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Genetic diversity of native Japanese dog breeds in the primitive type

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Abstract: *Genetic diversity of native Japanese dog breeds in the primitive type*. In the following paper the genetic distance among selected Japanese dog breeds (Akita, Kishu, Kai, Shiba, Shikoku, Hokkaido) was estimated. In order to determine genetic differentiation we analyzed a fragment of the mitochondrial control region and 10 microsatellite loci. We found that variation of the nuclear DNA was larger than that at the level of mitochondrial DNA. Within a fragment of 614 bp of control region, 13 haplotypes were identified. The highest diversity of mitochondrial DNA was found in Akita. Based on mitochondrial DNA analysis, genetic distance between breeds ranged from 0.003 to 0.018. The lowest genetic distance was observed between Shikoku and Kai (0.003), and the highest between Shiba and Akita (0.018). After the analysis of nuclear DNA, 65 alleles were identified. The mean percentage of polymorphic alleles in the applied microsatellite markers was 94.4% ±2.5%. The analysis showed that**,** the highest distance estimated on the frequency of microsatellite markers was found between Kai and Hokkaido (1.066), and the lowest between Shiba and Kai (0.212). Hokkaido and Shikoku were the only clearly distinguishing breeds.

Key words: *Canis lupus familiaris*, mtDNA, microsatellites, control region, primitive breeds

INTRODUCTION

Japan is famous for six native breeds of dogs in the primitive type (Fig. 1), which due to the size of the body, can be divided into three groups. Large breed includes Akita, medium breeds: Kishu, Shikoku, Kai and Hokkaido, while Shiba is classified as a group of small breeds. In 1928 in Japan an association was established (Nihon Ken Hozonkai – NIPPO) aimed at standardizing and establishing the only appropriate patterns of the six breeds and above all protecting these breeds as a National Heritage (Tanabe 1991, Anderson 2012). The first breed to be protected was Akita (1931) and successively: Kishu and Kai (1934), Shiba (1936), and Shikoku and Hokkaido (1937).

Over the years, researchers have developed a number of methods to distinguish dog breeds and to study genetic differentiation among them, as well as among individuals between the breeds. The comparative analysis of blood proteins initially used (Tanabe 1991) proved to be an insufficient tool and gave way to other, more polymorphic, genetic markers. Currently, to assess genetic variability in dogs' populations, analysis based on nuclear or mitochondrial DNA (mtD-
-NA) are used. These analyzes include i.a.: polymorphism analysis of the mitochondrial control region of DNA (Okumura et al. 1996, 1998, 1999, Webb et al. 2009, Sindičić et al. 2011, Sugiyama et al. 2013), microsatellite length polymorphism analyzes (Koskinen and Bred-

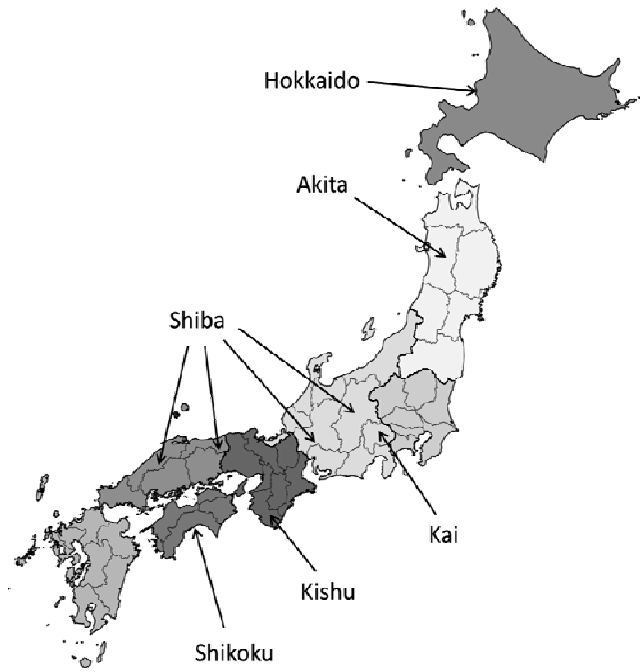

FIGURE 1. Map of Japan with division into the prefectures. The arrows indicate the origin of the breed

backa 2000, Kim et al. 2001, Irion et al. 2003, Schelling et al. 2004, Cho 2005, Ye et al. 2009, Phavaphutanon and Laop 2011), and analysis of coding sequences (Niimi et al. 2001).

