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DOCTORAL SCHOOL
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(SUMMARY OF Ph.D. THESIS)

MOLECULAR CHARACTERIZATION OF THE ROMANIAN SHEPHERD DOG BREEDS

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CONTENTS

INTRODUCTION

The domestic dog is the most widespread species from the Canidae family, counting around 400 million individuals, with the largest geographic distribution among domestic species. The domestic dog is remarkable due to its multitude of natural variations which it exhibits from a morphological and behavioural point of view. There are about 400 dog breeds worldwide, and their economic importance is substantial (Lindblad-Toh et al. 2005).

The molecular study of the domestic dog breeds has become a necessity along with the perfection of molecular genetics techniques. Understanding the origin and the phylogenetic relationships of the domestic dog represented the first step for a detailed knowledge of ”man’s best friend”. In the year 2005 the dog has become the fifth species whose genome was fully sequenced. The sequencing of the canine genome was performed in the hope that it will help scientists to understand the complex evolutionary mechanisms which form genes and genomes and also to provide a powerful tool for identifying the genetic factors that contribute to the well-being of man. It is expected that in future years the genome of the domestic dog may help to understand the genetic cause of many genetic inherited diseases, which dogs and humans have in common (Pires E.A. 2006).

Another research direction for the canine species is the biodiversity and phylogenetic studies, as the domestic dog presents the largest phenotypic variability among mammals. These studies analyze the the origin of breed diversity and the phylogenetic relationships between canine breeds and species and also help establishing the shared haplotypes and the genetic distances between and among populations. Molecular research on the Romanian shepherd dog breeds has not been yet performed. The molecular characterisation of these breeds is highly important for the detailed knowledge of our autochtonous dog breeds, the origin of each breed and the phylogenetic relationships between the Romanian breeds and other foreign dog breeds.

PART I. BIBLIOGRAPHIC STUDY

CHAPTER I. ROMANIAN SHEPHERD DOG BREEDS

This chapter is structured in two subchapters. The first subchapter presents general aspects regarding the domestic dog. The second subchapter presents a detailed description of the four Romanian shepherd dog breeds: Bucovina Shepherd Dog, Carpathian Shepherd Dog, Corb Shepherd Dog and Mioritic Shepherd Dog

CHAPTER II. RESEARCH REGARDING THE ORIGIN AND DIVERSITY OF THE DOMESTIC DOG

This chapter presents the most important studies regarding the origin and phylogenetic relationships between the domestic dog and other related animal species.

CHAPTER III. THE CURRENT STATUS OF CANINE RESEARCH

The third chapter of this Ph.D thesis shows the present day status of canine research. The second subchapter presents some phenotypic measurements and the numeric evolution until the uear 2009 of the Romanian shepherd dog breeds that have been homologated by the F.C.I (International Canine Federation); these breeds are the Bucovina Shepherd Dog, Carpathian Shepherd Dog, and Mioritic Shepherd Dog.

PART TWO. PERSONAL RESEARCH

THE AIM, THE OBJECTIVES AND THE IMPORTANCE OF THE RESEARCH

The aim of this Ph.D. thesis is to establish the genetic distances and phylogenetic relationships of the four Romanian shepherd dog breeds.

Objectives and activities

1. **Sampling and analysis of the mitochondrial DNA sequences for each shepherd dog breed**

In order to achieve this objective, the following activities have been undertaken:

- Blood sampling was performed only from individuals with pedigree which participated in dog shows
- Blood was sampled from the four Romanian shepherd dog breeds
- DNA extraction from whole blood was performed
- mtDNA samples obtained have been sequenced

2. **Molecular diversity indices and genetic distances estimation**

In order to achieve this objective, the following activities have been undertaken:

- Haplotype numbers and frequences estimation
- Nucleotide diversity indices estimation
- Genetic distance estimation

3. **Determination of the phylogenetic relationships for the Romanian shepherd dog breeds**

In order to achieve this objective, the following activities have been undertaken:

- Nucleotide sequences analysis and alignment for the four Romanian breeds
- Phylogenetic trees construction

The importance of the research resides in the fact that the results obtained in this study will contribute to a more detailed knowledge, at molecular level, of the Romanian shepherd dog breeds and also to establish the phylogenetic relationships between them. Until the present time, no such research at molecular level has been performed these Romanian breeds. This research will be part of the permanent homologation process for the Bucovina Shepherd Dog, Carpathian Shepherd Dog, and Mioritic Shepherd Dog

CHAPTER FOUR. MATERIALS AND METHODS

In order to obtain the mtDNA sequences from the Romanian shepherd dog breeds individuals, the following steps have been performed:

1) Blood sampling
2) DNA extraction from blood
3) PCR amplification
4) Agarose gel migration
5) Purification of DNA samples
6) Sequencing of DNA samples

In this study, a 887 bp nucleotide sequence was obtained from the four Romanian shepherd dog breeds. The sequence comprises a segment of the cytochrome b, the tRNA-Pro, the tRNA-Thr, and a segment of the control region. This sequence was chosen because of its high variability (figure 1).

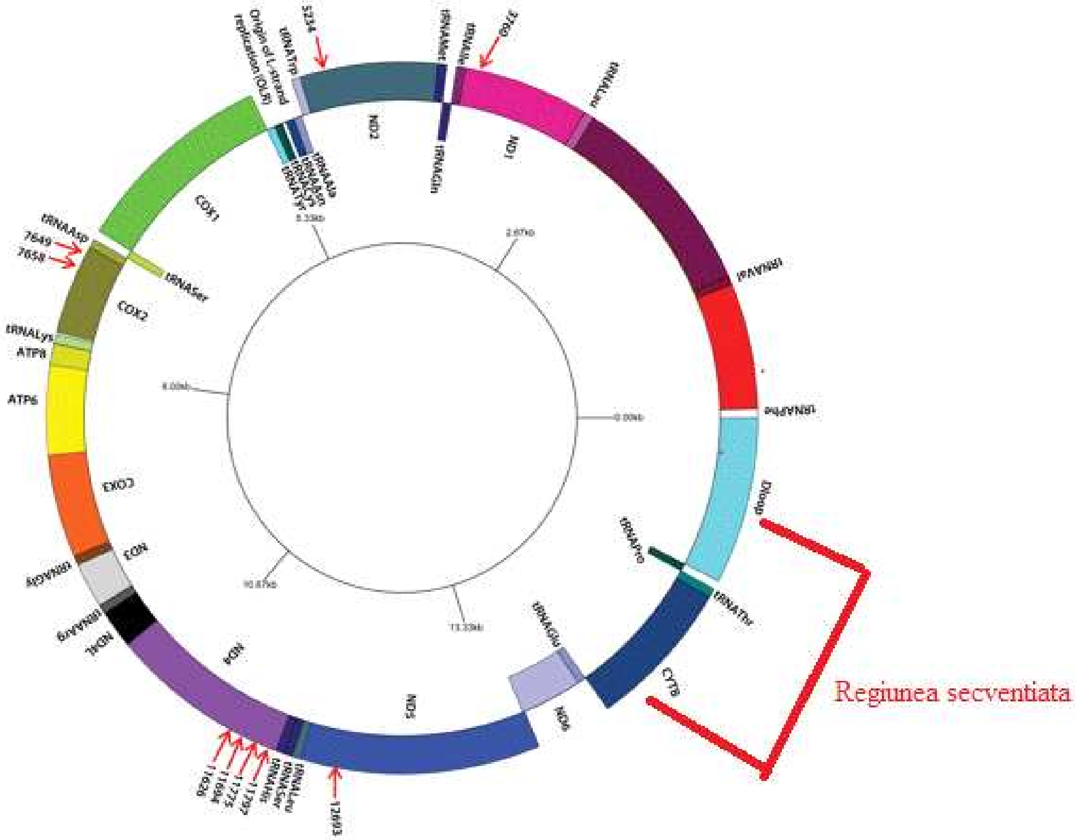


Figure 1. Map of the canine mitochondrial genome. The map has been drawn by Ryong Nam Kimet al. (2012), as a result of the complete sequencing of the whole mitocondrial genome belonging to a Korean Jindo individual.

The genetic analysis performed in this study contained the following aspects:

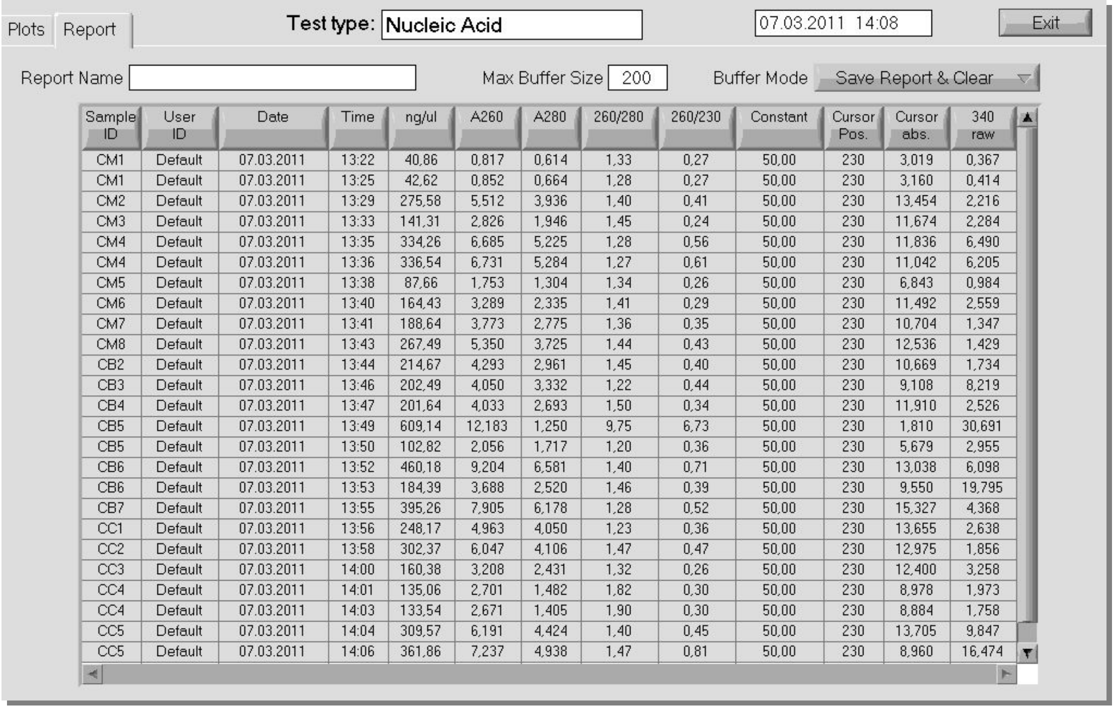
a) DNA sequence alignment
b) Molecular diversity indices estimation
c) Construction of nucleotide mismatch distribution graphics
d) Tajima's D test calculation
e) Genetic distance estimation
f) Analysis of Molecular Variance

The last part of this paper is the phylogenetic study of the four Romanian breeds. In order to construct phylogenetic trees, MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods (Tamura K. et al. 2011) software was used.

