Analysis of Genetic Variation in 28 Dog Breed Populations With 100 Microsatellite Markers

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Abstract

Dog breeds were created by man choosing for select phenotypic traits such as size, shape, coat color, conformation, and behavior. Rigorous phenotypic selection likely resulted in a loss of genetic information. The present study extends previous dog population observations by assessing the genotypic variation within and across 28 breeds representing the seven recognized breed groups of the American Kennel Club (AKC). One hundred autosomal microsatellite markers distributed across the canine genome were used to examine variation within breeds. Resulting breed-specific allele frequencies were then used in an attempt to elucidate phylogeny and genetic distances between breeds. While the set of autosomal microsatellites was useful in describing genetic variation within breeds, establishing the genetic relatedness between breeds was less conclusive. A more accurate determination of breed phylogeny will likely require the use of single-nucleotide polymorphisms (SNPs).

Breeds are defined as intraspecies groups that have relatively uniform physical characteristics developed under controlled conditions by man. Dog breeds were originally developed from canids indigenous to a country or geographic region, and breeding animals were selected for phenotypic traits such as size, coat color, structure, and behavior. Later breeds were in turn developed from existing breeds, each foundation breed providing a phenotypic trait that bred true. Based on available breed histories, the majority of extant dog breeds were developed in the 19th century. Thus, while there are exceptions, such as the greyhound and chow chow, the creation of most dog breeds is a recent event. Rapid phenotypic selection has resulted in canine breeds as diverse as the tall, refined borzoi and the short, stocky pug; no other species of animal displays the range of phenotypic diversity seen in purebred dogs. The strong and focused selection pressure inherent in the development of domestic breeds leads to loss of genetic variation, with some breeds potentially losing more than others owing to variation in breed histories and breeding practices.

Genetic polymorphism, heterozygosity, and phylogeny have been studied with a variety of genetic markers—autosomal microsatellites markers, Y chromosome markers, mitochondrial DNA (mtDNA), and more recently, single-nucleotide polymorphisms (SNPs). All of these marker types have been used to distinguish mammalian populations with varying degrees of success (Brinkman et al. 1998; Kittles et al. 1999; MacHugh et al. 1998; Redd et al. 2002; Rolf et al. 1998; Vila et al. 1999; Zhou and Lamont 1999). However, when used alone, each marker type has its limitations. Analysis of Y chromosome markers and mtDNA sequence variation limits study to a fraction of the total genetic material and to one gender. In addition, mtDNA has shown a 20-fold increase in mutation rate across the hypervariable regions relative to nuclear DNA (Kittles et al. 1999; Sigurgardottir et al. 2000), and Y chromosome microsatellites have equally high mutation rates to autosomal markers (Kayser et al. 2000). SNPs are abundant in the genome, have a lower mutation rate than microsatellite markers and mtDNA, and, once discovered, can be efficiently assayed and analyzed. However, the current paucity of SNPs available for canine (Brouillette et al. 2000) limits this approach in breed population studies.

Autosomal microsatellites have been used to study

Table 1. Number of dogs tested, heterozygosity (H_B), heterozygosity standard deviation (SD_H), and number of AKC registrations for the past 5 years per breed

