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Evaluation of Genetic Biodiversity in Farm-bred and Wild Raccoon Dogs in Poland*

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The aim of the study was to analyze the intra- and inter-group diversity in farm-raised and wild raccoon dogs with the use of molecular markers. Genetic differences between the particular raccoon dog groups were observed, accompanied by a relatively high intra-group genetic variation. It was noted that the wild raccoon dogs were characterized by the highest genetic diversity, compared to the three study groups of farm-bred raccoon dogs. Wild raccoon dogs and farm-bred raccoon dogs constitute separate phylogenetic groups. The results obtained suggest that farm breeding may lead to differentiation into a different phylogenetic lineage than that of the wild raccoon dogs. In each case, the genetic distance between the animals bred on the individual farms was lower than the distances between the farm-raised and wild animals. Since the Polish farm breeding is based entirely on phenotype ranking, the genotype of "native" animals is still closely related to that of wild animals.

Key words: *Nyctereutes procyonoides*, biodiversity, phylogenesis.

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Conservation of biological diversity is one of the key issues in breeding. Improvement of fur-bearing animals is achieved by selection based on the phenotypes of usability traits, in which relatedness between mating animals is avoided and genetic markers are not taken into account. Such practice usually leads to a reduction in genetic diversity and subsequent elimination of a certain gene pool, which is accompanied by the discarding of usability traits that are undesirable in breeding. Breeding should aim at conserving population biodiversity; therefore, it should make use of modern methods of diversity evaluation. The development of molecular methods has provided the possibilities of using DNA polymorphism in the analysis of animal diversity and has thus yielded new information about species. ARIF and KHAN (2009) point out that the RAPD markers are most frequently used in genetic diversity analyses in various animal species. STEPNIAK *et al.* (2002) confirmed the applicability of four RAPD markers in the phylogenetic analysis of four species of the family Canidae. The use of molecular markers has facilitated comprehensive analyses of the genetic structure of various animal species. It also allows monitoring of genetic diversity, the determination of which is an essential element of biodiversity conservation.

The raccoon dog (*Nyctereutes procyonoides procyonoides*) has been bred in Poland for thirty years. The Far East, and more specifically, the area of the Amur and Ussuri river basins, is its homeland. In 1927, approximately 9000 raccoon dogs were in-

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troduced into the forested areas in the north of the European part of the former USSR, where they became perfectly acclimated. They spread from that region and in the seventies they were found in Finland, Sweden and Poland. In 1971-1972, wild raccoon dogs were hunted and adapted to breeding. Successful breeding was started in Poland in 1979, when 200 raccoon dogs were imported from Finland. Thus, virtually all the animals bred both in Finland and in Poland nowadays (raccoon dogs are bred in these European Union countries only) originate from individuals from the 9000 animals introduced into the north of the European part of the former USSR.

The use of molecular markers facilitates determination of the hypothetical phylogenetic distance between farm-bred and wild raccoon dogs as well as the degree of preservation of biological diversity of raccoon dogs bred in Poland. Selection of many generations of farm-raised raccoon dogs aiming at the improvement of the quality of usability traits may have resulted in remarkable changes in the genotypes of these animals. Therefore, monitoring of animal genetic resources should include a comprehensive analysis of their genetic structure with the use of genetic markers. The aim of this work was to analyze the intra- and intergroup diversity in three groups of farm-raised raccoon dogs and wild raccoon dogs.

Material and Methods

The study material was blood taken from 121 farm-raised raccoon dogs bred in three fur-bearing animal farms located in the podkarpackie voivodship – farm A (40 individuals), świętokrzyskie voivodship – farm B (38 animals) and mazowieckie voivodship – farm C (41 animals). Blood, tissues or skin were sampled for analysis from 6 wild raccoon dogs living in the lubelskie and świętokrzyskie voivoidships. DNA from the total peripheral blood was isolated with the use of the QIAamp DNA Blood Mini Kit (QIAGEN), whereas the QIAamp DNA Blood Kit (QIAGEN) with our modifications was used for isolation of DNA from the skin and tissues. Quantitative and qualitative analyses of genetic diversity were performed by RAPD-PCR. The reaction mixture (sample volume – 30 μl) contained 80 ng DNA; 3 μl PCR buffer, 4.2 μl Q solution, 200 μM of each nucleotide, 0.2 μM arbitrary primer, 25 mM $MgCl_2$, 1 U Taq polymerase. In order to determine which markers generated the highest diversity in raccoon dogs, 8 animals were included in the preliminary analyses and amplification was performed with the use of 20 arbitrary primers: OPG01-OPG20 (*Operon Technologies, Inc.* KIT G; Table 1). The preliminary study was composed