CHAPTER FIVE. RESULTS AND DISCUSSIONS

5.1 RESULTS FOR DNA EXTRACTION FROM BLOOD

Two methods were used and compared for DNA extraction: a classic method and an extraction kit based method. Both methods gave satisfying results, but the classic method is more recommended for DNA extraction from whole blood. Figure 2 shows the DNA purity and concentration after the classic method extraction.



Test type: Nucleic Acid

07.03.2011 14:08

Report Name

Max Buffer Size 200

Buffer Mode Save Report & Clear

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
CM1	Default	07.03.2011	13:22	40,86	0,817	0,614	1,33	0,27	50,00	230	3,019	0,367
CM1	Default	07.03.2011	13:25	42,62	0,852	0,664	1,28	0,27	50,00	230	3,160	0,414
CM2	Default	07.03.2011	13:29	275,58	5,512	3,936	1,40	0,41	50,00	230	13,454	2,216
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 2. Quantification of the extracted DNA samples from the shepherd dogs. CC – Carpathian Shepherd Dog, CB - Bucovina Shepherd Dog, CM – Mioritic Shepherd Dog

The mean value for DNA quantity was 267,24 ng/µl, with a minimum value of 40,86 ng/µl and a maximum of 609,14 ng/µl. DNA purity ranged from 1,20 to 1,90 with a minimum value of 1,41

5.2 ELECTROPHORETIC MIGRATION OF THE DNA SAMPLES

The samples were migrated in an agarose gel in order to check the PCR amplification process performed earlier.

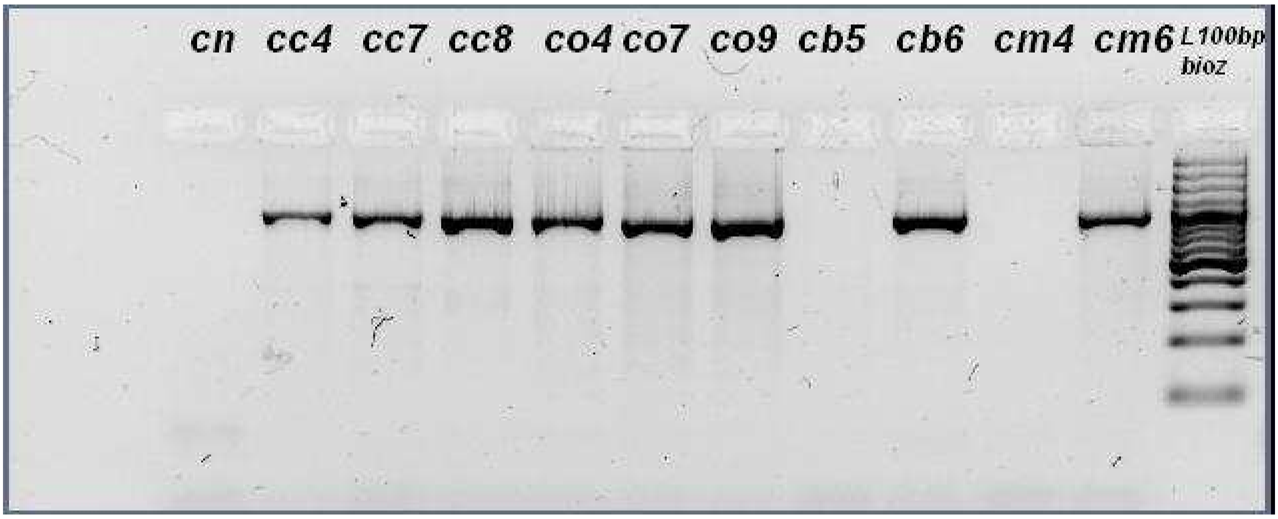


Figure 3. UV light visualisation of an agarose gel. The dark bands correspond to the 887 bp mtDNA fragment. CC – Carpathian Shepherd Dog, CB - Bucovina Shepherd Dog, CM – Mioritic Shepherd Dog, CO - Corb Shepherd Dog

5.3 *SEQUENCING OF THE MITOCHONDRIAL DNA SAMPLES*

The sequencing of the mtDNA samples was performed by the Macrogen corporation in Holland. 44 DNA samples have been sequenced, which means 11 samples for each breed. The mitochondrial DNA sequences obtained will be deposited in Genbank.

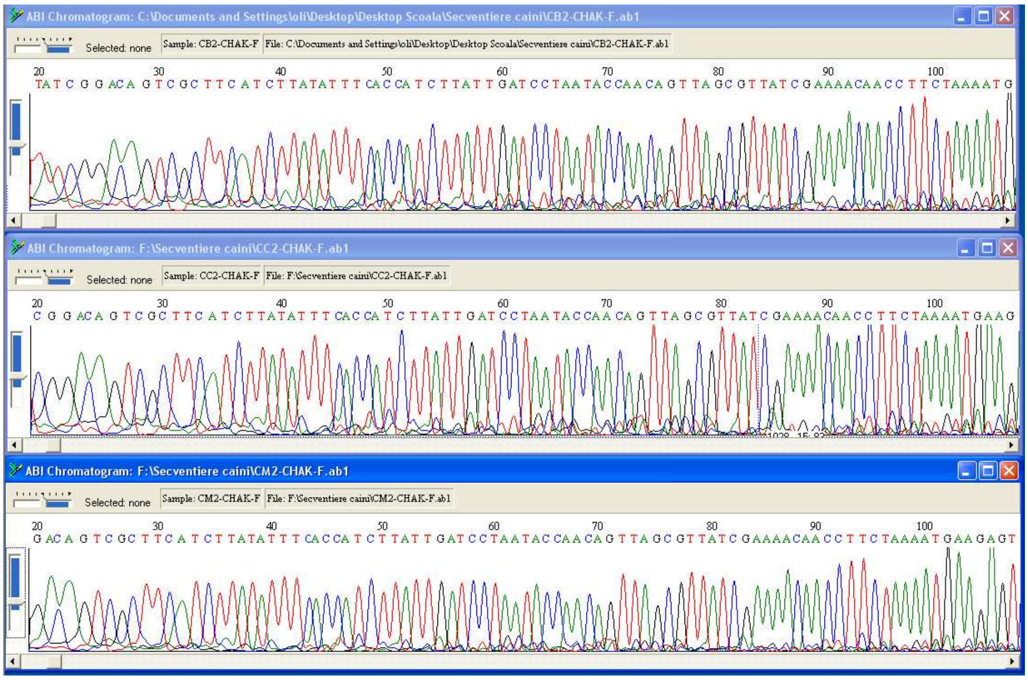


Figure 4. Chromatogram example. The chromatograms belong to mtDNA samples from the following breeds: CC2 – Carpathian Shepherd Dog, CB2 - Bucovina Shepherd Dog, CM2 – Mioritic Shepherd Dog. Each peak from the graphics corresponds to a nucleotide base. The nucleotide bases are coloured as follows: adenine – green, cytosine – blue, guanine – black, thymine - red

Table 1

Percentage content of the nucleotide bases for the four Romanian shepherd dog breeds (relative values). The nucleotide content has been estimated using Arlequin v. 3.5.

Breed	*Adenine* (%)	*Guanine* (%)	*Thymine* (%)	*Cytosine* (%)	AT (%)	GC (%)
Bucovina Shepherd Dog	28,31	15,24	29,74	26,71	58,05	41,95
Carpathian Shepherd Dog	28,19	15,32	30,03	26,47	58,22	41,78
Corb Shepherd Dog	28,26	15,23	29,90	26,61	58,16	41,84
Mioritic Shepherd Dog	27,96	15,85	29,64	26,55	57,60	42,40

Table 1 shows the nucleotide bases percentages for each shepherd dog breed. As expected, the nucleotide base composition is very similar for the four breeds. The AT percentages ranged between 57,60% and 58,22%, while the GC content was between 41,78% and 42,40%.

5.4 ALIGNMENT OF THE MITOCHONDRIAL DNA SEQUENCES

All the 44 obtained DNA sequences have been aligned using the BLAST alignment tool and compared to the most similar sequences in the Genbank. Multiple sequence alignment was performed using ClustalW2 form the EMBL-EBI database. The alignment results were used for building dendrograms.

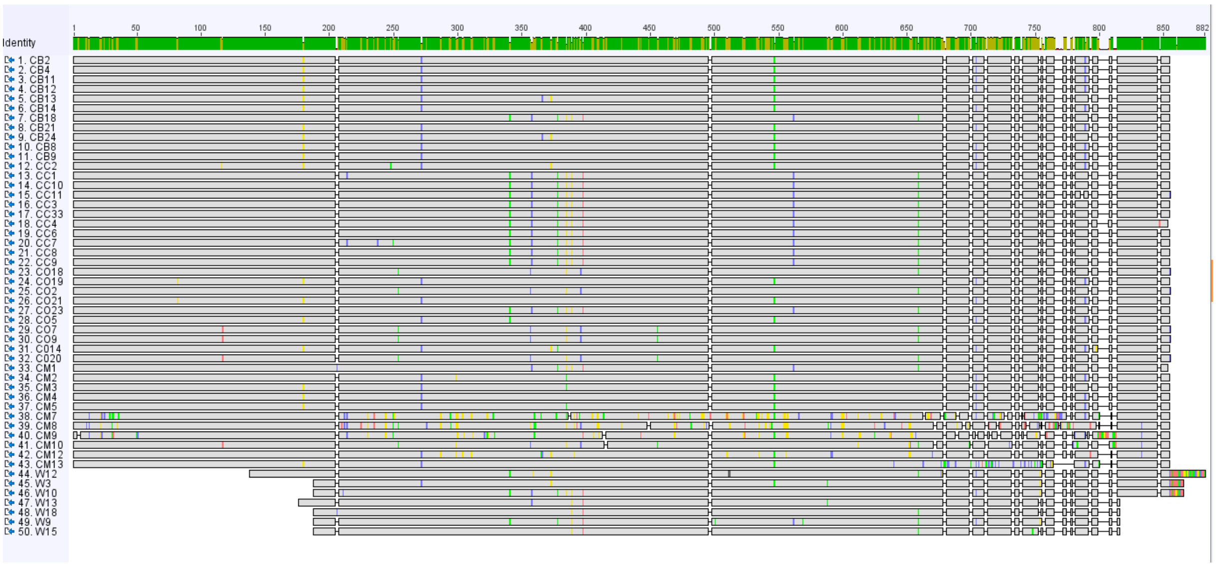


Figure 5. Alignment example of the Romanian shepherd dog sequences together with wolf sequences taken from the Genbank database. The wolf sequence names start with ”W”

5.5 ESTIMATION OF THE HAPLOTYPE FREQUENCE AND NUMBER

Among the 44 analyzed sequences, 18 haplotypes have been identified. Their distribution is shown in table 2.

