

Mitochondrial DNA haplotypes are associated with performance traits in Raccoon dogs*

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Given the important function fulfilled by mitochondria in cells, the relationship between mitochondrial DNA polymorphism and economically important traits has been investigated for many farm animal species. However, no such an information is available for farm fur-bearing animals. Therefore, we have evaluated three fragments of mitochondrial genes: *MT-COI* (cytochrome c oxidase subunit I), *MT-COII* (cytochrome c oxidase subunit II), and *MT-CYTB* (cytochrome b) for their association with performance traits in raccoon dogs. The results obtained in this study indicate that the candidate gene *MT-COII* can be involved in the development of hair coat. Hair coat quality was significantly associated with the m.A7446 polymorphism. Raccoon dogs of genotype m.G7446 had significantly better hair coat quality in comparison with animals of genotype m.A7446. In the case of the other eight polymorphisms identified, no significant differences were found in the values of the six assessed traits (body weight, body size and conformation, colour type, hair coat purity, hair coat quality, total score of conformation traits) between the alternative genotypes. Six polymorphisms were found in the *MT-COII* gene sequence and, accordingly, three haplotypes: A (AAGAAA - respectively in positions 7125, 7194, 7221, 7287, 7446, 7578), B (AAGAGA), and C (GCAGGG) were distinguished. Animals with haplotype B and C were characterised by a significantly better hair coat quality in comparison

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with raccoon dogs of haplotype A. Given the important function of mitochondria in biochemical processes related to economically important traits and animal healthiness, the investigations should be continued and extended on other functional and health traits.

KEY WORDS: mitochondrial DNA / performance traits / polymorphism / Raccoon dogs

Mitochondria play an extremely important role in metabolic processes, apoptosis, and aging. In the process of oxidative phosphorylation, mitochondria produce energy in the form of ATP and heat. The presence of own genetic material in the form of mitochondrial DNA (mtDNA) in these organelles is indispensable for maintenance of their function [Fernández *et al.* 2008, Grzybowska-Szatkowska and Ślaska 2014]. Mitochondrial DNA encodes 13 proteins, 2 rRNA molecules, and 22 tRNA molecules (tRNAs) [Sutarno *et al.* 2002, Chen *et al.* 2009]. Additionally, it contains a control region, which is involved in regulation of mtDNA replication and transcription [Reicher *et al.* 2012]. Mitochondrial DNA is present in thousands of copies per cell and exhibits a 5-10-fold higher mutation rate than that of nuclear DNA (nDNA). Changes occurring in mtDNA may be reflected in the phenotype. It has been evidenced that mutations in the mitochondrial DNA are the cause of many chronic diseases in humans and various animal species [Sutarno *et al.* 2002, Grzybowska-Szatkowska and Ślaska 2014].

The raccoon dog (*Nyctereutes procyonoides procyonoides*) is a representative of the Canidae family. It is a fur-bearing animal bred in farms for pelts used in production of fur coats [Ślaska *et al.* 2008, Ślaska and Grzybowska-Szatkowska 2011, Bugno-Poniewierska *et al.* 2013]. The value of pelts depends on their size and quality, which is determined by the colour and quality of hair coat [Lorek *et al.* 2001, Gugolek *et al.* 2002, Wierzbicki *et al.* 2004; Ślaska *et al.* 2007]. Therefore, selection on fur-bearing animal farms is targeted at improving the productivity of reared animals [Wierzbicki *et al.* 2004; Ślaska *et al.* 2007]. Extensive investigations into the genome of the raccoon dog conducted within the scope of molecular genetics [Ślaska *et al.* 2007, Ślaska and Jeżewska 2008, Ślaska *et al.* 2008, Ślaska and Grzybowska-Szatkowska 2011] and cytogenetics [Switonski *et al.* 2009, Bugno-Poniewierska *et al.* 2013] have recently enabled usage of genetic markers for animal selection in breeding programs [Ślaska *et al.* 2007, Ślaska and Jeżewska 2008, Ślaska *et al.* 2008, Grzes *et al.* 2011, Urbański *et al.* 2013].