Table 1

Characteristics of the OPG01-OPG20 primers in raccoon dogs

Primer	Nucleotide sequence 5'›3'	Annealing temperature (°C)	Size range of amplified bands (bp)**	Tnb*	Poly*
OPG01	CTACGGAGGA	36	170-2000	21	16
OPG02	GGCACTGAGG	36	250-700	8	1
OPG03	GAGCCCTCCA	36	280-950	10	2
OPG04	AGCGTGTCTG	36; 50	–	–	–
OPG05	CTGAGACGGA	36; 50	–	–	–
OPG06	GTGCCTAACC	36	170-1200	12	0
OPG07	GAACCTGCGG	36	280-900	6	5
OPG08	TCACGTCCAC	36	300-950	10	2
OPG09	CTGACGTCAC	50	180-900	10	2
OPG10	AGGGCCGTCT	50	170-1000	10	7
OPG11	TGCCCGTCGT	36	200-1500	14	12
OPG12	CAGCTCACGA	36	220-1500	12	12
OPG13	CTCTCCGCCA	36	200-950	13	6
OPG14	GGATGAGACC	50	170-1000	10	0
OPG15	ACTGGGACTC	50	300-750	8	0
OPG16	AGCGTCCTCC	36	150-800	11	7
OPG17	ACGACCGACA	50	150-1100	14	0
OPG18	GGCTCATGTG	36	150-1000	13	11
OPG19	GTCAGGGCAA	50	200-800	11	0
OPG20	TCTCCCTCAG	36	320-700	3	0

* – Tnb – total number of bands
* – Poly – number of polymorphic bands
** – Approximate values

of 320 analyses. Three (OPG01, OPG12 and OPG18) out of the twenty arbitrary primers were used in the subsequent study (375 analyses).

The amplification reaction consisted in preliminary denaturation (94°C, 5 min); 46 cycles of denaturation (94°C, 1 min), primer binding (36°C, 2 min) and lengthening of DNA strands (72°C, 1 min); terminal lengthening of primers (72°C, 10 min) and cooling to temperature 4°C. The RAPD-PCR products were fractioned in 2% agarose gel (using bromophenol blue loading buffer). The gels were analyzed in UV light (Transilluminator) and archived with the use of Scion Image software (Syngen Biotech). O'GeneRuler 50bp DNA Ladder and GeneRuler 100bp DNA Ladder, Fermentas, were used as markers of the volume of the DNA fragments. The RAPD analysis yielded amplification products of all the primers used in the study, both at the temperature of primer binding 36°C and at 50°C. OPG4 and OPG5 primers (at both temperatures) and OPG8 (at 50°C) were discarded due to their incompatibility with the criteria of efficiency and of the quality of the band pattern obtained the gel.

The use of the two values of primer binding temperature aimed at determination of the appropriate temperature in DNA amplification for *Nyctereutes procyonoides procyonoides*. According to ATIENZAR *et al.* (2000), the above-mentioned temperature for OPG1-OPG20 primers was 50-54°C. However, unsatisfactory quality of the band pattern in these temperature ranges advocated finding a solution to the aforementioned problem. In 12 (OPG01, OPG02, OPG03, OPG06, OPG07, OPG08, OPG11, OPG12, OPG13, OPG16, OPG18, OPG20) out of 17 primers analyzed in the present study, higher amplification efficiency was observed at 36°C, which is inconsistent with the literature reports (ATIENZAR *et al.* 2000).

Not all the studied primers generated polymorphic bands (Table 1). The highest H values were obtained in the case of OPG12, OPG01 and OPG18 (0.386, 0.340 and 0.319, respectively). Primers with the largest number of polymorphic loci and the highest diversity (OPG01, OPG12 and OPG18) were used for determination of the genetic diversity in the four raccoon dog study groups.

The statistical analyses of the band patterns in the separate raccoon dog groups comprised: the mean number of bands (mnb), band sharing index of similarity (BS) (NEI & LI 1979; LYNCH 1990), average percent difference (ADP), the unbiased estimation of the number of loci calculated from the band frequency (L) (STEPHENS *et al.* 1992), the mean heterozygosity (H), probability of identity (P_{ID}) (JEFFREYS & MORTON 1987) and the average variability of bands (V) (KUHNLEIN *et al.* 1989). The genetic distance and similarity between the 4 study groups were determined with the method adapted by LYNCH (1991).

