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Mitochondrial diversity in autochthonous cattle breeds from the Balkan Peninsula

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ABSTRACT: The Bulgarian Grey Cattle (BGC) and Shorthorn Rhodopean Cattle (SRC) are indigenous breeds from the Balkan Peninsula region. The Balkans, as part of Southeast Europe, is a crucial civilization crossroad of cultures, people, and livestock. This region is considered the civilization "cradle" of prehistoric times (around 6500 BC). The aim of the present study is to reveal the genetic profile and population structure of BGC and SRC according to displacement loop control region. The results showed that these ancient cattle breeds belong to the common T1, T2, and T3 haplogroups. Within the BGC population the T3a sub-haplogroup was predominant (about 80% – 31/39) with haplotypes BGC-4.1 (33% – 13/39) and BGC-4.2 (about 40% – 16/39). Mitochondrial DNA analysis of SRC population showed a heterogeneous structure consisting of five basic haplo- and sub-haplogroups, all based on twelve haplotypes with equal frequencies. Based on 173G polymorphic site newly named sub-haplogroup T3c was proposed. A detailed comparative analysis with other Balkan cattle populations was performed. Data showed multiple haplotype mtDNA profile with no phylogenetic relationships within.

Keywords: autochthonous cattle; domestication; D-loop region; genetic diversity

INTRODUCTION

The analysis of mitochondrial DNA (mtDNA) sequence diversity has provided important insights into the origin, domestication, and biogeography of modern cattle populations (Ludwig et al. 2013). Analyses of mtDNA displacement loop (D-loop) sequences have indicated that the two main domestic cattle species, the humpless taurine (*Bos taurus*) and humped zebu cattle (*Bos indicus*), have clearly distinguishable specific haplotype profiles (Loftus et al. 1994; Bradley et al. 1996; Mannen et al. 2004). This observation points towards two independent domestication events from genetically and geographically differentiated auroch (*Bos primigenius*) populations about 8000 years ago. The modern European domestic cattle are of a humpless taurine type and descend from the auroch populations domesticated in the Near East region (Bradley et al. 1996; Troy et al. 2001). The humped zebu cattle has been domesticated in the Indus Valley (today's Pakistan) (Meadow 1993; Chen et al. 2010) with primary spreading of these breeds in India and a more recent secondary male introduction to Africa (Bradley et al. 1998).

The mtDNA D-loop sequences of the modern taurine cattle populations form five basic haplogroups: T, T1, T2, T3, and T4, and non-T haplogroups: Q, R, and P (Troy et al. 2001; Mannen et al. 2004; Achilli et al. 2008, 2009; Bonfiglio et al. 2010) present in modern cattle populations in low frequencies. T1 is the most common African

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haplogroup, T2 is almost entirely found in Middle East and Anatolian breeds, T3 is the most frequent European haplogroup, and T4 is found only in East Asiatic breeds (Beja-Perejra et al. 2006).

Shorthorn Rhodopean Cattle (SRC) is one of the two indigenous breeds in Bulgaria. The breed is part of the Busha Brachicerous cattle in Southeast Europe – Ilyric Dwarf cattle (Albania), Busha cattle (Montenegro, Serbia, Croatia, and Bosnia and Herzegovina), Brachykeratiki (Greece) (Mason 1996) (Figure 1). A basic phenotypic characteristic for this group is the smallest body size among all European cattle (Ajmone-Marsan et al. 2010). Nowadays the population of SRC is with reduced size (only about 500 individuals) and is in endangered state of extinction due to the uncontrolled crossbreeding with modern breeds which have almost completely replaced it. The population occurs in the Rhodope Mountains (the southeastern Balkans). This breed (as part of the Busha cattle group) represents a valuable genetic resource for the entire Balkan region. Due to this fact, a national strategy has been developed targeting *in situ* conservation of this breed.

The Bulgarian Grey Cattle (BGC) is another local Bulgarian cattle breed represented by only about 1000 cows. It is a representative of the so-called Podolian cattle, which form a large group of grey cattle breeds, usually with long horns, whose origin is not well established. The name of the group indicates its possible origin from Podolia (nowadays a region in Ukraine). The spreading of this cattle type possibly started about 2000 years ago southward to Anatolia and westward to the Balkan and Italian Peninsulas (Beja-Pereira et al. 2006; Ivankovic et al. 2012; Maretto et al. 2012). It is the base of many local breeds (thus influenced their names) such as the Podolian cattle, Hungarian steppe grey, Romanian steppe, Turkish Grey, Katerini cattle (Soysal and Kok 2008) (Figure 1). BGC as well as SRC biodiversity is protected at national level in Bulgaria.

The aim of the research is to analyze the mitochondrial diversity of two autochthonous Balkan cattle populations (SRC and BGC) with respect to the D-loop region.

