Genetic Distinctiveness of Endangered Dwarf Blue Sheep (*Pseudois nayaur schaeferi*): Evidence From Mitochondrial Control Region and Y-Linked ZFY Intron Sequences

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To elucidate the controversial systematic relationship of blue sheep (*Pseudois nayaur*) and endangered dwarf blue sheep, we sequenced part of the mtDNA control region and Y-linked ZFY intron, and carried out phylogenetic analyses. Mitochondrial results revealed that the dwarf blue sheep is a strongly supported monophyletic group, with an average of 12.21% sequence divergence from the blue sheep. This is the first genetic evidence for the distinctness of the dwarf blue sheep. ZFY intron results showed an average of 0.51% sequence divergence, and one shared haplotype between the dwarf blue sheep and blue sheep. By analyzing an expanded data set that incorporated ZFY intron sequences of two additional *Ovis* (sheep) species—*O. nivicola* and *O. ammon*—we demonstrated that ZFY intron provides good resolution at the species and genus levels. The ZFY intron sequence divergence between dwarf blue sheep and blue sheep was comparable to that within the two *Ovis* species. Moreover, we found intraspecific sequence variation in ZFY intron for all three species examined. We propose that dwarf blue sheep be designated as a subspecies of blue sheep, *P. n. schaeferi.*

Blue sheep (*Pseudois nayaur*) is a geographically widespread species occupying the entire Tibetan Plateau (Hass 1990). Early in this century, Schafer (1937) discovered an endemic morph of blue sheep designated "dwarf blue sheep" in the upper Yangtze Gorge at the southeastern Tibetan Plateau. The primary morphological difference between blue sheep and dwarf blue sheep is body size, with dwarf blue sheep weighing approximate 50% less than blue sheep (Wang and Hoffmann 1987). Along the precipitous slopes of the upper Yangtze Gorge, dwarf blue sheep and blue sheep occupy disjunct habitats separated by a belt of subtropical forest. Dwarf blue sheep inhabit the arid and rocky slopes below the forest belt (elevation 2700–3200 m), whereas blue sheep occupy open steppe with abundant grass above the tree line (elevation ≥4500 m).

In sharp contrast with the abundant blue sheep (more than 25,000 individuals in the Himalayas alone; Nowak 1999), the single dwarf blue sheep population in the world has declined to a total of 70–200 individuals and a density of 0.5–1.0 individual/km^2 (Wang et al. 2000; Wu et al. 1990). The International Union for Conservation of Nature (IUCN) currently lists dwarf blue sheep as endangered (Nowak 1999). However, a controversy regarding the genetic distinctness of dwarf blue sheep from the common blue sheep has led to a lack of effective legislation for protecting this remaining population (Wang et al. 2000). The call for a national nature reserve for dwarf blue sheep in the Batang region (Wu et al. 1990) is still being debated.

The taxonomic position of dwarf blue sheep has been controversial since its discovery. Three different designations have been proposed. First, Allen (1939) did not recognize dwarf blue sheep as an independent subspecies because he considered the small size of dwarf blue sheep to be caused by a lack of pasturage in its habitat (the malnutrition hypothesis). Second, Englemann (1938) and Haltenorth (1963) considered dwarf blue sheep to be a subspecies of blue sheep, *P. n. schaeferi.* Third, Groves raised the rank to a full species, *P. schaeferi,* based on "the distinctiveness and isolation of Dwarf Blue Sheep, together with its geographic approach to the Greater form" (1978:182). He also argued that the environmental effect on body size alone is unlikely to account for the 50% size difference between the two forms.

To help clarify the confusion in taxonomy and provide crucial information for conservation management of the endangered dwarf blue sheep, we examined the

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phylogenetic relationship between dwarf blue sheep and blue sheep based on the mtDNA control region and nuclear Y-linked ZFY intron sequences. A strong monophyly of dwarf blue sheep in at least one of the gene phylogenies would indicate the genetic distinctiveness of dwarf blue sheep and thereby refute Allen's malnutrition hypothesis.