Here we investigated the genetic differentiation among six Japanese dog breeds by analysis of DNA diversity of the mitochondrial control region, and by using ten microsatellites to determine the genetic distance between them. We assumed that due to the available literature of researches conducted on Japanese dogs, the Japanese dog breeds will not demonstrate large genetic distinctiveness for the sake of originating from a specific, often isolated, geographical area. The presented research is the first one in which the interpretation of the results of the two types of genetic markers was undertaken to determine the distance of selected Japanese dog breeds.

MATERIALS AND METHODS

Hair samples and buccal swabs used in this study were obtained from 85 individual dogs, representing six breeds. The sample sizes were as follows: Akita (N = 27), Shiba (N = 24), Hokkaido (N = = 11), Kai (N = 9), Kishu (N = 8), Shikoku (N = 6). The biological samples were accompanied by pedigree data of each individual, which was used to select unrelated (to fifth generation) representatives of each of the investigated breeds. Selection of samples for further analysis was based on the measurement of concentration, purity and degree of DNA

degradation (electrophoresis in an agarose gel). Samples characterized by low DNA concentration, a significant degree of contamination or degraded were rejected. Taking into account dog relatedness, the low DNA content of extracted samples and quality of genetic material, final number of analyzed samples was 21 in the case of mitochondrial DNA analysis: Akita (N = 2), Shiba (N = 5), Hokkaido (N = 4), Kai (N = 5), Kishu (N = 2), Shikoku (N = 3), and 27 for microsatellite loci analysis Akita (N = 7), Shiba (N = 5), Hokkaido (N = 4), Kai (N = 3), Kishu (N = 4), Shikoku (N = 4).

Molecular genetic analysis

DNA was extracted using the GeneMATRIX Tissue & Bacterial DNA Purification Kit (EURx) according to the manufacturers' instructions. The purity and concentration of DNA was measured in Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). The average concentration was 15.1 ng/µl, while the average purity at wavelengths 260/280nm was 2.25. Samples for which a satisfactory result was obtained were subjected to electrophoresis in a 1.5% agarose gel to assess DNA integration. The material thus obtained was stored at –20°C.

Analysis of mitochondrial DNA control region was based on the primer's sequences described by Oskarsson et al. (2011). The PCR reaction mixture containing 5 µl of DNA, 2.5 µl of each 10 µM primers, 25 µl of PCR Master Mix (EURx) and 20 µl of milliQ water. The PCR reaction was performed under the following conditions: initial denaturation 95^{o}C for 4 min, followed by 30 cycles at 90°C for 30 s, 60°C for 45 s, 72°C for 45 s and final elongation 72°C for 15 minutes. The amplified products were purified using the QIAquick PCR Purification Kit (QIAGEN) with standard protocol. The Sanger sequencing PCR profile was: initial denaturation 96°C for 3 minutes, followed by 30 cycles at 96°C for 30 s, 55°C for 30 s, 60°C for 2 min using the BigDye Terminator set v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturers' instructions. The material thus obtained was once again purified (BigDye XTerminator™ Purification Kit – Applied Biosystems) and sequenced in an ABI 3500 Genetic Analyzer (Applied Biosystems).

For fragment length polymorphism analysis, the first microsatellite set for dog genotyping accepted by ISAG was used. The 10 used microsatellite loci (FH2010, FH2054, FH2079, PEZ1, PEZ3, PEZ5, PEZ6, PEZ8, PEZ12, PEZ20) were amplified using the StockMarks® Dog Genotyping Kit (Thermo Fisher Scientific) containing the AmpliTaq Gold™ 360 DNA Polymerase. Touchdown PCR cycles were used: initial denaturation 94°C for 10 minutes, followed by 20 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 1 min with a decrease of the annealing temperature of 0.1°C for each cycle, followed by 15 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min with a decrease of the annealing temperature of 0.2°C for each cycle, and 72°C final elongation for 30 min. The PCR products were subjected to electrophoresis in an ABI 3500 Genetic Analyzer (Applied Biosystems).

