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Mitochondrial control region diversity in Polish sheep breeds

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Abstract. The aim of the study was to determine the genetic variability of the Polish sheep breeds Świniarka, Wrzosówka, Pomorska, and Wielkopolska based on mitochondrial control region polymorphism. A comprehensive phylogenetic analysis and information about the genetic origin of the breeds were also obtained. The genetic variability of the breeds studied has been assessed based on the number of haplotypes, haplotype diversity, nucleotide diversity, the average number of nucleotide differences, the number of mutations, and phylogenetically informative sites. Sequence divergence between identified haplogroup A (HA) and haplogroup B (HB) was also calculated. Moreover, a neighbour-joining (NJ) haplotype tree was constructed based on Kimura's two-parameter genetic distance calculation. Finally, the history of the population was investigated by mismatch distribution and Fu's F statistics. The 559 bp long mitochondrial DNA (mtDNA) control region (CR) sequences of 143 sheep were analysed. The 65 haplotypes were defined by 45 parsimony informative sites. Among the four Polish breeds, Wrzosówka had the highest while Świniarka the lowest values of haplotype (H_d) and sequence diversity (π) ($H_d = 0.9735$ and $\pi = 0.0040$ for Wrzosówka; $H_d = 0.8975$ and $\pi = 0.0030$ for Świniarka). Five haplotypes were shared between breeds, whereas the remaining 60 were unique. The NJ phylogenetic tree has revealed that 61 haplotypes of all analysed breeds clustered into clade B while the remaining 4 haplotypes representing all but the Świniarka breed pooled together with clade A. None of the other reported mitochondrial haplogroups were identified. The haplotypes representing HB formed a star-like network with the single central haplotype, which in association with extensive haplotype sharing reveals a weak structure of Polish breeds and the existence of gene flow between the breeds studied.

1 Introduction

In animal tissues, mitochondrial DNA (mtDNA) is characterized by very high polymorphism within species, a lack of recombination, and maternal inheritance. Moreover, very often it is the only genetic material that can be obtained from degraded or archaeological biological material. The analysis of both the conserved sequence of mitochondrial genes and the non-coding part of mtDNA, called the control region or displacement loop (D-loop), has been applied inter alia in phylogenetic studies.

mtDNA has been used to investigate the history of modern domestic animals, i.e. to identify wild ancestors and to define domestication centres (Bruford et al., 2003). Studies of cattle (Loftus et al., 1994; Bradley et al., 1996), goats (Luikart et al., 2001), pigs (Giuffra et al., 2000), sheep (Hiendleder et al., 2002), and poultry (Rosenberg et al., 2001) have revealed that each has multiple maternal origins. The identification in modern breeds of different maternal lineages showed independent domestication events of originally wild populations. So far, five maternal lineages have been identified in sheep: haplogroup A originated from Asia, haplogroup B from Europe, and haplogroups C, D, and E from the Near East (Meadows et al., 2007).

The present study analyses four Polish sheep breeds which have different phenotypic characteristics. These breeds have been included in the genetic resources programme in Poland because they are threatened by extinction.

Table 1. Primers used for the amplification and sequencing of the D-loop of Polish sheep.

Primer name	Primer sequence 5′–3′	Breed*	PCR product size (bp)	References
1MTFR	F AGAAGCTATAGCCCCA R ACTTAAAGCAAGGCAC	pom, swin, wrz	600	Pedrosa et al. (2005)
WLKFR	F GGGGGAAGCGTGTTAAAAAT R TCCACAAGCCCACATAACAA	wlk	600	Designed according to the NCBI sequence No. AF010406

* pom – Pomorska; swin – Świniarka; wrz – Wrzosówka; wlk – Wielkopolska.

Świniarka is an old, native, and primitive sheep breed which used to be very common in central and western Europe. In the interwar period it formed the majority of the primitive sheep population and was used for developing the noble lines of crossbred sheep. Even such features of Świniarka as high resistance to various diseases (especially to foot rot), and the fact that they are undemanding in feed and well adapted to harsh living conditions did not prevent the breed from near extinction in the 1980s. Since then, Świniarka has been successfully restored from only a few individuals found in eastern Poland.

Another breed, Wrzosówka (Heath sheep), is one of the oldest surviving primitive Polish sheep breeds. Before the Second World War, Wrzosówka was present in large numbers in north-eastern Poland. After the war a rapid decline in the population of Wrzosówka was observed resulting in the near extinction of the breed, which was then successfully restored. According to data from 2014, the population of Polish Wrzosówka sheep comprises 8343 animals (NRIAP, 2015). Wrzosówka is prolific and reared for wool production as well as its delicate meat. It is also recognized to be disease resistant as well as adaptable to difficult conditions.