Although the mtDNA control region has proven useful in intraspecific phylogenetic studies (Avise 2000), both mitochondrial introgression and sex-biased dispersal can confound the results. Therefore we examined the final intron of the ZFY gene located in the nonpseudoautosomal portion of the Y chromosome in eutherian mammals (Johnston et al. 1998). The unique genetic features of the ZFY intron can complement mtDNA by providing information of paternal lineages (Cavalli-Sforza 1998). Studies (Cathey et al. 1998; Slattery and O'Brien 1998) have shown that the ZFY intron is phylogenetically informative at the species level and above, and that its substitution rate is lower than those of noncoding 12S and 16S mitochondrial rDNA in cats. The lower substitution rate of the ZFY intron may help resolve the subspecies/species controversy of dwarf blue sheep.

Materials and Methods

Sample Collection

Due to the isolation and small population size of dwarf blue sheep, it is very difficult to obtain samples. During 1996–1998, skin and liver samples of four dwarf blue sheep individuals were collected from the Yangtze Gorge in Batang County (approximately 99°E, 29°36′N) at the southeastern edge of the Tibetan Plateau. Skin and liver samples from five blue sheep individuals were collected from Dulan County (approximately 98°10′E, 35°50′N) and Subei County (approximately 95°50′E, 39°N), which are located in the northeastern Tibetan Plateau. The parapatric blue sheep population in Batang was inaccessible due to its high altitude. Serving as outgroups, skin samples of two species in genus *Ovis,* that is, snow sheep (*O. nivicola*) from Russia and argali sheep (*O. ammon*) from China, were also collected.

DNA Extraction

Genomic DNA was extracted from each sample using a standard proteinase K digestion and phenol/chloroform extraction method (Sambrook et al. 1989). A reagent blank control was performed along with each batch of extractions and was subjected to subsequent polymerase chain reaction (PCR; see below) to detect possible contamination.

Mitochondrial Control Region Amplification and Sequencing

The mtDNA control region was amplified from genomic DNA through PCR by using the modified universal primer pair for ungulates from Murray et al. (1995). PCR conditions were as follows: 20 μl total reaction volume containing 20–50 μg genomic DNA, 1× PCR buffer, 0.2 mM dNTPs, 0.2 mM of each primer, 3.0 mM $MgCl_2$, and 0.5 U *Taq* polymerase. The PCR program was 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The last cycle was followed by a 5-min extension at 72°C. The PCR product was purified from 2% agarose gel using the Qiaex kit (Qiagen). The portion of the control region proximal to $tRNA^{PRO}$ was sequenced via cycle sequencing using the Big-dye terminator kit (Perkin Elmer). Electrophoresis of labeled products was carried out on an ABI 377 automated sequencer at Guelph University. Randomly chosen samples were sequenced twice to ensure the fidelity of sequencing.

Nuclear Y-Linked ZFY Intron Amplification and Sequencing

ZFY intron was amplified from genomic DNA through PCR using primer pair LGL331/335 from Cathey et al. (1998). The conditions and program for the PCR were the same as those described above for the control region. PCR products consisted of the ZFY intron and the homologous ZFX intron on the X chromosome. These were separated by 2% agarose electrophoresis because the ZFY intron (~850 bp) is smaller in size than the ZFX intron (~950 bp). The ZFY intron band was cut from the gel and then purified using the Qiaex kit (Qiagen). We sequenced the ZFY intron following the protocol described above.

Sequences Alignment and Phylogenetic Analyses

We aligned unambiguous sequences using the Clustal X program (Thompson et al. 1997) under the default conditions, and verified visually using the SeqApp 1.9a program (Gilbert 1992). To assess the phylogenetic signal contained in the data, we used PAUP 4.0b2a (Swofford 1998) to evaluate the skewness of the tree length distribution (Hillis and Huelsenbeck 1992) and perform the permutation tail probability (PTP) test. We used PUZZLE 3.1 (Strimmer and Haeseler 1997) to obtain the likelihood mapping values. Multiple phylogenetic analyses including maximum parsimony (MP), maximum likelihood (ML), minimum evolution (ME), bootstrapping, and the Kishino–Hasegawa test (Kishino and Hasegawa 1989) were conducted with PAUP 4.0b2a.

