Microsatellite Evolution in Congeneric Mammals: Domestic and Bighorn Sheep

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We compared genotypes at eight (AC)n microsatellite loci in domestic sheep (*Ovis aries*) and wild Rocky Mountain bighorn sheep (*O. canadensis*). The domestic sheep had greater genetic variation, higher allele-size variances, and larger allele sizes than the wild sheep. Accumulating evidence from higher taxonomic comparisons shows that these parameters are biased if microsatellite loci are selected in one taxon and used in another. Our results demonstrate similar biases between congeneric species. We compared standard measures of genetic variation, differentiation, and distance within and between species (H, D, F_{ST}) to newer measures based on allele-size variance (S_W, S_B, R_{ST}). The size-based distances better detected species-level divergence, but standard measures better distinguished allopatric populations. Empirical calibration of these measures at the subspecies level is needed to establish their useful ranges.

Introduction

DNA microsatellites are genetic markers containing 2- to 4-base pair tandem repeats that provide a vast store of polymorphic genetic markers (Tautz 1989; Weber and May 1989). Evolutionary studies using microsatellite loci require knowledge of their mode of evolution. Microsatellites have been suggested to play a role in gene regulation or in recombination (Stallings et al. 1991), but no certain functions have been identified. Mutations in repeated DNA are thought to occur by unequal crossing over or replication slippage, but these mechanisms are not well understood, and they may differ among the several classes of repeated DNA. Microsatellite mutation rates are approximately 10^{-3}–10^{-4} (Dietrich et al. 1992; Weissenbach et al. 1992). Repeat length is weakly correlated with the number of alleles in several species (Beckman and Weber 1992; Buchanan et al. 1993; Ostrander et al. 1993), suggesting that mutation rate may be correlated with repeat size, although the structure of the repeat may also be important (Pepin et al. 1995).

Most microsatellite mutations involve changes in repeat number. Thus, the number of alleles is large but finite, and homoplasy is inevitable. Simulation studies show that allele distributions fit a stepwise mutation model better than they do an infinite alleles model (Harding et al. 1993; Shriver et al. 1993; Valdes et al. 1993). However, mutations of more than one step are reported for both microsatellites and minisatellites (Jeffreys et al. 1985; Valdes et al. 1993). The best model for evolution of these loci may be a finite alleles, stepwise model that allows jumps of multiple steps (Di Rienzo et al. 1994).

Neutral and unconstrained variation at microsatellite loci would result in random walks in which some loci would drift to very large or small repeat arrays. This apparently does not happen (Bowcock et al. 1994; Stephan and Cho 1994). The restriction of allele sizes may be due to general mechanisms governing repeat size (e.g., rates of replication slippage or unequal crossing over) or to unknown direct physical constraints on allele size. Any of these factors would especially affect population genetic and phylogenetic studies if they differed among taxa. Polymerase chain reaction (PCR) priming sites are generally conserved among related taxa (Moore et al. 1991), making possible evolutionary comparisons of repeat sizes.

Here we compare allele sizes at eight (AC)n microsatellite loci in domestic sheep (*Ovis aries*) and wild Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*). A previous study using the same loci (Buchanan et al. 1994) showed that allele frequencies are useful measures of genetic divergence between domestic sheep breeds and that mutation rates calibrated by breed divergence times were in the range cited above. By ex-

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Table 1
Microsatellite Loci Analyzed in Domestic Sheep and Wild Sheep

			Ovis aries (n = 150)			*Ovis canadensis* (n = 100)		
Locus	Chromosome	GenBank Number	Alleles	Range	Mean	Alleles	Range	Mean
MAF33	9	M77200	7	121–139	133.3	5	121–129	124.9
MAF48	*U*	M62645	6	122–138	132.1	7	122–134	127.0
MAF65	15	M67437	8	119–135	126.6	10	99–135	121.9
MAF209	17	M80358	10	109–135	124.6	7	109–123	116.7
OarFCB11	2	L01531	10	121–143	128.7	3	125–129	126.4
OarFCB128	2	L01532	9	99–127	116.4	5	113–121	118.7
OarFCB266	25	L01534	8	98–114	103.2	7	84–98	89.4
OarFCB304	19	L01535	14	142–192	167.6	5	136–144	138.1
Mean			9.0		129.1	6.1		120.4

NOTE.—Number of alleles and allele sizes are summarized for three breeds of domestic sheep (*Ovis aries*) and five wild populations of Rocky Mountain bighorn sheep (*O. canadensis canadensis*). Allele-size ranges and means are in DNA base pairs. Chromosome assignments are from Crawford et al. (1994; *U* = unassigned). The two markers assigned to chromosome 2 (OarFCB11 and OarFCB128) are more than 50 cM apart.

amining allele-size distributions at homologous loci in two congeneric mammal species, we test for differences in allele-size distributions, and we address the effects of these differences on measures of genetic variation and genetic distance.

