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'Sierra Nevada Bighorn Sheep': Are they an endangered 'metapopulation' due to disease Transmission from Domestic Sheep?

1. Are the Sierra Nevada Bighorn Sheep a Subspecies or even distinct Metapopulation?

Background: In 2002 NDOA, the Nevada Department of Wildlife (NDOW) and the University of Nevada, Reno (UNR) entered into a collaborative project to investigate disease transmission between domestic sheep and BHS. The project comprises domestic sheep sampling (6 flocks), state wide sampling of BHS during NDOW's capture and relocation events and hunter kill sampling. The project aims to elucidate causes and incidence of Pneumonia in BHS. The standard sampling protocol includes collection of nasal and pharyngeal swabs to facilitate isolation of *Pasteurella spp*., fecal sampling to evaluate lungworm burden and general parasite infestation and collection of a blood sample for sero-surveillance in live sheep (both domestic and BHS). Hunter kill sampling also involves collection of a lung sample to identify presence of lung pathogens. Lung tissues from hunter kill samples were used to isolate sheep genomic DNA. Genetic analysis of BHS ram DNA will provide information about the level of heterozygosity (genetic diversity) in BHS populations, both Desert and California BHS populations in Nevada and in the eastern Sierra Nevada.

Microsatellites are the markers of choice for a wide range of molecular genetic studies, including analysis of mating systems and population structure (Bruford & Wayne, 1993; Queller et al., 1993; Schloetterer & Pemperton, 1994). The objective of this ongoing study is to determine the genetic relationship between an individual from a reportedly isolated metapopulation, the Sierra Nevada BHS which are of Desert BHS origin with other BHS populations on the eastern slope of the Sierra Nevada and throughout the State of Nevada. Currently no publications exist on the genetic relationship between the SNBS population and its closest geographical neighbor and hence most likely closest genetic neighbor.

An extensive list of publications of genetic studies is incorporated in the SNBS Draft recovery plan (p 76-92). With the exception of the publication by Boyce et al. (1997) none of the studies emphasize or use microsatellite markers. None of these published studies used any DNA derived from Nevada Desert BHS. Population comparison based on microsatellite data is not only well established methodology (Ciofi et al., 2005; So et al., 2005; Mitchell et al., 2006) but must be considered for any population comparison to avoid misinterpretation of mitochondrial DNA data. Declaring the SNBS a 'distinct metapopulation' or a subspecies based on the data presented by the recovery team is impossible. The recovery team has completely ignored the concept of cytonuclear genomic dissociation (Roca et al, 2005). Mitochondrial DNA is inherited maternally, but genetic changes in the nuclear genome of a population is driven predominantly paternally (Amos & Harwood, 1998; Graves 2004, Wade & Shuster, 2004). Preliminary data presented in the attached poster (Rink, 2005, Appendix), and in this report clearly show that the nuclear genome of the SNBS ram which was tested is indistinguishable from Desert Bighorns in Nevada. This finding resembles very much the findings as dicribed by Roca et al. (2005), Thalmann et al. (2004) and Pakendorf and Stoneking (2005) who very clearly demonstrates how misleading and detrimental to any conservation effort genetic classification based solely on mitochondrial DNA is. Additionally, the subspecies concept currently pursued by the recovery team is out dated (Fischer et al., 2006).

Material and Methods: Genotyping was performed by the Veterinary Genetics Laboratory at UC Davis using a standard protocol. The microsatellite panel comprises 21 autosomal markers (BM203, BMC1009, CELJP23, IRBP, OarCP026, TGLA94, BM4107, BM6506, CELJP15, MAF209, OarFCB128, OarFCB304, MAF35, MAF36, MAF48, MAF65, ETH152 and the MHCII linked DRB3) and one X-linked marker (CELB9), to facilitate direct comparison to the currently available data set. Data analysis at a minimum will include calculations of a Standard Genetic Distance Matrix (Dps) using Microsatellite Analyzer (MSA) (Dieringer et al., 2002) under the assumption that alleles are unlinked, in Hardy-Weinberg equilibrium and mutate at a constant rate of 1.1 + 0.5 x 10-4(Crawford & Cuthbertson, 1996). Phylogenetic analysis will be performed using MEGA V3.0 (Kumar et al., 2004).