AKC group	Breed	n^a	$H_B{}^a$	$SD_H{}^a$	No. of AKC registrations/year
Herding	Pembroke Welsh corgi	45	.630	.017	9,340
	Belgian tervuren	42	.650	.017	479
	Border collie	44	.669	.018	1,572
	Australian shepherd	45	.696	.012	6,093
Hound	Borzoi	39	.605	.021	928
	Norwegian elkhound	45	.623	.015	1,179
	Rhodesian ridgeback	44	.647	.015	2,362
	Greyhound	44	.648	.017	183
Nonsporting	Bulldog	42	.581	.020	14,396
	Keeshond	36	.650	.015	1,588
	Chow chow	40	.666	.017	5,307
	American Eskimo dog	41	.686	.014	519
Sporting	Weimaraner	36	.614	.017	8,407
	Labrador retriever	44	.641	.016	162,020
	Golden retriever	39	.657	.016	65,458
	Brittany spaniel	44	.666	.014	9,261
Terrier	Bull terrier	44	.387	.021	1,029
	Miniature bull terrier	33	.474	.019	133
	Airedale terrier	41	.515	.020	3,110
	Jack Russell terrier	29	.758	.012	1,134
Toy	Pug	42	.566	.017	22,253
	Yorkshire terrier	45	.684	.018	42,093
	Papillon	43	.698	.013	3,646
	Pomeranian	39	.705	.014	34,709
Working	Boxer	43	.474	.023	37,046
	Doberman pinscher	38	.527	.017	14,925
	Bernese mountain dog	41	.543	.019	2,145
	Akita	42	.642	.018	7,138

Values ranked alphabetically by AKC breed group and by ascending H_B values.

^{a} Values averaged for the 100 microsatellite markers tested.

genetic diversity in several dog breeds, primarily for the purposes of determining the power of exclusion for parentage applications, match probability for forensic casework, and characterization prior to linkage analysis in specific breeds (Altet et al. 2001; Fredholm and Wintero 1995; Ichikawa et al. 2001; Koskinen and Bredbacka 1999; Mariat et al. 1996; Sutton et al. 1998; Zajc et al. 1997). In addition, Zajc and Sampson (1999) and Koskinen and Bredbacka (2000) have investigated phylogeny in three and five breeds, respectively, using sets of polymorphic microsatellite markers. As microsatellites are easy to test, abundant on the canine genetic map, and can be used for both genders, it is of interest to determine the results when applied to a larger set of breeds. One caveat to interpreting microsatellite data is that the results can be confounded by high mutation rates (Francisco et al. 1996; Landry et al. 2002).

The present study makes use of a data set of more than 114,000 dog genotypes generated using the genome screening panel developed for canine linkage studies at the Veterinary Genetics Laboratory (VGL) at the University of California, Davis (Eggleston et al. 2002). This data set was generated by typing 100 polymorphic microsatellite markers for 28 American Kennel Club (AKC) recognized breeds. The

present study investigates the efficacy of the data set to address intrabreed diversity and interbreed phylogeny.

Materials and Methods

Animal Selection

Breeds were selected from the seven AKC recognized groups (Table 1). The breeds screened were Australian shepherd, Belgian tervuren, border collie, Pembroke Welsh corgi (herding group); borzoi, greyhound, Norwegian elkhound, Rhodesian ridgeback (hound group); American Eskimo dog, bulldog, chow chow, keeshond (nonsporting group); Brittany spaniel, golden retriever, Labrador retriever, weimaraner (sporting group); Airedale terrier, miniature bull terrier, bull terrier, Jack Russell terrier (terrier group); papillon, Pomeranian, pug, Yorkshire terrier (toy group); Akita, Bernese mountain dog, Doberman pinscher, and boxer (working group). Samples and first-generation pedigrees were collected from dog owners and breeders from across the country. To avoid the possibility of testing related animals, care was taken to select dogs from various geographic regions; dogs with common ancestors within the

first generation or those with identical kennel names were not included in the study. To minimize the effect of potential second-generation relatives in the data set, we tested a large sample size of 29 to 45 dogs per breed (mean = 41). A larger sample size also avoided a skewed representation that may have resulted from choosing a small group of dogs from only one geographic area or from one or two kennel populations.

Marker Selection

The VGL genome screening panel, comprised of 100 autosomal microsatellite markers multiplexed into 12 sets (Eggleston et al. 2002), was used for this study. Elements of the panel were selected from the 1999 canine genetic linkage map (Neff et al. 1999) based on map location, reported polymorphism, and allele size ranges. Informativeness was the primary criterion in marker selection; ease of amplification and scoring were also taken into account.