Table 2

Number of haplotypes and number of individuals for each haplotype for the four Romanian shepherd dog breeds

Breed / *Haplotype*	*Bucovina Shepherd Dog* n = 11	*Carpathian Shepherd Dog* n = 11	*Corb Shepherd Dog* n = 11	*Mioritic Shepherd Dog* n = 11
Hap. 1	8	0	0	1
Hap. 2	2	0	0	0
Hap. 3	1	6	1	0
Hap. 4	0	1	0	0
Hap. 5	0	1	0	0
Hap. 6	0	1	0	0
Hap. 7	0	1	0	0
Hap. 8	0	1	0	0
Hap. 9	0	0	2	0
Hap. 10	0	0	2	0
Hap. 11	0	0	1	0
Hap. 12	0	0	3	0
Hap. 13	0	0	2	0
Hap. 14	0	0	0	1
Hap. 15	0	0	0	1
Hap. 16	0	0	0	3
Hap. 17	0	0	0	4
Hap. 18	0	0	0	1

For the Bucovina Shepherd Dog population, 3 haplotypes were identified (Hap. 1, 2 and 3). The other three shepherd dog breeds displayed 6 haplotypes each. From a total of 18 haplotypes, only two haplotypes were shared between breeds (hap.1 and hap. 3). Haplotype 1 is shared by the Bucovina Shepherd Dog and the Mioritic Shepherd Dog, with one copy per breed. Haplotype 3 is shared by the Carpathian Shepherd Dog and the Corb Shepherd Dog.

This haplotype presented the highest frequency within the Carpathian Shepherd Dog population, with six individuals sharing it.

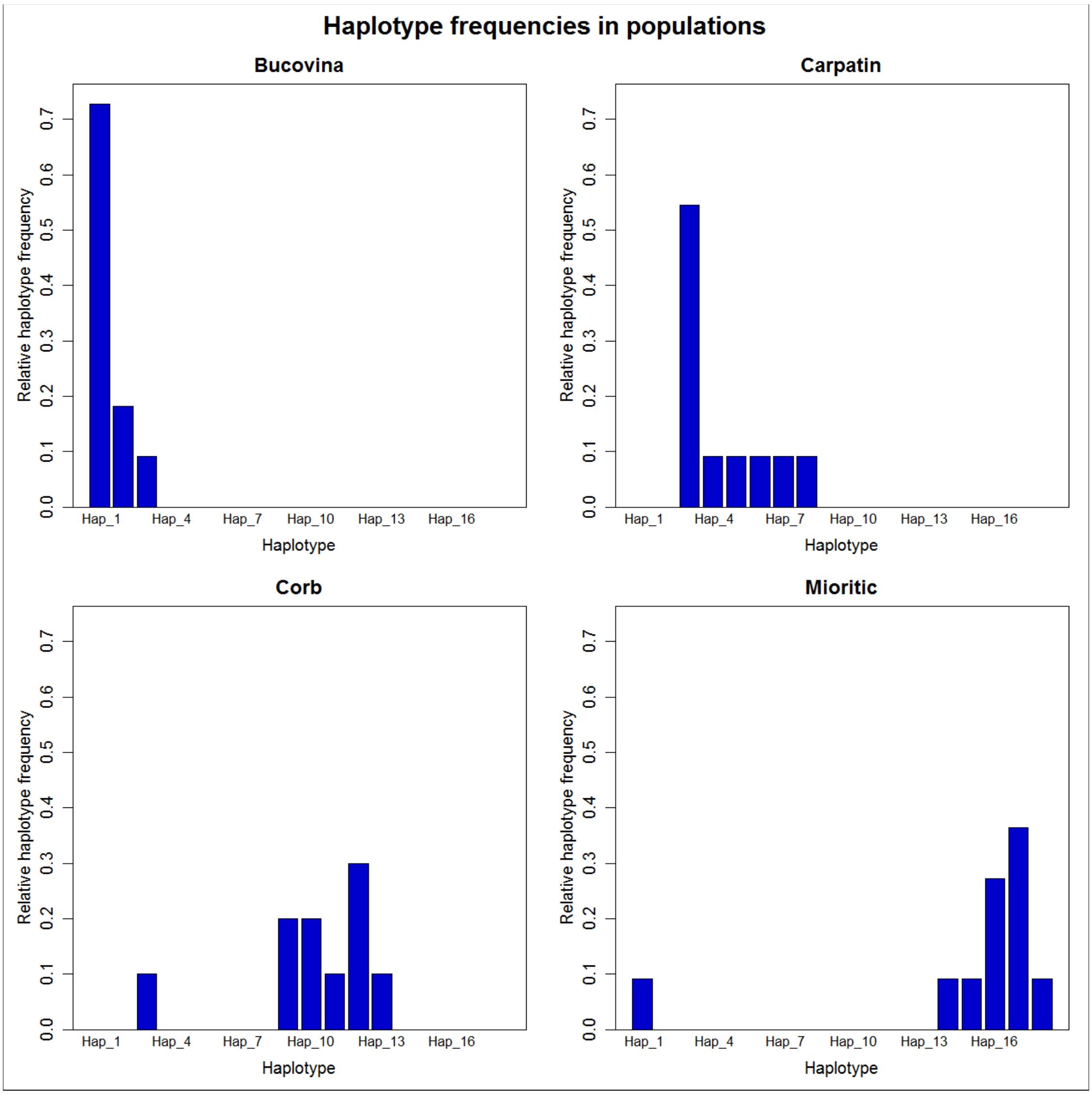


Figure 6. Haplotype frequences of the four Romanian shepherd dog breeds (relative frequences)

Figure 6 shows the relative frequencies of the 18 haplotypes identified. Haplotype 1 has reached the highest frequency (0,727), followed by haplotype 3 (0,545), haplotype 17 (0,364), haplotype 12 (0,300), and haplotype 16 (0,273). The rest of the haplotypes have been identified in one or two individuals only and exhibit frequencies ranging from 0,090 to 0,200.

Based on the nucleotide differences between the mtDNA sequences, Arlequin software was used to build distance matrixes between haplotypes for each shepherd dog population. These graphs are shown in figures 7, 8, 9 and 10.

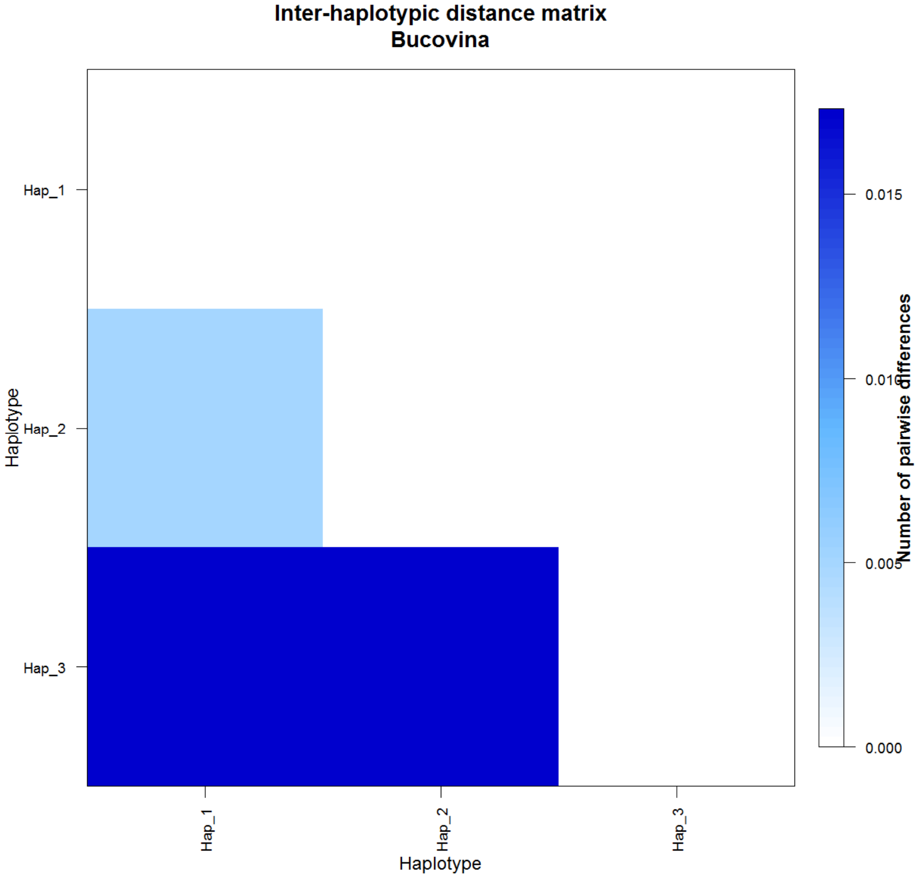


Figure 7. Inter - haplotypic distance matrix for the Bucovina Shepherd Dog population. The distances between the haplotypes have been calculated based on the number of nucleotide differences between the sequences. The intensity of the graphic's colour is directly proportional to the number of differences between the mtDNA sequences

The mean number of pairwise differences for the Bucovina Shepherd Dog was 3,759 +/- 2,051 (s.d.). Only three haplotypes have been identified for this breed. Haplotype 1 is shared between 8 of the 11 sampled individuals. The inter - haplotypic distance matrix shows the largest genetic distance is between haplotype 3 and haplotype 1 and 2. Haplotype 3 is shared with the Carpathian Shepherd Dog and the Corb Shepherd Dog, and it is not characteristic for the Bucovina Shepherd Dog.

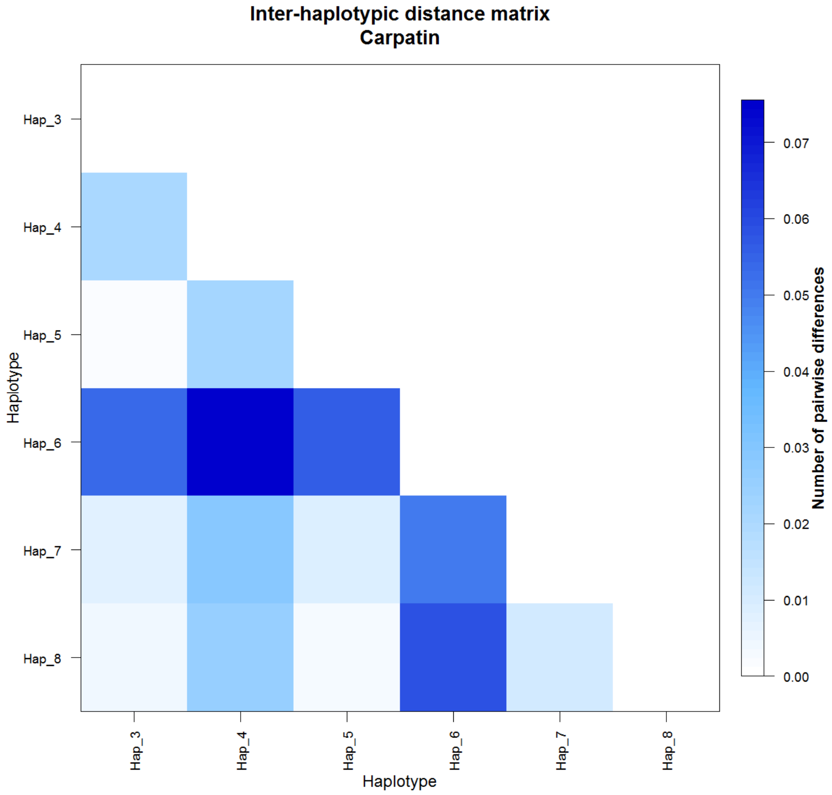


Figure 8. Inter - haplotypic distance matrix for the Carpathian Shepherd Dog population. The distances between the haplotypes have been calculated based on the number of nucleotide differences between the sequences. The intensity of the graphic's colour is directly proportional to the number of differences between the mtDNA sequences

Among the six haplotypes identified for the Carpathian Shepherd Dog breed, the highest estimated distance, was between haplotype 6 and the rest of the haplotypes. Mean pairwise differences number for this population was 12,896 +/- 6,300 (s.d.). The highest frequency was observed for haplotype 3 (0,545). This haplotype is shared with the Bucovina Shepherd Dog and the Corb Shepherd Dog.