MATERIAL AND METHODS

DNA extraction. Two sets of 20 and 39 nasal swab samples were collected from typical representatives and unrelated animals from different SRC and BGC herds, respectively. DNA was extracted by commercial GeneJET™ Genomic DNA Purification Kit (Fermentas/Thermo Fisher Scientific, Waltham, USA). The DNA concentration was determined spectrophotometrically and the quality of the DNA was examined by 1% agarose gel electrophoresis.

Figure 1. Geographic distribution of the Balkan Peninsula autochthonous cattle populations. The spreading of Podolian Grey Cattle is presented by light grey ovals. The dissemination of brachicerous cattle breeds is shown in dark grey rectangles

PC = Podolian Cattle, BGC = Bulgarian Grey Cattle, TGC = Turkish Grey Cattle, RGC = Romanian Grey Cattle, HGC = Hungarian Grey Cattle, CGC = Croatian Grey Cattle, IGC = Italian Grey Cattle, SRC = Shorthorn Rhodopean Cattle, BK = Brachykeratiki, BC = Busha Cattle, IDC = Illyric Dwarf cattle

mtDNA analysis. The D-loop region was used for analysis of the breeds' population structure. The D-loop region was amplified by PCR using the following primers: L-strand, 5'-CTGCAGTCTCACCATCAACC-3' and H-strand, 5'-GTGCCTTGCTTTGGGTTAAG-3' (sequence from positions 15792-363 of bovine mtDNA, Accession No. V00654) (Achilli et al. 2008).

All PCR reactions were performed in 50 µl volumes under the following conditions: initial denaturation at 94°C for 5 min, 35 cycles (denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s,

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extension at 72°C for 1 min), and final extension at 72°C for 10 min. PCR products were visualized on 1% agarose gel with GreenSafe (NZYtech, Lisboa, Portugal) under visible and UV light. The fragment size was determined using GeneRuler™ 100 bp Ladder Plus (Fermentas/Thermo Fisher Scientific). The amplified products for each animal were directly sequenced and the sequencing was performed by one direction using the L-strand primer (Eurofins MWG Operon, Ebersberg, Germany) (the H-strand primer showed sequencing failure). To avoid some PCR errors, the samples were twice sequenced independently.

Sequence analysis of the mtDNA region. 59 sequences were obtained and processed using MEGA software (Version 5.0, 2011). A fragment of 777 bp was aligned to the Bovine Reference Sequence (BRS, Accession No. V00654) using ClustalW2 software package (Version 2.0, 2007) and BLAST analysis (Altschul et al. 1990). Sequences were analyzed by polymorphic SNPs position and haplogroups were determined according to Achilli et al. (2009). The sequences from the investigation were deposited in Gene Bank under Accession No. KF373013–KF373031 (PopSet Accession No. 530540029).

Phylogenetic relationships of mtDNA haplotypes were explored with a Reduced Median (RM) network by using a NETWORK 4.5.1.6 (http://www.fluxus-engineering.com/sharenet.htm).

Statistical analysis. The overall mean of genetic distance was calculated using the Maximum Composite Likelihood model (Tamura et al. 2011). The standard error was estimated by a bootstrap procedure (500 replicates).

RESULTS

Twenty animals from SRC breed and 39 animals from BGC breed were analyzed. On the basis of polymorphic SNPs positions, seven and twelve haplotypes for BGC and SRC sets were defined, respectively (Table 1). The haplotypes were determined on the basis of 28 transitions and one transversion (16 057 G/C) and were included in haplogroups according to the classification of Achilli et al. (2009).

Five basic haplo/sub-haplogroups were defined: T1 (16113 C, 16255 C), T1a (16050 C, 16255 C), T2 (16057 C, 16185 A, 16255 C), T3a (169 G), and T3b (169 A) (Achilli et al. 2009). On the basis of polymorphic site at position 173G, a newly named sub-haplogroup labelled as T3c was defined (Table 1). This sub-haplogroup was characterized with motif 169 A/173 G or 169 G/173 G.

In the BGC breed, 13 polymorphic sites (1.6% nucleotide sequence diversity) were identified. They determined four haplo- and sub-haplogroups: T1a (5.1% – including the haplotype BGC-1), T2 (12.8% – BGC-2 haplotype), T haplogroup (2.6% – BGC-3 haplotype), and the predominant T3a (79.5%, consisting of four haplotypes – Table 1). T3a sub-haplogroup consists of two dominating basic haplotypes BGC-4.1 (33.3%) and BGC-4.2 (41%) determined by the SNP position 15965 T/C. This haplogroup is the root of another two haplotypes BGC-5.1 (2.6%) and BGC-5.2 (2.6%) which are defined by SNP at 16231 T observed only in two individuals (Figure 2A).

