Genetic distance between three breeds of dogs based on selected microsatellite sequences

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The aim of this study was to evaluate genetic diversity and genetic distance between three breeds of dogs: Bernese Mountain Dog (BMD), Chihuahua (Ch) and Caucasian Shepherd Dog (CSD), based on microsatellite sequences commonly used to confirm/exclude dogs' parentage. The study included 60 dogs (20 dogs per breed) from Czech kennels. One dog was randomly selected from a single litter while the litters were unrelated down to the second generation. A commonly used panel of microsatellite markers (PEZ1, FHC2054, FHC2010, PEZ05, PEZ20, PEZ12, PEZ3, PEZ6, PEZ8 and FHC2079) was applied in the study. *Loci* of each microsatellite revealed different polymorphism levels with an average of 5.1 alleles per *locus* (from 2 to 9 across breeds). PEZ3 and PEZ12 were the most informative markers (7.0 and 6.7 alleles, respectively). Specific alleles were detected within each breed. *Loci* PEZ5 and PEZ20 showed the lowest average number of alleles (3.7). Number of observed genotypes ranged from 3 to 14, but only few genotypes were common to all breeds. Average heterozygosity ranged from 0.49 in BMD to 0.72 in Ch. Within CSD, solely the PEZ20 *locus* was not highly polymorphic (PIC<0.5), while in BMD only half of the microsatellites were highly polymorphic (PIC>0.5). The lowest genetic distance was found between CSD and Ch, whilst relatively large genetic distance was estimated between Ch and BMD, as well as between the CSD and BMD, which may support the hypothesis of these breeds' separation at an early stage of domestication.

KEY WORDS: Bernese Mountain Dog / Chihuahua / Caucasian Shepherd Dog / genetic distance

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All dog (*Canis lupus f. familiaris*) breeds have the same ancestor, which is the grey wolf (*Canis lupus*). After the initial domestication of the wolf, selection started to improve functional traits and consequently to create different breeds and groups of breeds. However, only at the turn of the 19th century dog breeding became systematized by formation of breed associations promoting specific types (breeds) of dogs and organizing dog shows to nominate the perfect representatives of the breeds [Parker 2012]. Selection which was focused on desirable traits, while restricting natural selection, caused that dog breeds were becoming increasingly isolated from each other, and thus their gene pools became eventually more and more limited [Parker 2012] within a population.

An analysis of microsatellite sequences short tandem repeats (STR) can be used for genetic distance determination. Populations having many similar alleles (microsatellite sequences) are considered genetically close, which means that they share a proximate ancestor.

Out of the indicators of genetic variation within a breed, heterozygosity (H) and effective number of alleles at a *locus* (EA) are usually employed to describe a population. They demonstrate the degree of diversity based upon analyzed polymorphic *loci* within a breed. High heterozygosity and high effective number of alleles inform about a greater genetic diversity of a breed. In addition to those indicators, polymorphic information content (PIC) is widely used as a measure of genetic polymorphism within a *locus* used as a marker in the linkage analysis [Botstein *et al*. 1980].

The dog breeds selected for the present study are characterized by diverse origin resulting in morphological and behavioral traits. The BMD was used to herd cattle, and because of its size and strength, was also used as a draught dog. Its origin is not known in full. Some researchers suggest that this breed originates from the Tibetan dog, which came to Europe from Asia with the migrating men, while others, relying on archaeological excavations, propose an exclusively European ancestry of this breed [Räber 1965]. Furthermore, Wayne and Von Holdt [2012] placed BMD in the group of mastiffs, along with Rottweiler, Newfoundland, Boxer, Bulldog, and other large breeds, for which the common ancestor was a big dog of the contemporary mastiff type.

The origin of Chihuahua is not clear, either. Probably, its direct ancestor was a dwarf dog, Techichi, bred by the Toltec people that had lived on the territory of present Mexico [Van Asch *et al*. 2014]. The literature data also suggest Chinese roots of that breed, along with a common to the Chinese Crested Dog progenitor, which was brought to South America by merchants [Von Holdt *et al*. 2010, Wayne and Von Holdt 2012]. According to Van Asch *et al*. [2014], the Mexican Chihuahua breed comes directly from the Mexican pre-Columbian dogs. Only the origin of CSD is certain. It is an old breed of herding dogs from Caucasus area (Georgia, Armenia, and Azerbaijan) used to guard flocks of sheep and belongings. For centuries, these dogs had lived in a complete isolation; first specimens were brought to Europe only in 1969, and the breed was registered by the FCI in 1984 [Kopaliani *et al*. 2014].

The aim of the study was to confirm common or different parentage of the examined dog breeds which are spread all over the world (Chihuahua, Caucasian Shepherd, Bernese Mountain Dog), and for this purpose the genetic distance between three breeds was determined.