Results

mtDNA Control Region Phylogeny Inferences

We aligned 567 bp mtDNA control region sequences obtained from five blue sheep and four dwarf blue sheep. Eight different haplotypes were found: four for blue sheep and four for dwarf blue sheep. Base composition was not significantly different across taxa ($\chi^2 = 1.764$, df = 21, $P = 1.00$). Mean base frequencies were A = 0.387, C = 0.288, G = 0.097, and T = 0.228. Among 567 sites, 97 were variable (Figure 1A) and had only two states. The transition:transversion (ti:tv) ratio estimated by the maximum likelihood method was 6:1. The low number of base states at variable sites and the high ti:tv ratio indicate that transitional saturation is unlikely. Three different a priori tests were conducted to assess the nonrandomness of the data (Table 1), all of which indicated that the dataset contained a strong phylogenetic signal.

The single best tree obtained in the MP analysis (Figure 2) showed reciprocal monophyly of dwarf blue sheep and blue sheep, well supported by high bootstrap values and decay indexes. This analysis also showed that the in-group (i.e., dwarf blue sheep plus blue sheep) formed a strongly supported (bootstrap value of 100% and a decay index of 128) clade separated from the outgroups. Similar results were obtained using a ML approach. For ML analysis we first used a hierarchical model-fitting procedure with likelihood ratio tests (LRTs) (Cunningham et al. 1998; Huelsenbeck and Rannala 1997) to test the model of DNA substitution. In this procedure, only those parameters that significantly improved the likelihood of the tree were incorporated into the final sequence evolution model. For our dataset, the model-fitting results (Table 2A) indicated that the best-fit model was HKY85, which incorporated unequal base composition and unequal transition/transversion rates. Under the HKY85 model settings, a heuristic search yielded a tree with the same topology as the MP tree. Finally, we conducted ME analysis using ML distance (likelihood settings were the best-fit mod-

a.

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                  1111111111112222222223333333333333333444444444444444444444555555555555 5555555555555566666
                133577115566777999001344556012333355566889911112222234555777788990000011111222 2234455557789900111
Haplotype       847972837185846957969210512206303460582845582367012484446735781849013791246713 4640723898942746056
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Dw.bs1          ACG-CACCCCTTGTTCTTTGTGAGCGTATAGC-TGATGCCTCCTACCCATAGGATGACACGGCTAC-TGAAAACC-CT ATGGAGTTTC-ACAATTTC
Dw.bs2          -.....................................................................T...A.....-...-GA
Dw.bs3          ...G.....................................................C..............T..TA.....-...-.A
Dw.bs4          .T......GTA...A.CCCAC.G......GAT...GC...C...--........CA.T..AC.......GGGGT.CTC .CT..TA..T..-...-.A
BS1             .TA.TGTTCTTCACAT.....A.ATACGCGA.C-AG.ATTCTT..T.---.AATCAG.GAAC-CGTC-AG....T-.. GCTAGTACC..G--GC-.A
BS2             ATA-CGTTCTTCACAT.....A.ATACGCGA.C-AG.ATTCTT..T.---.AATCAG.GAAC.CGTC-AG....T-.. .CTAGTACC..G-..C-.A
BS3             ATA-CGTTCTTCACAT.....A.ATACGTGA.C-AG.ATTCTTC.T.---.AATCAG.GAAC.CATC-AG....T-.. GCTAGTACC.CG-.GC-.A
BS4             ATA-CGTTCTTCACAT.....A.ATACGTGA.C-AG.ATTCTT..T.---.AATCAG.GAAC.CATC-AG....T-.. G.TAGTACC..G-.G.-.A
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b.

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                    1111111122222222333333333334445555555555555555555555666
                  257016666795677888911225788899247245556677777777788888134
Haplotypes        909211234715069056734132804912743442378901234567801246304
-------------------------------------------------------------------------------
Snow1             ATCTA----GGTAATTCGGTT-G-TTGT-ATTCGTT-AATATTTTAATTG-AATAAT
Snow2             ..G.....................................................G......
Snow3             .....................................................T-.....
Snow4             .....................................................T......
Arg1              .CG.....A.....C.....G.....T......
Arg2              .CG.....AC..C..AC.....G.....T......
Arg3              .CG.T...CA.CCC..A.....G.....T......
BS1               TCG-.AAAC.A....AT..G..T.....G..GCC-A.----------AT.C....
BS4               TCG-.AAAC.A....AT..G..T.....G..GCC-A.----------AT..C-.-
Dw.bs1            TCG-.AAAC.A....AT.AGCATTCCCCTGT.GCC-A.----------AT......
Dw.bs2            TCG-.AAAC.A....AT..G..T.....G..GCC-A.----------AT......
BS5               TCG-.AAAC.A....AT..G..T.....G..GCC-A.----------AT....-.
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Figure 1. Sequence variation in **(A)** mtDNA control region sequence alignment of dwarf blue sheep ("Dw.bs") and blue sheep ("BS") haplotypes, and **(B)** expanded ZFY intron sequence alignment of snow sheep ("Snow"), argali sheep ("Arg"), blue sheep ("BS"), and dwarf blue sheep ("Dw.bs") haplotypes. Except for a single indel (position 630), haplotypes Dw.bs2 and BS5 are identical. Boxes in (B) highlight areas of intraspecific variation. Dots indicate a character state identical to the top sequence and dashes indicate indels.