Polymorphism identified in mitochondrial DNA is one of the most common genetic markers employed in population studies of many animal species [Mannen *et al.* 1998, Fernández *et al.* 2008, Chen *et al.* 2009, Głazewska *et al.* 2013], including farm fur-bearing animals [Ślaska and Grzybowska-Szatkowska 2011]. This is related to the high rate of mtDNA mutations and inheritance thereof almost exclusively in the maternal line. Analyses of mitochondrial DNA revealed intraspecific phylogenetic relationships in various populations of raccoon dogs [Ślaska and Grzybowska-Szatkowska 2011].

Results obtained by various authors have indicated relationship between polymorphisms both in the nuclear and mitochondrial genomes and the utility

traits of different animal species. Given the important role played by mitochondria in cells, researchers have attempted at determination of the association between mtDNA polymorphism and economically important utility traits in various animal populations, e.g. in cattle, swine, sheep, and donkeys [Mannen *et al.* 1998, Sutarno *et al.* 2002, Mannen *et al.* 2003, Yen *et al.* 2007, Fernández *et al.* 2008, Chen *et al.* 2009, Reicher *et al.* 2012]. In cattle, a relationship between changes in mtDNA and fertility [Sutarno *et al.* 2002] as well as carcass traits and meat quality [Mannen *et al.* 1998, Mannen *et al.* 2003] has been found. Similar analyses focused on the association between mtDNA polymorphism and meat quality traits were performed in a group of Iberian pigs [Fernández *et al.* 2008]. Chen *et al.* [2009] analysed three breeds of Chinese donkeys and reported statistically significant relationships between mtDNA polymorphism and rump width (Yunnan breed) and body weight (Dezhou breed).

Available literature does not provide information about the relationship between mtDNA polymorphism and performance traits in the raccoon dog. Since it is a fur-bearing animal, it is important to achieve the best utility traits, and recognition of the association between mtDNA polymorphism and economically important traits may provide valuable information in terms of knowledge and application.

Currently, there is increasing evidence that mtDNA polymorphisms (among others in genes of the IIIth and IVth of the respiratory chain complexes) are associated with performance and reproduction traits in various animal species. According to Ślaska and Grzybowska-Szatkowska [2011], analyses of mitochondrial DNA genes (cytochrome b, cytochrome c oxidase subunit I and subunit II) revealed four mitochondrial haplogroups in farmed raccoon dogs, which were not found in wild-living animals. The occurrence of new haplogroups in the farm animals indicates the appearance of adaptive mutations. The authors concluded that, the diversity of the haplogroups may have been one of the factors inducing changes in selected performance traits in the phenotype of farmed raccoon dogs within a relatively short time. Therefore, the aim of the study was to determine the relationships between the polymorphisms in fragments of the *MT-CYTB*, *MT-COI*, and *MT-COII* genes of the mitochondrial DNA of the raccoon dog and the performance traits.

Material and methods

The analyses were carried out on 346 raccoon dogs (48% of males and 52% of females) raised in 2005-2009 on a breeding farm in south-eastern Poland. Blood from each raccoon dog was sampled intravitaly into sterile vacuum tubes containing K₂EDTA (dipotassium ethylene diamine tetraacetic acid) anticoagulant. DNA was isolated with the use of an automatic system of nucleic acid isolation QIAcube (Qiagen, Hilden, Germany). Isolation from whole peripheral blood was done using the QIAamp DNA Blood Mini Kit (Qiagen). The primers used and the PCR reaction conditions were described in the literature on the genome of the raccoon dogs by Ślaska and Grzybowska-Szatkowska [2011]. The isolated DNA was used to amplify

