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Evaluation of the genetic conservatism of particular genes of the respiratory chain in the raccoon dog

Ocena konserwatyzmu genetycznego wybranych genów łańcucha oddechowego jenota

Summary. The aim of the present study was estimation of diversity of haplotypes of chosen mtDNA genes in the raccoon dog and determination of their conservatism level. The study material included DNA isolated from blood of farm-bred raccoon dogs. The PCR-RFLP reaction was performed for fragments of raccoon dog mitochondrial genes: cytochrome b (*cytb)* and cytochrome c oxidase subunits I and II (*COI*, *COII*). Two endonucleases: *Hinf*I and *Msp*I were used for the restriction analysis of each gene fragment. In each animal group haplotype AA was only observed in the case of *cytb* gene and *COI*. The restriction analysis of gene *COII* revealed presence of three haplotypes. The highest frequency was noted for haplotype BB (0.48). The frequencies of haplotypes AA and AB were 0.32 and 0.20, respectively. The studied mtDNA regions containing fragments of *cytb* and *COI* genes were conservative with respect to the occurrence of restriction sites; therefore, they are likely to be used for determination of species affiliation. Due to its variability, gene *COII* may be useful in genetic lineage reconstruction, phylogeography and identification of demographic changes in various raccoon dog groups.

Key words: *Nyctereutes procyonoides procyonoides*, mtDNA, haplotype

INTRODUCTION

Animal mitochondrial DNA is characterised by traits that determine its usability in numerous scientific disciplines. These include forensic medicine, population genetics, molecular evolutionism, molecular ecology, anthropology, archaeology, phylogeography, medical diagnostics and others.

Mitochondrial DNA differs substantially from the nuclear genome both in terms of its structure, function and resistance to environmental conditions (*post mortem*) and in its mode of inheritance, repair systems and many other factors. The use of mtDNA as a marker facilitates investigation of phylogenetic dependencies between animal groups, based on haplotype diversity; it also is of paramount importance in the search for ancestors of particular populations [Wayne *et al.* 1997, Zrzavy and Ricankova 2004, Agnarsson *et al.* 2010, Perini *et al.* 2010]. The methods of animal mtDNA analysis are the most popular procedures used in ecological, evolutionary and population studies [Randi 2000], due to the fact that the mtDNA structure is more resistant to degradation than that of the nuclear DNA. They are also used for investigation of hybridization of related species, e.g. within the family *Canidae* [Randi *et al.* 2000].

Raccoon dogs (*Nyctereutes procyonoides procyonoides*) both live in natural conditions and are reared in fur-bearing animal farms in Poland and Finland, taking into account the EU member states. Molecular analyses based on RAPD-PCR techniques of reared and wild-living raccoon dogs in Poland revealed that farm breeding practice could result in genetic distinctiveness between farm and wild-living raccoon dogs [Slaska *et al.* 2010].

It is therefore advisable that genes encoded in mtDNA should be used in analyses aiming at determination of the origin and phylogenetic relationships between populations of farm-bred and wild-living raccoon dogs; mitochondrial genes should also be used in determination of the phylogenetic position of animals within the family *Canidae*. Currently, the raccoon dog is one of the carnivorous species whose phylogenetic position has not been fully clarified [Wayne *et al.* 1997, Zrzavy and Ricankova 2004, Agnarsson *et al.* 2010, Perini *et al.* 2010]. Taking into consideration the above-mentioned information, we undertook a study to assess the diversity of mtDNA haplotypes of selected mtDNA genes in the racoon dog and to determine the level of their conservatism.