Statistical analysis

The sequencing results were analyzed and compared in the GeneDoc program (Nicholas et al. 1997). The Mega6 pro-

gram (Tamura et al. 2013) was used to obtain the number of haplotypes and nucleotides diversity. The mean genetic distance (Maximum Composite Likelihood model) between breeds was also estimated (Tamura et al. 2004, 2013).

Due to the very small number of individuals within the breed, in fragment length polymorphism analysis, only the average number of alleles per locus and the genetic distance based on Fst coefficient between the breeds were calculated (Weir and Cockerham 1984). We also checked whether the tested markers showed polymorphism. These analysis were performed using the GenAlex 6.5 software (Peakall and Smouse 2006, 2012). Similarities and differences between the individuals, including breed affiliation, were obtained using a plot created in Structure 2.3.4 software (Pritchard et al. 2000). The number of different groups was selected using the Structure Harvester (Earl and von Holdt 2012), searching the highest rate of change of the likelihood distribution, with K from 1 to 6 (Evanno et al. 2005).

RESULTS

Analysis of mitochondrial DNA

After the sequencing, a product with a length of 614 bp was obtained. 31 polymorphic sites for all breeds were identified (Table 1). It was observed that the vast majority were transitions – 87.1%, which consisted of replacing: cytosine for thymine (64.5%) and adenine for guanine (22.7%). Transversions were found only in 9.7% of cases (replacement of adenine to cytosine – 6.4%, adenine to thymine – 3.2%). In one case (3.2%) deletion was observed.

TABLE 1. The polymorphic sites observed in the sequenced control region

Position (nucleotide number)	Type of change
4, 29, 48, 116, 142, 151, 153, 230, 374, 445, 553	C → T
132, 133, 141, 156, 170, 185, 284, 333, 347, 348	T → C
40, 483	A → C
148, 155, 163, 172, 631, 611	A → G
159	A → T/G
466	G → deletion

Source: own research.

13 haplotypes were obtained. The average differentiation of haplotypes within the breed range 0.9% and overall 1%. For individual breed, the number of different haplotypes was respectively (in brackets): Akita N = 2 (2), Hokkaido N = 4 (2), Kai N = 5 (2), Kishu N = 2 (2), Shiba N = 5 (3) and Shikoku N = 3 (2). The phylogenetic distance illustrates the similarity of the breeds in terms of the analyzed mitochondrial control region (Table 2).

TABLE 2. Genetic distance (Maximum Composite Likelihood model) between breeds, estimated based on mitochondrial control region.

	Akita	Hokkaido	Kai	Kishu	Shiba	Shikoku
Akita						
Hokkaido	0.016					
Kai	0.016	0.006				
Kishu	0.015	0.006	0.006			
Shiba	0.018	0.008	0.010	0.010		
Shikoku	0.015	0.005	0.003	0.005	0.009	

The number of base substitutions per site from averaging over all sequence pairs between groups are shown in Table 1. Analyses were conducted using the Maximum Composite. Values between breeds ranged from 0.003 to 0.018 (Table 2). The lowest genetic distance estimated on mitochondrial control region analysis was observed between Shikoku and Kai (0.003), and the highest between Shiba and Akita (0.018). The most diverse haplotypes and the greatest distance between breeds showed Akita sequences.

Analysis of nuclear DNA

We successfully amplified 10 microsatellite loci in 27 dogs. We identified 65 alleles. The mean percentage of polymorphic alleles in the applied microsatellite markers was 94.4% ±2.5%. The average number of alleles identified in the breed was 3.3 per locus, and the average number of effective alleles in the breed was 2.5. The genetic distance estimated based on the Fst coefficient (Table 3), showed that the most consolidated and the least similar to the compared breeds are Shikoku and Hokkaido. The pairwise population matrix showed that highest distance (1.066) was found between Kai and Hokkaido. Lowest distance was found between Shiba and Kai (0.212).