The vast majority of microsatellite markers which are currently used for population studies in wild animals were derived from domestic ruminants (Kemp et al., 1996) markers used in nonhuman primates are usually derived from humans (Ryder 2005., Lau et al., 2004). Transfer of these markers can lead to loss of information, e.g markers developed in cattle distributing a high level of polymorphism often fail to do so when applied in sheep in goat DNA genotyping and vice versa. This phenomenon is called ascertainment bias (Crawford & Cuthbertson, 1996). All microsatellite markers currently used for BHS genotyping were developed in domestic sheep and cattle. Since microsatellite markers are anonymous markers and in most genomic regions are not subject to selection they are usually taken as a representative for a larger gene segment than the microsatellite locus itself (Weber 1990). All genotyping approaches using anonymous markers are based on this principle. The larger the number of highly polymorphic markers used, the better the representation of the entire genome. The 22 markers used in this preliminary study and suggested for use in this proposed study represent the marker set used at the UC Davis Veterinary Genetics Laboratory. These markers show minimal if any ascertainment bias and where chosen based on those grounds (Dr. Cecilia Penedo, personal communication). Nine of these markers are currently used by the SNBS recovery team for nuclear genotyping (J. Weyhausen, personal communication).

Results and Discussion: From the currently existing data set of 101 genotypes a Standard Genetic Distance Matrix (Dps) was generated using individual pair wise proportion of shared alleles (POSA). All pair wise distances are based on the same number of loci in each individual. The overall average of shared alleles is 0.576.

Heterozygosity values, allele numbers and size ranges have been determined in domestic sheep for 13 of the 22 markers used in this study and differ considerably between domestic sheep and BHS for individual markers. However, the average number of alleles per marker in this population of 101 BHS is similar (7 in BHS, 8 in domestic sheep) to the average number of alleles determined in the MARC-USDA mapping flock on ca. 1000 animals. This indicates that genome wide allele loss/heterozygosity loss through the population bottleneck was limited and no ascertainment bias exists. Sheep microsatellites have a low mutation rate of ca. $1.1 \pm 0.5 \times 10^{-4}$ (Crawford and Cuthbertson 1996); it is there for unlikely that any of the alleles observed in any of the individuals in this study is due to a spontaneous mutation.

An individual in a genetically distinct population segment should be identifiable by either a specific allele, a specific allele combination at a locus or a distinct genotype. The genotype of one Sierra Nevada BHS ram was compared to the genotype of ca. 70 Desert BHS rams and ca. 30 California BHS rams. Unweighted pair-group method using arithmetic averages (UPGMA) was used to construct a phylogenetic tree of 101 BHS rams. This preliminary analysis shows that

the Sierra Nevada BHS ram falls into a cluster with 15 other Desert BHS rams, several of which were harvested in central Nevada Hunt Units.

Sample submissions from Nevada BHS Hunters are always accompanied by the tag number of the harvested animal. With very few exceptions the tag number is restricted to one specific Nevada Hunt Unit. NDOW verifies location of harvest with each hunter upon trophy registration. Many hunters record the GPS location of the kill or give a description of the terrain within the hunt unit which is recorded by NDOW personnel. All sample collection bags are then forwarded from NDOW offices to the ADL. According to my records there have not been any out of state takes entered into this study. No BHS of California origin have ever been used to supplement NV populations (Mike Cox, personal communication). Considering the relatively large number (15) of rams which is closely related to the SNBS ram it is highly unlikely that all of these rams were SNBS rams which migrated from California to Nevada.

All Desert BHS in Mineral and Esmeralda Counties, originate within the State of Nevada. Herds in Esmeralda County have been supplemented primarily with Clark County animals from various locations within Clark County. Herds in Mineral County have been supplemented on more than 20 occasions with animals captured in Esmeralda County and Clark County, beginning in 1968. The analysis of 100 BHS genotypes clearly reflects these movements. This also explains the very close relationship of the SNBS ram with a Desert BHS taken in Lincoln Co. The fact that members of previously distinct populations have been moved around the state and caused multiple potential 'founder effects' (Galbreath & Cook 2004, DeYoung et al., 2003) is discernable by microsatellite analysis (Rink, unpublished date, 2005 poster). The close genetic relationship of the SNBS ram with 15 other Desert BHS ram is an indication that this population has had genetic influx after 1968. Since genetic ties to the Nevada relocations have already been established it is both appropriate and important to establish if this one ram of SNBS phenotype was a genetic outlier or represents a 'typical' SNBS microsatellite genotype (Table 1). The genotyping result is also an indication that the repopulation history of Nevada BHS habitat is significant, because it has clearly influenced the genetics of the SNBS.