Marker selection for the phylogenetic tree analysis was based on the total number of alleles observed. Thirty-four loci with a total number of alleles of more than 18 were eliminated from analysis, owing to the theoretically high probability of mutation (Brohede et al. 2002; Webster et al. 2002). The observed mutation frequency for all 100 markers was 1.1×10^{-2}, and nearly fourfold lower, at 2.9×10^{-3}, for the 66-marker subset used to construct the tree (Irion et al. 2002, unpublished data).

Sample Preparation and Polymerase Chain Reaction

All samples used in this study were derived from buccal cells obtained from bristle cytology brushes (Medical Packaging Corp., Camarillo, CA). Buccal swabs were collected by owners and submitted directly to the laboratory. DNA was extracted by heating a single swab for 10 min at 95°C in 400 μl 50 mM NaOH and then neutralizing it with 140 μl 1M Tris-HCl, pH 8.0. A 2 μl aliquot of this extraction was then used in each polymerase chain reaction (PCR). Forward primers were synthesized and labeled with the Fam, Hex, or Tamra dyes (Applied Biosystems, Foster City, CA). Reverse primers were synthesized by Operon (Alameda, CA). Each primer pair was tested with a PCR reagent mix of 1× PCR buffer (ABI), 2.5 mM MgCl$_2$, 200 μM of each deoxynucleotide triphosphate (dNTP) (Hoffmann-La Roche, Nutley, NJ), 0.7 unit AmpliTaq (ABI), and 2% dimethyl sulfoxide (DMSO). Thermal cycler parameters differed depending on the annealing temperature used. All PCRs were performed with MJ Research PTC-100 thermal cyclers (Waltham, MA).

Gel Electrophoresis Conditions and DNA Fragment Analysis

One ml aliquots of PCR product were mixed with 2 μl fluorescent ladder (CXR) (Promega 400) or internal lane standard (Promega 600; Promega, Madison, WI), denatured for 3 min at 95°C, then held at 5°C or placed on ice for at least 1 min. Two ml aliquots were loaded onto a 6% denaturing polyacrylamide gel and run on an ABI 377 auto-mated sequencer using ABI 10″ × 7⅛″ short plates (12 cm). Gels were run at a voltage of 1.10 kV, 60.0 mA variable current, 200 W (constant) power, 51°C, and 40.0 mW (constant) laser power for up to 2 h when using Promega 400 and up to 3 h using Promega 600. DNA fragment analysis was performed with STRand software (Hughes 1998). These data were then transferred to a statistical database compatible with STRand.

Statistical Analysis

Marker polymorphism was determined by the relative number and frequency of alleles for a specific locus within each breed (Lingaas et al. 1996; Zajc et al. 1997), where allele number and frequencies were determined by direct counting. The fixation index, F_{ST} (often symbolized as G_{ST} when there are more than two alleles at a locus), was used to provide a measure of genetic differentiation, where $F_{ST} = (H_T - H_S)/H_T$. H_T is the measure of the total heterozygosity for a locus (i.e., the probability that two gametes chosen at random from the total population will carry different alleles) and H_S is the subpopulation heterozygosity (i.e., the average heterozygosity among subpopulations). Calculation of heterozygosity was made using public domain software, DISPAN (genetic distance and phylogenetic analysis; Ota 1993). Heterozygosities were then averaged for all 100 markers for each breed.

Hardy–Weinberg equilibrium tests were conducted with GENEPOP (version 3.3). This is an updated version of the software first presented by Raymond and Rousset (1992). Exact p values, along with their standard errors, were calculated using a Markov chain algorithm (Guo and Thompson 1992) with 1000 dememorization steps for 100 batches and 1,000 iterations per batch.