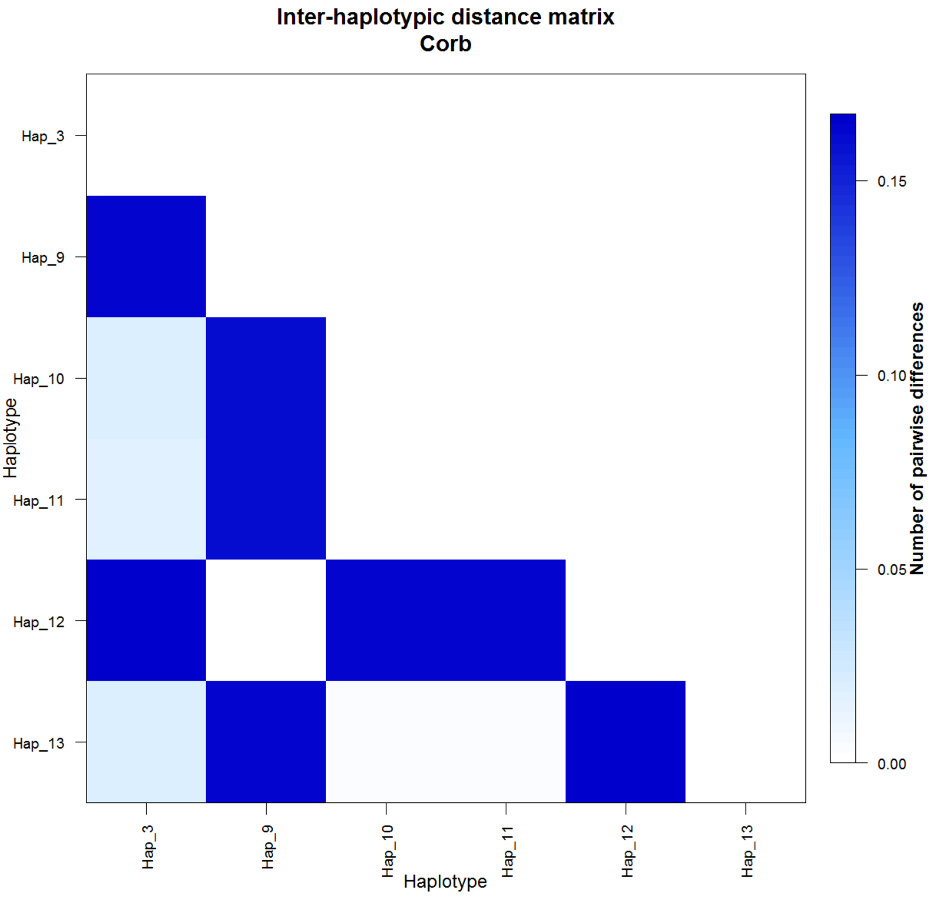


Figure 9. Inter - haplotypic distance matrix for the Corb Shepherd Dog population. The distances between the haplotypes have been calculated based on the number of nucleotide differences between the sequences. The intensity of the graphic's colour is directly proportional to the number of differences between the mtDNA sequences

The largest distance for Corb breed population (figure 9) was estimated between and the other five haplotypes observed. The mean number of pairwise differences for this populations is 76,817 +/- 36,217 (s.d.)

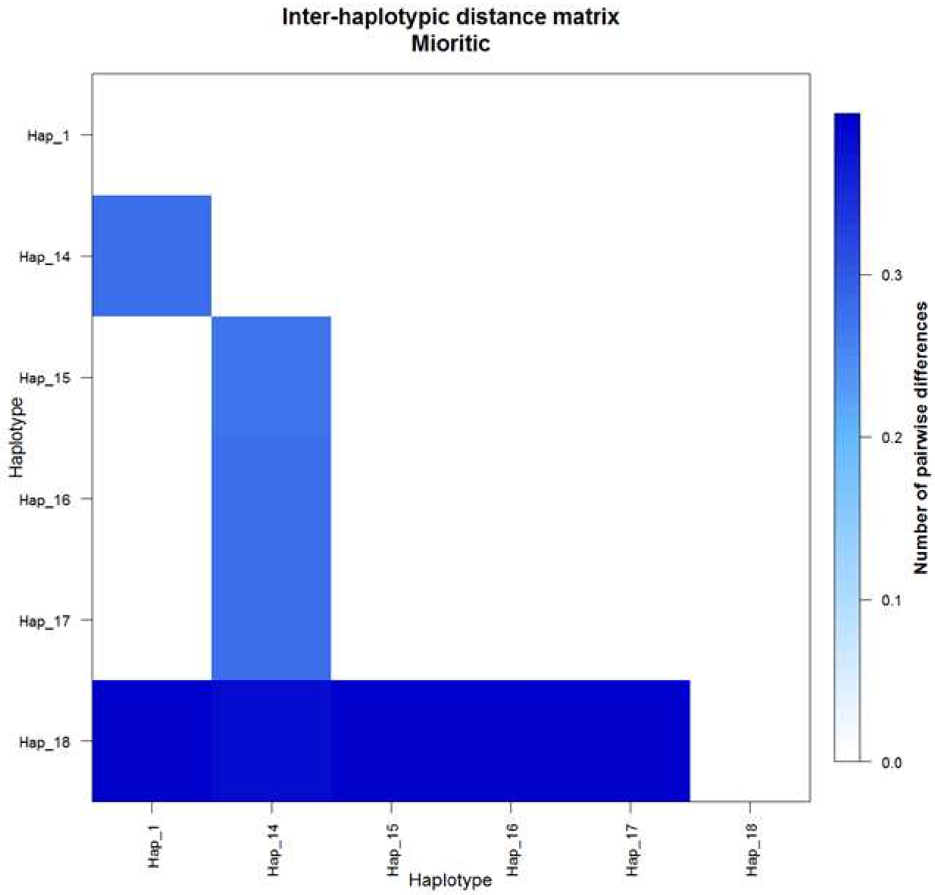
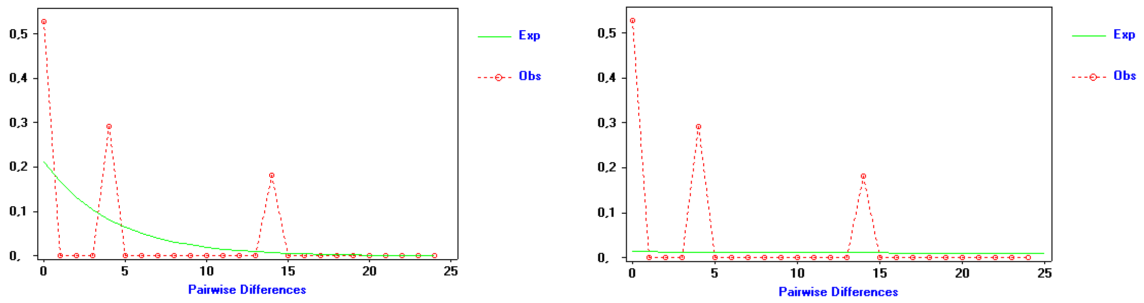


Figure 10. Inter - haplotypic distance matrix for the Mioritic Shepherd Dog population. The distances between the haplotypes have been calculated based on the number of nucleotide differences between the sequences. The intensity of the graphic's colour is directly proportional to the number of differences between the mtDNA sequences

For the Mioritic Shepherd Dog, the mean number of pairwise differences was 97,205 +/- 45,335, which is the highest number of pairwise differences observed for the four shepherd dog breeds

5.6 MISMATCH DISTRIBUTION GRAPHICS FOR THE ROMANIAN SHEPHERD DOG BREEDS

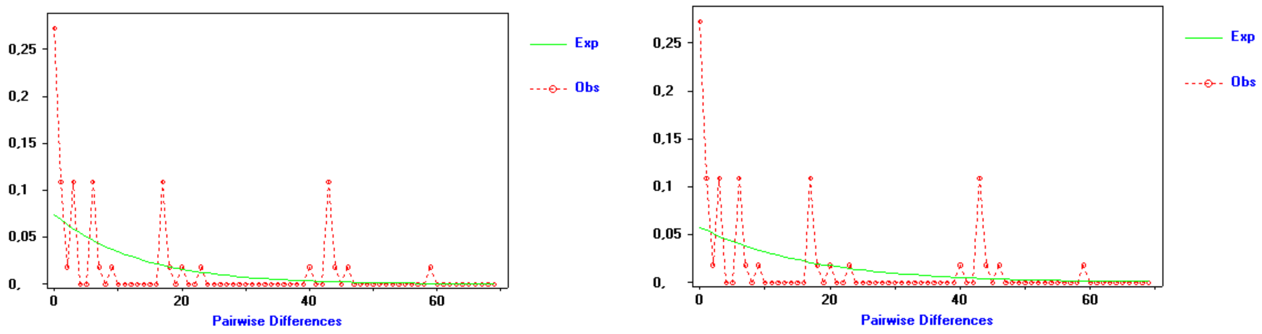
The mismatch distribution graphics have been cosntructed for two scenarios: a constant population size and a population going through a demographic expansion process. The graphs exhibit the observed and the expected nucleotide pairwise differences for the two cases.