Material and methods

The DNA was sampled from saliva. Samples of 20 individuals from each of the three breeds: Chihuahua, Caucasian Shepherd Dog and Bernese Mountain Dog were selected from a large collection of biological material of various breeds of dogs kept in the Czech Republic. Dogs came from breeders from across the country, and the criterion for selection was belonging to a breed and to a different family (unrelated for two generations) within a breed.

DNA isolation from buccal epithelial cells employed the conventional phenol/chloroform method [Sambrook and Russell 2001]. Concentration of the extracted DNA was determined spectrophotometrically (BioPhotometer, Eppendorf). The stock DNA was stored at -20°C.

Ten microsatellite *loci* (*PEZ01*, *FHC2054*, *FHC2010*, *PEZ05*, *PEZ20*, *PEZ12*, *PEZ03*, *PEZ06*, *PEZ08* and *FHC2079*) were genotyped by the StockMarks®Dogs Genotyping kit (Applied Biosystems, USA), under conditions recommended by the manufacturer.

Frequencies of alleles and genotypes at the *loci* of 10 microsatellite sequences in each of the three breeds were calculated. To further analyze the genetic variation, the effective number of alleles (EA), the expected heterozygosity (H) and the polymorphic information content (PIC) indicators were computed. The above statistics were calculated for each *locus* separately for each breed or for all the *loci* within each breed. To determine the genetic distance between the breeds the Nei formula was used [Nei 1972].

Results and discussion

There was no monomorphic *loci* in any of the three breeds. Observed, however, were alleles specific to a single breed, while a case when an allele was present in two breeds and was missing in the remaining breed was not recorded. The number of alleles ranged from 2 to 9. PEZ3 and PEZ12 were found to be the most informative markers, because they showed the highest number of alleles (average number of alleles: 7.0 and 6.7, respectively). The lowest average number of alleles (3.7) was detected at the PEZ5 and PEZ20 *loci* (Tab. 1).

The number of genotypes ranged from 3 to 14 (Tab. 2). Although a large number of genotypes was found in all breeds, not many genotypes were common: FHC2010 – 4, FHC2054 – 3, FHC2079 – 2, PEZ1 – 2, PEZ5 – 1, PEZ6 – 4, PEZ8 – 4, PEZ12 – 1, PEZ20 – 3. The PEZ3 *locus* showed no common genotype. *Locus* FHC2054, which had the highest number of genotypes in two breeds, had only three genotypes common to all the breeds.

Table 1. Expected size of alleles and number of alleles at each *locus* in three dog breeds

Locus	Expected size (bp)	Dog breed			
		Chihuahua	Caucasian Shepherd Dog	Bernese Mountain Dog	Average for 3 breeds
FHC2010	92-136	5	4	4	4.3
FHC2054	140-183	7	6	4	5.7
FHC2079	210-260	4	4	3	3.7
PEZ1	97-121	7	4	4	5.0
PEZ3	170-201	9	7	5	7.0
PEZ5	250-320	5	4	2	3.7
PEZ6	95-154	7	6	4	5.7
PEZ8	164-214	7	5	5	5.7
PEZ12	222-260	7	7	6	6.7
PEZ20	263-299	4	4	3	3.7
Mean		6.2	5.1	4.0	5.1

Table 2. Highest and lowest number of genotypes in chosen *loci* in three dog breeds

Number of genotypes	Locus	Dog breed		
		Chihuahua	Caucasian Shepherd Dog	Bernese Mountain Dog
Lowest	FHC2010	7		
	PEZ5		4	3
Highest	FHC2054, PEZ1, PEZ12	13		
	FHC2054		14	
	PEZ8			8
Average	all 10 *loci*	10.6	8.5	5.4

The H values ranged from 0.49 in BMD to 0.72 in Ch (Tab. 3).

The highest values of EA, H and PIC were recorded for *loci* PEZ12 and FHC2054 in Ch, for PEZ3 and FHC2054 in CSD and for PEZ8 in BMD (Tab. 3). All the microsatellites in Ch were highly polymorphic (PIC and H > 0.5) and in CSD only *locus* PEZ20 was not highly polymorphic (PIC < 0.5). In turn, in BMD merely half of the microsatellites were highly polymorphic (FHC2054, PEZ1, PEZ6, PEZ8 and PEZ12). The lowest values of EA, H and PIC indices were obtained for Ch and CSD for *locus* PEZ20 and for BMD for *locus* PEZ5. Thus, the lowest variability was observed at *loci* PEZ20 and PEZ5. The highest polymorphism was found at *loci* PEZ3, PEZ8 and PEZ12, in every breed.

