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GENETIC DIFFERENTIATION OF COMMON FOX *VULPES VULPES* (LINNAEUS, 1758) ON THE BASIS OF THE INSULIN-LIKE GROWTH FACTOR 1 (*IGF1*), MYOSIN-XV (*MYO15A*) AND PAIRED BOX HOMEOTIC 3 (*PAX3*) GENES FRAGMENTS POLYMORPHISM*

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Abstract

Single-nucleotide polymorphism (SNP) was analysed for selected fragments of three genes – insulin-like growth factor 1 (*IGF1*), myosin-XV (*MYO15A*) and paired box homeotic gene 3 (*PAX3*) – in farm and wild red foxes from two continents. The study was undertaken in order to verify whether the SNP characteristics of these genes enable farm-bred foxes to be distinguished from free-living foxes. The greatest number of changes were detected in the *IGF1* gene. For each of the genes investigated specific SNP profiles characteristic only for farm foxes and only for wild foxes were noted. At the same time, specific SNP profiles were noted for wild foxes from North America and from Europe. The frequency of SNP (bases per SNP) in the gene fragments examined was 22 bp for *IGF1*, 34 bp for *PAX3* and 56 bp for *MYO15A*. Single-nucleotide polymorphism is a very good molecular marker enabling characterization of nucleotide variation in the genes investigated between wild and farm individuals.

Key words: SNP, *IGF1*, *MYO15A*, *PAX3*, *Vulpes vulpes*

Based on the latest systematics, 45 local varieties or subspecies of red fox (*Vulpes vulpes*) are distinguished. Three genes were selected for the study, influencing body mass, hearing, and organ and tissue formation. The *IGF1* gene encodes a specific protein (insulin-like growth factor 1, somatomedin C) whose structure and function are similar to those of insulin, included in the family of proteins that significantly affect growth and development (Rotwein et al., 1986). Research on mice, humans, and canids has demonstrated that the *IGF1* gene significantly influences body size in

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mammals. Mice with a damaged *IGF1* gene have been shown to attain a very small size, while humans with a deletion in this gene are born with a body length considerably below the norm (Sutter et al., 2007). The type of arrangement determines the size of the individual. The arrangement of the gene together with its regulatory sequence occurs in two forms, termed I and B. All small breeds of dog have the I form, while large breeds have the B form. The only representatives of large breeds with the same DNA sequence as small dogs are Rottweilers. This is because they have other gene sequences that significantly influence their size (Sutter et al., 2007). The *MYO15A* gene is involved in the production of a protein included in the group of motor proteins known as myosins (Nal et al., 2007). Mutation in the *MYO15A* gene leads to malfunctioning of myosin XVA and to hereditary hearing impairment in humans, mice and dogs (Kikkawa et al., 2005). The *PAX3* gene plays a key role in the formation of individual organs and tissues in the initial stages of embryonic development, and in maintaining normal cell functions after birth. In the dog it is localized in 37q16-q17 (Krempler et al., 2000). The protein produced by *PAX3* is essential during the formation of the myotome (it induces expression of two MRFs – muscle regulatory factors) and acts together with the protein produced by the *PAX7* gene (Lamey et al., 2004). During embryonic development and in the muscles of the adult organism, these two proteins – *PAX3* and *PAX7* – take part in myotome formation and in muscle growth and regeneration. Mutants without a functional *PAX3* gene – splotch mutants – are characterized by defects in the structure of the neural tube and limb muscles (Relaix et al., 2004).