el found earlier) and heuristic search algorithm. The resulting tree has the same, well-resolved topology as the MP and ML trees (Figure 2). The average pair-wise maximum likelihood distance (HKY85 model) between dwarf blue sheep and blue sheep (12.21%) was significantly (t tests, $P < .01$) greater than that within blue sheep (0.79%) and that within dwarf blue sheep (3.47%).

Thus a single topology was generated by all analyses using different optimality criteria, strongly suggesting that the obtained tree represents the true mtDNA phylogeny. The monophyly of dwarf blue sheep was consistently supported by high bootstrap values whenever bootstrapping analysis was performed. Therefore mtDNA control region sequence analyses indicated that dwarf blue sheep is a genetically distinct group separated from blue sheep by an average sequence divergence of 12.21%.

The malnutrition hypothesis predicts that dwarf blue sheep are not a genetically distinct monophyletic group. To test this hypothesis, we conducted a priori Kishino–Hasegawa tests with in-group sequences for both MP and ML analyses. The dwarf blue sheep haplotypes were constrained to be monophyletic. In MP analysis the shortest trees that were not consistent with the constraint (two trees of length 156, CI = 0.699, RI = 0.722, RC = 0.504) were significantly longer ($P < .0001$) than the shortest trees found under the constraint (three trees of length 114, CI = 0.956, RI = 0.970, RC = 0.928). In ML analysis, the likelihood of the best tree found under the constraint ($-\ln L = 1129.0719$) was significantly higher ($P < .0001$) than the likelihood of the best tree in which dwarf blue sheep was nonmonophyletic ($-\ln L = 1210.7563$). Therefore the malnutrition hypothesis was rejected by our mtDNA control region sequence data.

ZFY Intron Sequences Analyses

We aligned 639 bp of ZFY intron sequence from three blue sheep and two dwarf blue sheep. Disregarding indels, four different haplotypes were found, with one haplotype being shared between dwarf blue sheep and blue sheep (Figure 1B). Among the 639 sites, only 8 (1.25%) were variable (6 transitions and 2 transversions), and most of these (6 of 8) occurred in one dwarf blue sheep haplotype. We found no fixed variations to distinguish dwarf blue sheep from blue sheep (Figure 1B). Due to this low level of variation, little phylogenetic signal existed in the ZFY intron sequences of blue sheep and dwarf blue sheep. Thus, further sequencing of additional samples was precluded. The observed lack of differentiation warranted further investigation regarding the window of phylogenetic resolution of ZFY intron sequence.

The ZFY intron sequence has been shown to provide good phylogenetic resolution at the species level and above, but to exhibit low intraspecific variation probably due to its low evolutionary rate (Cathey et al. 1998; Slattery and O'Brien

Table 1. Results of testing for phylogenetic signal contained in data sets

Test	mtDNA control region sequence data	Expanded ZFY intron sequence data
Skewness of tree length distribution (*g1* from 10,000 random trees)	$g1 = -0.347$[a]	$g1 = -0.524$, $P < .01$
Permutation tail probability (PTP) test (*P* from 1000 replicates)	$P = .01$	$P = .01$
Likelihood mapping (frequency of quartets in treelike regions)	91.5%	100%[b]

[a] There were 8 taxa and 62 informative characters in the control region dataset. The critical *g1* value is −0.37 for 8 taxa, 50 characters, and −0.31 for 8 taxa, 100 characters. Therefore the *g1* value obtained from the test was marginal.

[b] Sequences were grouped in four clusters, that is, a = snow sheep, b = argali sheep, c = blue sheep, d = dwarf blue sheep. Without the grouping, the value was 83.4%.