Genetic distances between the three breeds, based on 10 microsatellite *loci*, are presented in Table 4. Genetic distance between the Ch and CSD breeds was the closest. A relatively large distance was estimated between Ch and BMD, as well as between CSD and BMD.

Ten popular microsatellite sequences were used in this study. The same or similar microsatellite sequences have been studied by several authors [e.g. De Nise *et al.* 2004, Cho 2005, Pribánová *et al.* 2009, Dimitrijevic *et al.* 2013] in populations of

Table 3. Effective number of alleles (EA), heterozygosity (H) and polymorphic information content (PIC) in three dog breeds

Locus	Chihuahua			Caucasian Shepherd Dog			Bernese Mountain Dog		
	EA	H	PIC	EA	H	PIC	EA	H	PIC
FHC2010	3.33	0.70	0.65	2.56	0.61	0.55	1.69	0.41	0.38
FHC2054	5.33	0.81	0.79	4.49	0.78	0.74	2.61	0.62	0.55
FHC2079	2.64	0.62	0.55	2.77	0.64	0.57	1.36	0.27	0.25
PEZ1	4.49	0.78	0.75	3.56	0.72	0.67	2.37	0.58	0.51
PEZ3	4.02	0.75	0.72	5.33	0.81	0.79	1.87	0.47	0.42
PEZ5	3.00	0.67	0.63	2.84	0.65	0.59	1.30	0.23	0.20
PEZ6	3.57	0.72	0.68	4.30	0.77	0.73	2.40	0.58	0.53
PEZ8	4.12	0.76	0.72	3.20	0.69	0.64	3.42	0.71	0.66
PEZ12	5.44	0.82	0.79	3.29	0.70	0.66	2.23	0.55	0.51
PEZ20	2.62	0.62	0.57	2.37	0.58	0.49	1.89	0.47	0.38
Mean	3.86	0.72	0.68	3.47	0.69	0.64	2.11	0.49	0.44

Table 4. Genetic distance between the Chihuahua, Caucasian Shepherd Dog and Bernese Mountain Dog breeds

Breed	Caucasian Shepherd Dog	Bernese Mountain Dog
Chihuahua	0.33	0.50
Caucasian Shepherd Dog	–	0.44

dogs of various breeds. These sequences are commonly used to confirm or exclude parentage [Halverson and Edwards 2000].

Our results showing a high polymorphism at *loci* FHC2054, PEZ1, PEZ12 and PEZ3 are consistent with results obtained by De Nise *et al*. [2004]. The authors recognized these *loci* as the richest in information basing on a research conducted on 9561 dogs of 108 breeds. De Nise *et al*. [2004] indicated *locus* PEZ3 as the most informative, due to its highest average number of alleles (6.7) per breed. Also in our study, this sequence had the highest number of variants. Our results are also in accordance with a research on the Yugoslav Shepherd Dog breed (Šarplanina) [Dimitrijevic et al. 2013]. *Locus* PEZ3 had the highest number of alleles in that breed, but it was characterized by lower values of the basic diversity indicators. Similarly low H and PIC values were found for *locus* PEZ3 in the present study, but only for BMD. The least informative *loci*, in the Yugoslav Shepherd Dog, were FHC2010 and PEZ5, which is coherent with our results for all three breeds and with several previous studies [De Nise *et al*. 2004, Pribánová *et al*. 2009, Dimitrijevic *et al*. 2013].

The lowest variability, as described by the EA, H and PIC indices, was observed in the BMD breed, and the highest in the Ch breed. CSD breed took an intermediate position, however its statistics were closer to those of Ch. The representatives of the BMD breed were characterized by the lowest average number of alleles at a *locus* and the lowest H value (0.49), which points to a small variability within the breed, caused probably by an intensive selection based only upon phenotypic evaluation.

Similar results were obtained by Cho [2005], Irion *et al*. [2003] and Kopaliani *et al*. [2014]. Research conducted by Cho [2005], in which comparable microsatellite *loci* in six dog breeds in Korea were used, confirm high values of expected heterozygosity and PIC in the Ch breed. The author found the highest H and PIC values in the native Jindo breed (0.796 and 0.755, respectively) and in the Ch breed (0.713 and 0.665, respectively), while in Poongsan and German Shepherd breeds these values were the lowest (Poongsan – 0.550 and 0.502; German Shepherd – 0.567 and 0.538). Irion *et al*. [2003] obtained varying H indicators for 28 dog breeds, including terriers (from 0.387 for Bull Terrier up to 0.758 for Jack Russell Terrier), and moderate H value (0.543) for BMD (23rd position in 28 analyzed breeds). Kopaliani *et al*. [2014] estimated lower expected heterozygosity in wolves (0.78) comparing to CSD (0.83), whereas values of the observed heterozygosity were similar (0.74 and 0.73, respectively).