Material and Methods

Microsatellite Loci

Details of microsatellite loci developed by cloning (AC)n repeats in domestic sheep have been published previously (Buchanan et al. 1993, 1994; Crawford et al. 1994). Segregation analysis of the eight loci in nine three-generation domestic sheep pedigrees showed them to be unlinked Mendelian markers. All but one of these markers (MAF48) have been placed on the sheep genetic linkage map (table 1).

Populations

The three domestic breeds (Buchanan et al. 1994) have ancient origins and have been selected by people for different uses. The Awassi samples are from an Israeli flock comprising at least five breeding lines. The Merino and Romney sheep are from New Zealand flocks originating from multiple importations from South Africa, England, and Australia. Microsatellite data for 50 sheep of each of these breeds have been previously described (Buchanan et al. 1994).

Rocky Mountain bighorn sheep were sampled from throughout the subspecies' range, from Alberta to Colorado (table 2). The Sheep River sample (N = 50) is from a large, native Alberta herd that retains the possibility of genetic exchange with neighboring populations. The Bison Range herd was begun with the introduction of 12 bighorn sheep from Banff, Alberta, to the National Bison Range, Montana in 1922. It endured a population bottleneck of less than 20 sheep for about 12 yr, and it was genetically isolated for 63 yr. The Bison Range sample (n = 20) represents about one-half of the sheep alive in 1985, before a program of artificial im-

Table 2
Summary of Genetic Variation at Eight Microsatellite Loci in Domestic Sheep and Wild Bighorn Sheep

Population Species	Breed/Herd	Origin	n	A	H_o	H_e	S_W
1 *Ovis aries*	Awassi	Israel	50	6.0	0.657	0.641	47.0
2 *Ovis aries*	Merino	Spain	50	6.8	0.672	0.693	56.7
3 *Ovis aries*	Romney	Britain	50	5.6	0.658	0.678	55.9
Domestic breeds pooled			150	9.0	0.662	0.733	65.8
4 *Ovis canadensis*	Sheep River	Alberta	50	4.6	0.607	0.585	22.9
5 *Ovis canadensis*	Bison Range	Alberta	20	2.1	0.438	0.429	9.7
6 *Ovis canadensis*	Sun River	Montana	10	4.5	0.575	0.591	18.2
7 *Ovis canadensis*	Whiskey B.	Wyoming	10	4.0	0.650	0.598	21.0
8 *Ovis canadensis*	Tarryall	Colorado	10	3.4	0.525	0.549	28.8
Bighorn herds pooled			100	6.1	0.566	0.681	22.5

NOTE.—n, number of sheep sampled; A, average number alleles per locus; H_o, observed (direct count) heterozygosity; H_e, expected heterozygosity (unbiased estimate); S_W, twice the mean allele-size variance over eight loci (see Methods).

migration began. The Sheep River and Bison Range samples are adult sheep that are not known to be related. Smaller numbers of sheep ($n = 10$) were sampled from three widely distributed, large, native U.S. herds: (1) Sun River, Montana; (2) Whiskey Basin, Wyoming; and (3) Tarryall, Colorado.

DNA Isolation, PCR Reactions, and Genotyping

Genomic DNA was purified from 5 ml blood or 50–100 mg skin or muscle tissue by protease K digestion and phenol/chloroform extraction (Ausubel et al. 1989). Alternatively, 5 mg of tissue or 5 µl of blood were prepared by autoclaving for 5 min in 200 µl 5% Chelex 100 resin (Bio Rad Laboratories) and pelleting the resin in a 10-kg microcentrifuge for 10 min.