Constant population size

Demographic expansion

Figure 11. Mismatch distribution graphs for the Bucovina Shepherd Dog breed



Constant population size

Demographic expansion

Figure 12. Mismatch distribution graphs for the Carpathian Shepherd Dog breed

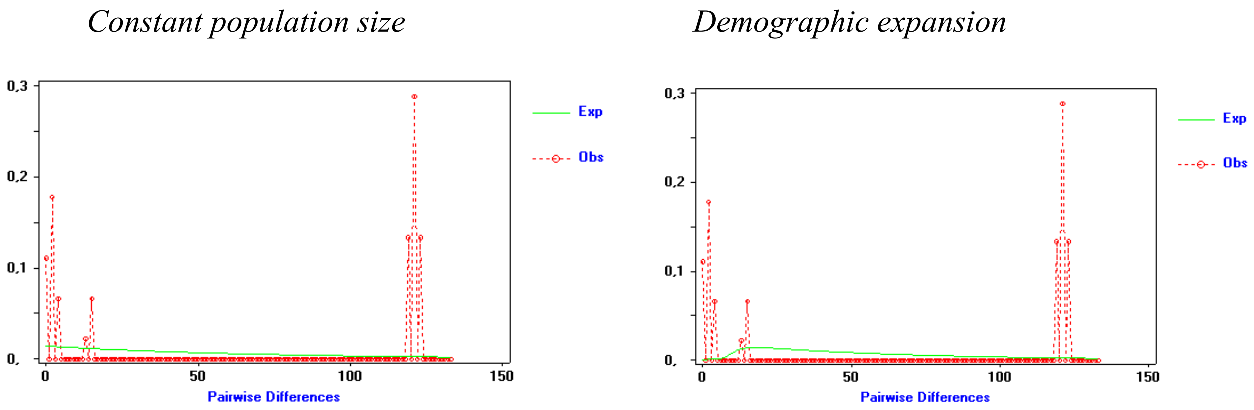

Constant population size *Demographic expansion*

Figure 13. Mismatch distribution graphs for the Corb Shepherd Dog breed

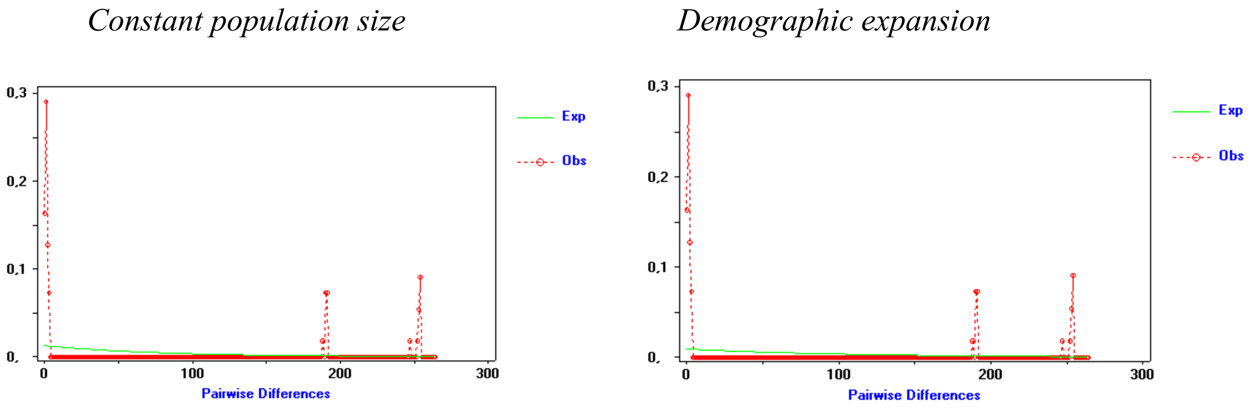

Constant population size *Demographic expansion*

Figure 14. Mismatch distribution graphs for the Mioritic Shepherd Dog breed

The two graphics were compared for each of the four studied breeds. For the Bucovina Shepherd Dog breed the constant population size graph shows a greater match between the nucleotide pairwise differences, which might suggest a recent demographic expansion of this population. The mismatch distribution graphs for the Carpathian Shepherd Dog are almost identical, therefore inconclusive regarding the recent demographic history of this breed. For the Corb Shepherd Dog breed the constant evolution model shows a higher degree of matching between the observed and expected pairwise differences. The mismatch graphs for the Mioritic Shepherd Dog the graphs are too similar in order to draw a conclusion.

5.7 TAJIMA'S D STATISTICAL TEST

The *D* test discriminates between randomly evolving DNA sequences and those under the effect of a randomized process such as purifying or directional selection , demographic expansion etc.

Table 3

D test values for the Romanian shepherd dog breeds

Breed *Statistics*	*Bucovina Shepherd Dog*	*Carpathian Shepherd Dog*	*Corb Shepherd Dog*	*Mioritic Shepherd Dog*
No. of individuals	11	11	11	11
S	16	62	131	327
Π	0,004	0,015	0,084	0,094
D test	-1,430	-2,115	2,394	-1,817
Statistical significance	n.s., p>0.01	**, p< 0.01	**, p< 0.01	*, p< 0.05

The *D* test values were negative for the Bucovina Shepherd Dog, the Carpathian Shepherd Dog and the Mioritic Shepherd Dog. Only for the Corb Shepherd Dog was this value positive. The negative value show an excess low frequency polymorphisms compared to the expected values, which indicates a growth in the size of these populations and/or negative selection. The positive value of the *D* test for the Corb Shepherd Dog shows a decrease in the size of the population and/or stabilizing selection.

5.8 MOLECULAR DIVERSITY INDICES

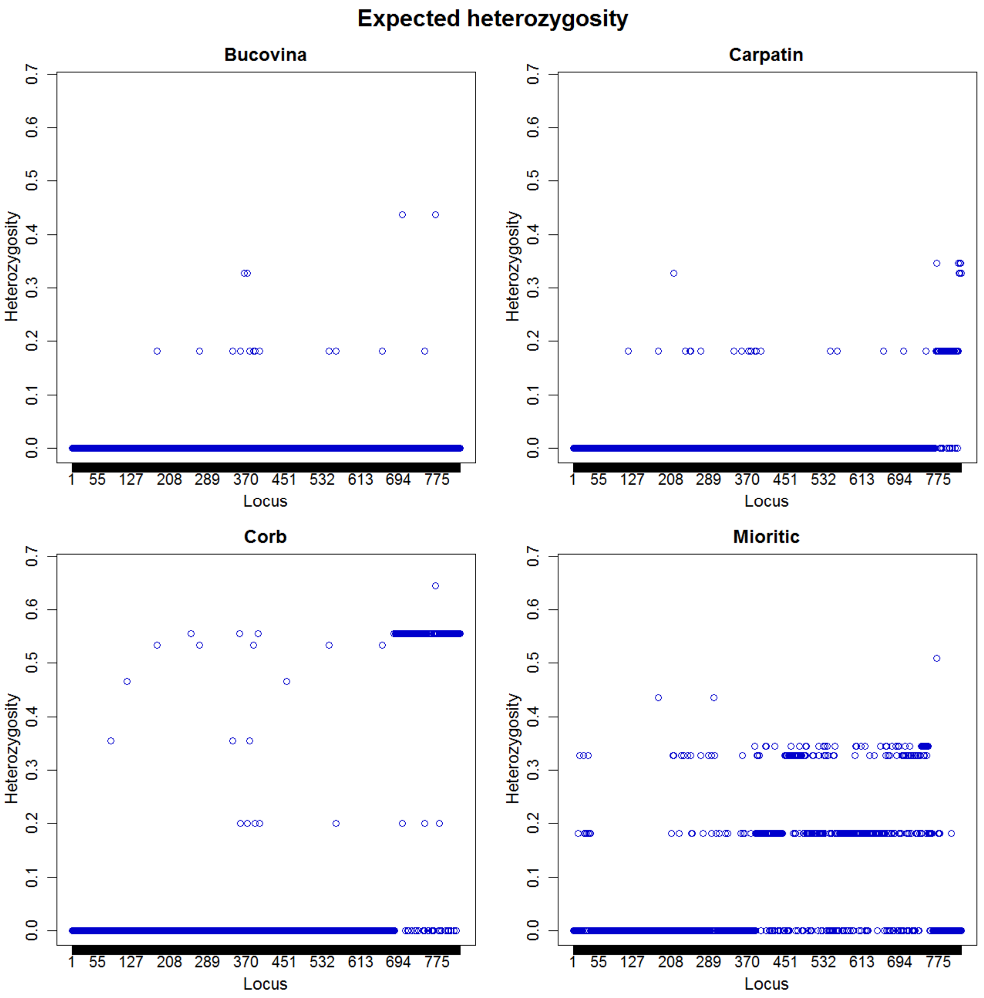


Figure 15. Gene diversity degree of the four Romanian shepherd dog breeds

Figure 15 shows that the highest gene diversity was observed for the Corb Shepherd Dog breed with a value of $G_{ST} = 0.888$ +/- 0.075(s.d.). The values for the Mioritic Shepherd Dog are very close to those for the Corb Shepherd Dog, with $G_{ST} = 0.836$ +/- 0.088 (s.d.). The values obtained for the Romanian shepherd dog breeds are similar to those reported by other authors for different dog breeds.

Table 4

Molecular diversity indices for the four Romanian shepherd dog breeds

Breed / *Statistics*	*Bucovina Shepherd Dog*	*Carpathian Shepherd Dog*	*Corb Shepherd Dog*	*Mioritic Shepherd Dog*
Observed no. of transitions	16	32	59	141
Observed no. of transversions	0	64	73	224
No. of substitutions	16	66	132	365
Observed no. of transition sites	16	31	59	133
Observed number of transversion sites	16	31	59	133
No. of polymorphic sites	16	62	131	327
Mean number of pairwise differences *(π)*	3,709	12,509	69,133	77,855

5.9 ESTIMATION OF THE GENETIC DISTANCES

Fixation index (F_{ST}) estimation

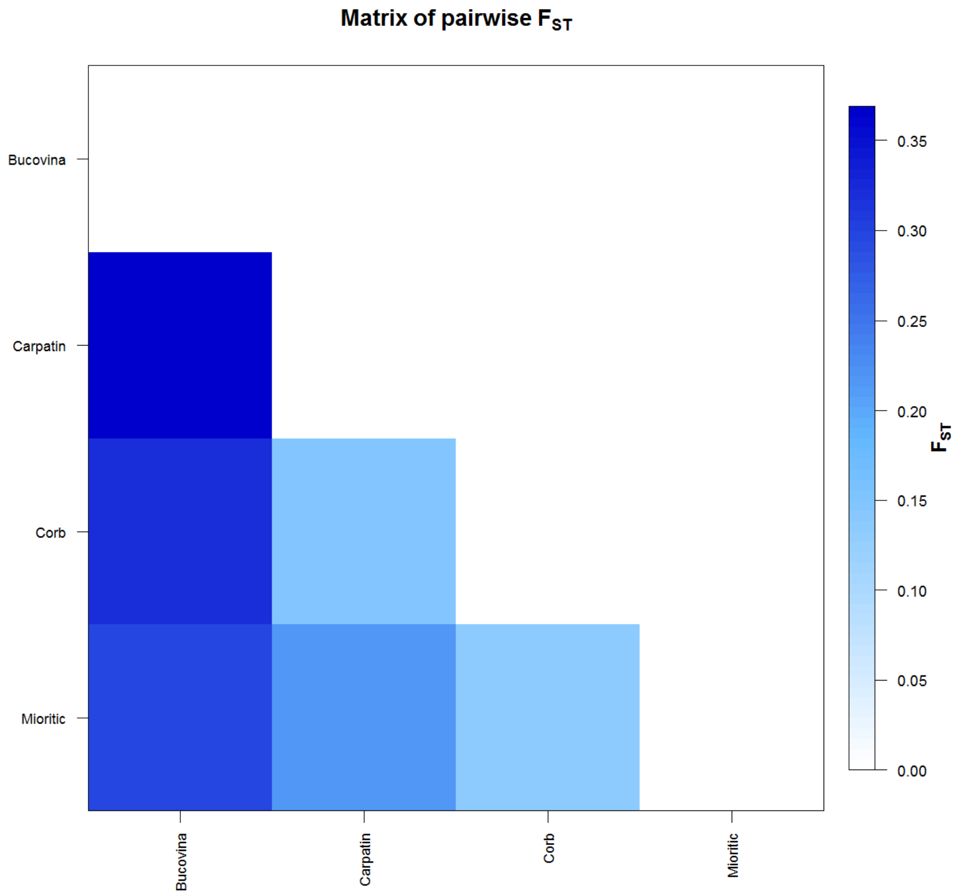


Figure 16. Matrix of pairwise F_{ST} between the Romanian shepherd dog breeds. The colour intensity of the graphic is directly proportional to the value of the genetic distance between the breeds.