H values in this study were comparable with values estimated for other breeds: 0.46 – German Shepherd, 0.63 – Kunming Wolfdog, 0.63 – Labrador Retriever, 0.66 – English Springer Spaniel, 0.75 – Belgian Shepherd, 0.76 – Tibetan Mastiff [Ye *et al*. 2009] and 0.43 – Yugoslav Shepherd Dog [Dimitrijevic *et al*. 2013].

Pribánová *et al*. [2009] analyzed genetic variability of 6 dachshund breeds and showed that the average observed heterozygosity ranged from 0.58 to 0.70. Similar values for expected (0.62-0.79) and observed (0.70-0.72) heterozygosity were found in four Turkish breeds [Altunok *et al*. 2005].

The PIC value can provide a better information piece on the degree of variability, as it depends on both, polymorphism and frequency of alleles, contrary to heterozygosity. The higher the value of PIC, the more information can be obtained from a marker [Ciampolini *et al*. 2011]. In our study, the highest PIC values characterized Ch and the lowest BMD. Similar results were obtained by Ye *et al*. [2009] for several breeds: German Shepherd – 0.49, Kunming Wolfdog – 0.62, Labrador Retriever – 0.69, English Springer Spaniel – 0.7, Belgian Shepherd – 0.72 and Tibetan Mastiff – 0.74. A similarly high PIC value (0.66) was also found for the Yugoslav Shepherd Dog by Dmitrijevic *et al*. [2013].

Our estimates of genetic distance were consistent with those obtained by Cho [2005]. The author used similar microsatellite *loci* (FHC2010, FHC2054, FHC2079, PEZ1, PEZ6, PEZ8, PEZ10, PEZ11, PEZ12, PEZ15 and PEZ17) to determine genetic distance between five breeds in Korea (Chihuahua, Jindo, Miryang, Poongsan and German Shepherd). The highest genetic distance was found between German Shepherd and Miryang breeds (1.244), and the lowest between Chihuahua and Jindo breeds (0.154). Ye *et al*. [2009] carried out a research on six breeds (Tibetan Mastiff, Kunming Wolfdog, German Shepherd, Belgian Shepherd, Labrador Retriever and English Springer Spaniel), using the same 10 microsatellites as in our study, but estimated much lower values of the genetic distances. The closest breeds were Labrador Retriever and English Springer Spaniel (0.099) and the most distant were German Shepherd and Labrador Retriever (0.254).

A relatively high genetic distance was estimated between Ch and the BMD, as

well as between CSD and BMD, which may suggest that phylogenetically Ch and CSD breeds have been separated from BMD at an early stage of domestication. A flat topology of phylogenetic tree created by Parker *et al*. [2012] largely confirms a common founder of dog breeds and an extensive gene flow between breeds with very different phenotypes, even before the advent of kennel associations and restrictions on breeding due to determination of the breed standards.

The Ch and CSD breeds were characterized by the lowest genetic distance and also by the highest heterozygosity, which is in accordance with the research reported by Leroy *et al*. [2009].

Von Holdt *et al*. [2010] analyzed the shared proportion of microsatellite haplotypes characteristic for four populations of the gray wolf (Middle East wolf, European wolf, Chinese wolf and North American wolf) in genotypes of dogs of 80 breeds. The contribution of haplotypes of the Middle East and Chinese wolves in Ch and BMD genotypes were similar (35% and 20%, respectively), while these breeds differed with regard to the proportion of haplotypes of the European and North American wolves (Ch – 30% and 15%, respectively; BMD – 35% and 10%). The authors did not analyze CSD, but according to Kopaliani *et al*. [2014] this breed has approximately 68% of haplotypes characteristic to East Asian wolf. Hence, the greater genetic similarity between Ch and CSD breeds and a larger genetic distance between the both breeds and BMD may be explained.

The presence of the same alleles in 10 microsatellite *loci* observed in all three dog breeds is an evidence of their belonging to one species. However, in each breed alleles not found in the other two breeds have been detected, which differentiates them.

Data on microsatellite polymorphism of dogs may be useful in studies concerning the segregation of microsatellite sequences associated with quantitative traits or genetic diseases. Because inbreeding occurs often in purebred dogs, genetic markers should be as much polymorphic as possible, which would increase chances of detection of markers associated with genetic diseases. The lowest EA, H and PIC values obtained for *loci* PEZ5 and PEZ20 indicate a low degree of variability and a low suitability of these microsatellite sequences to examine the origin and genome mapping of dogs.

Genetic diversity among the studied breeds may reflect genetic isolation, and thus the differences in gene flow between them in the past. The large variation of these breeds of dogs may also result from selection by man. The large values of genetic distance between BMD *versus* Ch and CSD breeds support the hypothesis of their separation at an early stage of domestication.

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