Diversity of phenotypes occurring in both wild and farm animals is an important factor allowing for the differentiation of groups belonging to the family Canidae. The hypothesis was that there is the SNPs differentiation in *IGF1*, *MYO15A* and *PAX3* genes in the farm-bred and wild living population of common fox from Europe (Poland) and North America (north-eastern regions of the United States and Canada). Although we know the localization of genes on chromosomes and their nucleotide composition, it is not always clear how they are inherited and the way they affect a given trait is not fully understood (e.g. complementary interaction or epistasis). The aim of the study was to determine the nucleotide sequence of fragments of the genes *IGF1*, *MYO15A* and *PAX3* in farm and wild individuals of the Canidae family from two continents, and identify any polymorphisms occurring in the nucleotide sequence. In the future, this will contribute to a better understanding of the role of these genes in heredity of morphometric traits in species of this family. The study was undertaken in order to verify whether the SNP characteristics of these genes enable farm-bred foxes to be distinguished from free-living foxes.

Material and methods

Animals

The material for the study consisted of the blood of farm red foxes (*Vulpes vulpes*) from Poland (20 individuals) and raw skins of wild foxes from North America –

north-eastern regions of the United States and Canada (20 individuals) and Poland (20 individuals).

Genetic and data analysis

Total genomic DNA was extracted from each sample using a commercial QIAgen extraction kit (QIAamp DNA Blood Mini Kit or DNeasy Blood and Tissue Kit) following the protocol provided in the QIAcube. The primers were designed using Primer3Plus software (Rozen and Skaletsky, 2000) to amplify each region of *IGF1*, *MYO15A* and *PAX3* (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) (Table 1).

Fragments were selected of genes responsible for different traits (body weight, the hearing process, development of the central nervous system and melanocytes). The genes were selected because these traits are important both physiologically and in terms of livestock selection.

Table 1. Primers used for PCR of the three genes

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Size (bp)
IGF1	AAGTAGCCTGAGTAAGATTTGACT	AGCAATCTACCAACTCCAGGACCA	305
MYO15A	TTTCCACATCCACTCTCACG	GAAGGGGGAGAAGCAGACTT	168
PAX3	CGACCTTGCAGCTGCTTGGGT	AGTGTGGGATGCCCGCAGTG	303

The first amplification was performed by polymerase chain reaction (PCR). The reactions (25 µL total volume) contained 2 µl DNA (with the DNA concentration 50 ng/µl) and 1.0 U Taq polymerase (Ampli Taq Gold 360 DNA Polymerase, Applied Biosystems) in the manufacturer's buffer, adjusted to a final concentration of 2.5 mM MgCl2, 0.2 mM of each dNTP and 0.1 mM of each primer. PCR cycling conditions were 95°C for 10 min; 30 cycles of 95°C for 30 s, 52°C for 60 s (*IGF1* and *MYO15A*) or 63°C for 60 s (*PAX3*), 72°C for 60 s; and 72°C for 20 min. (Labcycler, SensoQuest). To confirm the PCR products, gel electrophoresis was carried out using 2% agarose gel with ethidium bromide (EtBr). The PCR product was purified using an ExoSAP-IT kit (Affymetrix). The second amplification (sequencing PCR) – bidirectional sequencing – was carried out according to the BigDye® Terminator v3.1 CycleSequencing Kit (Applied Biosystems). PCR products were purified using a DyeEx Spin Kit (Qiagen) in the QIAcube. PCR products were sequenced using a 3100 Genetic Analyser (Applied Biosystems). The sequences were assembled into consensus sequences using DNA Baser (Heracle Biosoft; http://www.DnaBaser.com). Sequencing results were aligned using BLAST. The sequencing data were then compared with canine reference sequences for the genes *IGF1* (NCBI – ID: 610255), *MYO15A* (EMBL – AJ428858) and *PAX3* (NCBI – ID: 488544) registered in the NCBI database. The SNP positions of farm and wild individuals were compared using MEGA4 software. The frequency of individual SNP profiles was calculated with the SAS statistical package and ARLEQUIN v.3.5. SNP profile was defined as

a set of single nucleotide polymorphisms (SNPs) obtained through sequencing with respect to each of the gene fragments investigated.

Results

The following genes were analysed: insulin-like growth factor 1 (*IGF1*); myosin-XV (*MYO15A*) and paired box homeotic gene 3 (*PAX3*).