All 150 domestic sheep and 30 bighorn sheep were genotyped from purified genomic DNA as described by Buchanan et al. (1994) in the lab of A. Crawford. Fourteen of these bighorn sheep and 70 more were genotyped in the lab of S. Forbes with minor modifications: PCR reactions contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 2 µg/ml BSA, 0.2 mM each dNTP, 4 pmol of each unlabeled primer, 0.2 pmol of labeled primer, 0.5 U Taq DNA polymerase (Perkin-Elmer Cetus), and either 50–100 ng purified genomic DNA or 1 µl Chelex tissue preparation in a total volume of 10 µl. Annealing temperatures for bighorn sheep were the same as for domestic sheep (Buchanan et al. 1994). Allele sizes were standardized using the original clone for each locus or the M13 phage control DNA in the Sequenase™ DNA sequencing kit (United States Biochemical). Fifty-one genotypes distributed over all loci were analyzed in both laboratories to confirm consistent allele scoring. Seventy-seven genotypes compared in Chelex preparations and genomic DNA preparations from the same animal confirmed identical allele scoring from both template types.

Statistical Analysis

Population genetic data were calculated using BIOSYS-1 (Swofford and Selander 1989). We tested allele frequency differences using the Monte Carlo simulation in the REAP program package (McElroy et al. 1991). Genetic distance dendrograms were made using the Gendist and Fitch programs from PHYLIP 3.5 (Felsenstein 1993).

Variance-based genetic distances were calculated according to Slatkin (1995), using

$$\bar{S} = \frac{2n-1}{2nd_s-1} S_W + \frac{2n(d_s-1)}{2nd_s-1} S_B \qquad (1)$$

$$R_{ST} = \frac{\bar{S} - S_W}{\bar{S}} \qquad (2)$$

where $\bar{S}$ is twice the variance in allele size in the populations together, S_W is twice the average of the variances of allele size within each population, d_s is the number of populations (2), and n is the sample size. S_B is the mean square difference between alleles drawn one from each of two populations, and R_{ST} is the proportion of the total variance due to differences between populations. This method requires equal sample sizes; original data sets were randomly sampled once without replacement to create the largest possible samples of equal size for each pairwise comparison. S_W and S_B are equivalent to D_0 and D_1 of Goldstein et al. (1995).

Results

Genetic Variation

We used eight (AC)n dinucleotide repeat loci that were cloned from domestic sheep and chosen for their high allelic diversity and lack of linkage in domestic sheep (table 1) (Buchanan et al. 1994). All loci were also polymorphic in bighorn sheep (table 2). We tested for deviations from expected Hardy-Weinberg genotype proportions in two ways. First, all alleles except the most common one were pooled, and genotype proportions were tested with standard two-allele χ^2 tests (Swofford and Selander 1989). Second, two-class χ^2 tests were used after pooling all homozygotes and all heterozygotes (Lessios 1992). No single population had genotypes differing from binomial expected proportions with either test when the number of tests was considered. The pooled species groups (table 2) had heterozygote deficits at all loci that were highly significant under both tests, as expected under the Wahlund effect (all $P < 0.01$).

Alleles that do not amplify due to PCR priming site mutations are frequently reported (Weissenbach et al. 1992; Callen et al. 1993; Kemp et al. 1993) and are likely a greater concern in transfers of markers between species. However, we found no evidence for null PCR alleles in bighorn sheep using domestic sheep primers. In bighorn sheep parentage studies where the mother is known and the father found by population-wide paternity exclusion, we found no evidence of null alleles and two apparent mutations in 1,212 ewe/lamb/locus tests (unpublished data).

The domestic sheep had more alleles per locus, higher heterozygosities, and higher allele-size variances than did the bighorn sheep (tables 1, 2). This was uniformly true in comparisons of single populations and of pooled species groups. The Bison Range, which had known population bottlenecks of fewer than 20 sheep (see Methods), had the lowest variation by all measures.

All eight populations had significantly different allele frequencies in all pairwise tests (all $P < 10^{-4}$; Monte Carlo tests combined over loci; tests not shown). P values were consistently very low in these tests despite sample

sizes as small as $n = 10$ for several of the bighorn sheep herds. Genetic differentiation among the three domestic breeds was substantial ($F_{ST} = 0.085$); among the five bighorn herds it was even greater ($F_{ST} = 0.224$).

