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Origin and genetic diversity of Romanian Racka sheep using mitochondrial markers

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ABSTRACT

The current study investigates the genetic diversity of Racka sheep from Romania and its relationship with other breeds having different geographic origin. Mitochondrial markers (D-loop and cyt b gene) were sequenced in 40 unrelated individuals. A number of 12 distinct haplotypes, with haplotypes diversity (Hd) of 0.897 and nucleotide diversity (Pi) of 0.00437 was highlighted. The results were comparable with the ones from other sheep breeds and revealed that the Racka population has a good genetic variability. To infer the origin of Racka sheep, a number of 113 mtDNA sequences from different breeds were included into the data set. The network profiles showed that sheep breeds from different geographic regions intermixed. The sequences grouped within the network in clusters correspondent to the five haplogroups A, B, C, D, and E, with the Racka sequences distributed in A and B lineages. The genetic variation indicates that the Romanian Racka is an important reservoir of diversity and the presence of A and B haplotypes within the population is accordance with the findings for different breeds from Zackel group, in which Racka is considered to be included. In conclusion, our findings demonstrate that up to present the Romanian Racka breed was properly managed and has a good potential for conservation.

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1. Introduction

The process of sheep domestication was very important for the development of human civilization, and the diversity of the breeds that appeared after domestication raises problems of origin and classification.

The origin and name of the local Racka sheep are extremely controversial. Also, the relationship between the stocks of Racka sheep from Hungary, Romania and Serbia is still unclear. Thus, regarding the origin, Drăgănescu (2005) proposed 2 hypotheses: Racka breed is either part of the old Egyptian sheep with corkscrew horns, or it is descended from the Mesopotamian sheep, where, however, only the rams had corkscrew horns, the sheep being hornless. The Balkan Peninsula is an important area for sheep breeds with primitive features, but unfortunately the majority of these are endangered. Thus in the former Yugoslav area (Kosovo, Montenegro and Serbia) there is at least one sheep breed resembling Racka sheep, and probably related to it, the Balusha sheep (Drăgănescu, 2005). Presently, parts of the Zackel sheep are accepted as Racka sheep, including several breeds from the Balkan region, northern Greece, the former Yugoslav countries (Serbia, Montenegro, Bosnia and Herzegovina, Slovenia, Macedonia, Croatia) Albania, Bulgaria, Romania and Hungary. Generally, the sheep from Zackel group are dual purpose sheep and, although their productivity is moderate, they are very resilient to the harsh environmental conditions from the mountain areas where they live (Drăgănescu, 2007; Savic et al., 2013).

In terms of stocks, Racka breed is in a good state of conservation in Hungary, in critical state in Serbia (Savic et al., 2011), while in Romania it lost importance in favor of other breeds, being now bred in just a few locations in Banat area at the border with Serbia. Possible explanations for the low interest in this breed include that the breed is not economically competitive and that the productions of wool, milk and meat are lower than those of the sheep breeds that underwent breeding programs. According to the current estimations, in the five villages where it is still reared, there are some 4000 sheeps, of which just 500 are pure breed (Savic et al., 2013). In Romania there are two varieties of the breed: white (brown face and white wool) and black (black face and black wool), but it has been assimilated as variety of Tsurcana sheep up to present (Drăgănescu, 2005). The rearing of Racka sheep is now subsidized through the program of animal genetic resources conservation.

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In Hungary, starting with 1960 significant imports of sheep from Romania was done and the breed was exposed to an intensive process of selection and conservation. However, the selection was, however, more efficient in Hungary since there only sheep with "V" shaped horns can be identified, while in Romania, there are still sheep with side-oriented horns. Additionally, the Romanian Racka sheep are larger and have longer, thicker wool (Drăgănescu, 2005; Supplementary material Fig. S1 in the online version at DOI: 10.1016/j.smallrumres.2016.10.016).

The name of the breed has many regional variants. Thus, in Serbia, this breed is also named "Vlachko-Vitoroga", in Hungary "Raczka" or "Hungarian Zackel", and in Romania "Rața". Due to the multiple names given to the breed, and to the resemblance to other breeds from the Balkans, the Racka sheep breed has probably been mistaken or assimilated over time with other groups of sheep, in Romania being considered as a variety of Tsurcana sheep. Presently, the formal name of the breed is Vlashko-Vitoroga Zackel according to "FAO Domestic animal Diversity Information Service" and it is being considered a trans-border breed (Savic et al., 2013).

Currently, three groups of wild sheep living in Eurasia, the mouflons (*Ovis orientalis*), urial (*Ovis vignei*) and argali (*Ovis ammon*), are considered to be the ancestors of the domesticated sheep, each of them having a contribution to the formation of breeds. The literature has several theories on the origin of the modern domesticated sheep. Thus, in 2002, Hiendleder et al. (Hiendleder et al., 2002) hypothesized that all the current breeds of domesticated sheep evolved from two different subspecies, each determining dam descendance (A and B). B haplotype is predominant in the sheep populations from Europe, being also the only haplotype observed in the European mouflon, but has a low frequency in the native breeds from Eastern Eurasia (Hiendleder et al., 1998a, 2002; Guo et al., 2005; Meadows et al., 2005). In 2013, Singh et al. (Singh et al., 2013) after surveying several studies on the origin and domestication of sheep, said that the process of sheep domestication is very complex and that it involves two main maternal lines (A and B) and three minor lines (C, D and E). The maternal haplotypes C, D and E appear to be more frequent in the sheep breeds form Near East.