Phylogenetic Tree Construction

Allele frequencies from a subset of 66 markers were used to compute a matrix of genetic distances (Nei 1987); this matrix was used to construct a phylogenetic tree of relationships among dog breeds. Genetic distances and the phylogenetic tree were computed with PHYLIP (version 3.6 for Linux; Felsenstein 2001). To provide an evaluation of the reliability of the tree, 1,000 bootstrap samples of the data were generated for distance computations (using the SEQBOOT program of PHYLIP). A matrix of Nei's (1987) genetic distance was computed for each generated sample (using GENDIST), followed by the construction of a tree by neighbor joining (using NEIGHBOR) for each sample. One thousand trees were generated by random sampling of portions of the entire data set. The 1000 generated trees were then used to create the final, consensus phylogenetic tree with the majority rule algorithm using CONSENSE (Margush and McMorris 1981).

Results

Analysis of genotypes obtained from 100 microsatellite loci in 28 purebred dog populations yielded several findings.
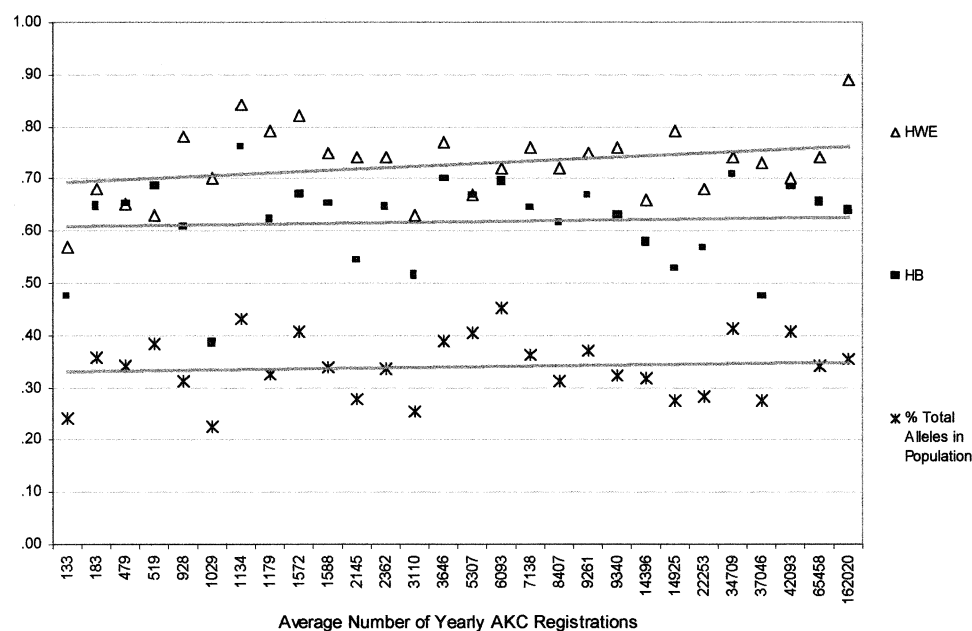
Figure 1. Percentage of loci in Hardy–Weinberg equilibrium (HWE), average heterozygosity (H_B), and percentage of the total observed alleles for each breed in order of average number of AKC registrations per year. Trends for each data series are presented in gray.

Table 1 presents average breed heterozygosities (H_B) for all 100 microsatellite loci for the 28 breeds under investigation. Clearly the amount of genetic variation is considerable, with values that are similar to those of other investigators (Fredholm and Wintero 1995; Zajc et al. 1997). Total heterozygosity (H_T) for all the breeds was high (0.618), with a range of 0.387 to 0.758 between the breeds (Table 1). Only three breeds fell below 0.500 H_B; bull terrier, miniature bull terrier, and boxer. The average standard deviation for H_B was 0.017, with a range of 0.012 to 0.023. Significant differences were found between the least and most heterozygous breeds in each of the seven groups, with the terrier group showing the most divergence.

Not presented are the fixation indices (F_{ST}) for the 100 loci, where values ranged from a low of 0.12 (for FH2165) to a high of 0.46 (for AHT136) in this set of 28 dog breeds. The average value of F_{ST} for all loci was 0.23.