1998). If this is also true for our data, we predicted that a dataset incorporating ZFY intron sequences from other closely related species should both resolve the higher-level phylogeny and show a low level of variation within each species. Dwarf blue sheep and blue sheep are the only members of the genus *Pseudois.* The phylogenetic relationships of the two genera, *Ovis* (sheep) and *Pseudois,* as well as the two congeneric species [snow sheep (*O. nivicola*) and argali sheep (*O. ammon*)] are well defined by morphology and 12S rDNA sequence analyses (Ludwig and Fischer 1998). Thus to test our prediction, we sequenced the ZFY intron of different subspecies within *O. nivicola* and *O. ammon,* and added them to the ZFY intron dataset described above for subsequent phylogenetic analyses.

The expanded ZFY intron sequence dataset included a 652 bp sequence of four snow sheep, three argali sheep, three blue sheep, and two dwarf blue sheep. Similar phylogenetic analyses as those described in mtDNA sequence analyses were carried out. Base composition was homogeneous across taxa ($\chi^2 = 1.633$, df = 33, $P = 1.00$), and mean base frequencies were A = 0.3173, C = 0.1336, G = 0.1730, and T = 0.3760. A strong phylogenetic signal was detected by all three a priori tests (Table 1). Among 652 nucleotide sites, 57 sites were variable and 34 were parsimony informative. The single best tree obtained in MP analysis (Figure 3) revealed three well-resolved monophyletic clades representing *O. nivicola, O. ammon,* and dwarf blue sheep plus blue sheep. Sequence variations were revealed within each clade, and the amount was consistently low (0.3–1.4%). The amount of variation was similar in clades *Pseudois* and *O. ammon.* Haplotypes of *O. nivicola* and *O. ammon* further formed a well-supported monophyletic clade (*Ovis*). This tree topology was in agreement with the phylogeny defined by morphology and 12S rDNA sequence analyses (Ludwig and Fischer 1998). Both the ML analysis under the best-fit model (HKY85; see Table 2B) and the ME analysis using the ML distance yielded the same tree topology as the MP tree. Therefore, as predicted, the expanded dataset fully resolved the species phylogeny and exhibited a low level of intraspecific variation. This is concordant with the reported resolution window of ZFY intron sequences. Moreover, our analysis showed that weakly differentiated dwarf blue sheep and blue sheep ZFY intron haplotypes formed a strong monophyletic clade (*Pseudois*), within which the amount of sequence variation was similar to that within the species *O. ammon.* These results support the view that dwarf blue sheep may not be a full species separate from blue sheep.