Genetic profiles of all examined dogs were compared to each other and then the Structure Harvester Software estimated the most probable number of genetic groups (k). For the six compared breeds, four genetic groups ($k = 4$) were identified, of which only two corresponded to a

TABLE 3. Pairwise Population Matrix of Genetic Distance based on Fst coefficient between breeds

	Akita	Hokkaido	Kai	Kishu	Shiba	Shikoku
Akita						
Hokkaido	0.437					
Kai	0.507	1.066				
Kishu	0.505	0.931	0.609			
Shiba	0.417	0.579	0.212	0.646		
Shikoku	0.875	1.047	0.613	0.662	0.485	

Source: own research.

given breed. The plot (Fig. 2) accurately shows, that dogs belonged to Hokkaido and Shikoku breeds were assigned to two different groups according to their breed. The individuals from other breeds have different genotypes, but the breed recognizing is no longer possible – their genotypes represent mostly two genetic groups.

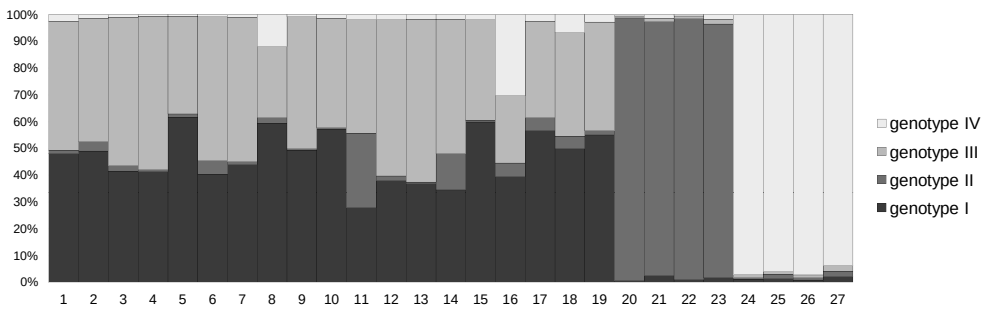

FIGURE 2. Distribution of four typed groups among the tested individuals from six breeds

DISCUSSION

This research was aimed at characterization the genetic differentiation among six Japanese dog breeds. The DNA variation of the mitochondrial control region and fragment length polymorphisms were used to estimate genetic relationship between studied breeds.

The analysis showed that in the analyzed control region (Table 2) differences between breeds are small and shows low genetic variation. Identified different haplotypes in each breed is not surprised, despite the fact, that investigated animals were unrelated and came from different places. In 1996, phylogenetic analysis within the non-coding region of mitochondrial DNA showed that individuals native to Japanese breeds cannot be unambiguously identified. The occurrence of the same haplotypes was observed in individuals assigned to different clusters, which may indicate a small difference and genetic variability between these breeds (Okumura et al. 1996). Moreover, the same author (Okumura et al. 1998) analyzed the variability within the cytochrome b gene. The results again did not yield an answer, the breeds were not clearly identified, which indicates that these breeds were probably crossed in the past. From breeders standpoint, in general, but also in Japan, it is popular to bred dogs not only in the purity of the breed, but also in the cleanliness of the line. Despite the fact that mitochondrial DNA is not subject to selection as such, due to the use of precisely selected (usually by specific phenotypic traits) maternal lines, certain haplotypes could be fixed. This process may be reflected in existing dogs. Our findings showed that the lowest genetic distance was observed between Shikoku and Kai (0.003). The highest genetic distance was observed between Shiba and Akita (0.018). The analysis carried out were not confirmed by the relation between Akita and Shiba