To estimate for each breed population size, the number of dogs registered per year by the AKC was averaged over the past 5 years (http://www.akc.org/breeds/regstats2001.cfm). The average number of new registrations per year was 16,373 with a range of 133 (miniature bull terrier) to 162,020 (Labrador retriever), representing a more than 100-fold difference between the smallest and largest estimated breed population sizes (Table 1). To determine the effect of this wide range on heterozygosity, H_B values were plotted against estimated population size (Figure 1). For all 28 breeds studied, only a slight correlation was found between the estimated population size and H_B (~3%). A stronger correlation was found between date of breed recognition by a registry and H_B, with more recently recognized breeds

showing approximately 19% higher H_B than the earlier recognized breeds (Figure 2).

It was also of interest to determine if the number of alleles per breed differed relative to the totality of alleles observed in all breeds, and to what extent this was influenced by population size and time since registry recognition. The total number of alleles observed for all breeds and loci was 1,780. Within each breed, a range of 399 to 805 alleles per breed was found, with an overall average of 605 alleles (Table 1). The number of alleles per breed mirrored the level of heterozygosity (Figures 1 and 2). As a function of population size, the breeds with smaller populations had about 6% fewer alleles than the breeds with larger populations. When plotted as a function of time since recognition by a registry, the numbers of alleles observed per breed was lower for the earlier recognized breeds by about 7%.

Assessment of Hardy–Weinberg equilibriums found that an average of 27% of markers per breed were out of equilibrium. The values ranged from 11% (Labrador retriever) to 43% (miniature bull terrier). When the average Hardy–Weinberg equilibrium values for all 28 breeds were plotted against their estimated population size, a trend of an approximate 10% increase in Hardy–Weinberg equilibrium occurred as population size increased. When plotted against time since registry recognition, the number of loci in Hardy–Weinberg equilibrium tended to be about 4% higher in the recently recognized breeds.

Phylogenetic analysis using the more stable (less mutable) 66-marker panel revealed two significant relationships among the 28 breeds. First, bull terriers and miniature bull terriers grouped in 100% of the trees generated for the final consensus
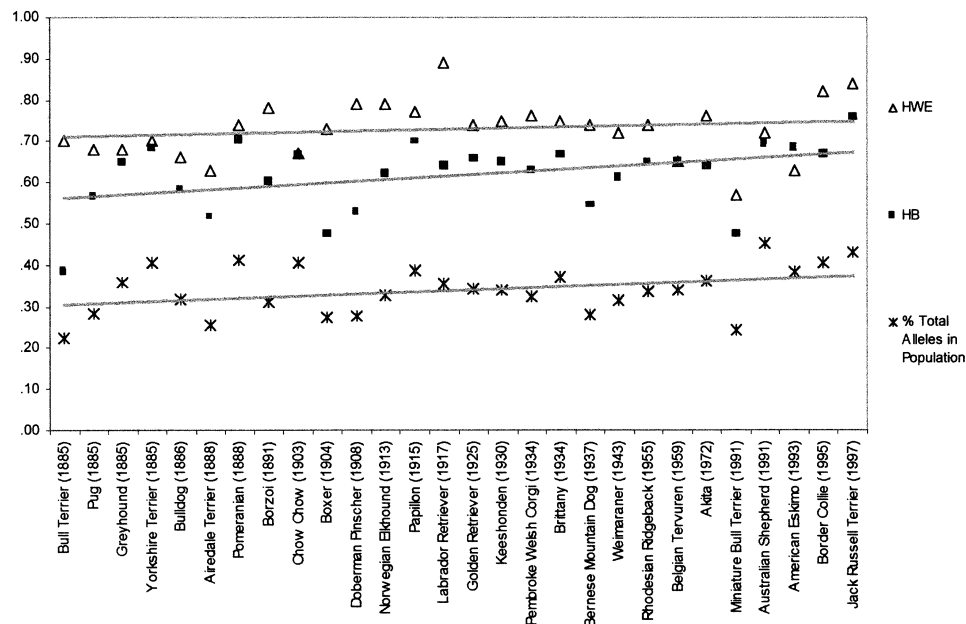
Figure 2. Percentage of loci in Hardy–Weinberg equilibrium (HWE), average heterozygosity (H_B), and percentage of the total observed alleles for each breed from earliest to most recent breed registrant. Trends for each data series are presented in gray.