The Pomorska (Pomeranian sheep) breed is reared at the Baltic Sea in Pomerania and Mecklenburg. This long-wool-type sheep is characterized by low nutritional requirements, resistance to many diseases, and good adaptation to tough environmental conditions. Such features allow Pomeranian sheep to be kept in cool and damp coastal areas. Pomeranian sheep are suitable for the traditional breeding system based on grazing in small flocks. Due to its versatile utility, it is mainly bred for meat, milk, or wool. In 2014 the population of Pomeranian sheep, comprising 7621 animals, was the second largest of all native breeds (NRIAP, 2015).

Another common Polish native sheep breed is Wielkopolska, with over 7000 animals in 2014, participating in the genetic resources programme (NRIAP, 2015). The Wielkopolska sheep was bred in the years 1948–1976, based on the primary population of white Świniarka and improved Świniarka. This lowland wool- and meat-type sheep was intended for farming and less intensive breeding in either small or large flocks. The most important advantage of Wielkopolska sheep is good wool quality, rapid weight gain, and, unlike Merino sheep, low nutritional requirements.

To date, the majority of surveys on the genetic diversity among Polish sheep have been conducted on blood groups and blood plasma proteins polymorphisms (Rychlik et al., 2007, 2009, 2011; Rychlik and Krawczyk, 2009, 2010) as well as autosomal short tandem repeat variations (STR, microsatellites) (Radko et al., 2006; Kawęcka and Piórkowska, 2011). Few inquiries have been undertaken on the mitochondrial cytochrome b gene (Karpiński et al., 2006; Prusak et al., 2005).

The aim of the study was to determine the genetic variability of four Polish sheep breeds based on mitochondrial control region polymorphism. Moreover, a comprehensive phylogenetic analysis of the sheep breeds was conducted and information was obtained about their genetic origin.

2 Materials and methods

2.1 Sample collection and DNA isolation

A total of 143 blood samples was taken from sheep belonging to four Polish sheep breeds: Wrzosówka (wrzos, $n = 33$; ewes: 22; rams: 11; 2 flocks), Świniarka (swin, $n = 35$; ewes: 29; rams: 6; 2 flocks), Pomorska (pom, $n = 40$; ewes: 32; rams: 8; 3 flocks), and Wielkopolska (wlk, $n = 35$; ewes: 32; rams: 3; flock). The blood samples were taken by an authorized person observing routine procedure for ovine pedigree verification, for which the permission of the Polish Ethics Committees for Experiments with Animals is not required. The individuals were unrelated according to breeding documentation. Total genomic DNA was extracted from blood samples using a DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturers instruction.

2.2 Polymerase chain reaction and sequencing

In Table 1 three sets of primers are given, which were used for both polymerase chain reaction (PCR) and sequencing. The first one (1MTF 5′AGAAGCTATAGCCCCA and 1MTFR 5′ ACTTAAAGCAAGGCAC), according to Pedrosa et al. (2005), was used for samples of all but the Wielkopolska breed. The second set of primers (WLK_F 5′ GGGGGAAGCGTGTTAAAAAT, WLK_R 5′ TCCACAAGCCCACATAACAA) was designed using the Primer3 program (Koressaar and Remm, 2007; Untergasser et al.,

Table 2. PCR cycling conditions used for the amplification of the control region of sheep.

Step	Primer name					
	MTFR			WLKFR		
	Temp [°C]	Time	No. of cycles	Temp [°C]	Time	No. of cycles
Initial denaturation	95	15 min	1	95	15 min	1
Denaturation	94	40 s	30	94	40 s	30
Annealing	57	40 s		60	40 s	
Elongation	72	1 min		72	1 min	
Final elongation	72	10 min	1	72	10 min	1

Table 3. Genetic diversity parameters and Fu's F statistics in analysed sheep.

Breed	N^a	H_n^b	H_d^c	Π^d	k^e	M_s^f	P_s^g	F_s^h
Wrzosówka	33	21	0.9735	0.0040	5.8674	39	35	−8.385**
Świniarka	35	14	0.8975	0,0030	2.7462	17	10	−5.199**
Pomorska	40	20	0.9269	0.0041	5.5794	32	26	−5.678*
Wielkopolska	35	17	0.9345	0,0036	3.4386	30	13	−7.227**
All breeds	143	65	0.9720	0.0037	4.7476	55	45	

* $p<0.05$; ** $p<0.01$. [a] Number of sequences. [b] Observed haplotypes (H_n). [c] Haplotype diversity. [d] Population nucleotide diversity. [e] Average number of nucleotide differences. [f] Mutation sites. [g] Phylogenetically informative sites. [h] Fu's F_s statistics.