Substitutions

Fourteen transitions and transversions (SNPs) were observed in the *IGF1* gene, ranging from 3 to 303 bp in the region examined (intron 5, exon 6 and intron 6), including eight transitions, at positions c.26037 (A>G), c.26144 (G>A), c.26194 and c.26246 (C>T), c.26251 and c.26258 (A>G), c.26282 (T>C) and c.26334 (G>A), and six transversions, at c.26032 and c.26200 (T>A), c.26043, c.26249, c.26275 (G>C) and c.26324 (C>A). In the case of the *MYO15A* gene fragment, three SNPs were noted, including two transitions, at c.426 (A>G) and c.463 (G>A), and one transversion, at c.578 (C>A). For the *PAX3* gene, 9 SNPs were identified, including 6 transitions, at c.11212, c.11260, c.11395 (C>T), c.11284 (T>C), c.11437 and c.11485 (G>A), and 4 transversions, at c.11198 (T>A), c.11273 (G>T), c.11476 (G>C) and c.11485 (G>C). Both transition and transversion occurred at position 302, which is an example of a very rare triallelic SNP locus. The frequency of occurrence of SNPs in the gene fragments was 1 SNP every 22 bp for IGF-1, 34 bp for PAX3 and 56 for MYO15A.

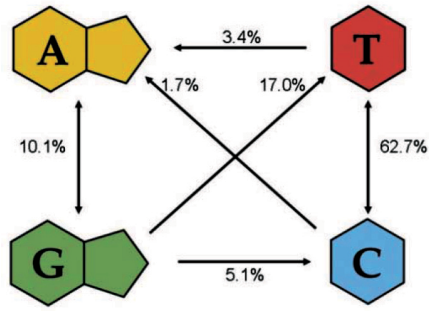

Figure 1. Distribution of transitions and transversions among SNPs for the genes investigated

Transitions accounted for 72.8% and transversions for 27.2% of the SNPs analysed. The transition/transversion ratio (R) for the genes ranged from 2.59 to 3.0: RIGF1=2.88; RMYO15A=3.00 and RPAX3=2.59 (Figure 1).

Table 2. The types and frequency of SNP profiles in the genes *IGF1*, *MYO15A* and *PAX3* in foxes (*F – farm, WP – wild Poland, WNA – wild North America)

Gene		SNP position														Frequency of SNP profiles*			
IGF1		c. 26032	c. 26037	c. 26043	c. 26144	c. 26194	c. 26200	c. 26246	c. 26249	c. 26251	c. 26258	c. 26275	c. 26282	c. 26324	c. 26334	All	F	WP	WNA
SNP profiles	A	A	A	C	G	C	T	C	G	A	A	G	C	A	G	0.050		0.150	
	B	A	A	G	G	C	T	C	G	A	A	G	C	C	G	0.070			0.200
	C	T	A	G	G	C	A	T	C	A	G	G	C	C	G	0.050		0.150	
	D	T	A	G	G	C	T	C	C	A	A	G	C	C	G	0.050		0.150	
	E	T	A	G	G	C	T	C	G	A	A	G	C	C	G	0.610	0.850	0.400	0.600
	F	T	A	G	G	C	T	C	G	A	A	G	Y	C	G	0.050	0.150		
	G	T	A	G	G	C	T	C	G	A	G	C	C	C	G	0.050		0.150	
	H	T	G	T	A	T	T	C	G	G	A	G	T	C	A	0.070			0.200
Genotype frequency	AA	0.120	0.940		0.070		0.050			0.930	0.900			0.050	0.120				
	TT	0.880		0.070		0.070	0.950	0.050					0.070		0.880				
	CC			0.050		0.930		0.950	0.100			0.050	0.880	0.950					
	GG		0.060	0.880	0.930				0.900	0.070	0.100	0.950							
	TC												0.050						
Allele frequency	A	0.120	0.940		0.070		0.050			0.930	0.900			0.050	0.120				
	T	0.880		0.070		0.070	0.950	0.050					0.095		0.880				
	C			0.050		0.930		0.950	0.100			0.050		0.950					
	G		0.060	0.880	0.930				0.900	0.070	0.100	0.950	0.905						

Table 2 – contd.

