washington), and the samples were drawn from effectively one population each and during one sampling period. Repeated samples from these populations and neighboring sites from both the Washington and Idaho localities are needed to confirm these conclusions:

(1) The Washington population (Nespelem and Dyre Hill samples) has reduced genetic variability as measured by observed heterozygosity, and this reduction is a function of inbreeding. Although this population appears to be inbreed, the total number of alleles, representing some measure of genetic diversity, is similar to the outbreed population in Idaho.

(2) The Washington and Idaho populations are significantly differentiated genotypically, which suggests that there is little or no gene flow between these two sites and the populations are currently on different evolutionary trajectories.

In addition to these two conclusions, the following is a set of tentative conclusions based on the results concerning all five populations in the analysis. Because the sample sizes for the Alaska, Powell County (wMT), and Lewis and Clarke County (eMT) populations are very small, these conclusions should be used with caution, and it is very likely that the conclusions may change with additional samples.

(3) The Powell County, Montana (wMT) samples appear most similar genetically to the Washington samples (and secondarily to the Idaho samples, although this relationship is not entirely clear based on Figure 3). These wMT samples are not genetically similar to the Lewis and Clark County (eMT) samples, which suggest that the wMT samples were drawn from a Columbian Sharp-tailed Grouse population rather than a Plains Sharp-tailed Grouse population.

(4) The current Sharp-tailed Grouse taxonomy is not reflected in the coancestry coefficients and the minimum evolution tree, based on these coefficients. That is, the Idaho Columbian Sharp-

tailed Grouse appears more similar genetically to either the Alaska Sharp-tailed Grouse or the Plains Sharp-tailed Grouse, than to the Washington Columbian Sharp-tailed Grouse. However, the large sample size for the Idaho populations and the small sample sizes for the wMT, eMT, and Alaska samples may be affecting this analysis.

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TABLE 1. Origin of each microsatellite locus used in this study. Accession # refers to the nucleotide database in Genbank, except the AF303097, which refers the European Molecular Biology Laboratory (EMBC) database.

Locus	Developed in	Reference	Accession #
ADL146	Domestic Chicken (*Gallus gallus*)	Cheng and Crittenden (1994); Bouzat et al. (1998a,b)	G01571
ADL162	Domestic Chicken (*Gallus gallus*)	Cheng and Crittenden (1994); Bouzat et al. (1998a,b)	G01586
ADL230	Domestic Chicken (*Gallus gallus*)	Cheng and Crittenden (1994); Bouzat et al. (1998a,b)	G01650
LLSD3	Red Grouse (*Lagopus lagopus*)	Piertney and Dallas (1997)	X99053
LLSD4	Red Grouse (*Lagopus lagopus*)	Piertney and Dallas (1997)	X99054
LLSD7	Red Grouse (*Lagopus lagopus*)	Piertney and Dallas (1997)	X99057
TTD6	Black Grouse (*Tetrao tetrix*)	Caizergues et al. (2001)	AF303097

TABLE 2. Sample sizes and number of alleles for each microsatellite locus per locality. wMT and eMT refer to the Powell County, Montana and Lewis and Clark County, Montana samples, respectively.

	Sample Size					Number of Alleles				
	WA	**ID**	**wMT**	**eMT**	**AK**	**WA**	**ID**	**wMT**	**eMT**	**AK**
ADL146	14	31	2	6	3	3	5	3	3	2
ADL162	13	31	3	6	3	5	2	1	3	2
ADL230	18	31	3	6	3	7	6	5	5	3
LLSD3	17	31	3	6	3	5	5	3	4	2
LLSD4	20	31	3	6	3	11	14	4	7	4
LLSD7	19	31	3	6	3	7	8	5	5	5
TTD6	19	31	3	4	3	5	4	1	3	2
Overall	17.1	31.0	2.9	5.7	3.0	6.14	6.29	3.14	4.29	2.86

TABLE 3. Expected and observed heterozygosities and deviations from Hardy-Weinberg (heterozygote deficit).