MATERIAL AND METHODS

The analyses involved 40 raccoon dogs from the basic stock, reared on one of the farms in the Podkarpackie region. Blood from each raccoon dog was intravitally sampled into sterile tubes (Medlab) containing K2EDTA as an anticoagulant. DNA was isolated from whole peripheral blood using the QIAamp DNA Blood Mini Kit (QIAGEN) for DNA

Table 1. Primer sequences of the analysed loci
Tabela 1. Sekwencje starterowe analizowanych loci

Locus	Primer H Starter H	Primer L Starter L
cytb	H15013 TTCCTATTCGCCTACGCCAT	L15312 TTCGATGATACCGATGGTTG
COI	H6055 TTCTTTGGACATCCTGAGGTT	L6634 AGTGTTGAGGGAAGAAAGTCA
COII	H7048 TACCCTTTCCAACTCGGATT	L7713 GGCAGATCAGGTTTCGAAAT

isolation. The PCR-RFLP (restriction fragment length polymorphism) reaction was performed for the following fragments of raccoon dog mitochondrial genes: cytochrome b (cytb) gene, cytochrome c oxidase subunit I (COI) gene and cytochrome c oxidase subunit II (COII) gene. On the basis of gene sequences (*cytb*, NCBI, Accession Number: AF028173; *COI,* NCBI: AF028197; *COII*, NCBI: AF028221), primers (Tab.1) containing fragments thereof were designed (respectively: 300, 580 and 666 bp.).

Table 2. Conditions of the PCR reaction for *cytb, COI* and *COII*
Tabela 2. Warunki reakcji PCR dla *cytb, COI* i *COII*

Stage Etap	Temperature (°C) Temperatura (°C)	Time Czas
Initial denaturation Denaturacja wstępna	95	10 min
Denaturation Denaturacja	95	60 s
Annealing of the primers Przyłączanie starterów	57.8; 62.4; 56.7 for *cytb, COI, COII*, respectively 57.8; 62.4; 56.7 – odpowiednio dla *cytb, COI, COII*	60 s
Extension of the primers Wydłużanie starterów Number of cycles – 35 Liczba cykli – 35	72	60 s
Terminal extension of the primers Końcowe wydłużanie starterów	72	20 min

Temperatures of annealing of the primers and amplification conditions were established for the particular genes (Tab. 2)

The products of gene fragment amplification were subjected to digestion (in separate reactions) using restriction enzymes: *Hinf*I and *Msp*I (*Hpa*II) (Fermentas). The total of 240 analyses was performed with the use of the PCR-RFLP technique for the gene fragments in question. Incubation was carried out according to the manufacturer's procedure. Visualization of the results was performed in 2% agarose gel (using a loading buffer containing bromophenol blue). The size of the fragments after the restriction analysis was determined using a size standard – GeneRuler 50bp DNA Ladder (Fermentas). The gels were analyzed under UV light (Transilluminator) and archived.

An *in silico* analysis of the restriction sites of the study gene fragments was performed with the use of the complete sequence of the mitochondrial genome of the raccoon dog [Wayne *et al.* 1997, Zhang and Chen 2010] and the coding sequences for each analysed gene (NCBI, Accession Numbers: AF028173, AF028197, AF028221). The size of the particular fragments obtained within each of the analyzed genes was determined with the use of a program for restriction site detection [Heiman 1997] and the raccoon dog mtDNA sequences contained in the GenBank database. In the case of restriction sites resulting from incompatibility of the analyzed sequences with the reference sequences, the fragment length was a rough estimate.

RESULTS AND DISCUSSION

The results of digestion of the *cytb*, *COI* and *COII* gene fragments in two separate restriction analyses were the basis for determination of the haplotypes. Table 3 presents the results of the restriction analysis performed on the raccoon dog genes. The Figures show examples of electrophoregrams of the *COII* gene fragment after the restriction analysis performed with the use of *Hinf*I (Fig. 1) and *Msp*I (Fig. 2) endonucleases.

Table 3. Length of DNA fragments obtained after the PCR-RFLP analysis of the study mitochondrial gene fragments in the raccoon dog
Tabela 3. Długości odcinków DNA otrzymanych po przeprowadzeniu reakcji PCR-RFLP badanych fragmentów genów mitochondrialnych jenota

Restriction enzyme Enzym restrykcyjny	Haplotype Haplotyp	Lengths of DNA fragments (bp)* Długości fragmentów DNA (pz)*		
		cytb 300	*COI* 580	*COII* 666
*Hinf*I	A	250 50	101 134 345	317 349
	B			317 ~239 ~110
*Msp*I (*Hpa*II)	A	205 95	513 77	666
	B			~ 441 ~ 225
Haplotype Haplotyp		AA	AA	AA BA BB

* ~ rough estimates / wartości szacunkowe

The RFLP-PCR analysis of the *cytb* gene yielded two DNA fragments after digestion with both *Hinf*I and *Msp*I. The occurrence of a single restriction site in the case of each of the endonucleases used is consistent with the results presented by Zhang and Chen [2010]. Haplotype AA has only been reported in the animal group in question.