MYO15A		c. 426	c. 463	c.578	All	F	WP	WNA
SNP profiles	A	A	A	C	0.017		0.050	
	B	A	G	A	0.050	0.150		
	C	A	G	C	0.883	0.850	0.900	0.900
	D	A	R	C	0.017		0.050	
	E	G	G	C	0.033			0.100
Genotype frequency	AA	0.970	0.020	0.050				
	CC			0.950				
	GG	0.030	0.960					
	GA		0.020					
Allele frequency	A	0.970	0.030	0.050				
	C			0.950				
	G	0.030	0.970					

Table 2 – contd.

PAX3		c. 11198	c. 11212	c. 11260	c. 11273	c. 11284	c. 11395	c. 11473	c. 11476	c. 11485	All	F	WP	WNA
1		2	3	4	5	6	7	8	9	10	11	12	13	14
SNP profiles	A	A	C	Y	K	T	Y	G	G	G	0.017			0.050
	B	T	C	C	G	C	C	G	G	G	0.017			0.050
	C	T	C	C	G	T	C	A	G	G	0.017			0.050
	D	T	C	C	G	T	C	G	G	A	0.017			0.050
	E	T	C	C	G	T	C	G	G	G	0.100	0.200		0.100
	F	T	C	C	G	T	T	G	G	G	0.033		0.050	0.050
	G	T	C	C	G	T	Y	G	G	G	0.033	0.100		
	H	T	C	C	G	Y	C	G	G	G	0.100	0.150		0.150
	I	T	C	C	K	Y	Y	G	G	G	0.017			0.050
	J	T	C	T	G	C	C	G	G	G	0.017	0.050		
	K	T	C	T	G	T	T	G	G	G	0.017			0.050
	L	T	C	T	G	T	Y	G	C	C	0.017		0.050	
	M	T	C	T	K	T	C	A	G	G	0.017		0.050	
	N	T	C	T	K	T	C	G	G	G	0.100		0.250	0.050
	O	T	C	T	K	T	Y	G	G	G	0.017		0.050	
	P	T	C	T	K	Y	Y	G	G	G	0.062		0.200	
	R	T	C	T	T	T	C	G	G	G	0.017			0.050
	S	T	C	T	T	Y	C	G	G	G	0.050	0.050	0.100	
	T	T	C	Y	G	T	C	G	G	G	0.017			0.050

Table 2 – contd.

1		2	3	4	5	6	7	8	9	10	11	12	13	14
	U	T	C	Y	G	Y	C	G	G	G	0.017	0.050		
	W	T	C	Y	K	T	C	G	G	G	0.017		0.050	
	X	T	C	Y	K	Y	C	G	G	G	0.017			0.050
	Y	T	C	Y	K	Y	Y	G	G	G	0.017			0.050
	Z	T	C	Y	T	T	Y	G	G	G	0.050		0.150	
	A1	T	C	Y	T	Y	C	G	G	G	0.017		0.050	
	B1	T	C	Y	T	Y	Y	G	G	G	0.033			0.100
	C1	T	T	C	G	T	C	G	G	G	0.033	0.100		
	D1	T	Y	C	G	T	C	G	G	G	0.033	0.100		
	E1	T	Y	C	G	T	Y	G	G	G	0.033	0.100		
	F1	T	Y	Y	G	T	C	G	G	G	0.017			0.050
	G1	T	Y	Y	G	Y	C	G	G	G	0.017	0.050		
	H1	T	Y	Y	K	Y	C	G	G	G	0.017	0.050		
Genotype frequency	AA	0.020						0.030		0.020				
	TT	0.980	0.030	0.320	0.170	0.600	0.050							
	CC		0.850	0.430		0.030	0.650		0.020	0.020				
	GG				0.530			0.970	0.980	0.960				
	TC		0.120	0.250		0.370	0.300							
	GT				0.300									
Allele frequency	A	0.020						0.030		0.020				
	T	0.980	0.090	0.445	0.320	0.785	0.050							
	C		0.910	0.555		0.215	0.650		0.020	0.020				
	G				0.680		0.300	0.970	0.980	0.960				