The high F_{ST} values are largely due to alleles present in one or a few populations and absent in others (data not shown). Furthermore, the most common allele in a population was often absent in others. This was especially true in the bighorn sheep. Of the 40 single most common alleles at each locus in each herd, 17 were absent from one or more other bighorn samples. Private alleles were even more apparent in species comparisons. Seventy-one percent of all alleles found in either species were entirely absent in one or the other species (67 of 94; fig. 1).

Allele-Size Distributions

By sampling from many distinct breeds and wild herds, we strove for species-specific allele distributions that were not strongly affected by drift in local populations. There was no discernible pattern in the shapes of allele distributions compared between species or between loci. For some loci distributions were highly centralized, while others were skewed, flat, or multimodal (fig. 1).

Allele-size ranges and means were both significantly larger in domestic sheep than in bighorn sheep. Domestic sheep had wider allele-size ranges than did bighorn sheep at seven of eight loci (table 1) (sign test, $P < 0.04$). Consistent with this, allele-size variances within populations (S_W) were, on the average, three times higher in domestic sheep (table 2).

The mean allele size in *Ovis aries* exceeds the mean in *O. c. canadensis* at seven of eight loci by a mean difference of 8.7 base pairs per locus (table 1) (Wilcoxon signed-ranks test, $P < 0.01$; Sokal and Rohlf 1981). No such tests within species were significant; all comparisons between populations show less than 1 base pair per locus difference. Consistent with the allele-size differences, of the 44 alleles found only in domestic sheep, 39 (89%) fell above the allele-size range in bighorn sheep (fig. 1).

There is no indication that small samples from skewed distributions biased allele-size comparisons between species. Small samples from historically large bighorn populations undoubtedly underestimated total allele numbers. Nevertheless, the shapes of allele distributions (fig. 1) do not suggest that either large or small alleles were consistently rare. If such biases were occurring, it would be hard to distinguish the effects of small experimental samples from a similar bias caused by natural sampling variance (genetic drift).

Genetic Distances

Nei's D values between sheep species range from 1.35 to 2.78 (table 3). Nei's Ds between bighorn herds were, on the average, twice as large as between domestic breeds. Figure 2 shows a dendrogram compiled from all pairwise Nei's D values. The tree is biogeographically accurate. The European domestic breeds are expected to be more similar than either is to the Middle Eastern Awassi breed. The Whiskey Basin (Wyoming) and Tarryall (Colorado) bighorn herds are increasingly distant geographically from the other Rocky Mountain bighorn herds.

We also computed genetic variation and genetic distance using the allele-size variance method (Slatkin 1995; Goldstein et al. 1995). The variance-based distances between populations (S_B) were higher among the domestic breeds than among bighorn herds, opposite to

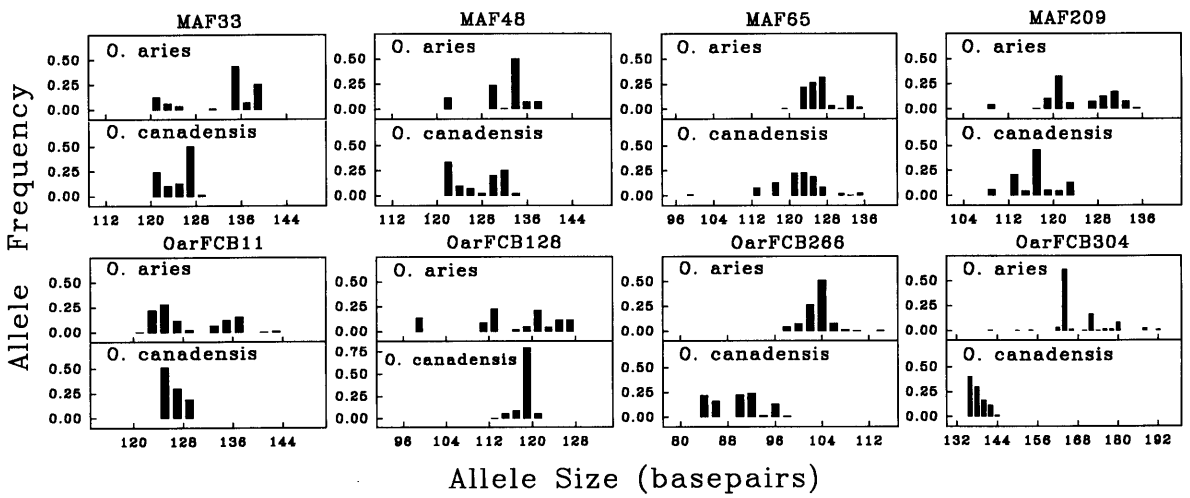

FIG. 1.—Allele-size distributions at eight microsatellite loci in domestic sheep (*Ovis aries*, $n = 150$) and bighorn sheep (*O. canadensis*, $n = 100$).