The *cytb* gene sequence is considered to be one of the most conservative sequences, in which variability within the species is very low, if any [Wayne *et al.* 1997]. The results presented by Prusak *et al.* [2004] indicate the cytochrome b gene sequences may be regarded as representative of the species, which allows using it in species identification in cases where the origin of the biological material is unknown.

The results of restriction analysis of *COI* gene facilitated identification of two cleavage sites when *Hinf*I was used and a single site in the case of *Msp*I. The study animals displayed haplotype AA only. The results of the restriction analysis were consistent with those presented by Zhang and Chen [2010].

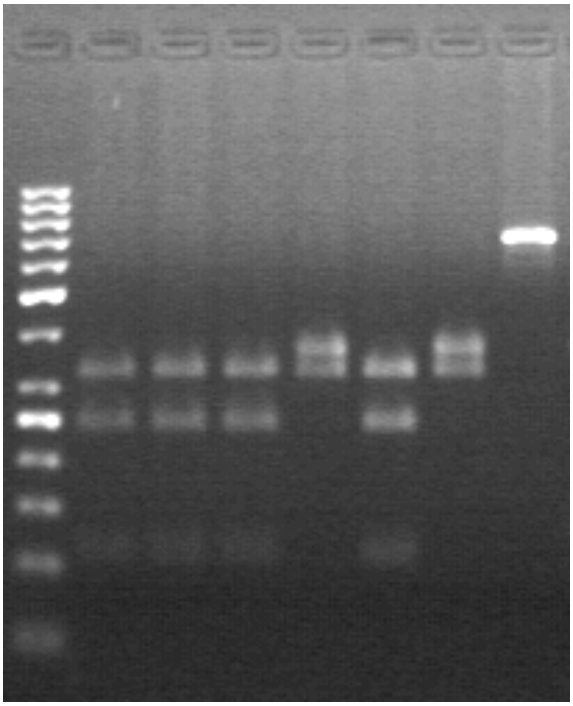

Fig. 1. Electrophoregram of a *COII* gene fragment after the restriction analysis (*Hinf*I) (the first lane from the left – the size standard, lanes 5 and 7 – haplotype A, lanes 2–4 and 6 – haplotype B, lane 8 – a *COII* gene fragment not subjected to digestion)
Rys. 1. Elektroforegram fragmentu genu *COII* po analizie restrykcyjnej (*Hinf*I) (pierwsza ścieżka od lewej – standard wielkości, ścieżka 5 i 7 – haplotyp A, ścieżki 2–4 i 6 – haplotyp B, ścieżka 8 – fragment genu *COII* niepoddany trawieniu)

The cleavage of the mitochondrial genes of cytochrome b and cytochrome c oxidase subunit I with the *Hinf*I and *Msp*I restriction enzymes yielded fragments of equal lengths in all the studied individuals. This may indicate monomorphism within the gene sequences recognized by the aforementioned restriction enzymes. This demonstrates absence of mutations of the sequences mentioned above and conservatism of the *cytb* and *COI* genes.

In some individuals, the analysis of the fragment of the cytochrome c oxidase subunit II gene with the use of restrictase *Hinf*I yielded two 317 bp and 340 bp fragments, which was in agreement with the *in silico* analysis of the *cytb* gene fragment in the raccoon dog [Zhang and Chen 2010]. In the study animal group, an additional cleavage site for *Hinf*I was also reported within a 349 bp fragment, which was possibly a consequence of a mutation. Interestingly, the GenBank contains a sequence encoding the *cytb* gene of the raccoon dog [Wayne *et al.* 1997]. It was observed on the basis of the *in silico* analysis performed that there were two cleavage sites for *Hinf*I in the aforementioned sequence within the analysed *COII* gene fragment.