The values for the genetic distances based on the F_{ST} are shown in figure 16. The highest value was reported between the Bucovina Shepherd Dog and the Corb Shepherd Dog (F_{ST} = *0,368*). The lowest value was between the Mioritic Shepherd Dog and the Corb Shepherd Dog (F_{ST} = *0,137*).

Nei 's genetic distance estimation

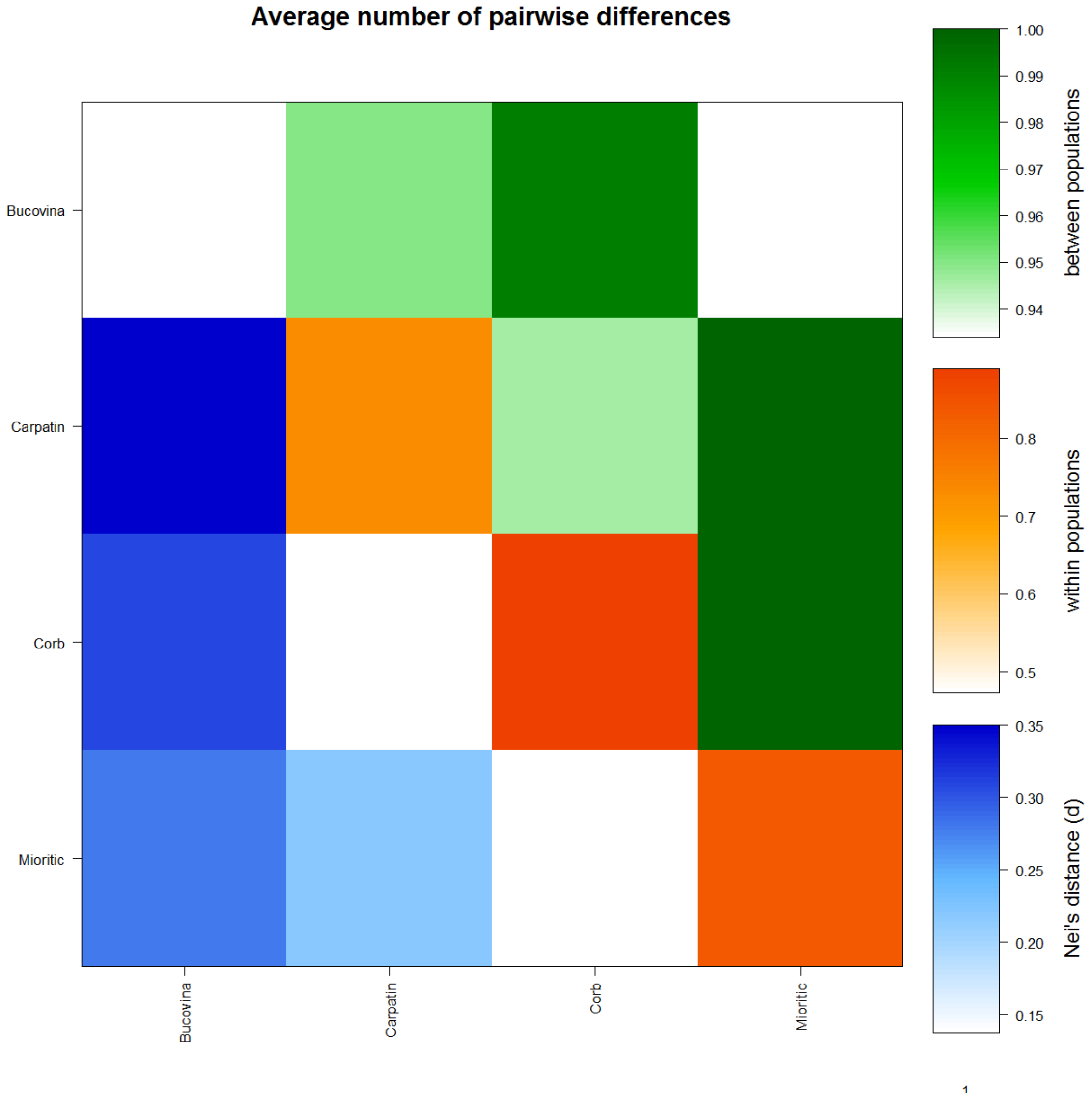


Figure 17. Average number of pairwise differences between (green squares) and within populations (orange squares). Nei 's distance is coloured in blue.

The genetic distance within populations reached values ranging from 0,472 for the Bucovina Shepherd Dog and 0,888 for the Mioritic Shepherd Dog. Nei’s D_A distance reached a highest value of 0,350 between the Bucovina and Carpathian populations. The other distances were lower than 0,280. These values are similar to those reported by other authors for other dog breeds.

5.10 ANALYSIS OF MOLECULAR VARIANCE (AMOVA)

Table 6

Results of the Analysis of Molecular Variance

Source of variation	*Degrees of freedom*	*Sum of squares*	*Expected square means*	*Percent of variation*
populations	3	4,981	0.12069	24.92
Within populations	40	14.182	0.36364	75.08
Total	43	19.163	0.48432	

The AMOVA analysis was performed based on the number of mutations between the molecular haplotypes, 400 polymorphic loci were considered. The percent of variation is 24.92% due to inter-racial diversity, while the rest of 75.08% is due to intra-breed diversity.

5.11 PHYLOGENETIC TREES

The phylogenetic trees in figures 18 and 19 show a cluster grouping based on breed affiliation, of the sequences from the four Romanian breeds. Each breed has its own cluster, which shows a good degree of genetic diversity.

The internal nodes of the trees indicate that between the four shepherd dog breeds there are common hypothetical ancestors. The fact that breed clusters are mingled with sequences from other Romanian shepherd dog breeds is a sign of gene flow between these populations (before controlled breeding started).

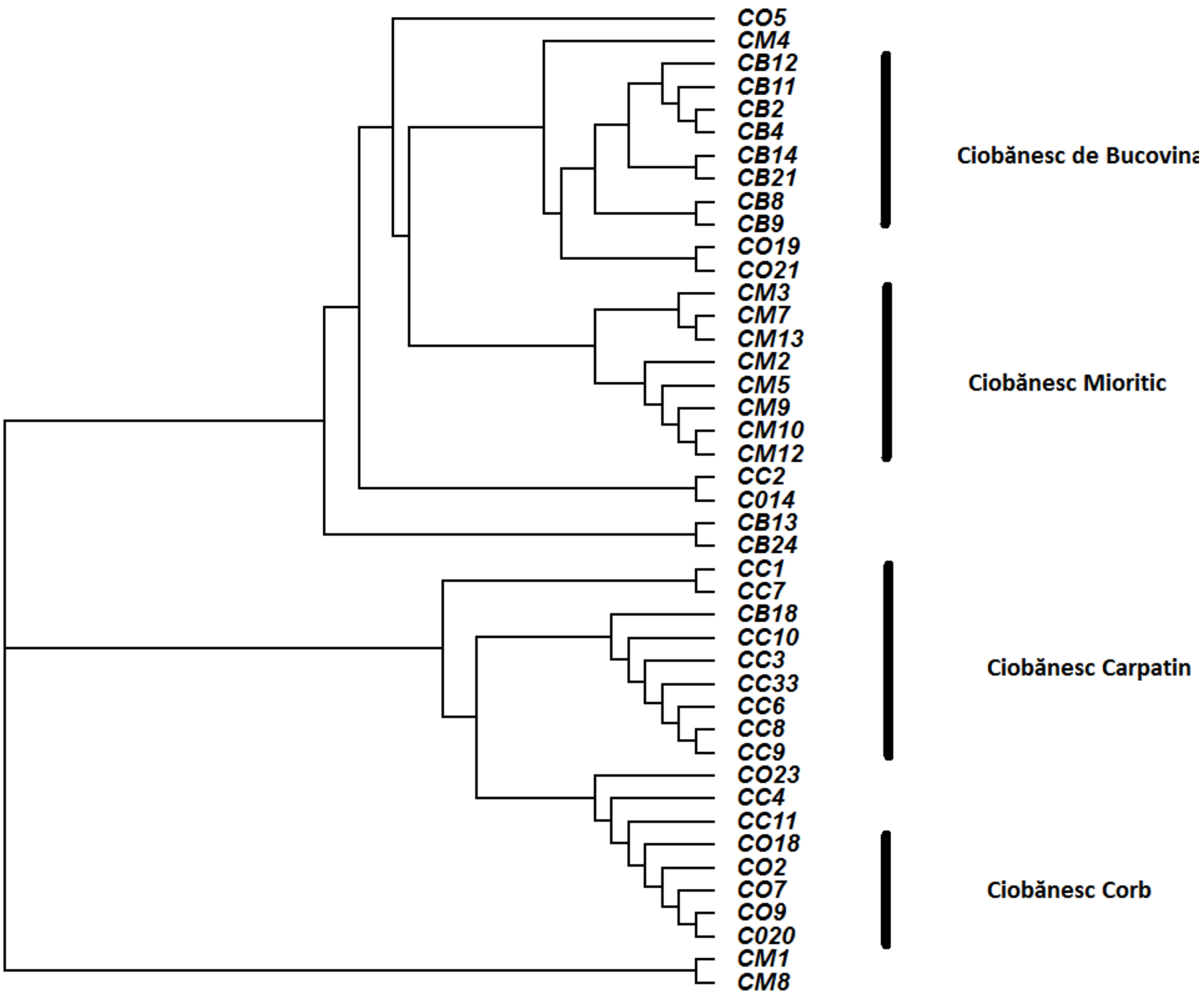


Figure 18. Maximum likelihood phylogenetic tree using the Kimura 2 parameter substitution model. The tree comprises nucleotide sequences from the four Romanian shepherd dog breeds. Abbreviations: CB - Bucovina Shepherd Dog, CC – Carpathian Shepherd Dog, CO – Corb Shepherd Dog, CM – Mioritic Shepherd Dog