three fragments of mitochondrial genes: *MT-COI* (cytochrome c oxidase subunit I), *MT-COII* (cytochrome c oxidase subunit II), and *MT-CYTB* (cytochrome b). Amplicons were sequenced using a Terminator Cycle Sequencing kit (Applied Biosystem, Foster City, CA) in the GeneAmp PCR system 9700 (Applied Biosystem). Samples were subsequently purified on CentriSep columns according to the manufacturer's protocol or precipitated with ethanol and sodium acetate according to the protocol of the BigDye kit manufacturer. Extension products were separated on an ABI 377 automated sequencer (Applied Biosystem). The nucleotide sequences obtained were subjected to bioinformatics analyses in order to determine mutation and polymorphic sites within the analysed mtDNA fragment [Huang and Madan 1999, DNA Baser Sequence Assembler v 3.2 2012]. Haplotypes of examined genes according to the nomenclature by Ślaska and Grzybowska-Szatkowska [2011] were established. The coding sequences of the raccoon dog *MT-CYTB*, *MT-COI*, *MT-CO2* genes are archived in the GenBank (JN703387, JN703388 and JN703389, respectively).

The pedigree data of animals and their genotypes at 3 gene fragments (*MT-COI*, *MT-COII*, *MT-CYTB*) were used to estimate the relationship between the genotypes and exterior traits (body weight, body size and conformation, colour type, hair coat purity, hair coat quality, total score of conformation traits). Animals were weighed at the age of 19 weeks when they reached fur maturity and evaluated for conformation traits in accordance with the evaluation standard (20-point scale) developed by the Central Animal Breeding Office [CSHZ, 1997]. The pedigree data and the phenotypic characteristics of the population of the raccoon dogs came from breeding documentation. Mean phenotypic values of raccoon dog conformation traits were as follow: body weight (g) $\bar{x} = 10916 \pm 1402$; body size $\bar{x} = 5.73 \pm 0.56$; colour type $\bar{x} = 2.99 \pm 0.12$; colour purity $\bar{x} = 2.54 \pm 0.52$; hair coat quality $\bar{x} = 6.00 \pm 1.04$; total score $\bar{x} = 17.24 \pm 1.46$.

The traits were analysed using the following mixed model:

$$y_{ijklmn} = Year_i + Sex_j \times Genotype_k + sire_l + dam(sire)_{lm} + e_{ijklmn}$$

where:

y_{ijklmn} – dependent variables;

$Year_i$ – year of birth;

$Sex_j \times G_k$ – effect of genotype and sex;

s_l – random effect of sire;

d_{lm} – random effect of dam nested within sire;

e_{ijkl} – the residual effect.

On the basis of explanatory analyses, the model takes into account factors that affect significantly the studied traits. Analyses were performed in the GLIMMIX procedure of SAS 9.4 (SAS Institute, Cary NC). Appropriate pre-planned orthogonal contrasts were performed for the sex $\times$ genotype effect to evaluate the differences

between the genotypes. The *P*-values for the estimated differences were subjected to Bonferroni correction to account for multiple comparisons. Percentages of variance explained by the genotype was calculated as the proportion of residual sum of squares due to the genotype effect on the residual sum of squares excluding the genotype effect.

Results and discussion

The values of heritability coefficients of the conformation traits appeared to be relatively high (0.35-0.48), which indicated that the variability of fur traits observed in the population was to a high degree conditioned by the additive effect of the individual's genotype (Tab. 1). Genetic correlations between conformation traits were mostly positive, but their values were low (Tab. 1).