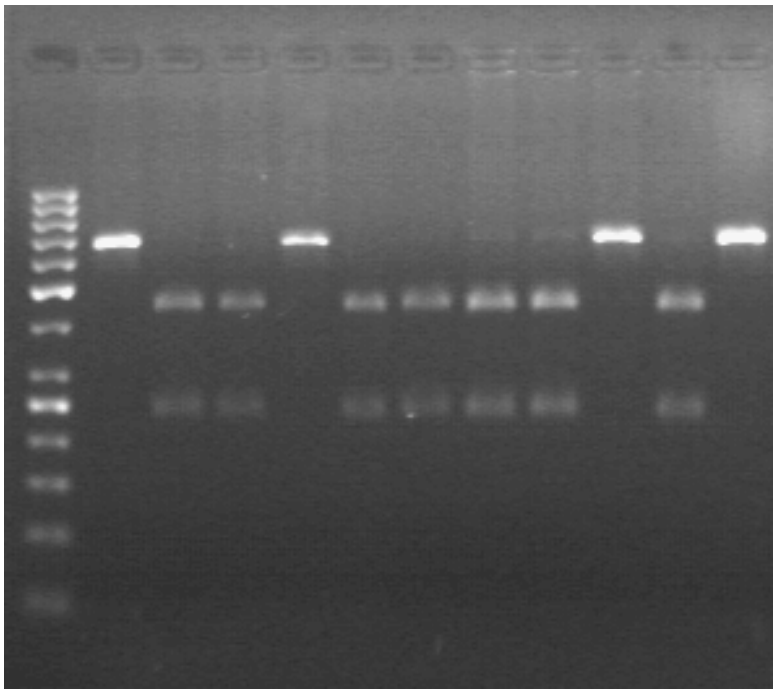

Fig. 2. Electrophoregram of a *COII* gene fragment after the restriction analysis (*Hinf*I) (the first lane from the left – the size standard, lanes 2, 5 and 10 – haplotype A, lanes 3, 4, 6–9 and 11 – haplotype B, lane 12 – a *COII* gene fragment not subjected to digestion)
Rys. 2. Elektroforegram fragmentu genu *COII* po analizie restrykcyjnej (*Msp*I) (pierwsza ścieżka od lewej – standard wielkości, ścieżki 2, 5 i 10 – haplotyp A, ścieżki 3, 4, 6–9 i 11 – haplotyp B, ścieżka 12 – fragment genu *COII* niepoddany trawieniu)

In some proportion of the individuals, the restriction analysis of the *COII* gene performed with the use of *MspI* revealed lack of cleavage of the PCR product of the gene fragment, which is consistent with the *in silico* analyses of sequences deposited in the GenBank [Wayne *et al.* 1997, Zhang and Chen 2010]. However, in some individuals, the restriction analysis showed a cleavage site for *MspI*, which is not present in the gene reference sequences.

The restriction analysis of the *COII* gene demonstrated three haplotypes. Haplotype BB displayed the highest frequency (0,48). The frequencies of haplotype AA and BA were 0.32 and 0.20, respectively.

The results of the restriction analysis performed with the *Hinf*I and *MspI* enzymes indicate variability in gene *COII,* which may cause abnormal energy metabolism in the mitochondria, due to the encoding nature of the sequence in question. Since each functional mutation of polypeptide encoding genes can disturb the functions of respiratory chain protein complexes, it is advisable that further COII gene analyses should be carried out in order to determine the type of mutation and its possible impact on the structure of the polypeptide encoded by the cytochrome c oxidase subunit II.

A characteristic feature of *cytb* is a slow pace of evolution; it is also an evolutionarily conserved gene [Wayne *et al.* 1997]. The study results obtained indicate that the *cytb* and *COI* genes display a slow pace of evolution, i.e., they are evolutionarily conservative genes. However, the differences determined in the *COII* gene sequence indicate occurrence of polymorphic sites within the gene sequence.