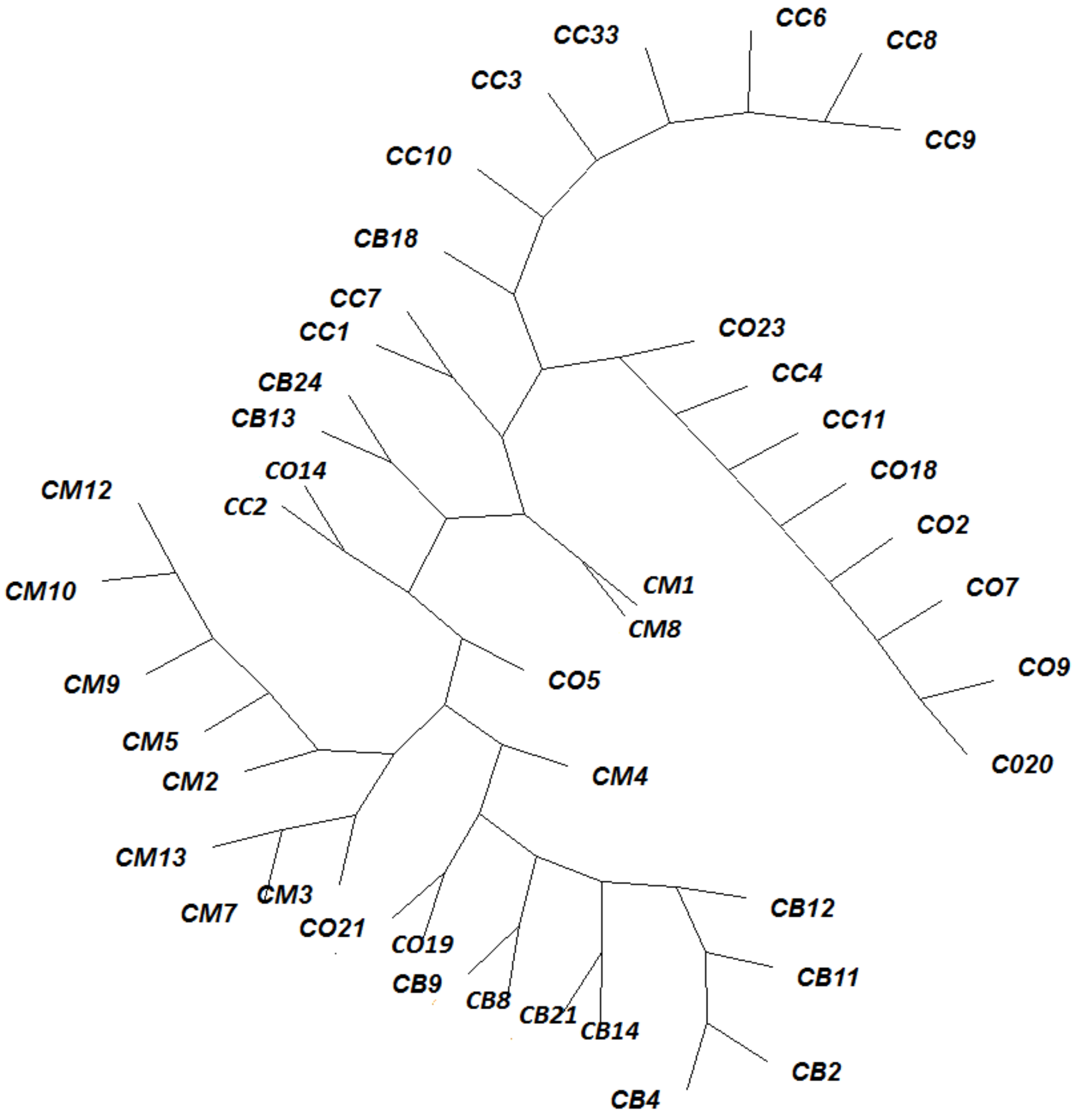


Figure 19. Unrooted UPGMA phylogenetic tree using the Kimura 2 parameter nucleotide substitution model. The tree comprises nucleotide sequences from the four Romanian shepherd dog breeds. Abbreviations: CB - Bucovina Shepherd Dog, CC – Carpathian Shepherd Dog, CO – Corb Shepherd Dog, CM – Mioritic Shepherd Dog

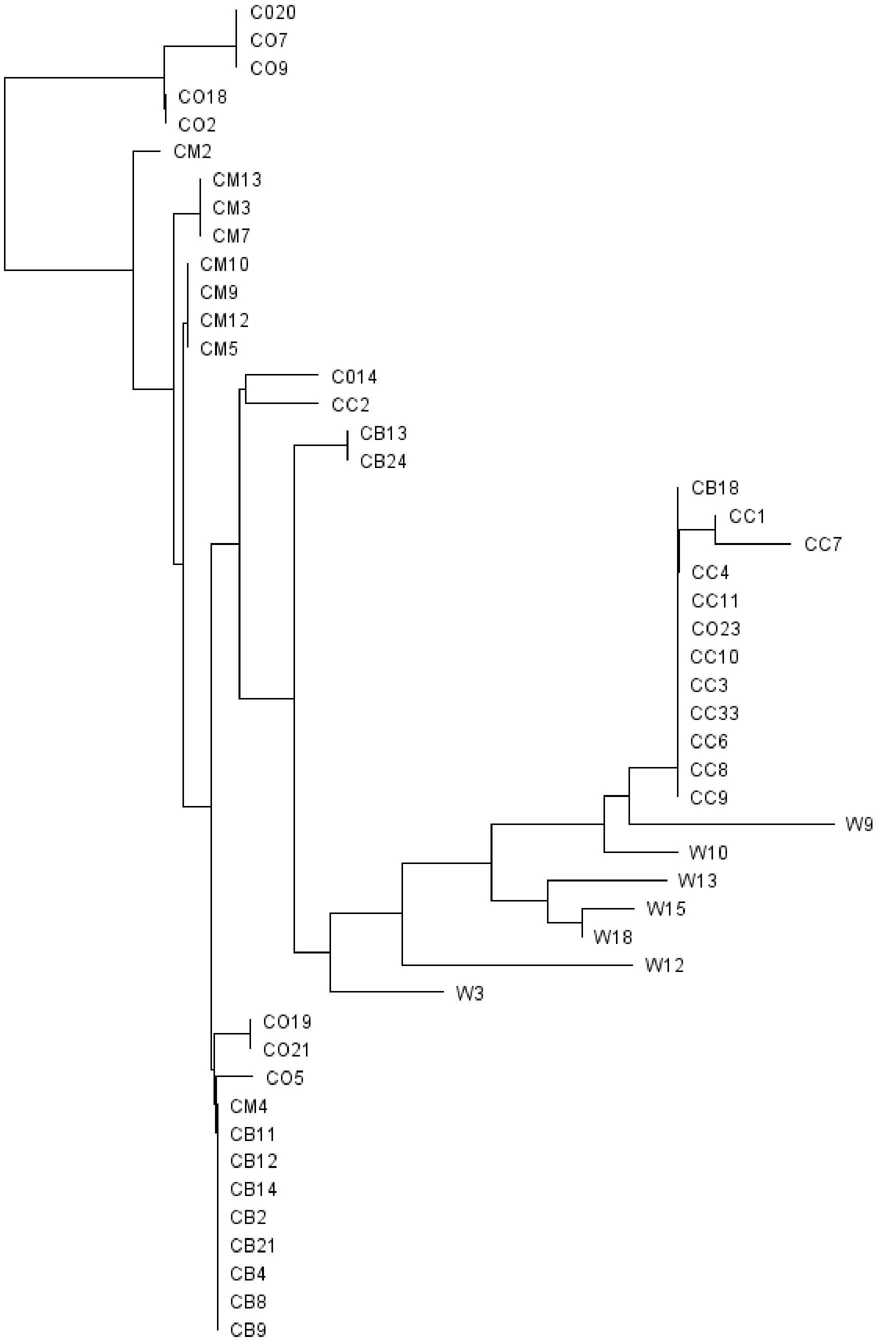


Figure 20. Neighbor joining phylogenetic tree using the Tamura – Nei nucleotide substitution model. The tree comprises nucleotide sequences from the four Romanian shepherd dog breeds

The phylogenetic tree above is constructed together with wolf nucleotide sequences an indicates a higher similarity between the Carpathian breed and the wolves compared to the other Romanian breeds. The sequences from the Bucovina, Corb and Mioritic Shepherd Dog show a very high nucleotide divergence from the wolf sequences.

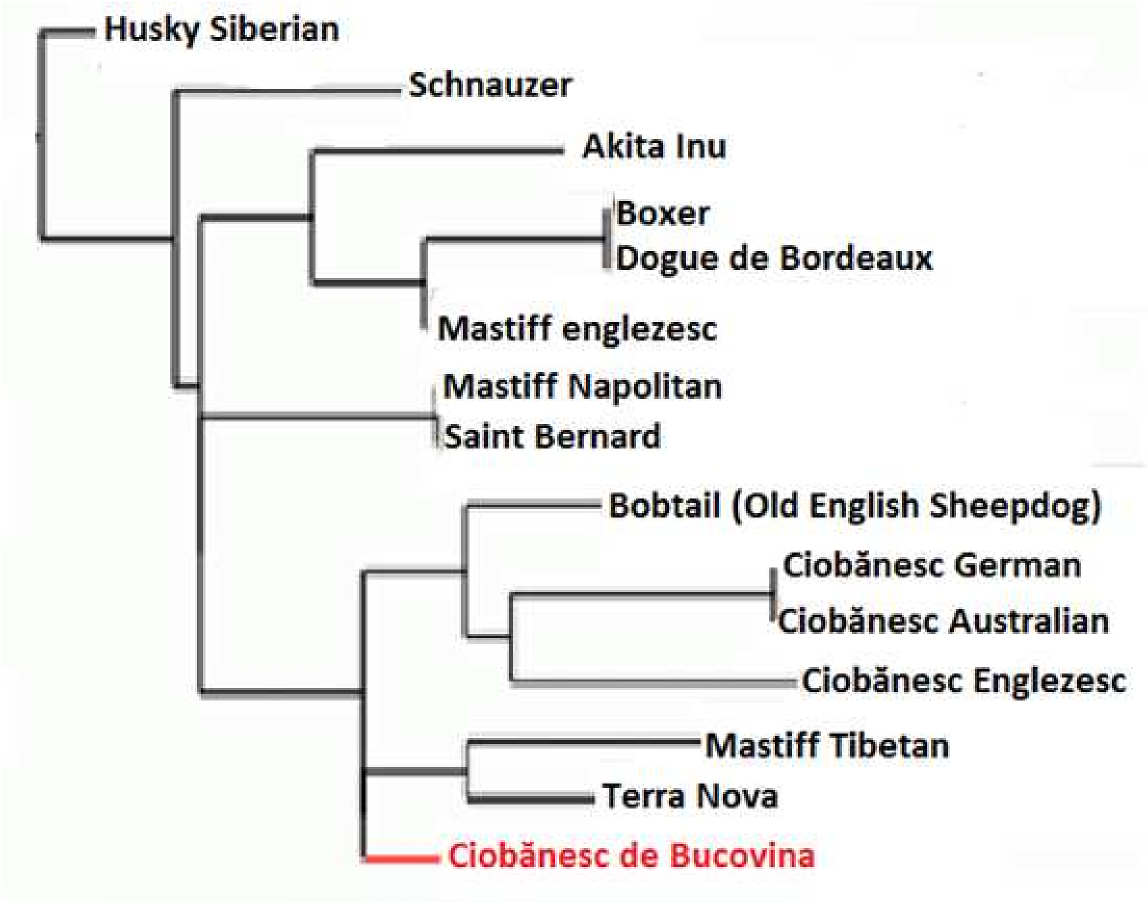


Figure 21. Neighbor joining phylogenetic tree based on the BLAST alignment between a Bucovina Shepherd Dog nucleotide sequence and nucleotide sequences of other large dog breeds