tree. The second significant observation was that Australian shepherds significantly diverged from the rest of the 27 AKC breeds (95.9% confidence). Finally, the Akita/chow chow grouping approaches significance at 91.1% confidence.

Discussion

Analysis of Genetic Diversity

The results of this study illustrate that population substructure in dog breeds is complex, especially when studying the question with microsatellite markers specifically chosen for their polymorphism as linkage markers. Multiple factors contribute to the degree of heterogeneity observed.

As one would expect, heterozygosity (H_B) and Hardy–Weinberg equilibrium tended to decrease as population size decreased and as length of time in a registry increased. Counterintuitive to this was the finding that the miniature bull terrier had a 22.5% higher H_B value than the bull terrier. The miniature bull terrier originated from the bull terrier in the late 19th century and has a population size one tenth that of the bull terrier. In this case, it may indicate that outcrossing occurred in the miniature bull terrier or that the bull terrier experienced a genetic bottleneck since the two breeds diverged.

Analysis of how many loci are in Hardy–Weinberg equilibrium is another method by which to analyze the results of population substructure. Hardy–Weinberg equilibrium results from a random mating population free from outside forces such as mutation, migration, and selection. We

found, on average, 27% of loci to be out of equilibrium, with population size having a greater impact than the length of time in a registry. These findings may indicate that most breeds were somewhat homogeneous prior to being officially recognized by a breed registry. Indeed, breed clubs have to demonstrate a well-documented history and a well-described conformation standard prior to recognition of their breed by a registry. However, forces such as founder effects and bottlenecks (as a result of popular sires, severe changes in population sizes, and intense phenotypic selection) will continue to contribute to a decrease in genetic diversity after registry recognition.

The high level of heterogeneity across breeds, regardless of widely varying population size, must also be evaluated in light of marker selection. The markers used in this study were selected for high polymorphism values for use in genome screening (Eggleston et al. 2002). Of the 100 markers tested, 99 have an average H_T of 0.50 or higher and 89 have an average H_S of 0.50 or higher. Further, they have an observed mutation frequency of 1.1×10^{-2} (Irion et al. 2002, unpublished data), which is an order of magnitude higher than that seen in humans (Ellegren 2000). This comparatively high mutation frequency will give rise to new alleles or a higher incidence of previously rare alleles in each breed over time. At this rate, 12,995 mutations would be expected among the approximately 1 million AKC dogs registered each year. Certainly the frequent mutations observed in this set of microsatellite loci may cause even those breeds subject to strict selection to appear more heterogeneous than their pedigrees suggest.

Phylogenetic Analysis

In an attempt to establish interbreed genetic distances, phylogenetic analysis was performed by determining genetic distances from allele frequencies and then creating 1,000 different trees (Nei 1987). The 1,000 trees were then combined to create one consensus tree. The effect of this method is to minimize the impact of a few unstable markers on the final resulting tree. Mutations in just a few loci will result in weak bootstrap values unless allele frequencies in a majority of the other loci are statistically powerful enough to compensate. As mutation events go both ways (divergent and convergent), the effect on genetic distance is difficult to predict and involves complex statistical estimates (Landry et al. 2002). Thus the best way to minimize the effect of mutation events on allele frequencies is to select the most stable microsatellites from within the typed set. To that end, a subset of 66 more stable markers was selected from the 100 marker set. This subset had an observed mutation frequency of 2.9×10^{-3}, nearly fourfold lower than the 1.1×10^{-2} frequency observed in the 100-marker set (Irion et al. 2002, unpublished data).

