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Aspects regarding the molecular characterisation of the Romanian Shepherd dog breeds

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Abstract. There are four shepherd dog breeds at the present time in Romania: the Bucovina Shepherd dog, the Carpathian Shepherd dog, the Mioritic Shepherd dog and the Corb Shepherd dog. The first three breeds have been provisory homologated by the F.C.I. The Corb Shepherd dog breed is only recognized in Romania. In this article we present the steps we have performed in order to obtain the mitochondrial DNA nucleotide sequences for several individuals belonging to the three homologated Romanian dog breeds, in order to be used for later phylogenetic studies.

Key Words: mtDNA, Romanian Shepherd dog breeds, nucleotide.

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Introduction

The Romanian Shepherd dog breeds have not been seriously taken into account by breeders as far as the 1980's. Since then people started to recognize the importance of the autochthonous dog breeds and the great value they provide for our national heritage. Since no molecular studies have been performed so far on these dog breeds it is imperative that they are also characterised from the molecular point of view.

Mitochondrial DNA has been successfully used so far for estimating the phylogenetic structure, genetic diversity and recent history of domestic animals (Bruford *et al* 2003; Kim *et al* 1998). A drawback of this technique resides in the fact that it is limited, as it can provide data concerning only the female lineage (Pires *et al* 2006). The most comprehensive study based on mtDNA sequencing (Savolainen *et al* 2002) included samples from various dog breeds belonging to four continents: Africa, Asia, Arctic America and Europe. The study concluded that at least five wolf matrilines stand at the foundation of the domestic dog populations. This conclusion is based on the fact that from the six clades which resulted from the phylogenetic analysis (A, B, C, D, E, and F), except clade F, are intermingled with wolf mtDNA sequences (see also Lindblad-Toh *et al* 2005).

Materials and Methods

In order to obtain the mtDNA sequences for the Bucovina Shepherd dog, Carpathian Shepherd dog and Mioritic Shepherd dog we have performed the following steps:

• collecting the blood samples from individuals belonging to the three Romanian Shepherd dog breeds;
• mtDNA extraction from the collected blood samples;
• sequencing of the mtDNA.

The blood samples have been collected from a number of 78 unrelated individuals at dog shows and from breeding kennels (Table 1). The selection of the animals was based on their ancestry in order to exclude related animals and also based on the morphological standards for each breed.

Table 1. Number of sampled individuals from each breed (male and female)

No.	Breed	Males	Females	Total (males+ females)
1	Bucovina Shepherd dog	22	15	37
2	Carpathian Shepherd dog	8	6	14
3	Mioritic Shepherd dog	10	17	27
4	Total	40	38	78

The blood samples were collected into vacutainers containing ethylenediaminetetraacetic acid (EDTA) and kept frozen until processed. The blood quantity collected in each vacutainer was about 2-3 mL. The DNA extraction was performed from whole blood using a quick blood extraction protocol proposed by the Laboratory of Veterinary Genetics, University of California, Davis, CA 95616, modified by Yves Amigues, INRA, Jouy-en-Josas programme. The DNA purity and quantity were assessed using the Nanodrop ND-1000UV/VIS spectrophotometer. The amplification of the DNA fragments was performed using the Bioline Amplification kit in a Biorad IQ5 thermocycler. The primers that we used are L15210 5'-ACA TGA ATT GGA GGA CAA CCA GT-3' (a shortened version of Shields & Kocher's (1991) L15774 primer) and H16097 5'-TAT GTC CTG TGA CCA TTG ACT GA-3' (S. Funk, Institute of Zoology, London). This primer pair was also used by A. E. Pires *et al* in 2006, during a phylogenetic study on the Portuguese native dog breeds. The electrophoresis was carried out in an 2% agarose gel with T.A.E. 0.5x buffer solution (Tris 242 g, EDTA 18.6g pH 8.00, H2O 700 mL, and glacial acetic acid 90mL pH 7.8 for a 1000 mL final solution volume). We also used: 3 µL Smart ladder and 2 µL Loading dye (see Figure 2).