	Expected Heterozygosity					Observed Heterozygosity				
	WA	**ID**	**wMT**	**eMT**	**AK**	**WA[a]**	**ID**	**wMT**	**eMT**	**AK**
ADL146	0.519	0.576	0.833	0.439	0.333	0.357	0.581	1.000	0.500	0.333
ADL162	0.351	0.275	0.000	0.318	0.333	0.308	0.323	0.000	0.333	0.333
ADL230	0.852	0.746	0.933	0.788	0.733	0.611****	0.774	1.000	0.833	1.000
LLSD3	0.775	0.675	0.600	0.803	0.333	0.529***	0.677	0.667	0.833	0.333
LLSD4	0.860	0.873	0.867	0.924	0.800	0.650*	0.839	0.667	1.000	0.667
LLSD7	0.785	0.818	0.933	0.788	0.933	0.737	0.806	1.000	0.667	1.000
TTD6	0.670	0.673	0.000	0.679	0.333	0.421**	0.613	0.000	0.750	0.333
Overall	0.688	0.662	0.595	0.677	0.543	0.516	0.659	0.619	0.702	0.571

[a] Observed heterozygosity significantly less than expected heterozygosity (heterozygote deficit), using an estimate of p-values based on Markov-chain method (Genepop)

* p = 0.20 (adjusting for experimentwise error rate; actual probability is p = 0.04)
** p = 0.10 (adjusting for experimentwise error rate; actual probability is p = 0.02)
*** p = 0.05 (adjusting for experimentwise error rate; actual probability is p = 0.01)
**** p = 0.01 (adjusting for experimentwise error rate; actual probability is p = 0.002)

TABLE 4. Allele frequencies, with alleles measured in numbers of basepairs. Inbreeding coefficient (*f*) is the loss of heterozygosity resulting from inbreeding. The overall *f* is the average across all loci, with the upper and lower bounds from 1000 bootstrap runs, resampling across loci.

	WA	ID	wMT	eMT	AK
ADL146					
188	8	36	1	9	5
190	18	9	2	1	1
192	2	17	1	2	-
f	0.32	-0.01	0.00	-0.33	-0.15
ADL162					
93	-	-	-	-	1
117	-	10	-	1	-
119	1	-	-	1	-
121	21	52	6	10	5
137	2	-	-	-	-
139	1	-	-	-	-
155	1	-	-	-	-
f	0.13	-0.18	0.00	0.00	-0.05
ADL230					
100	-	-	-	-	1
106	2	19	1	1	-
108	6	-	-	-	-
110	2	1	1	-	3
112	-	21	-	3	2
114	-	-	1	1	-
116	9	14	1	2	-
118	6	4	2	5	-
120	5	3	-	-	-
122	6	-	-	-	-
f	0.29	-0.04	-0.50	-0.09	-0.06

	WA	ID	wMT	eMT	AK
LLSD3					
121	-	2	-	-	-
123	-	-	-	3	-
127	3	-	-	-	-
131	12	22	1	2	1
133	6	3	-	-	-
135	-	8	-	3	-
137	9	27	4	-	5
139	-	-	1	4	-
141	4	-	-	-	-
f	0.32	0.00	0.00	-0.14	-0.04
LLSD7					
156	-	5	1	1	-
158	-	-	1	-	-
160	14	7	1	3	-
162	2	11	-	1	1
164	7	9	-	-	-
166	1	1	-	-	-
168	8	21	1	2	2
170	5	5	-	5	-
172	-	3	-	-	-
174	1	-	2	-	1
180	-	-	-	-	1
182	-	-	-	-	1
f	0.06	0.01	-0.09	-0.09	0.17

TABLE 4. Continued.

	WA	ID	wMT	eMT	AK
LLSD4					
181	-	1	-	-	-
185	5	4	-	-	-
187	-	2	1	-	-
189	12	-	-	-	-
191	1	-	-	-	-
195	-	2	-	-	-
197	1	-	-	-	1
199	1	-	-	-	-
201	-	5	2	2	-
203	4	9	1	-	-
205	1	3	-	-	3
207	4	17	2	2	-
209	1	2	-	2	-
211	4	9	-	-	1
213	6	5	-	1	-
215	-	1	-	2	1
217	-	1	-	2	-
223	-	1	-	-	-
225	-	-	-	1	-
f	0.25	0.04	0.20	0.27	-0.09
TTD6					
112	2	9	-	-	-
116	17	25	-	3	5
118	14	24	6	-	1
120	2	-	-	4	-
122	3	4	-	1	-
f	0.38	0.09	0.00	0.00	-0.13