Results and Discussion

Table 2 presents the indices of biodiversity estimation in the studied raccoon dog groups. The mean number of bands ranged from 7.88 (farm C, OPG01) to 12 (wild raccoon dogs); the unbiased estimation of the number of loci (L) ranged between 5.39 (OPG18; wild) and 9.33 (farm C; OPG12). The lowest values of the band sharing index of similarity (BS) in the analyzed primers were

Table 2

Statistical characteristics of the selected RAPD primers in relation to the analyzed raccoon dog groups

Primer	Raccoon dogs	Statistical indices						
		mnb	BS	ADP (%)	L	H	P_{ID}	V
OPG01	Farm A	9.919	0.572	28.601	6.421	0.538	8.61e-06	0.973
	Farm B	10.325	0.711	35.540	7.624	0.350	4.56e-04	0.975
	Farm C	7.878	0.693	34.670	5.950	0.321	1.85e-03	0.976
	Wild	10.000	0.335	16.756	5.929	0.605	2.26e-08	0.750
OPG12	Farm A	8.216	0.755	37.766	5.891	0.391	6.61e-03	0.973
	Farm B	9.025	0.804	40.206	6.766	0.331	1.43e-02	0.975
	Farm C	10.049	0.931	46.547	9.328	0.077	2.26e-01	0.976
	Wild	12.000	0.588	29.425	8.202	0.405	1.36e-06	0.750
OPG18	Farm A	9.000	0.791	39.528	6.711	0.337	1.03e-02	0.973
	Farm B	8.750	0.833	41.633	7.127	0.225	3.23e-02	0.975
	Farm C	9.512	0.857	42.841	8.029	0.183	4.39e-02	0.976
	Wild	8.000	0.548	27.381	5.386	0.432	4.57e-05	0.750

Table 3

Nei's genetic similarities (above the diagonal) and distances (below the diagonal) between the particular raccoon dog groups

	Farm A	Farm B	Farm C	Wild
Farm A	---	0.935	0.894	0.828
Farm B	0.067	---	0.881	0.812
Farm C	0.112	0.126	---	0.835
Wild	0.211	0.208	0.179	---

obtained in the wild raccoon dogs (BS from 0.34 to 0.59); these values were only slightly higher in animals from farm A. A various degree of intra-group diversity was observed with regard to: the average percent difference (ADP), the average variability of bands (AVB) and the mean heterozygosity (H). The lowest values of the ADP and AVB indices among the analyzed primers were characteristic for wild raccoon dogs and, in the case of ADP, for animals from farm A. The highest mean heterozygosity (H) in the primers in question was observed in the wild raccoon dogs (between 0.41 and 0.61), and among the farm-bred raccoon dogs in farm A animals (from 0.34 to 0.54). Irrespective of the marker used, the lowest probability of identity (P_{ID}) was noted in the wild raccoon dogs (from 2.26e-08 to 4.57e-05). The lowest P_{ID} value in the farm-raised animals was observed on farm A (Table 2).

The results indicate genetic differences between the particular animal groups; however, the raccoon dog groups from Poland displayed a relatively high intra-group genetic diversity.

Genetic distances and similarities were determined between the particular groups of individuals (Table 3).

On the basis of the genetic distances (Table 3) calculated from the RAPD analysis profiles, a tree diagram was generated to illustrate the phylogenetic relations in the four groups of raccoon dogs (Fig. 1). It should be mentioned that no wild raccoon dogs were introduced into any of the farms. The dendrogram is unrooted and contains no out groups; yet, the phylogenetic distance between the wild raccoon dogs to the three groups of farm-raised animals seems to be most obvious. Hypothetically, selection of farm-bred raccoon dogs has resulted in their genetic divergence. It is probable that today farm-raised and wild raccoon dogs constitute separate phylogenetic groups. In each case, the genetic distance between the animals from different farms is lower than that between the farm-bred and wild individuals (Table 3).

CHEN *et al.* (1998) performed analyses to distinguish raccoon dog subspecies. They used RAPD marker analysis in order to determine the genetic relationships of the raccoon dogs living on the territory of the People's Republic of China. On the basis of the molecular phylogenetic trees, they distinguished four groups: *Guangxi*, *Anhui*, *Shaanxi* and *Yunnan – Vietnam* raccoon dogs. They suggested that each group should be granted the status of a subspecies in the phylogenetic classification.