2012) from the complete ovine mtDNA sequence (Gene Bank Acc No. AF010406; Hiendleder et al., 1998) exclusively for samples of the Wielkopolska breed since non-specific PCR products had been obtained using the 1MTF/R primer set. Amplification reactions were carried out in a 25 µL volume PCR mix containing 1 × PCR buffer, 2.5 mM MgCl2, 200 µM dNTPs, 0.5 U Taq DNA polymerase (HotStar Taq, Qiagen), 0.125 µmol of each primer, and 20 ng of DNA template. PCR thermal conditions were specified in Table 2. PCR products were bidirectionally sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA) and underwent capillary electrophoresis on a 3130xl Genetic Analyser (Life Technologies, USA).

2.3 Data analysis

The obtained sequences were inspected manually and aligned with an *Ovis aries* reference sequence of haplogroup B (AF010406) (Hiendleder et al., 1998) using BioEdit. For each breed, DnaSP 5.0 (Librado and Rozas, 2009) was used to sort haplotypes, haplotype diversity (H_d), nucleotide diversity (π), the average number of nucleotide differences (k), the number of mutations (M_s), and phylogenetically informative sites (P_s). In addition, the average number of nucleotide differences (K) and the average number of nucleotide substitutions per site (D) between two identified haplogroups were calculated.

MEGA 6 software (Tamura et al., 2013) served to evaluate Kimura's two-parameter genetic distance (Kimura, 1980) and construct the neighbour-joining (NJ) haplotype tree. The population expansion was investigated with mismatch distribution (DnaSp 5.0) and Fu's F_s statistics (Fu, 1997) computed by Arlequin (Excoffier et al., 2005). Only phylogenetically informative polymorphism was calculated in the survey. However, all mutation types including singletons remain in the sequences submitted to GenBank under the accession numbers KT158312–KT158460 (NCBI, 2015).

3 Results

3.1 mtDNA variation in Polish sheep

In the survey 559 bp long mtDNA control region (CR) sequences were obtained spanning *Ovis aries* reference sequence AF010406 (Hiendleder et al., 1998) from 15959 to 16518 bp. The analysis of combined data sets of 143 sequences revealed 55 polymorphic sites, of which 45 were phylogenetically informative, while the remaining 10 were singletons. The structure of transversion to transitions was 105 : 4, and no indel was noticed. As it is depicted in Table 3, the overall values for haplotype and nucleotide diversities were 0.9720 and 0.0037 respectively. Among the four Polish breeds, Wrzosówka showed the highest values of genetic diversity in terms of haplotype (0.9735) and nucleotide (0.0040) diversity, the average number of nucleotide differences (5.8674), the number of mutations (39), and phylogenetically informative sites (35). The lowest values of these parameters were reported for Świniarka ($H_d = 0.8975$; $\pi = 0.0030$; $k = 2.7462$; $M_s = 17$; and $P_s = 10$). Pomorska and Wielkopolska were similar in

Table 4. Control region diversity between two mtDNA lineages.

Population	H_n[a]	K[b]	D[c]	Shared mutations
Haplogroup A	4	0.2706	14.885	6
Haplogroup B	61			

[a] Number of haplotypes.
[b] Average number of nucleotide substitutions per site between the haplogroups.
[c] Average number of nucleotide differences between the haplogroups.

terms of haplotype diversity (0.9269 and 0.9345 respectively) but very different regarding other genetic diversity assessments ($k = 5.5794$, $M_s = 32$, and $P_s = 26$ for Pomorska and $k = 3.4386$, $M_s = 30$, and $P_s = 13$ for Wielkopolska). The analysis of two mtDNA lineages (type A and B) revealed high sequence diversity ($K = 0.2706$, $D = 14.885$), which is shown in Table 4.