IGF1

The *IGF1* gene fragment studied in the red foxes consists of 305 bp. The statistical analysis showed 14 mutations, allowing 8 SNP profiles (A-H) to be distinguished. Profile F was found exclusively in farm animals, while profiles A, B, C, D, G and H were present in wild animals. B and H were found in North American wild foxes, and the remaining profile in Polish wild foxes, which may have been due to the animals' different living conditions and adaptation to climate. Only profile E was present in all of the populations, which suggests that this set of SNP is inherited in both farm and wild animals (Table 2).

MYO15A

The *MYO15A* gene fragment analysed was 168 bp in length. Three SNPs were noted (two mutations in wild foxes and one in farm animals) and five SNP profiles (A-E). Profiles A and D were present only in foxes living in their natural habitat in Poland. Profile B was found only in farm animals. Only profile C was noted in all individuals and had the highest frequency. Profile E occurred with relatively low frequency, and only in North American wild foxes.

PAX3

The *PAX3* gene fragment analysed in the red fox was 303 bp in length. The statistical analysis showed 32 different SNP profiles, caused by 9 single-nucleotide polymorphisms. There were 8 characteristic SNP profiles noted for the farm foxes and 2 for the wild foxes (North American and Polish). At the same time, 12 SNP profiles were noted in the North American wild foxes (with a frequency of 5% to 10%), and 7 for Polish wild foxes (with a frequency of 5% to 20%). No SNP profile was noted that was characteristic of all three groups of foxes (Table 2).

Frequency of genotypes and alleles for specific SNP are shown in Table 2, and the gene diversity and nucleotide diversity between any two DNA sequences are shown in Table 3.

Table 3. Gene diversity and nucleotide diversity of the genes *IGF1*, *MYO15A* and *PAX3* in foxes (*F – farm, WP – wild Poland, WNA – wild North America)

Gene	Gene diversity in population*			Nucleotide diversity (π) (average over loci) in population*		
	F	WP	WNA	F	WP	WNA
IGF1	0.2684	0.7895	0.5895	0.019173	0.178195	0.192481
MYO15A	0.1895	0.1947	0.1895	0.089474	0.031579	0.063158
PAX3	0.9316	0.8947	0.9737	0.049123	0.054386	0.093567

Discussion

The completion of the dog genome sequencing project made it possible to find SNPs in the genomes of animals of the Canidae family. Sacks and Louie (2008) used

40 different primer pairs designed using random fragments of the dog genome to sequence 80–88% of loci in these fragments in the coyote (*Canis latrans*), the gray fox (*Urocyon cinereoargenteus*) and the red fox (*Vulpes vulpes*). In order to investigate the genetic basis for size variation in canids, Sutter et al. (2007) searched for SNPs in a region composed of 15 million base pairs. They discovered 302 SNPs and 34 indels in large and small Portuguese Water Dogs (PWD). The *IGF1* gene was shown to have a significant influence on individual size. Haplotypes B and I were identified. Dogs that were homozygous with haplotype B have a smaller mean body size than homozygotes with haplotype I, while heterozygous dogs are medium-sized. In a study of 122 SNPs spanning chromosome 15 in dogs representing 14 small and 9 giant breeds, a decrease in heterozygosity was observed in the small breeds, which may be due to selective breeding resulting in smaller and smaller dogs. Research concerning SNPs in the *IGF1* gene has also been carried out in other animal species. SNPs have been detected in the coding sequence of *IGF1* among five breeds of pig (Berkshire, Duroc, Landrace, Yorkshire and Korea Native Pig) (Niu et al., 2013), and SNPs have been found to be associated with growth, development, and fertility in cattle (Holstein-Friesian) (Mullen et al., 2011).