The Sierra Nevada BHS genotype consists of mostly frequent alleles and common allele combinations (Table 1, Appendix). This preliminary comparison of one individual against 100 Nevada BHS does not indicate genetic uniqueness either through selection after the population bottleneck or isolation and novel mutation.

A comparison of a larger number of individuals will show the level of genetic relatedness of Sierra Nevada BHS and Nevada Desert BHS. Genetic comparison using a large number (22) of microsatellite markers will give significant insight into the current genetic status of the Sierra Nevada Bighorn Sheep metapopulation and will give important information as to the level of genetic proximity to geographically adjacent Nevada Desert BHS populations. This study based on the analysis of the nuclear genotype can reduce the threat of extinction by identifying closely related individuals and such potential candidates for capture and relocation or population substitution if this need arises in the future.

The data set of 100 genotypes was offered to the Recovery team in exchange for a dataset of similar size generated from SNBS DNA in February of 2005. A response from the recovery team has not been received to date.

This preliminary data shows that the nuclear genotype of the Sierra Nevada Bighorn Sheep Ram is indistinguishable from Desert Bighorn Sheep in Nevada. If the antropogenic isolation which according to the recovery plan started in 1850 ever existed is questionable. To date there is

evidence of admixing with neighboring populations in Nevada. This situation does not qualify as a metapopulation or as a subspecies. Additional genetic comparisons of the SNBS population with Nevada Desert Bighorn Sheep populations are clearly indicated.

1. **Disease transmission from Domestic Sheep to the Sierra Nevada Bighorn Sheep Population:**

Currently the main concern amongst the majority of Wildlife Biologists tasked with the management of Bighorn Sheep (BHS) populations throughout the western US is that of disease transmission from domestic livestock, primarily sheep, to BHS. The perception is that any contact between domestic sheep (DS) and BHS will invariably lead to disease and death in the BHS. For more than a century this legend was perpetuated until in the 1990s a scientific approach was attempted to rule DS in or out as a cause for BHS disease events and die-offs.

Not a single report has been published where disease transmission from DS to BHS was proven to be the cause for morbidity and mortality in BHS in their natural habitat.

The Sierra Nevada Bighorn Sheep Recovery Plan (SNBSRP) on the other hand is still based to a large extend on the perception that the single biggest risk to the SNBS metapopulation is a potential direct contact with DS or even use of BHS habitat by DS. The same perception is the basis for the Risk Analysis of Disease Transmission between Domestic Sheep and Bighorn Sheep on the Payette National Forest.
According to the Sierra Nevada Bighorn Sheep (SNBS) recovery plans (2003, 2006) a minimum of 54.5% of BHS deaths are due to predation. In the same chapter (2003) the authors state “That predators take some BHS does not imply that these losses will limit BHS populations”. This argument is hard to comprehend. Furthermore the plan states that within the last 25 years (in the presence of DS grazing in the Sierras) there was no BHS die off, nor was there a die-off before that time. It is therefore quite surprising that DS are considered to pose the biggest disease risk to BHS.

Both the Draft (2003) and the Final SNBSRP (2006) are full of statements which warrant further explanation, for example (2003):
P14: Lungworm burdens are too low to be a risk – what data is this based on?
Lungworm burdens are a severe problem in BHS in Nevada, and had a significant part in the 2004 Santa Rosa die-off.
P15: Human disturbance has no detrimental effect/Conclusions of human effects must be limited to situations studied.
Why not apply that to disease studies, too?
P19: Specific causes of most population losses in the Sierra Nevada (historically) are unknown.
If the members of the recovery team know that, on what basis were DS identified as the single most significant cause?
After P20: Herds are stable at 40, not expanding, and no domestic sheep contact?
Were these animal placed in the wrong habitat?
P25: Death losses due to disease or predation- no proof, not one single report of unambiguously traced pathogens or even verification of contact, no verification if a single pathogen, multiple disease outbreaks and no diagnostic workup was performed.

The tenor of both versions of the SNBSRP even though somewhat modified in 2006, stays essentially the same: Contact with domestic sheep will invariably lead to a disease, most likely pneumonia outbreak which will result in catastrophic BHS losses.