Table 3
Nei's Normalized Genetic Identity (*I; above diagonal*) and Nei's Standard Genetic Distance (*D; below diagonal*) at Eight Microsatellite Loci between Domestic Sheep Breeds (populations 1–3) and Bighorn Sheep Herds (populations 4–8)

Population	1	2	3	4	5	6	7	8
1 Awassi	...	.696	.685	.231	.156	.207	.260	.166
2 Merino	.363	...	.775	.198	.210	.192	.248	.215
3 Romney	.378	.255	...	.109	.110	.062	.136	.110
4 Sheep River	1.464	1.620	2.214	...	.633	.698	.714	.303
5 Bison Range	1.856	1.561	2.208	.457	...	.686	.660	.243
6 Sun River	1.576	1.649	2.779	.360	.377	...	.738	.437
7 Whiskey B.	1.346	1.395	1.997	.337	.415	.304	...	.548
8 Tarryall	1.793	1.535	2.206	1.193	1.414	.827	.601	...

the result using Nei's D (fig. 3). The higher S_B values among domestic breeds correspond to the higher total allele-size variances in domestic sheep; when adjusted for total variance using R_{ST} (the proportion of variance that is between groups), domestic breed differences are generally lower than bighorn population differences (fig. 4).

Nei's D and F_{ST} appear more sensitive to population history than the variance-based measures. Pairs of bighorn herds with widely varying Ds have uniform S_Bs, and F_{ST} also varied more within species than R_{ST}. Conversely, the variance-based measures (S_B and R_{ST}) more clearly distinguish intraspecies versus interspecies comparisons. With the standard measures (D and F_{ST}) the ranges of these comparisons overlap.

Discussion

Microsatellite Differences between Species

Genetic variation, mean allele sizes, and allele-size variances were all higher in domestic sheep than in bighorn sheep. Differences in allele size and polymorphism among taxa may be explained by bias in the cloning and characterizing microsatellite loci (FitzSimmons et al. 1995; Pepin et al. 1995). The range of sizes found for simple repeat sequences depends on the method used to detect them. Many microsatellites are found by sequencing known genes (GenBank sequences), but increasingly they are detected by genomic library screening. Comparison of these methods for (AC)n microsatellites shows that the GenBank sequences have larger proportions of short repeats than are found in cloned repeats (Weber 1990; Beckman and Weber 1992). Repeats of less than 24 bp are very poorly detected by library screening. Further, allele size and allelic diversity at dinucleotide repeat loci are positively correlated in several species (Beckman and Weber 1992; Buchanan et al. 1993; Ostrander et al. 1993), so that the choice of highly

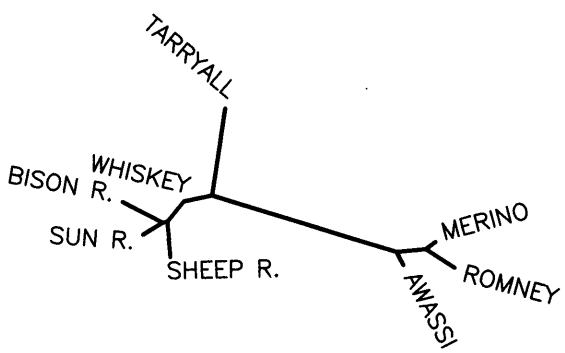

FIG. 2.—Genetic distance (Nei's D) dendrogram generated by the Fitch-Margoliash algorithm. The bighorn sheep species clade is on the left, and the domestic sheep species clade is on the right.