Table 1. Heritability coefficients (diagonal) and genetic correlations (below diagonal) and their standard errors (in bracket) of the conformation traits

Trait	Body weight	Body size	Colour type	Colour purity	Hair coat quality
Body weight	0.48 (0.17)				
Body size	0.65 (0.19)	0.43 (0.14)			
Colour type	-0.10 (0.34)	-0.06 (0.41)	0.35 (0.16)		
Colour purity	0.07 (0.36)	0.03 (0.34)	0.15 (0.44)	0.36 (0.14)	
Hair coat quality	0.02 (0.37)	0.14 (0.41)	0.16 (0.30)	0.06 (0.39)	0.42 (0.18)

The results concerning the differences in the values of the performance traits of the raccoon dogs depending on the genotype in the loci of genes *MT-CYTb* and *MT-COI* have been computed (not tabulated data). Two polymorphisms were identified in the *MT-CYTb* gene sequence: m.A15039G and m.C15045T. Two haplotypes were distinguished: haplotype A (m.A15039/m.C15045) and haplotype C (m.G15039/m.T15045) according to the nomenclature established by Slaska and Grzybowska-Szatkowska[2011]. The *MT-COI* gene sequence exhibited polymorphism at m.A6159G. No significant differences were found in the values of any of the performance traits analysed depending on the genotype/haplotype in the *MT-CYTb* and *MT-COI* loci.

The results of the statistical analysis comparing the genotype effects of the polymorphic loci of gene *MT-COI* are presented in Table 2. Six polymorphic loci have been noted (m.A7125G, m.A7194C, m.G7221A, m.A7287G, m.A7446G, m.A7578G). Significant differences in hair coat quality were only found for the polymorphism at m.A7446G. Raccoon dogs with genotype m.G7446 were characterised by a significantly higher (by 1.20 points) score of hair coat quality, compared with that in animals with genotype m.A7446. The polymorphism m.A7446/m.G7446 explains 1.7% of the phenotypic variance. The polymorphisms identified in the other five loci had no statistically significant effect on any of the analysed traits (Tab. 2).

Table 2. Differences between m.A7446 and m.G7446 alleles of the *MT-COII* gene and selected statistical parameters of studied performance traits

Trait	Difference	Standard error	P-value	Confident limits (95%)	
				lower	upper
Body weight (g)	945	719	0.190	-472	2362
Body size (scores)	0.20	0.26	0.447	-0.32	0.72
Colour type (scores)	0.00	0.05	1.000	-0.10	0.10
Colour purity (scores)	0.27	0.26	0.294	-0.24	0.79
Hair coat quality (scores)	-1.20	0.53	0.025	-2.25	-0.15
Total score	-0.52	0.73	0.478	-1.95	0.92

In the studied raccoon dog population, six polymorphisms were found in the *MT-COII* gene sequence (Tab. 2) and, accordingly, three haplotypes: A (AAGAAA - respectively in positions 7125, 7194, 7221, 7287, 7446, 7578), B (AAGAGA), and C (GCAGGG) were distinguished. The effect of the *MT-COII* gene haplotypes on the performance traits are presented in Table 3.

Significant differences were found between the hair coat quality in animals of haplotype A and B as well as A and C (Tab. 3). The scores of hair coat quality in raccoon dogs with haplotypes B and C were lower (Tab. 3). Animals with both haplotype B and haplotype C had a significantly higher hair coat quality than raccoon dogs with haplotype A (by 1.23 and 1.24 points, respectively – Tab. 3). In the case of raccoon dogs with a significantly higher hair coat quality (haplotype B and C), genotype m.G7446 was identified in the *MT-COII* gene, which was the only *locus*

Table 3. Differences and selected statistical parameters of performance traits in relation to haplotypes of the *MT-COII* gene