3.2 Phylogenetic analyses

The 65 haplotypes were defined by 45 parsimony informative sites. Only 5 haplotypes were shared between breeds (hap_1: pom/wrz; hap_3: swin/wlk/pom; hap_6: swin/wlk; hap_3: swin/wlk/pom and hap_39: wrz/wlk), whereas the remaining 60 were unique. As is shown in Fig. 1, the NJ phylogenetic tree revealed that 61 haplotypes of Polish sheep sequences clustered into clade B and the remaining 4 into clade A. These four haplotypes, which pooled together with cluster A, included two sequences of Wrzosówka, five sequences of Pomorska, and one sequence of Wielkopolska breed. All haplotypes of Świniarka clustered only into haplogroup B. Type B haplotypes formed a star-like tree with one central haplotype (hap_13). The mismatch analysis of all haplotype distributions gave significantly negative results. The histograms of mismatch distribution revealed two distinct peaks for all but the Świniarka breed (data not shown). The F_s (Fu, 1997) neutrality test for all breeds gave a significant ($p < 0.05$) negative value.

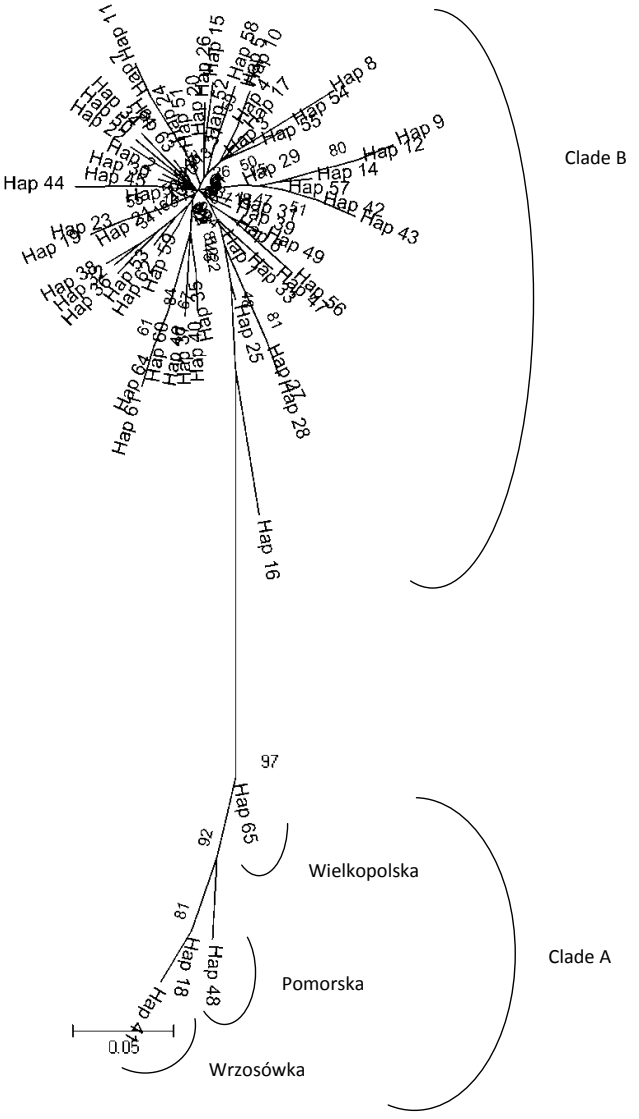

Figure 1. Unrooted neighbour-joining haplotype tree constructed from Kimura two-parameter distance of the 65 haplotypes of the Polish sheep breeds studied; bootstraps of 1000 replicates were used to test the robustness of the tree.

4 Discussion

The exclusive presence of two mtDNA lineages (i.e. haplogroups A and B) is characteristic of Polish sheep breeds, which is in agreement with the results from most other European populations sampled to date (Tapio et al., 2006; Meadows et al., 2007). The vast majority of Polish breed haplotypes represent haplogroup B. A few samples also showed the presence of maternal lineage A, suggesting introgression from Asian breeds. It is worth noting that no other reported haplogroups were observed in the investigated Polish sheep population. However, so far, the presence of haplogroup C in breeds from Europe has been found only among the Portuguese breeds (Pereira et al., 2006).

These two haplogroups are sufficiently divergent in the Polish population as it has been depicted in Table 4. This is confirmed by a high average number of mutational changes and sequence divergence between groups which were similar to that observed by Pardeshi et al. (2007) but unlike the results of Meadows et al. (2007).