In Appendix B of the 2006 SNBSRP (p 88ff) the authors state that the all-age losses of BHS in the late 1800s and early 1900s coincided with the introduction of DS for grazing. Psoroptic scabies and respiratory disease are mentioned as the presumed cause of the die-offs. The authors fail to mention that BHS have their own species of Psoroptic mites which they harbor until today, whereas in DS Psoroptic mange has been eradicated. The authors then go on to cite several *Pasteurella* pneumonia transmission studies using 'captive well adapted BHS'. Bighorn Sheep do not adapt well to captivity, which is the reason they do not breed well in captivity and their stress levels should be considered to be elevated throughout captivity (Jack Ryan, USDA-WS, personal communication). One of the most frequently cited studies on disease transmission (Foreyt et al., 1994) used 5.3 x 10(8) to 8.6 x 10(11) colony forming units to inoculate BHS. Seven of eight inoculated bighorn sheep died from acute pneumonia within 48 hr of inoculation. The infectious dose for the majority of bacterial pathogens lies somewhere in the order of 1 x 10(1) to 10(4).

In BHS/DS disease transmission studies in Nevada between 2002 and 2004 several hundred Pasteurella isolates were cultured from BHS and DS. To date more than 200 strains of *Pasteurella multocida* and *trehalosi*, as well as *Mannheimia hemolytica* have been genotyped using Amplified Fragment Length Polymorphism. Genetic diversity is significant in both BHS and DS derived isolates. None of the isolates were shared between BHS and DS (Rink et al, unpublished).

Pasteurella pneumonia in domestic livestock is called 'Shipping fever'. The upper respiratory tract of most domestic and wild ungulates is colonized by Pasteurella spp (Ward et al., 1997), under stressful conditions, such as shipping; the pathogen can overwhelm the host's immune system. Drs. ACS Ward and GC Weiser, Caine Veterinary Teaching and Research Center, The Caine Pasteurella Research Laboratory, Caldwell, Idaho have published widely on phylogenetic diversity, pathogenicity, transmission and identification of *Pasteurellacea*. In the 2003 version of the SNBSRP their work had not been quoted. The majority of their work has not found consideration in the 2006 Version either, with unfortunate results.

Almost 100% of Sierra Nevada Bighorn Sheep are culture positive for at least one *Pasteurella* species (Dr. Ben Gonzales, personal communication). In light of this fact it seems appropriate to acknowledge that SNBS populations already harbor all factors which could potentially lead to an enzootic pneumonia.

Eliminating domestic sheep grazing will probably have zero impact on Bighorn Sheep populations in the Western United States.

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Appendix

Table 1: Summary of allele distributions of 22 microsatellite markers with special reference to a Sierra Nevada Bighorn Sheep genotype.

Marker	# Alleles observed	# Alleles possible	Size Range	SNBS Allele A /Frequency	SNBS Allele B /Frequency	Rarest Allele observed/Frequency
BM203	16	17	213-247	217/0.054455	225/0.108911	239/0.00495
BMC1009	5	5	274-284	274/0.173267	276/0.034653	284/0.029703
CELJP23	9	9	240-256	240/0.346535	244/0.089109	252/0.009901
OarFCB193	7	7	104-118	114/0.034653		118/0.00495
IRBP	5	16	258-290	290/0.465347		284/0.009901
OarCP026	11	14	133-161	133/0.247525		137/0.00495
TGLA94	2	2	130-132	130/0.980198		132/0.019802
BM4107	2	2	147-149	147/0.782178		149/0.217822
BM6506	10	10	199-219	199/0.351485		219/0.00495
CELB9 (X-linked)	10	10	231-251	235/0.336634		231/0.009901
CELJP15	5	5	160-170	160/0.569307		164/0.009901
MAF209	8	8	109-123	121/0.539604		111/0.00495
OarFCB128	2	2	115-117	117/0.841584		115/0.158416
OarFCB304	6	6	134-144	136/0.386139		144/0.00495
OarFCB11	4	5	121-129	125/0.173267	129/0.321782	121/0.00495
OarFCB266	7	8	90-104	102/0.420792		92/0.00495
MAF36	8	8	93-109	101/0.188119	109/0.009901	95/0.00495
MAF33	5	5	123-	125/0.331683	127/0.20297	133/0.00495

			133			
MAF48	8	8	124-138	124/0.544554	128/0.054455	134/0.00495
MAF65	7	7	115-137	117/0.331683	131/0.094059	125/0.00495
DRB3	7	37	155-229	189/0.034653		177/0.024752
ETH152	9	9	203-221	211/0.351485		219/0.024752