Trait	Haplotype difference		Difference	Standard error	P-value	Confident limits (95%)	
						lower	upper
Body weight (g)	A-B	AAGAAA-AAGAGA	857	731	0.242	-583	2298
	A-C	AAGAAA-GCAGGG	1040	730	0.155	-398	2478
	B-C	AAGAGA-GCAGGG	183	232	0.432	-274	640
Body size (scores)	A-B	AAGAAA-AAGAGA	0.16	0.27	0.543	-0.36	0.69
	A-C	AAGAAA-GCAGGG	0.22	0.27	0.411	-0.31	0.75
	B-C	AAGAGA-GCAGGG	0.06	0.08	0.457	-0.09	0.21
Colour type (scores)	A-B	AAGAAA-AAGAGA	-0.01	0.05	0.845	-0.11	0.09
	A-C	AAGAAA-GCAGGG	0.01	0.05	0.873	-0.09	0.10
	B-C	AAGAGA-GCAGGG	0.02	0.01	0.187	-0.01	0.04
Colour purity (scores)	A-B	AAGAAA-AAGAGA	0.24	0.26	0.373	-0.28	0.76
	A-C	AAGAAA-GCAGGG	0.29	0.26	0.268	-0.23	0.81
	B-C	AAGAGA-GCAGGG	0.06	0.08	0.492	-0.10	0.22
Hair coat quality (scores)	A-B	AAGAAA-AAGAGA	-1.23	0.55	0.025	-2.31	-0.16
	A-C	AAGAAA-GCAGGG	-1.24	0.54	0.023	-2.32	-0.17
	B-C	AAGAGA-GCAGGG	-0.01	0.17	0.941	-0.34	0.32
Total score	A-B	AAGAAA-AAGAGA	-0.65	0.74	0.384	-2.12	0.82
	A-C	AAGAAA-GCAGGG	-0.58	0.74	0.437	-2.04	0.88
	B-C	AAGAGA-GCAGGG	0.07	0.22	0.739	-0.35	0.50

with a statistically significant effect on the hair coat quality (Tab. 2). The haplotype polymorphism in the *MT-CO2* gene explains 4.2% of the phenotypic variance.

Many research teams have attempted to determine the association between mtDNA polymorphisms and economically important utility traits in populations of various animal species.

The majority of those investigations has been conducted on cattle. Sutarno et al. [2002] studied the relationship of the polymorphisms identified in gene *MT-ND5* and in the control region (*D-loop*) with the reproduction traits in cattle. Statistically significant differences between the rate of calving and mitochondrial haplotypes were reported. Mannen et al. [1998] reported statistically significant differences between different mitochondrial types and the longissimus muscle area and beef marbling score. Mannen et al. [2003] also investigated the association between polymorphisms in the mtDNA coding regions and meat quality in Japanese Black cattle. A significant effect of substitution in 16S rRNA on the longissimus muscle area and beef marbling score was found. Similarly, the present investigations have revealed polymorphisms in protein encoding genes in mtDNA. As in the study carried out by Mannen et al. [2003], no relationships have been found between the polymorphisms identified in the gene encoding *MT-CYTB* and *MT-COI* and the performance traits of the raccoon dogs.

The present analyses of the six *MT-COII* gene polymorphisms revealed significant differences between the raccoon dogs of different genotypes in locus m.A7446G only in terms of hair coat quality (Tab. 2). Raccoon dogs with genotype m.G7446 were characterised by a significantly higher (by 1.20 points) score of hair coat quality, compared with animals with genotype m.A7446 (Tab. 2). According to Slaska and Grzybowska-Szatowska [2011], four haplotypes, A, B, C, and E, were identified in gene *MT-COII* in farm raccoon dogs. The current investigations have revealed three *MT-COII* haplotypes (Tab. 3). Both animals of haplotype B and those of haplotype C had a significantly better hair coat quality than the raccoon dogs of haplotype A (Tab. 3). These results are consistent with the significant differences reported between the hair coat quality and genotypes in locus m.A7446G (Tab. 2).