The history of the population has been investigated by mismatch distribution and Fu's F statistics (Fu, 1997). Assuming that population growth affects the distribution of nucleotide changes among homologous DNA sequences the study of histograms of mismatch distribution should give an insight into the history of the population. The mismatch distribution of the population which underwent a recent burst of

expansion is illustrated in the form of a bell-shape histogram, whereas the histogram of the steady-state population with a constant population size is ragged (Hartl, 2004). The analysis of mismatch distribution of the worldwide sheep population has shown that the haplogroups A and B have each experienced a recent population expansion from a one-line pedigree (Guo et al., 2005). The double-pick histograms of mismatch distribution for all but the Świniarka breed confirm descent from two maternal lineages of Wrzosówka, Pomorska, and Wielkopolska.

The results regarding Świniarka and Wrzosówka are in accordance with Tapio et al. (2006), who studied mitochondrial control region and cytochrome b polymorphism in 406 sheep from Europe and Asia. Among them four samples of the Wrzosówka and two of the Świniarka breed were found. All of them were pooled together with haplogroup B with the exception of one sample belonging to the Wrzosówka breed. This breed is descended from short-tailed sheep formerly present on the Scandinavian peninsula and in Great Britain and Ireland. The sheep population from this region also represents two types of CR sequence (Meadows et al., 2005). The ancestors of Polish Wrzosówka may have migrated to Poland by land along the Scandinavian peninsula, Finland, and Lithuania, Latvia, and Estonia. Sheep representing the Asian type of CR sequence to Pomeranian sheep – the breed which was reared and is still kept in the coastal region of Poland may have been introduced in a similar way. The most surprising results are for Wielkopolska and Świniarka sheep, from which Wielkopolska originates. The presence of both haplogroups in the Wielkopolska breed could be explained in two ways. Firstly, during the development of the Wielkopolska breed or later, females of breed(s) other Świniarka which represented haplogroup A were probably introduced. Secondly, the primary Świniarka breed was probably much more genetically variable and may have had both mtDNA sequence types. It is highly likely that the individuals of haplogroup A were lost due to a rapid reduction in the breed population in the 1980s. Consequently, the few individuals from which the breed was restored represented only haplogroup B.

Another test for population expansion is Fu's F test of neutrality, which serves to identify the number of mutations in a population under expansion. In the surveys performed elsewhere, the results of F statistics were negative for haplogroups A, B, and C, but statistically significant only for the haplogroup A (HA) and haplogroup B (HB; Guo et al., 2005; Meadows et al., 2007), which means that these last two lineages are not a constant population regarding mtDNA. Fu's test of neutrality for the population of Polish breeds is in accordance with these results. The results of both mismatch distribution and Fu's statistics reflect the strong reduction in sheep number in Poland which occurred in the 1990s.

The NJ phylogenetic tree formed two separate clades representing haplogroups B (more frequent) and A. The haplotypes representing HB formed a star-like network with a single central haplotype, which in association with extensive haplotype sharing reveals a weak structure of Polish breeds and the existence of gene flow between the breeds studied.

The mean values of haplotype and nucleotide variation in all Polish sheep breeds are higher compared to the rate of European breeds. However, they are not as high as those of Asian populations (Pardeshi et al., 2007). The low genetic variation in the Wrzosówka and Pomorska breeds assessed by blood groups, serum proteins (Rychlik et al., 2007, 2009; Rychlik and Krawczyk, 2010), and microsatellites sequences (Radko et al., 2006; Kawęcka and Piórkowska, 2011) contrasts with these results. This discrepancy may result from differences between the genetic markers used, such as much higher polymorphism of mtDNA than nuclear DNA. It also reflects the female majority compared to a limited number of males used for reproduction. The lowest nucleotide variability expressed by blood groups, serum proteins (Rychlik et al., 2009), and mitochondrial control region was found for Świniarka sheep. This may be caused by a relatively small number of sheep and rigorous selection for breed characteristics during the period when the Świniarka breed was restored.

5 Conclusions

This study has provided comprehensive information about the genetic origins of Polish local sheep breeds and conducted a phylogenetic analysis of them. Two maternal lineages (haplogroup A and B) from which the Polish sheep population descended were identified. None of the other reported mitochondrial haplogroups were identified. Moreover genetic diversity of all but the Świniarka breed is higher than that obtained for European populations but lower than that of Asian sheep breeds. The lowest genetic diversity was found for Świniarka.

Data availability

Data obtained during this research are openly available through the National Center for Biotechnology Information (NCBI, 2015) under accession numbers KT158312–KT158460: http://www.ncbi.nlm.nih.gov/nuccore/KT158312 to KT158460. Data regarding the number of sheep included in Genetic Resources Programme in Poland are publicly available through The National Research Institute of Animal Production (NRIAP, 2015) at http://owce.bioroznorodnosc.izoo.krakow.pl.

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