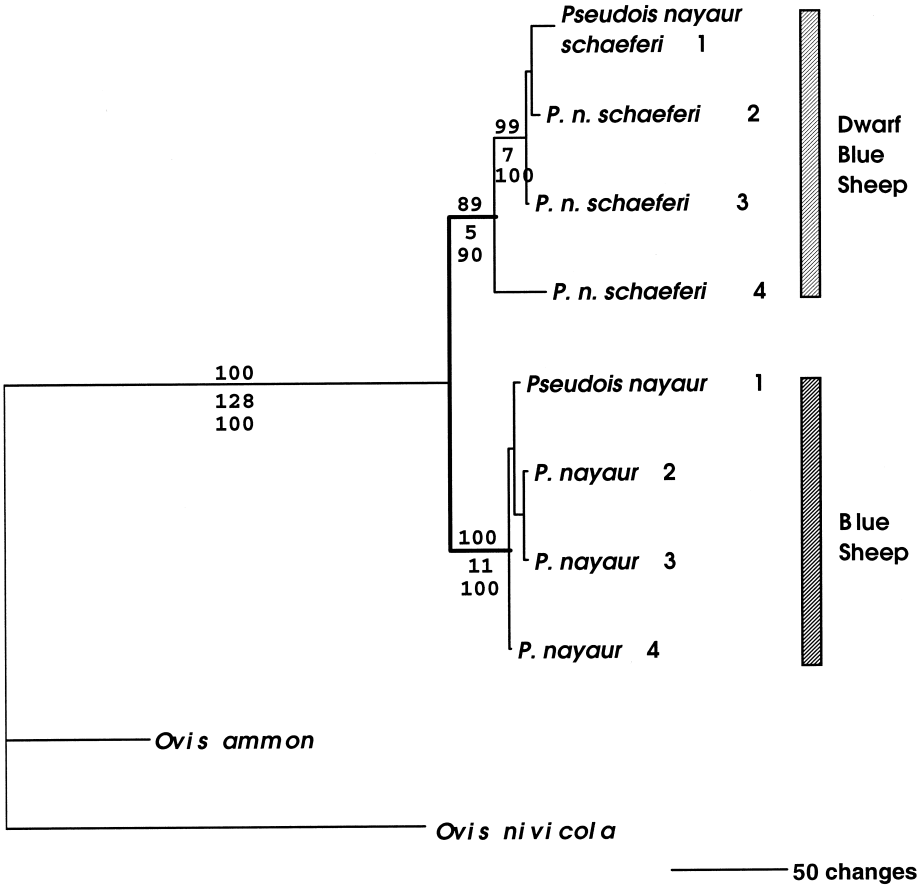

Figure 2. The single optimal MP phylogram of mitochondrial control region sequences obtained using branch and bound search algorithm (gaps were treated as "fifth state"; ti:tv ratio was 6:1; snow sheep and argali sheep were outgroups). Tree length was 554 (CI = 0.926, RI = 0.895). Identical topology was yielded in ML and ME analyses. Numbers at branches indicate statistical support for internal nodes. From top to bottom, they are MP bootstrap values above 70% (1000 replicates, branch and bound search algorithm), decay indexes, and ME bootstrap values above 70% (1000 replicates, heuristic search algorithm).

Discussion

Genetic Distinctiveness of Dwarf Blue Sheep

Other than body size, dwarf blue sheep and blue sheep are very similar to each other morphologically, which has led to confusion and debate regarding the taxonomic distinctiveness of these taxa. Our mtDNA control region phylogeny revealed strong monophyly of dwarf blue sheep. This provides the first molecular genetic evidence for the intrinsic genetic distinctiveness of dwarf blue sheep (at least in maternal lineages). Moreover, the mtDNA control region of dwarf blue sheep showed an average of 12.21% sequence divergence from blue sheep. This level of sequence

Table 2. Results of hierarchical likelihood ratio tests (LRTs) for model fitting in maximum likelihood analysis

Models	Estimated parameter	Additional df	Likelihood	*P* value
A. Mitochondrial control region sequence data				
JC			2330.92	
F81	Base composition	3	2279.28	<.001
HKY	Two substitution classes	1	2143.57	<.001
HKY + INV	Percent of invariable sites	1	2140.02	>.999
HKY + I + G	Among sites rate variation	1	2138.01	>.025
GTR/INV	Six substitution classes	4	2137.27	>.603
B. Expanded ZFY intron sequence data				
JC			1138.97	
F81	Base composition	3	1089.41	<.001
HKY	Two substitution classes	1	1086.09	.009
HKY + INV	Percent of invariable sites	1	1085.98	.620
HKY + I + G	Among sites rate variation	1	1085.98	.635
GTR/INV	Six substitution classes	4	1079.86	>.200

Model in bold is the optimal model.

Note: Details of listed models can be found in Huelsenbeck and Crandall (1997).

divergence exceeds that among many subspecies and some species of other large mammals (Douzery and Randi 1997; Garcia-Rodriguez et al. 1998; Taberlet and Bouvet 1994; Wooding and Ward 1997). Moreover, it also exceeds sequence divergence among examined subspecies of argali sheep living in a much larger geographic range including the entire Tibetan Plateau (Feng J, unpublished data). Finally, the malnutrition hypothesis was rejected by our mtDNA data. These results suggest that dwarf blue sheep is not only a distinct geographic population, but also qualifies as at least a subspecies ranking as well.

The large sequence divergence in the mtDNA control region between dwarf blue sheep and blue sheep implies a prolonged isolation of the dwarf blue sheep population. Although blue sheep occur in the same mountain range where dwarf blue sheep also occur, their habitats are separated by a belt of subtropical forest spanning 1000 m in elevation. These organisms avoid high vegetation to protect themselves from predators, and only enter the outer edges of the forest in winter looking for food (Hass 1990; Wang and Hoffmann 1987), suggesting ecological isolation and a likely lack of gene flow between these parapatric populations. Although molecular dating can be weak and vulnerable to violations of a molecular clock, it is still informative regarding the overall time frame of an evolutionary event. The local molecular clock for the blue sheep control region is unknown. Thus we followed Hiendleder et al. (1998) and used a rate of 6–12%/million years for the control region in sheep. Although only a rough estimate, this suggests a divergence time of approximately 1–2 million years ago between dwarf blue sheep and blue sheep.