In the case of raccoon dogs, i.e. farm fur-bearing animals, no investigations of the relationship between mtDNA polymorphisms and performance traits have been carried out. As in other farm animal species, nuclear quantitative trait loci (QTL) of conformation traits were determined also in raccoon dog populations. Genotypes of microsatellite markers localized in 5 linkage groups were used for the mapping. The results of that pioneering study identified the first hypothetical QTL regions in the raccoon dog. Scanning results showed that the LG01 and LG05 groups might contain loci determining hair coat quality in raccoon dogs. The authors concluded that the FH3922 and REN230G12 markers used in mapping QTL regions in raccoon dogs could be useful in selection and genetic improvement of a conformation trait such as hair coat quality in raccoon dogs [Slaska et al. 2007]. The goal of every breeder is to produce the largest and best-quality pelts. In accordance with the 20-point scale evaluation standard, hair coat quality in fur-bearing animals receives the highest

scores (0-8 out of 20 points) according to the Polish grading standard [CSHZ, 1997]. Hence, this trait is of the greatest importance to breeders who attempt at producing pelts that will be competitive in the global fur market.

Yen *et al.* [2007] investigated the relationship between D-loop polymorphisms and reproductive traits in Meishan pigs. They reported significant differences due to haplotype alleles and in body weight of piglets on day 21. Similarly, Fernández *et al.* [2008] analysed the association between mitochondrial genome polymorphism and utility traits (longissimus muscle composition) in pigs. The authors found that polymorphisms identified in gene *COIII* and 12S rRNA might be related to the meat quality traits in pigs.

According to Ślaska and Grzybowska-Szatkowska [2011], farm raccoon dogs had three *MT-CYTB* gene haplotypes A, B, and C. In the present study, two mitochondrial haplotypes (A and C) have been identified in the *MT-CYTB* gene fragment. However, no statistically significant differences were found between the haplotypes and the score of the examined utility traits. Chen *et al.* [2009] performed analysis of the relationships between polymorphisms in the mitochondrial gene *CYTB* and growth traits in donkeys. Their analyses included three breeds: Yunnan, Dezhou, and Liangzhou. Gene *CYTB* was shown to be useful as a genetic marker in selection of the Yunnan breed towards rump width. Reicher *et al.* [2012] carried out investigations of the relationship between polymorphism in e.g. gene *CYTB* and utility traits in Afec-Assaf sheep. Analysis of birth weight, growth rate of lambs, and productivity revealed no association between the mitochondrial haplogroup and female longevity, the perinatal survival rate, birth weight, and daily growth rate in lambs. However, significant differences among the haplogroups were found for prolificacy of ewes.

The present study has shown significant differences between the hair coat quality with different mitochondrial *COII* gene haplotypes. The differences were significant between animals with haplotypes A and B ($P_{\text{Boni}}=0.025$) as well as A and C ($P_{\text{Boni}}=0.023$) (Tab. 3).

Identification of mtDNA polymorphisms associated with economically important performance traits in raccoon dogs and development of molecular tests for detection thereof will allow breeders to implement new breeding strategies. Given the very important role of mitochondria in organism cells, changes in mitochondrial DNA may have a significant impact on the animal's breeding value. This is particularly important in the case of raccoon dogs due to the phenomenon of multipaternity [Ślaska and Jeżewska 2008] and lack of precise information about the breeding value of raccoon dogs on Polish farms.

In conclusion, the association data obtained in this study indicates that the candidate gene - *MT-COII* is involved in the development of hair coat. Gene *COII* is involved in the process of oxidative phosphorylation (OXPHOS) and is associated with energy production. It is not excluded that in the past the mtDNA haplotypes differentiated depending on the environmental conditions, with a predisposition to thinner coat in warm southern temperate climate zone, and to denser, in a cool temperate climate.

Therefore, the mutation in the *COII* gene may affect the development of hair coat. The presented results indicate that the analysis of the mtDNA gene polymorphism may be useful in selection aimed at improving the value of hair coat quality in raccoon dogs. The relationship between haplotype *MT-COII* and trait values in our study in the raccoon-dog may also be true for other fur-bearing Canidae species – e.g. the red and polar foxes. Continuation of similar studies in the future on other Canidae could shed more light on the association of mtDNA haplotypes with important fur animal traits. Given the important function of mitochondria in biochemical processes related to economically important traits, reproduction, and healthiness of fur-bearing animals, the investigations should be continued and extended on other functional and health traits.

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