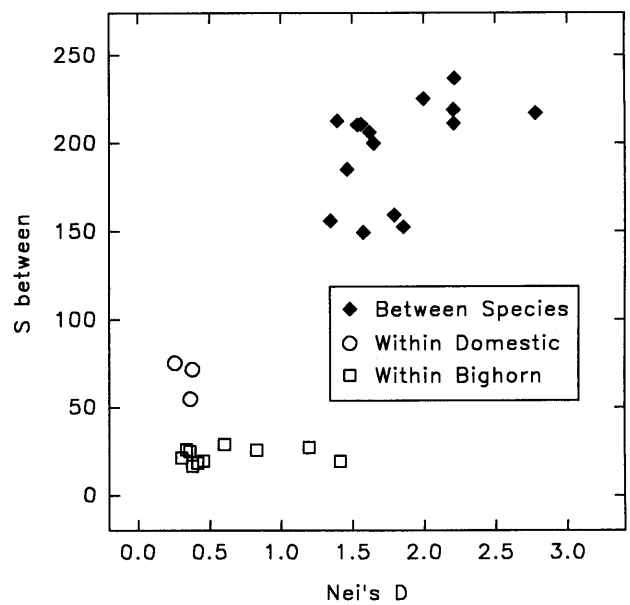

FIG. 3.—Comparison of genetic distance based on allele-size variance (S_B, Slatkin 1995; D_1, Goldstein et al. 1995) to Nei's standard genetic distance (D) within and between sheep species.

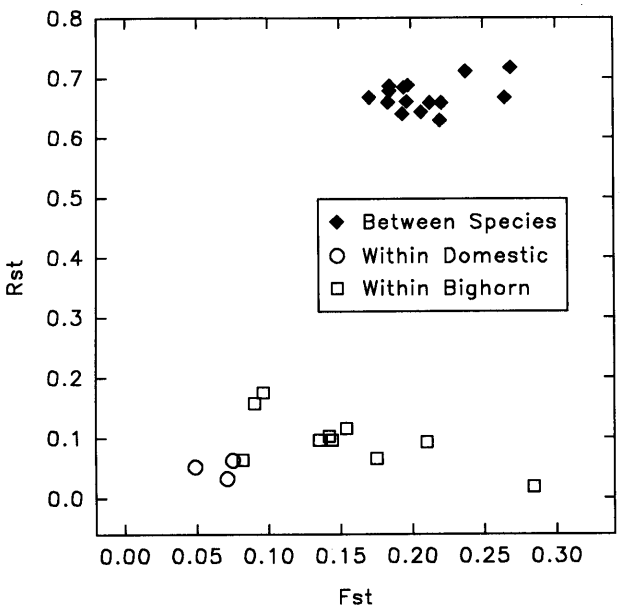

FIG. 4.—Comparison of genetic differentiation based on allele-size variance (R_{ST}, Slatkin 1995) to differentiation based on variance in allele frequencies (F_{ST}) within and between sheep species.

informative loci may also select larger repeat sizes. Therefore, the subset of all (AC)n repeats found by library screening in domestic sheep will have larger-than-average repeat lengths, while homologous loci in congeners will have smaller or altered repeats. If true, this bias would apply to any interspecies transfer of microsatellite markers, and it could be readily tested by reversing the procedure. We would expect a set of polymorphic microsatellites cloned in bighorn sheep to have smaller allele sizes in domestic sheep.

Data from comparisons of higher taxa support this explanation. For example, Bowcock et al. (1994) tested 10 human microsatellite loci on chimpanzees (*Pan troglodytes*), gorillas (*Gorilla gorilla*), and orangs (*Pongo pygmaeus*). We analyzed these data as we did the *Ovis* results. In the three great apes, mean allele sizes were smaller than in humans by 4.0, 5.3, and 9.9 base pairs per locus in chimps, gorillas, and orangs, respectively. The human-chimp and human-orang differences were significant at the $P < 0.005$ level (Wilcoxon signed-rank tests; Sokal and Rohlf 1981, p. 449). The human-gorilla test was not significant.

If microsatellite polymorphism is associated with repeat size and repeat size varies among taxa, a serious implication is that mutation rates at homologous loci may differ among taxa. However, the effect of mutation rate variation on population-genetic parameters will be difficult to assess. For comparisons at the species level or higher, the effect of the origin and application of the markers should be considered and tests for allele-size differences performed. These tests are simpler to interpret than comparisons of polymorphism between taxa, since the latter may be confounded by differences in population history.