Results of the phylogenetic analysis (not shown) revealed only two significant groupings for the 28 breeds tested. A group of populations was considered monophyletic only when they were found in the same branch more than 95% of the time (Weir 1996). Only bull terriers and miniature bull terriers were close enough for such a declaration. As would be expected, bull terriers and miniature bull terriers grouped together in 100% of the trees making up the final consensus phylogram. The bull terrier is an old breed that originated in England in the late 19th century. During the same period, the miniature bull terrier breed was developed from the bull terrier breed by selecting for dogs of diminutive stature. Over time, a significant size difference was developed and maintained.

A separate branching (95.9%) was seen between Australian shepherds and the rest of the AKC breeds tested. This divergence may be geographic in origin, as these dogs were found only in Australia as of 100 years ago. American ranchers imported them for their livestock tending skills and developed the breed with minimal crossbreeding to other herding breeds.

The relationship between Akitas and chow chows had a suggestive bootstrap value of 91%. Again, this grouping may be geographic in origin, as both breeds are of Asian descent. Chow chows are one of the most ancient breeds (more than 2,000 years old). It has been speculated that Akitas descended from the chow chow.

The bootstrap values in the remaining classifications represented by the tree were only loosely configured. Again, the widely recognized high mutation rates among microsatellites (Ellegren 2000; Francisco et al. 1996) may be a major cause. When limiting the tree to the 66 less polymorphic loci, there were still 220 predicted mutations among the 75,768 genotypes studied. Mutation patterns are also complex, occurring in some loci more than others and in larger alleles more often than their smaller counterparts (Ellegren 2000;

Takezaki and Nei 1996). Furthermore, the "evolutionary" time scale of each breed can differ by more than three orders of magnitude in dog breeds, as population sizes vary greatly (Table 1). This tends to further exacerbate the effect of microsatellite mutations when comparing populations (Goldstein et al. 1995). As a result, a frequently bred population may be more heterogeneous than phenotypic uniformity suggests.

Mutation rate is just part of the explanation for the lack of correlation between the allele frequencies for the 66-marker set and breed phenotypes. While some microsatellites may be closely linked to the phenotypes under selection, other microsatellites may be selectively neutral. It may be that several of the loci in this study are too distant from selected traits to provide good breed distinction. Further, it is estimated that just 0.2% of the genome differs between the domestic dog and the gray wolf (Wayne 1993). Extrapolated to the domestic dog, just a small fraction of the genome would be responsible for breed differences. It would be necessary to use DNA sequence data and ultimately have genetic markers tightly linked to the genes responsible for selected phenotypes to determine phylogeny. For this reason SNPs are now being used to elucidate close historical relationships in human populations (Redd et al. 2002). SNPs will likely be required to determine the phylogeny of dog breeds as well.

Despite the limitations inherent to microsatellite markers, they may still be of use for assessing genetic diversity, though less useful for establishing phylogeny relationships. As SNPs become available on the canine map, they will become the preferred choice for determining phylogeny. Presently microsatellite markers have multiple advantages, such as ease of use, availability, high polymorphism relative to SNPs, and can be used for both sexes. Care must be taken, however, to exclude from study microsatellites with a high mutation potential (Landry et al. 2002). Webster et al. (2002) has reported a fivefold increase in mutation rate with dinucleotide repeat lengths greater than 18 bp and a near 10-fold increase in tetranucleotide repeats greater than 18 bp. Brohede et al. (2002) reported that mutation rate increased by 0.1% per repeat unit over 10 repeat units. Using these observations, it is likely that dinucleotides with fewer than 10 alleles are quite stable and useful for population studies. The results of this study support previous findings that a wide genetic variation exists between current dog breeds, though determining exact phylogeny from such variation is hampered by the mutability of the microsatellite markers studied. Incorporation of DNA sequence analysis with other informative genetic markers should greatly improve the accuracy of interbreed genetic distance and intrabreed diversity estimates.

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