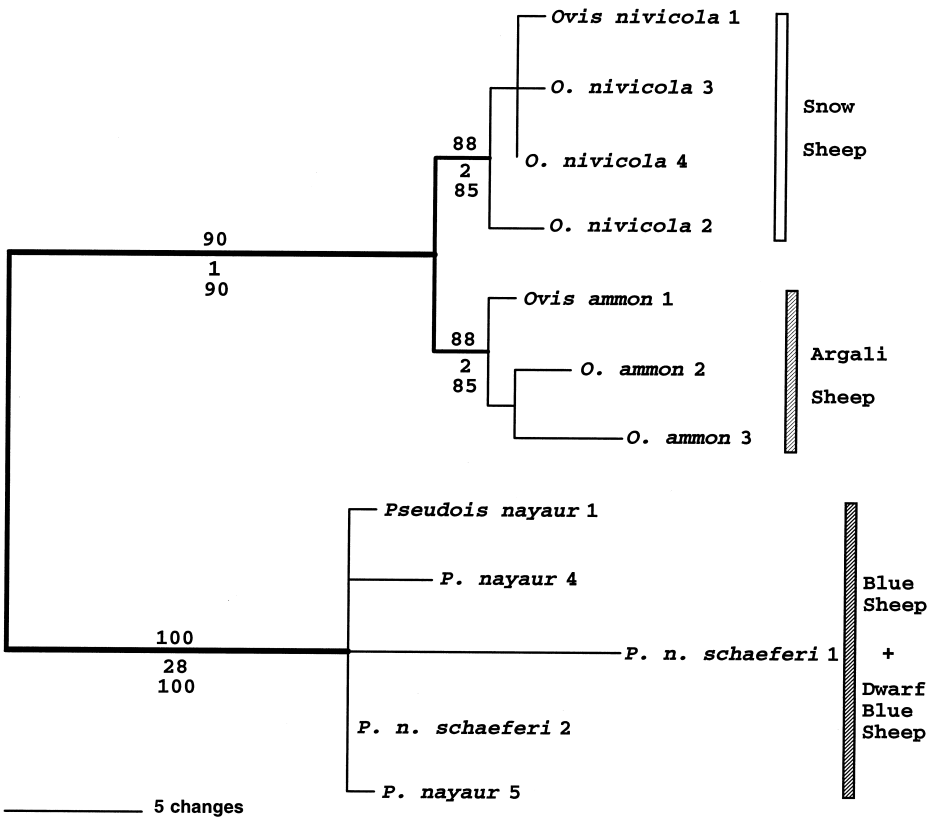

Figure 3. The single optimal MP phylogram of Y-linked ZFY intron sequences obtained using branch and bound search algorithm (gaps were treated as "fifth state"; ti:tv ratio was 1:1). Tree length was 60 (CI = 1.0, RI = 1.0, RC = 1.0). Identical topology was yielded in ML and ME analyses. Numbers at branches indicate statistical support for internal nodes. From top to bottom, they are MP bootstrap values above 70% (1000 replicates, branch and bound search algorithm), decay indexes, and ME bootstrap values above 70% (1000 replicates, heuristic search algorithm).

Subspecies or Species

Among subspecies in other large mammals, the level of sequence divergence in the control region ranges from 4.2% to 15.05% (Arctander et al. 1999; Douzery and Randi 1997; Garcia-Rodriguez et al. 1998; Matsuhashi et al. 1999; Taberlet and Bouvet 1994; Wooding and Ward 1997). In studies using the *Cyt b* gene, a comparable range of sequence divergence was found among subspecies in two bovine species (Kikkawa et al. 1997; Schreiber et al. 1999) and many other mammals (reviewed in Avise et al. 1998). We observed a 12.21% sequence divergence in the mtDNA control region between dwarf blue sheep and blue sheep, which fell in the higher end of this range and thus supported at least a subspecies ranking for dwarf blue sheep.