described by previous research (Tanabe 1991). Researcher showed that one of the varieties of local Shiba breed is probably closely related to Akita, which may probably ensue from the fact that these dogs were found in neighboring prefectures in Japan. The most diverse haplotypes and the greatest distance between breeds was showed by Akita. It can be speculating that this result is caused by an American Akita line among ancestors of one of the analyzed Akita individuals (belonging to the Japanese Akita breed). The American Akita breed is a separate dog breed, in which creation dogs from the Japanese Akita were involved. The second part of the research consist of the length polymorphism analysis in 10 microsatellite loci. Sixty-five alleles were identified. All these microsatellite markers are routinely used for the control of origin, which is why in groups of low-related individuals should show a large polymorphism. We should note, that investigated groups are small and the number 3.3 alleles per locus is probably not in accordance with expectations for such polymorphic loci. Therefore, it turned out that the lowest distance (0.212) was found between Shiba and Kai. The close links may result from the region of origin, where the Gifu and Nagano prefectures (Shiba) are adjacent to the Yamanashi prefecture (Kai). The highest distance (1.066), was found between Kai and Hokkaido, what may be the result of island isolation of Hokkaido. Moreover, results showed that Shikoku and Hokkaido are the homogenous and the most differentiated from the other compared breeds. After identification of 4 characteristic genetic groups among six breeds (Fig. 2), these two breeds were the only one ones, which correspond to identified groups. This distinction from other dogs most likely is caused by the fact that these breeds remained isolated on the islands (Fig. 1). This may prove that both breeds share a certain part of the microsatellite loci that are characteristic for them. On the contrary, it is clearly visible that remained breeds present high similarity in investigated alleles frequency.

First of all, we should pay attention to a small sample size in our research which does not allow for a detailed comparison. The obtained results of microsatellite loci analysis do not fully correspond to Kim et al. (2001) results. Authors were analyzed the microsatellite panel in selected dog breeds, but only in four Japanese (Akita, Hokkaido, Kishu and Shiba). The lowest genetic distance estimated by Kim et al. (2001) was 0.1510 between Akita and Hokkaido, and the highest was 0.2923 between Kishu and Akita. Comparing our results, it was, respectively, 0.437 and 0.505 for these breeds. The authors stated that Japanese breeds showed low level of variation, but it might be an effect of inbreeding and crossing among breeds. In addition, Shiba was characterized by the lowest genetic diversity among the tested Japanese breeds. The outcomes may suggest that Japanese breeds come not from one, but from several different populations, and then they crossed with each other.

To conclude, intensive breeding work lead to the consolidation of individual phenotypic traits in the breed, which in turn leads to high diversification in nuclear DNA between breeds. The great attention should be placed on the purity of

the breed, but it should be kept in mind that this might affect the inbreeding problem (Rooney 2009, Leroy 2011).

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Streszczenie: *Różnorodność genetyczna rodzimych ras psów japońskich w typie pierwotnym.* W poniższym artykule oszacowano dystans genetyczny pomiędzy wybranymi rasami psów japońskich (Akita, Kishu, Kai, Shiba, Shikoku, Hokkaido). W celu określenia zróżnicowania genetycznego przeanalizowaliśmy fragment mitochondrialnego regionu kontrolnego i 10 mikrosatelitarnych loci. Stwierdziliśmy, że zmienność jądrowego DNA była większa niż na poziomie mitochondrialnego DNA. W obrębie fragmentu 614 bp regionu kontrolnego zidentyfikowano 13 haplotypów. Największą różnorodność DNA mitochondrialnego stwierdzono w rasie Akita. Na podstawie analizy mitochondrialnego DNA dystans genetyczny między rasami wahał się od 0,003 do 0,018. Najmniejszy dystans genetyczny zaobserwowano pomiędzy rasami Shikoku i Kai (0,003), a największy pomiędzy rasami Shiba i Akita (0,018). Po analizie jądrowego DNA zidentyfikowano 65 alleli. Średni procent polimorficznych alleli w zastosowanych markerach mikrosatelitarnych wynosił 94,4% ±2,5%. Analiza wykazała, że największą odległość oszacowaną na podstawie częstotliwości markerów mikrosatelitarnych stwierdzono między rasami Kai i Hokkaido (1,066), a najmniejszą pomiędzy rasami Shiba i Kai (0,212). Hokkaido i Shikoku były jedynymi wyraźnie wyróżniającymi się rasami.

Słowa kluczowe: Canis lupus familiaris, mtDNA, sekwencjonowanie, markery mikrosatelitarne

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