DNA sequencing was performed at Macrogen Europe (Amsterdam, The Netherlands). The primers used for sequencing were the

Plots | Report

Test type: Nucleic Acid | 07.03.2011 14:08 | Exit

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Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
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CM3	Default	07.03.2011	13:33	141,31	2,826	1,946	1,45	0,24	50,00	230	11,674	2,284
CM4	Default	07.03.2011	13:35	334,26	6,685	5,225	1,28	0,56	50,00	230	11,836	6,490
CM4	Default	07.03.2011	13:36	336,54	6,731	5,284	1,27	0,61	50,00	230	11,042	6,205
CM5	Default	07.03.2011	13:38	87,66	1,753	1,304	1,34	0,26	50,00	230	6,843	0,984
CM6	Default	07.03.2011	13:40	164,43	3,289	2,335	1,41	0,29	50,00	230	11,492	2,559
CM7	Default	07.03.2011	13:41	188,64	3,773	2,775	1,36	0,35	50,00	230	10,704	1,347
CM8	Default	07.03.2011	13:43	267,49	5,350	3,725	1,44	0,43	50,00	230	12,536	1,429
CB2	Default	07.03.2011	13:44	214,67	4,293	2,961	1,45	0,40	50,00	230	10,669	1,734
CB3	Default	07.03.2011	13:46	202,49	4,050	3,332	1,22	0,44	50,00	230	9,108	8,219
CB4	Default	07.03.2011	13:47	201,64	4,033	2,693	1,50	0,34	50,00	230	11,910	2,526
CB5	Default	07.03.2011	13:49	609,14	12,183	1,250	9,75	6,73	50,00	230	1,810	30,691
CB5	Default	07.03.2011	13:50	102,82	2,056	1,717	1,20	0,36	50,00	230	5,679	2,955
CB6	Default	07.03.2011	13:52	460,18	9,204	6,581	1,40	0,71	50,00	230	13,038	6,098
CB6	Default	07.03.2011	13:53	184,39	3,688	2,520	1,46	0,39	50,00	230	9,550	19,795
CB7	Default	07.03.2011	13:55	395,26	7,905	6,178	1,28	0,52	50,00	230	15,327	4,368
CC1	Default	07.03.2011	13:56	248,17	4,963	4,050	1,23	0,36	50,00	230	13,655	2,638
CC2	Default	07.03.2011	13:58	302,37	6,047	4,106	1,47	0,47	50,00	230	12,975	1,856
CC3	Default	07.03.2011	14:00	160,38	3,208	2,431	1,32	0,26	50,00	230	12,400	3,258
CC4	Default	07.03.2011	14:01	135,06	2,701	1,482	1,82	0,30	50,00	230	8,978	1,973
CC4	Default	07.03.2011	14:03	133,54	2,671	1,405	1,90	0,30	50,00	230	8,884	1,758
CC5	Default	07.03.2011	14:04	309,57	6,191	4,424	1,40	0,45	50,00	230	13,705	9,847
CC5	Default	07.03.2011	14:06	361,86	7,237	4,938	1,47	0,81	50,00	230	8,960	16,474

Figure 1. The results of the DNA quantification using the Nanodrop ND-1000UV/VIS spectrophotometer. CB - Bucovina Shepherd dog, CC - Carpathian Shepherd dog, CM - Mioritic Shepherd dog

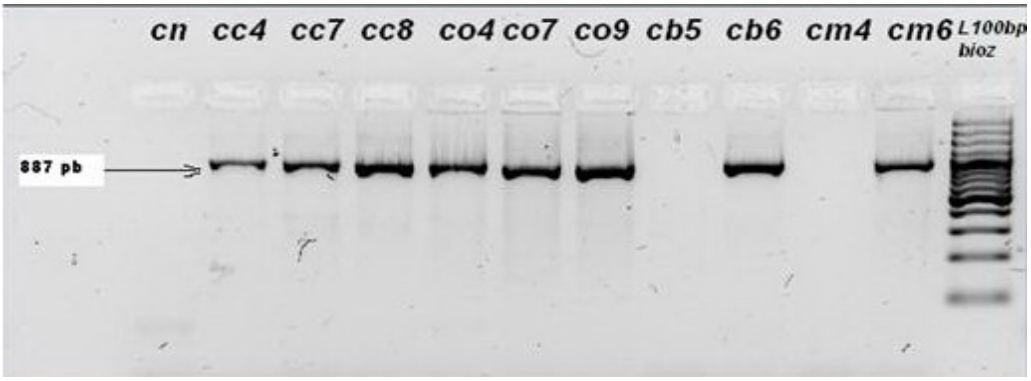

Figure 2. The migration profiles of the DNA samples after the electrophoresis in agarose gel (CC - Carpathian Shepherd dog, CB - Bucovina Shepherd dog, CM - Mioritic Shepherd dog)