The sequences of cytochrome b gene (*cytb*) and cytochrome c oxidase subunit I and II (*COI* and *COII*) were used in investigations with the aim of determination of the phylogenetic relationships between 23 [Wayne *et al.* 1997] and 37 species [Zrzavy and Ricankova, 2004] belonging to the family *Canidae*. The above-mentioned genes were used to determine the pace of evolution and the common ancestor of 27 endemic *Canidae* species in South America [Perini *et al.* 2010]. On the basis of *in silico* sequence analyses of cytochrome b, Agnarsson *et al.* [2010] identified phylogenetic relationships in 243 carnivorous species, including canids. There are attempts to use the 648 bp fragment of the cytochrome c oxidase (*COI*) gene as a genetic identification code (*barcode*) in animals [www.barcodinglife.com]. In principle, it could be used for identification of new species, or verification of the phylogenetic systematics of species already known and described.

According to Wayne *et al.* [1997], mitochondrial genes encoding cytochrome b and cytochrome c oxidase subunits I and II have evolved in a similar manner, therefore they can be used for determination of the phylogenetic relationships in various animal species. However, it should be noted that, depending on the markers used (morphological, cytogenetic, or allozymes), in comparison with the „molecular tree" constructed on the basis of specific genetic distances using mitochondrial sequences, the phylogenetic tree contains several disputable points with regard e.g. to the raccoon dog [Wayne *et al.* 1997].

Given the encoding nature of cytochrome c oxidase subunit II, the analysis should be continued in order to determine the type of mutation and its possible impact on the structure of the polypeptide encoded by *COII.*

CONCLUSIONS

1. The results obtained indicate that the cytochrome b and cytochrome c subunit I genes are characterized by a slow pace of evolution, i.e., they are evolutionarily conservative genes. The mitochondrial DNA regions including fragments of *cytb* and *COI* genes were conservative in terms of restriction sites; therefore, it cannot be excluded that they may be used in determination of species affiliation.

2. The different haplotypes within the cytochrome c subunit II mitochondrial gene, obtained after the restriction analysis, indicate occurrence of polymorphism within sequences recognized by endonucleases *Hinf*I and *Msp*I. Therefore, due to the diversity observed, the *COII* gene may be useful in reconstructing the genealogical lines, phylogeography and identification of demographic changes in various groups of raccoon dogs.

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www.barcodinglife.com

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Streszczenie. Przeprowadzone badania miały na celu ocenę różnorodności haplotypów wybranych genów mtDNA jenota oraz ustalenie poziomu ich konserwatyzmu. Materiał do badań stanowiło DNA wyizolowane z krwi jenotów hodowlanych. Reakcję PCR-RFLP przeprowadzono dla fragmentów genów mitochondrialnych jenota: cytochromu b (*cytb)* oraz podjednostki I i II cytochromu c (*COI*, *COII*). Do przeprowadzenia analizy restrykcyjnej każdego z fragmentów genów wykorzystano dwie endonukleazy: *Hinf*I i *Msp*I. W badanej grupie zwierząt odnotowano występowanie wyłącznie haplotypu AA w przypadku genu *cytb*, jak również *COI*. Analiza restrykcyjna genu *COII* ujawniła obecność trzech haplotypów. Największą częstość występowania odnotowano w przypadku haplotypu BB (0,48). Frekwencje haplotypu AA i BA wynosiły odpowiednio: 0,32 i 0,20. Badane w pracy regiony mtDNA obejmujące fragmenty genów: *cytb* i *COI* były konserwatywne pod względem występowania miejsc restrykcyjnych, nie jest więc wykluczone, iż mogą służyć do określania przynależności gatunkowej. Natomiast gen *COII*, ze względu na odnotowaną zmienność, może być przydatny w odtwarzaniu linii genealogicznych, filogeografii i identyfikacji zmian demograficznych różnych grup jenotów.

Słowa kluczowe: *Nyctereutes procyonoides procyonoides*, mtDNA, haplotyp