Mutation Models and Variation within Species

Microsatellite variation within species can also be assessed using either standard methods or allele-size variance. Effective population size (N_e) estimates depend too much on largely unknown mutation rates and patterns to be informative about population size. However, different results using different measures address the effect of the mutation model on the results. We first estimated N_es based on heterozygosity (H) and the classical stepwise mutation model ($H = 1 - [1/(1 + 8\,N_e\mu)^{0.5}]$; Ohta and Kimura 1973). We omitted the Bison Range bighorn herd because of its known bottlenecks and consequent low genetic variation. Assuming a mutation rate of 10^{-3}–10^{-4}, expected H_e values (table 2) gave N_e estimates of 1,000–10,000 for domestic sheep and 600–6,000 for bighorn sheep. We then estimated N_es using the stepwise model based on allele-size variance ($E[S_W] = 4\,N_e\mu$; Goldstein et al. 1995; Slatkin 1995). Using the same mutation rates, mean S_W values (table 2) gave N_e ranges of 16,000–160,000 in domestic sheep and 6,000–60,000 in bighorn sheep. These estimates are 10–16 times larger than those based on heterozygosity.

The likely reason for this discrepancy is that the variance-based model is incorporating larger, multistep mutations (as well as insertions and deletions in DNA flanking the repeat) as if they were large numbers of single-step mutations. Thus, single-step, variance-based models overestimate N_e to the extent that larger mutations actually occur. In the model of Di Rienzo et al. (1994), most of the allele-size variance is due to a small number of these larger mutations. This "two-phase" model (Slatkin 1995) is much less restrictive than a pure stepwise model because the complete absence of larger mutations would be difficult to prove. Research on the proportions of one-step mutations, multistep mutations, and larger rearrangements is needed.

Ovis Divergence Times and Genetic Distances

Biogeography (Spillett and Bunch 1979; Valdez 1982) and mtDNA analyses (Ramey 1993) provide an approximate divergence time between domestic sheep and bighorns of 2 million yr. Within-species divergence times are roughly 2,000–10,000 yr for both domestic sheep breeds and bighorn sheep populations. The Romney and the Merino breeds diverged about 2,000 yr B.P.; the Awassi breed is considerably older (Buchanan et al. 1994). Bighorn sheep are believed to have survived in a southern refugium during the last ice age, followed by

recolonization of the Rocky Mountains as the ice retreated 10,000–15,000 yr B.P. (Geist 1985). The estimate of 2 million yr divergence between species is therefore 100- to 1,000-fold larger than intraspecies divergence times.

We directly compared standard versus allele-size-based genetic distances methods. Allele frequency differentiation (F_{ST}) and standard genetic distance (e.g., Nei's D) readily resolve relationships among closely related sheep populations. Buchanan et al. (1994) studied genetic distances among a set of European domestic sheep breeds with moderate levels of divergence (Nei's $D = 0.08$–0.50). Using the eight loci reported here, they found a mean gene replacement rate of $\alpha = 2.74 \times 10^{-4}$, assuming a divergence time of 2,000 yr between the Merino and the British breeds and a generation time of 4 yr ($D = 2\alpha t$). This rate also gave a reasonable divergence time between the more closely related Australian and New Zealand Merino stocks.

With the same mutation rate and generation time, our within-species D values give reasonable divergence times between bighorn herds (2,200–8,700 yr). However, the between-species divergence time (mean $D = 1.813$; $t = 3{,}250$ generations, or 13,000 yr) is impossibly short. This discrepancy is easily explained. Nei's D loses its linear relation to time at about $D = 0.20$ (or $I = 0.82$) if there is either back-mutation or variation in α among loci (Nei 1987, p. 236). For allozyme mutation rates ($\alpha = 10^{-7}$), this threshold is at about 10^6 generations. For the higher microsatellite mutation rates the threshold is only 365 generations, or about 1,500 yr ($D = 0.20$; $\alpha = 2.74 \times 10^{-4}$). All the distances in the present study are larger than this.

Genetic distance based on allele-size variance (S_B) is relatively greater between species than is Nei's D, but even S_B is not linear out to 2 million yr. However, these data do not indicate the useful range of either measure because there are no comparisons with divergence times between 10,000 and 2 million yr. More studies with large and accurately known divergence times are needed.

Nei's D and F_{ST} are more sensitive to differences between allopatric or semi-isolated populations, but S_B and R_{ST} better detect longer historical separations. There is not yet a single genetic divergence or distance measure for microsatellites that is equally sensitive to between-population and between-species differences. Until such a comprehensive measure is developed, studies of taxa in the middle range of divergence will do well to use and compare both classical and variance-based measures.

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