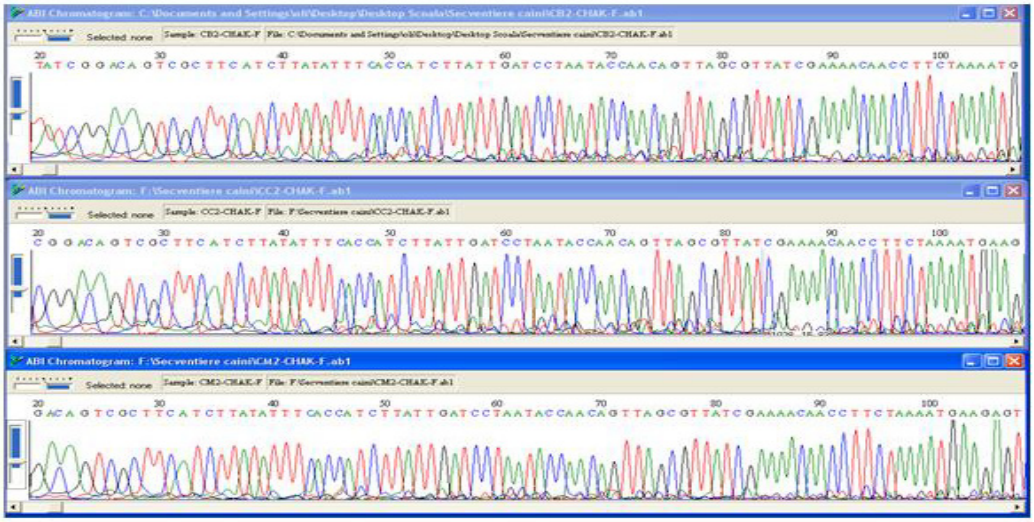

Figure 3. The sequencing chromatograms for the three Romanian dog breeds: CC - Carpathian Shepherd dog, CB - Bucovina Shepherd dog, CM - Mioritic Shepherd dog

same as the amplification primers. The target mtDNA sequence has a length of 887 base pairs and consists of a segment of the cytochrome b, the tRNA-Thr, the tRNA-Pro, and a segment of the control region.

Results and Discussion

The DNA extraction results show that the extraction protocol we used is well suited for extracting DNA from canine blood. The mean value for the DNA quantity was 267.24 ng/µL, ranging between 40.86 ng/µL (lowest value) and 609.14 ng/µL (highest value). Regarding DNA purity the lowest recorded value was 1.20 and the highest value was 1.90. The mean value for DNA purity was 1.41 (Figure 1). The sequencing of the 887 bp mtDNA has provided us the chromatograms corresponding to the mtDNA sequences for the Carpathian Shepherd dog, Bucovina Shepherd dog and the Mioritic Shepherd dog (Figure 3).

The alignment of the mtDNA sequences was performed with the Clustal W2 alignment tool available at the EMBL-EBI database. The alignment data has shown the following: there were only 3 nucleotide substitutions between the Bucovina Shepherd dog sequence and the Carpathian Shepherd dog sequence, between the Bucovina Shepherd dog sequence and the Mioritic Shepherd dog there were 14 nucleotide substitutions, while between the Carpathian Shepherd dog sequence and the Mioritic Shepherd dog sequence there were 16 nucleotide substitutions. The small number of substitutions between the first two sequences is due to the fact that these dog breeds were considered as one (Carpathian Shepherd dog) and were bred together, until the first breed standard for the Bucovina Shepherd dog was elaborated in 1982. The large number of substitutions between the Mioritic Shepherd dog mtDNA sequence and the sequences from the other two breeds is supported by the phenotypical characteristics of this breed, which has a typical body structure and coat and can be very easily distinguished from the other Romanian dog breeds.

The nucleobase composition of the mtDNA sequences is very similar. The AT (adenine-thymine) percentage was between 58,0 and 58,5, while the GC (guanine-cytosine) percentage ranged between 41,5 and 42,0. These results can be observed in Table 2. The nucleotide content was determined using the DNA Baser computer software (http://www.DnaBaser.com).

Table 2. The nucleotide structure of the analysed mtDNA sequences

Breed	Adenine (%)	Guanine (%)	Thymine (%)	Cytosine (%)	AT (%)	GC (%)
Bucovina Shepherd dog	28.8	15.5	26.4	29.6	58.5	41.5
Carpathian Shepherd dog	28	15.5	26.5	30	58	42
Mioritic Shepherd dog	27.9	15.3	26.3	30.4	58.4	41.6

Three mtDNA sequences have been deposited in Genbank, one for each Romanian Shepherd dog breed. The sequences have received the following accession numbers: HE687017 (Bucovina Shepherd dog), HE687018 (Carpathian Shepherd dog) and HE687019 (Mioritic Shepherd dog).

Conclusions

The laboratory protocols we used for obtaining the mtDNA have showed good results, both the extraction protocol and the PCR amplification. The primers we used for amplification were the same as the ones used for sequencing and have worked very well in both cases. The primers have been constructed as specified by A. E. Pires *et al* (2006). These results help us continue our characterisation of the Romanian Shepherd dog breeds and use the protocols presented above for the processing of the canine blood samples collected from these dogs.

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