The dendrogram displaying the phylogenetic relations between the raccoon dogs (Fig. 1) indicates that the animals from farm A and B share the same phylogenetic branch. We can therefore conclude that the groups are related to each other more than the raccoon dogs from farm C. This may be explained by the fact that the animals had been imported to farm A (several times) and farm B (once) from Finnish farms. The animals from farm C may be called "native". Although they originate from the animals imported from Finland in 1979, the breeding procedures performed on the farm led to the differentiation of a distinct phylogenetic lineage in relation to animals from other farms (Fig. 1). Simultaneously, the so-called "native" raccoon dogs are phylogenetically closer to the wild individuals. Breeding on Polish farms is based entirely on phenotype ranking. The results obtained dem-

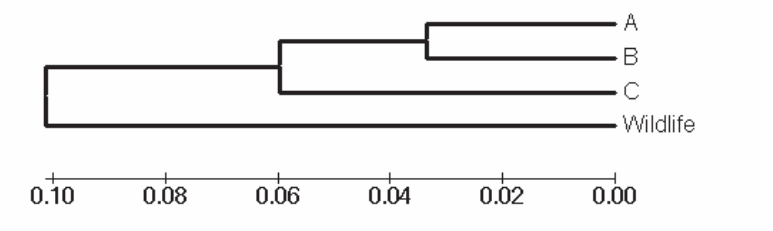

Fig. 1. Dendrogram of the hypothetical phylogenetic relations between the farm-bred raccoon dogs (farm A, B and C) and the wild raccoon dogs.

onstrate that such practice is ineffective in genetic improvement of animals, as the "native" raccoon dogs are still highly related to the wild individuals as far as their genotype is concerned.

As reported by ŚLASKA (2001) in her research conducted in 1997-1999, the mean body weight of native raccoon dogs was significantly lower than that of the raccoon dogs which had Finnish raccoon dog genes (62.5-100%) and amounted to 8.75 kg. A similar dependency was observed in the total conformation estimation. A significantly lower total conformation was noted in native raccoon dogs (16.07 points) in comparison with the value of this parameter in the raccoon dogs with varied participation of Finnish raccoon dog genes.

Simultaneously, it should be emphasized that the genetic variability observed in the present experiment resulted in a change in the phenotype over a relatively short time. The measureable parameters which underwent significant changes during several years include the body weight and the total conformation estimation (mainly characterising the coat traits). According to ŚLASKA [2001], the mean body weight in 1997 was 8.8 kg and it differed significantly from the mean body weight noted in 1999 (9.2 kg). Similarly, the total conformation evaluation was significantly lower in 1997 (16.37 points) in comparison with that in 1999 (16.62 points). These upgraded traits were a result of the import (to the study farm) of 30 Finnish raccoon dogs, which already at that time displayed higher body weight and higher conformation values. During subsequent years, until 2009, selection was performed on the study farm for bigger body weight and upgrading of the animal coat traits with concomitant, sporadic import of raccoon dogs from Finland. The mean body weight of the animals as well as the total conformation estimation were increasingly upgraded to reach 11.9 kg (at the maximal weight – 15.3) and 17.8 points, respectively, in 2009 (own unpublished study, 2009). Therefore, the genetic differences observed in the present study were accompanied by a distinct change in the animal phenotype as far as the raccoon dog selected usability traits are concerned.

Animal selection in Scandinavian countries is based on the breeding value of individuals, which undoubtedly contributes to the genetic progress of the improved traits and to an equalized level of the population phenotypic traits; this results in decreased genetic diversity in farm-raised populations. It appears that the import of raccoon dogs from Finland leads to an increased genetic distance between the farm-bred and wild animals. An unequivocal confirmation of this conclusion necessitates further studies that would include farm-raised animals bred in Finland.

The intra-group biostatistical analyses performed in the present study show that the wild raccoon dogs are characterized by the highest level of genetic diversity, compared to the three groups of the farm-raised animals. Selection conducted on farms reduces genetic diversity; however, the decline seems to be related to the system of evaluation and animal selection on farms. The choice of individuals for the basic stock based exclusively on the level of the phenotypic traits decreases genetic diversity to a lesser extent, leading to higher similarity between farm-bred animals, compared to the wild individuals. The results obtained imply that, compared to the wild raccoon dogs, farm breeding results in the differentiation of a new phylogenetic line.

The results of the RAPD technique used provide new information, useful in prevention of the loss of genetic diversity in the population of raccoon dogs.

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