In this context, the purpose of our study was to evaluate the genetic diversity and to clarify the origin of the Romanian Racka sheep using the analysis of the mitochondrial markers D-loop and cytochrome b. By comparative analysis of specific mitochondrial markers and complex molecular phylogeny, this study aims to identify the clear descendance and interrelation of the Romanian Racka sheep with other sheep breeds.

2. Materials and methods

2.1. DNA extraction

DNA samples were collected from 40 unrelated Racka sheep, of both sexes, from two private farms located in South-Western Romania (Caraş-Severin County), at the border with Serbian Republic. Blood samples were collected on EDTA anticoagulant and DNA was extracted using the Wizard Genomic DNA Extraction Kit (Promega) according to manufacturer's specifications.

2.2. PCR amplification and sequencing

For the phylogenetic analysis we amplified fragments from the gene that encodes for cytochrome b and from the mitochondrial D-loop area. The reactions of PCR amplification were performed using a GeneAmp 9700 PCR System (AppliedBiosystems), in a final volume of 25 μl which contained 10× PCR Buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM of each primer (cytochrome b F: 5′-gatctcccagctccatcaaa-3′; R: 5′-tgaggggagtgttaagtgg-3′; D-loop F: 5′-acccggagcatgaattgtag-3′; R: 5′-gggggaagcgtgttaaaaat-3′), 0.5 units of AmpliTaq Gold DNA Polymerase (5U/μl), 50 ng of DNA template and nuclease-free water. The amplification reaction was done during 40 cycles, each one including denaturation at 95 °C (30 s), hybridization at 58 °C (30 s) and extension at 72 °C (60 s). Furthermore, we also performed a step of initial denaturation at 95 °C for 10 min and a stage of final extension at 72 °C for 15 min. The primers were thus set as to amplify a fragment of D-loop region bordered by positions 16,345–16,520 within the sheep mitochondrial genome (NC001941, Hiendleder et al., 1998b), and a fragment from cytochrome b gene bordered by positions 14,216–14,958 (NC001941, Hiendleder et al., 1998b). The amplified fragments had a dimension of 175 bp for the control region and 743 bp for *cyt b* gene.

The amplification products were thereafter purified with the Wizard PCR Preps DNA Purification System Kit (Promega). The purified fragments were further amplified in with the purpose of sequencing with the ABI Prism®BigDye Terminator Cycle Sequencing Reaction Kit (AppliedBiosytems) and then run on the automatic ABI Prism 3130 Genetic Analyzer. The sequences were processed using DNA Sequencing Analysis 5.1 Software (AppliedBiosytems), and the nucleotide sequence was aligned and edited using BioEdit software (Hall, 1999). The partial sequences obtained for Racka sheep breed were deposited into the GenBank, under the access numbers KP710097- KP710176.

2.3. Data analysis

Both control region and *cyt b* sequences were aligned and compared with similar sequences from GenBank isolated in sheep breeds with different geographic origin from Europe, Asia and Middle and Near East (Supplementary material Table S1 in the online version at DOI: 10.1016/j.smallrumres.2016.10.016). The sequences were truncated to 175 bp for D-loop and respectively 743 bp for *cyt b* and for a higher accuracy of the analysis were concatenated to 918 bp dataset. The data set comprised 153 sequences isolated in different sheep breeds, including our 40 sequences from Romanian Racka. The sequences alignment was performed with ClustalW algorithm implemented in MEGA 5 (Tamura et al., 2011). The genetic diversity in terms of number of haplotypes, nucleotide diversity, haplotype diversity, average number of nucleotide differences and average number of nucleotide substitutions (Dxy) per site were calculated using DnaSP v5.1 (Librado and Rozas, 2009).

Degree of genetic differentiation and gene flow among Racka breed and different haplogroups were determined by F-statistics using the same software. Mismatch distribution, as well as Fu's F_S (Fu, 1997) and Tajima's D (Tajima, 1989) neutrality tests implemented in ARLEQUIN 3.5 (Excoffier et al., 2005) were used to assess departures from neutrality. DNA Alignment software v1.3.3.1 (http://www.fluxus-engineering.com) was used to convert the aligned sequences into RDF binary format. A haplotype network of mitochondrial sequences using a median-joining algorithm with default settings ($\varepsilon = 0$) and the variable sites weighted equally (Weight = 10) was inferred with NETWORK v4.6.1.1 (Bandelt et al., 1999). The species *Ovis orientalis anatolica* was used as an outgroup species.

3. Results

3.1. mtDNA variation in Romanian Racka. Comparative analysis with other sheep breeds

For the 153 mtDNA sequences, 66 haplotypes were identified, with an overall value of haplotypes diversity (H_d) of 0.960 and a

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