The tree from figure 21 shows that the highest similarity with the sequence of the Bucovina Shepherd Dog is exhibited by sequences from the Terra Nova, Tibetan Mastiff and Bobtail breeds.

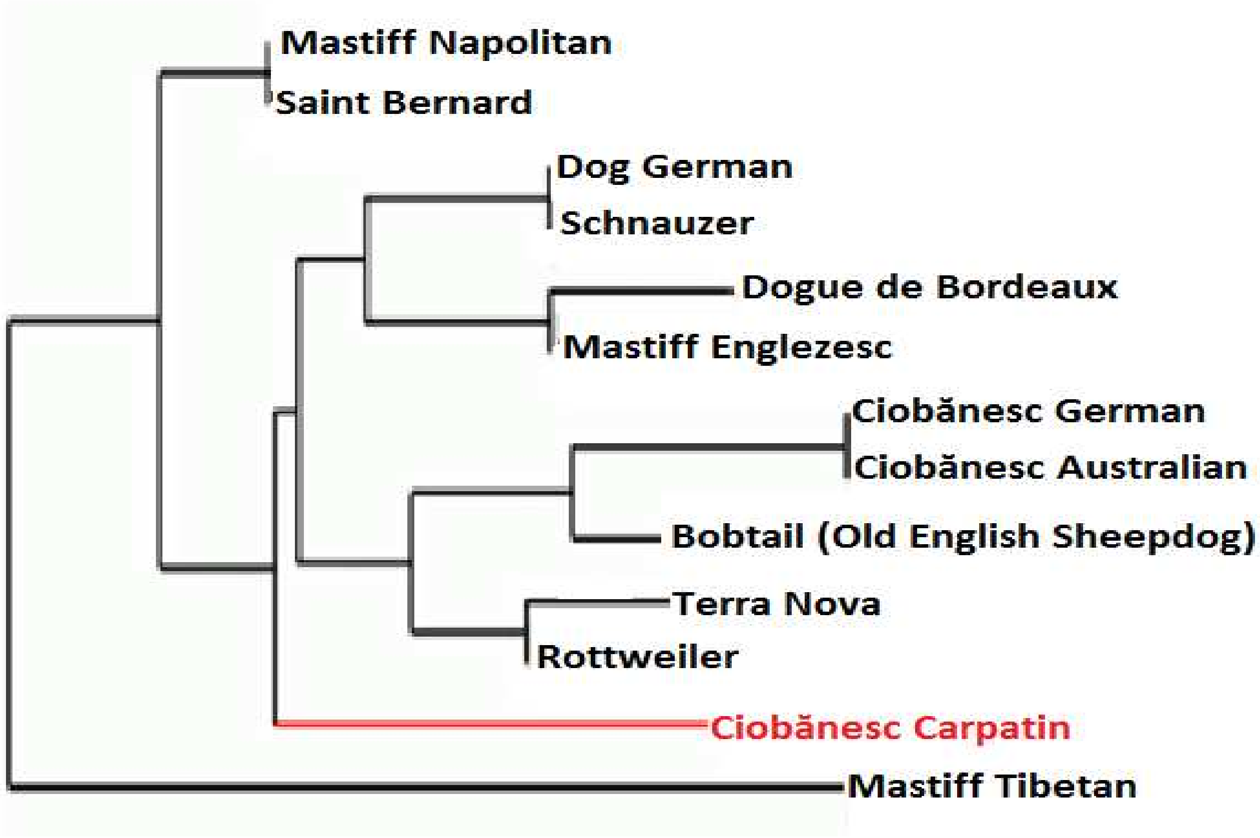


Figure 22. Neighbor joining phylogenetic tree based on the BLAST alignment between a Carpathian Shepherd Dog nucleotide sequence and nucleotide sequences of other large dog breeds

As indicated in figure 22, the most similar breeds with Carpathian Shepherd Dog (based on mtDNA) are the Tibetan Mastiff, Saint Bernard and Neapolitan Mastiff breeds.

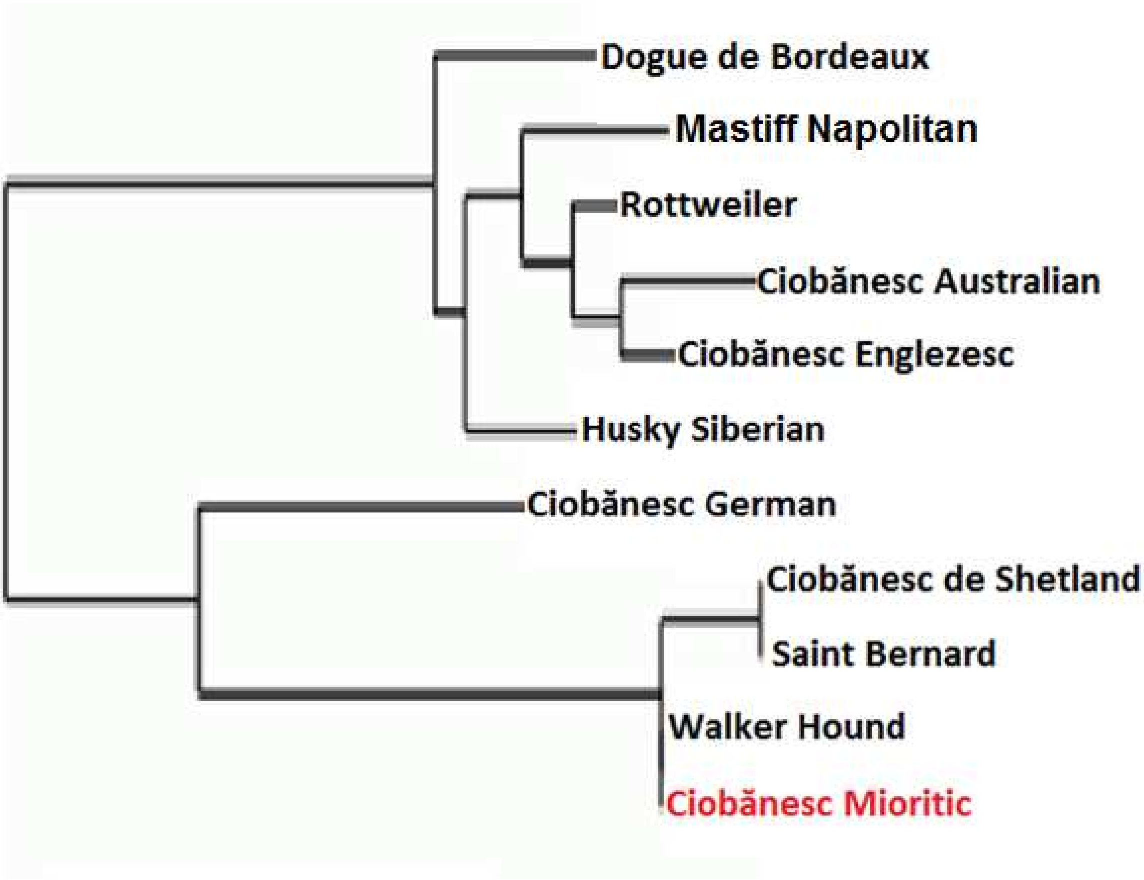


Figure 23. Neighbor joining phylogenetic tree based on the BLAST alignment between a Mioritic Shepherd Dog nucleotide sequence and nucleotide sequences of other large dog breeds

The Mioritic breed shows a great similarity with the Walker Hound, Saint Bernard and Shetland sheepdog. The sequences in all the phylogenetic trees that are from foreign breeds have been taken from the Genbank database.

Figure 24. Neighbor joining phylogenetic tree based on the BLAST alignment between a Corb Shepherd Dog nucleotide sequence and nucleotide sequences of other large dog breeds

The organization of the branches for the tree in figure 24 indicates a great genetic distance between the Corb Shepherd Dog sequence and the other sequences. The most similar sequence is that of the Australian Shepherd. Next, with a lower similarity are the sequences from the Rottweiler, English Sheepdog and Bobtail breeds.

CHAPTER VI. CONCLUSIONS

1. The haplotype distribution and frequencies for the Carpathian, Corb and Mioritic shepherd dogs indicate high haplotype diversity in these breeds.
2. Molecular diversity indices have reached the highest values for the Mioritic Shepherd Dog, followed by the Corb Shepherd Dog. The lowest diversity was reported for the Bucovina Shepherd Dog breed, which is more geographically restricted than the other three breeds.
3. The value of the genetic distance, estimated with the fixation index F_{ST} was highest between the Bucovina Shepherd Dog and the other three Romanian shepherd dog breeds, and indicates very great genetic differentiation. A great genetic differentiation was observed between the Carpathian Shepherd Dog and the Mioritic Shepherd Dog ($F_{ST}>0.15$). There is moderate genetic differentiation between the Corb Shepherd Dog and the Carpathian and Mioritic shepherd dog breeds ($F_{ST}<0.15$).
4. The genetic distances calculated using Nei's distance indicate the same high level of genetic differentiation.
5. All the phylogenetic trees indicate a cluster grouping for each of the four Romanian breeds.
6. The phylogenetic arrangement of the sequences of the four breeds, together with the data from the alignment process, indicate a constant gene flow between the four shepherd dog populations in their past histories, before controlled selection of the breeds started.
7. The molecular level characteristics, the genetic distances and the phylogenetic relationships of the four Romanian shepherd dog breeds show enough differences and diversity in order to conclude that each breed as distinct from the other three and has a good genetic variability.

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