	WA	ID	wMT	eMT	AK
Overall					
f	0.26	0.01	-0.06	-0.04	-0.07
upper	0.33	0.04	0.14	0.04	0.10
lower	0.17	-0.04	-0.24	-0.11	-0.28

TABLE 5. Two measures of population differentiation. Coancestry coefficient, θ, (above the diagonal) measures the degree to which the genotypes have differentiated between each pair of populations. A higher coancestry coefficient, the greater the genetic differentiation ($\theta = 0$ indicates no differentiation). Below the diagonal are p-values testing the null hypothesis that the genotypic distribution is identical between each pair of populations (genotypic differentiation test in Genepop). Populations that have significantly different genotypes are shown in bold[a]

	WA	ID	wMT	eMT	AK
WA		0.08	0.05	0.11	0.12
ID	**<0.00001**		0.07	0.09	0.08
wMT	0.02459	**0.00079**		0.19	0.21
eMT	**<0.00001**	**<0.00001**	0.03529		0.17
AK	**0.00014**	**0.00001**	0.60245	**0.00496**	

[a] $p < 0.05$ is considered significant; however, alpha is adjusted for experimentwise error rate resulting in $p < 0.005$ required to reject null hypothesis

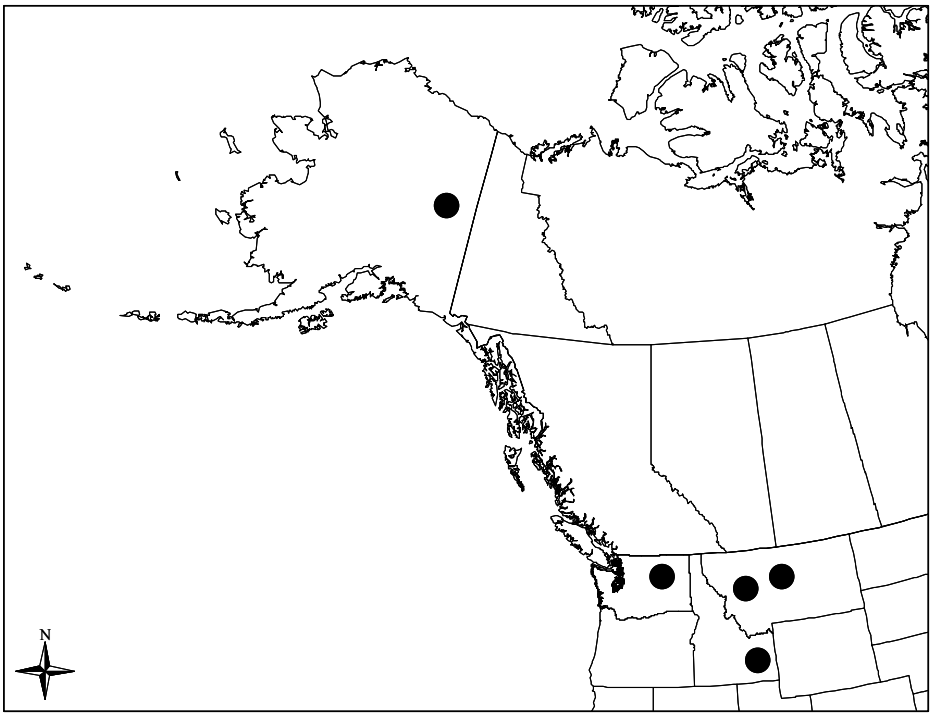

Figure 1. General localities of Sharp-tailed Grouse samples

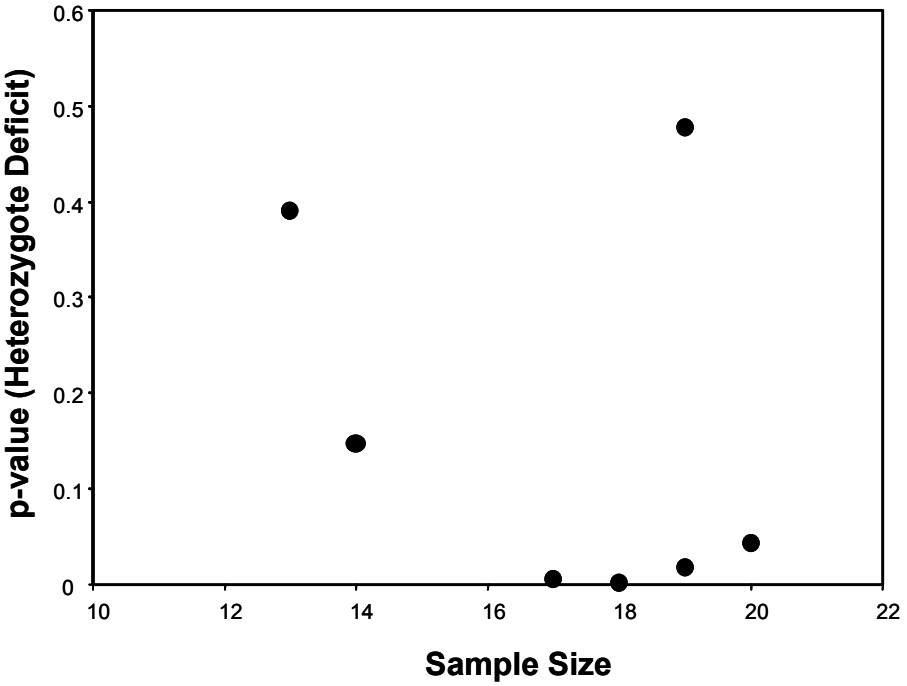

FIGURE 2. Sample size versus p-value for heterozygote deficit for each locus

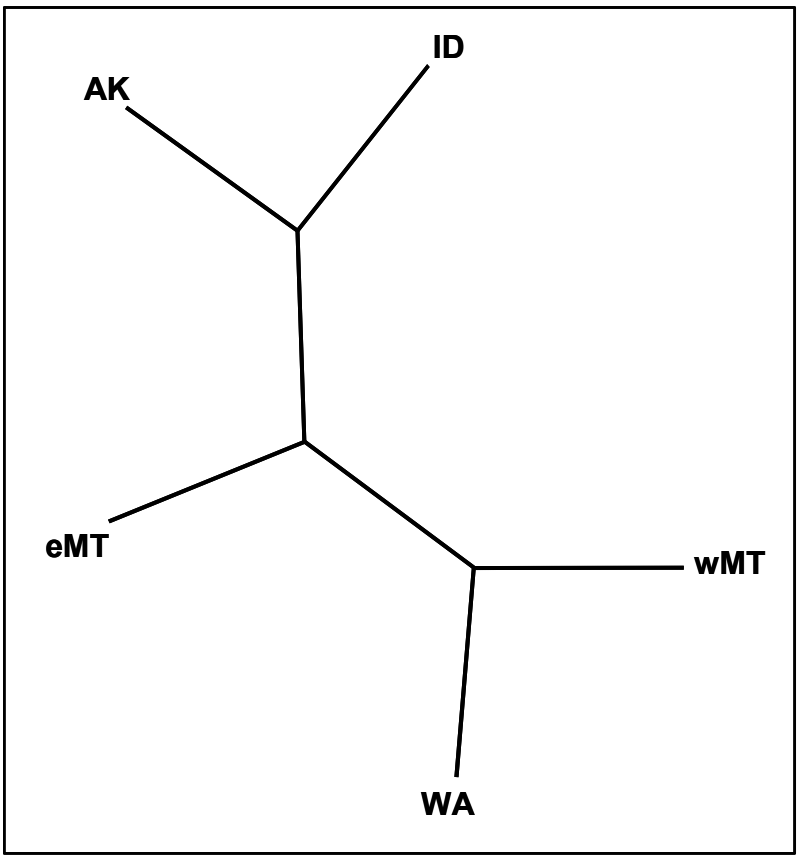

FIGURE 3. Unrooted minimum evolution tree described from the coancestry coefficients (Table 5)