In contrast with the mtDNA results, the ZFY intron data showed weak differentiation between dwarf blue sheep and blue sheep, that is, one shared haplotype and no fixed substitutions. Nevertheless, the expanded dataset demonstrates that ZFY intron provides good phylogenetic resolution at the species and genus levels in *Ovis* and *Pseudois,* and intraspecific variability was consistently low in the species analyzed. This window of resolution is in agreement with that reported in other studies (Cathey et al. 1998; Slattery and O'Brien 1998). The ZFY intron haplotypes of dwarf blue sheep and blue sheep formed a monophyletic clade *Pseudois* in

the higher-level phylogeny, and the sequence divergence between the two was close to that seen within *O. ammon.* The most likely explanation for these results is that blue sheep and dwarf blue sheep may belong to one species.

The contrasting phylogenetic pattern revealed by the mtDNA control region and the nuclear ZFY intron can be explained by a difference in the evolutionary rate of the two molecular markers, and/or by male-mediated gene flow between the parapatric dwarf blue sheep and blue sheep populations. Due to the lack of direct evidence regarding the presence or absence of gene flow, the possibility of male-mediated gene flow causing homogenization of ZFY intron haplotypes cannot be excluded. However, we feel that male-mediated gene flow is less likely for two reasons. First, the habitats of dwarf blue sheep and blue sheep are physically separated by a belt of forest spanning 1000 m in elevation. It is documented that these organisms, regardless of sex, only enter the outer edges of the forest in winter seeking food (Hass 1990; Wang and Hoffmann 1987). Groves (1978) considered the forest belt to be an ecological barrier that makes gene flow across it unlikely. Second, in cats, the ZFY intron has been found to have a resolution window at the species level and above, and its evolutionary rate is lower than that of mitochondrial 12S rDNA (Slattery and O'Brien 1998). Our analysis of the expanded dataset showed a similar resolution window for ZFY intron and the sequence divergence between dwarf blue sheep and blue sheep is close to that seen within another *Ovis* species. Thus, although significant divergence between dwarf blue sheep and blue sheep was detected by the rapidly evolving control region, it is likely that insufficient time has passed for mutation accumulation and lineage sorting at the ZFY intron locus. Therefore, not excluding the possibility of male-mediated gene flow, we consider the difference in substitution rates to be a more likely explanation for the contrasting pattern revealed by mitochondrial and nuclear markers. Nevertheless, both scenarios are consistent with a subspecies ranking of dwarf blue sheep.

By noting that the small endemic dwarf blue sheep population occurs at the edge of the vast distribution range of blue sheep, our findings support the view that the dwarf blue sheep is in the process of speciation through peripheral isolation (Wang and Hoffmann 1987). This peripheral isolation hypothesis can be further tested by examining the phylogeographic pattern of blue sheep populations in Asia and the positioning of dwarf blue sheep population in the tree.

In addition to providing molecular evidence for a subspecies ranking of dwarf blue sheep, our study revealed intraspecific variation in the ZFY intron for all three species examined. This was unexpected, and in sharp contrast with other studies reporting little ZFY intron variation within humans (Jaruzelska et al. 1999; Nachman 1998), cats (Slattery and O'Brien 1995), and North American deer (Cathey et al. 1998). However, except for the human studies, the intraspecific sampling was limited in these studies (i.e., two individuals per species). Therefore the reported lack of variation in species other than humans may be an artifact of small sample size. The variation difference between humans and the species we examined cannot be attributed to a sampling effect because more than 200 individuals from all over the world and 729 bp sequences were examined in those studies. There are four possible, nonmutually exclusive explanations that require further investigation: First, *Homo sapiens* may be a younger species than the Caprini species we examined. Second, the human population may have undergone severe historical bottlenecks and rapid recent expansion that the Caprini populations have not. Third, the substitution rate of ZFY intron may be higher in wild sheep and blue sheep than in humans. Finally, the human ZFY intron may be affected by natural selection, either via genetic hitchhiking or background selection. However, studies by Nachman (1998) and Jaruzelska et al. (1999) detected insignificant or weak (if any) selection on the human ZFY intron.

In conclusion, our mtDNA control region analysis indicated the genetic distinctiveness of dwarf blue sheep, supporting at least a subspecies ranking for this critically endangered population. The ZFY intron results support the view that dwarf blue sheep may not be a full species independent from blue sheep. Therefore we consider the dwarf blue sheep to be a subspecies of blue sheep endemic to the upper Yangtze Gorge on the Tibetan Plateau.

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