Mutations in the *MYO15A* gene cause congenital deafness in humans (*DFNB3*) and in mice (Shaker-2). In mice, the hair cells affected by Shaker-2 deafness are arranged normally, but their length is markedly reduced. Probst et al. (1998) compared healthy and Shaker-2 individuals and determined that the cause of the hearing loss was a mutation in the *MYO15A* gene. The G>A substitution causes changes in the motor domain of the protein involving a Cyst/Tyr substitution, leading to shortening of the stereocilia. Rak et al. (2002) used human cDNA to design primers specific for dogs, which were then used for RH mapping. PCR yielded a product of 201 bp containing exclusively canine DNA. In addition, FISH (fluorescence *in situ* hybridization) was carried out in order to map the position of *MYO15A* on chromosome 5 in dogs (*CFA5*). This made it possible to ascertain that mutations in the *MYO15A* gene in dogs can cause hereditary deafness. The present study on red foxes demonstrated the presence of three SNP-type polymorphisms in the *MYO15A* gene. In Polish wild foxes two SNP profiles were distinguished – A and D, while only North American wild foxes had SNP profile E.

Due to the importance of the processes involving proteins encoded by *PAX3*, mutations in the genes encoding them can lead to very serious disturbances in embryotic development, and in extreme cases can even be lethal. There are currently no publications concerning mutations of the *PAX3* gene in animals of the Canidae family, but we have identified 9 polymorphisms of the SNP type in individuals from this family, allowing 32 different SNP profiles to be distinguished. The greatest number of animals tested had profile H, which occurred in the farm and the North American wild foxes, and profile N, which was characteristic of Polish and North American wild foxes. At the same time, SNP profiles were identified among wild foxes that were characteristic only for North American animals and only for European ones. The most likely cause of the development of separate SNP profiles was the geographical barrier and divergent evolutionary paths of the species *Vulpes vulpes* in Europe and North America (Aubry et al., 2009; Kutschera et al., 2013).

There is greater probability of nucleotide transitions than transversions, as the substitution takes place between structurally similar compounds, while transversions involve compounds of different structure. Another factor during substitution that should be considered is the varied rate of evolution between particular sites within codons, resulting from degeneration of the genetic code (Wondji et al., 2007). The very high proportion of C↔T transitions (Figure 1) may result from C-methylation in the CpG dinucleotide (Gryzińska et al., 2013 a, b). 5mC is spontaneously deaminated to T, causing the formation of a mismatched T:G pair and often a C:G→T:A transition (Holliday and Grieg, 1993). Although in humans TDG (thymine-DNA glycosylase) preferentially removes T from the T:G mismatch, the frequency of deamination exceeds its capacity, which leads to suppression of the amount of CpG. The transition/transversion ratio is similar to that obtained for *Drosophila* and humans (Brookes, 1999; Moriyama and Powell, 1996). The frequency of occurrence of one SNP was from 22 to 56 bp – lower than in *Anopheles gambiae* (Morlais et al., 2004) and in humans (Aquadro et al., 2001).

Single-nucleotide polymorphism is a very good molecular marker enabling characterization of variation between wild and farm individuals. The confirmation of genetic differentiation between wild and farm-bred animals of the Canidae family are reports by Bugno-Poniewierska et al. using the FISH technique (Bugno-Poniewierska et al., 2012, 2013). Variation studies of different species of fur animals of the family Canidae, free-living and farm-bred, have been and are carried out on several levels: diversity of morphological, physiological parameters, phenotypic traits, as well as at the molecular and cytogenetic level (Gugołek et al., 2012; Jeżewska-Witkowska et al., 2012; Ślaska et al., 2010). However, to date in world literature there are no papers considering the above-mentioned differences using sequence of nuclear gene fragments: insulin-like growth factor 1 (*IGF1*), myosin-XV (*MYO15A*) and paired box homeotic 3 (*PAX3*).

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