In the SRC breed 23 SNPs were identified, which represents 2.9% of nucleotide sequence diversity.

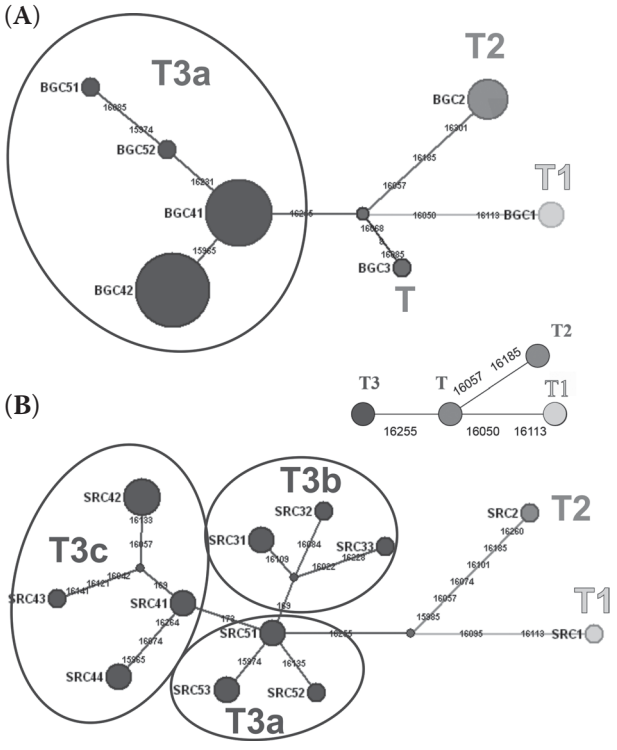

Figure 2. The Reduced Median network of the main mtDNA haplotypes from Bulgarian Grey Cattle population (**A**) and Shorthorn Rhodopean Cattle population (**B**). The sequence variations and codes of the haplotypes are from Table 1. Circle areas are proportional to haplotype frequencies. Haplogroups defined by polymorphic sites are shown in the skeleton network (Troy et al. 2001; Mannen et al. 2004)

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Table 1. SNPs variation detected in 59 D-loop sequence in two Bulgarian indigenous cattle breeds

Breed	Frequency, number	Frequency (%)	Haplogroup	Haplotype	15965	15974	15985	16022	16042	**16050 (T1a[b])**	**16057 (T2[b])**	16068	16074	16084	16085	16095	16101	16109	**16113 (T1[b])**	16121	16133	16135	16141	**16185 (T2[b])**	16228	16231	**16255 (T[b])**	16260	16264	16301	8	**169 (T3a/b[b])**	**173 (T3c[c])**	GenBank Accession No.
Bovine reference sequence[a]					T	C	T	G	T	C	G	T	T	C	T	A	T	T	T	G	T	T	T	G	A	C	T	C	G	C	G	A	A	V00654
Bulgarian Grey Cattle	2	5.13	T1**a**	BGC-1	.	.	.	.	.	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	C	.	.	.	.	G	.	KF373013
	5	12.82	T2	BGC-2	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	C	.	.	T	.	G	.	KF373014
	1	2.56	T	BGC-3	.	.	.	.	.	.	.	C	.	.	C	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	A	G	.	KF373015
	13	33.33	T3a	BGC-4.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	KF373016
	16	41.03	T3a	BGC-4.2	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	KF373017
	1	2.56	T3a	BGC-51	.	T	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	G	.	KF373018
	1	2.56	T3a	BGC-5.2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	G	.	KF373019
Shorthorn Rhodopean Cattle	1	5.00	T1	SRC-1	.	.	.	.	.	.	.	.	.	.	.	G	.	.	C	.	.	.	.	.	.	.	C	.	.	.	.	G	.	KF373020
	1	5.00	T2	SRC-2	.	.	C	.	.	.	C	.	C	.	.	.	C	.	.	.	.	.	.	A	.	.	C	T	.	.	.	G	.	KF373021
	2	10.00	T3b	SRC-3.1	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	KF373022
	1	5.00	T3b	SRC-3.2	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	KF373023
	1	5.00	T3b	SRC-3.3	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	KF373024
	1	5.00	T3c	SRC-4.3	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	.	.	.	.	.	.	G	KF373027
	4	20.00	T3c	SRC-4.2	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	G	KF373028
	2	10.00	T3c	SRC-4.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	KF373025
	2	10.00	T3c	SRC-4.4	C	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	G	G	KF373026
	2	10.00	T3a	SRC-5.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	KF373029
	1	5.00	T3a	SRC-5.2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	G	.	KF373031
	2	10.00	T3a	SRC-5.3	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	KF373030

[a]*Bos taurus* reference sequence (GenBank Accession No. V00654) (Anderson et al. 1982)
[b]polymorphic sites characterizing the main haplogroups (bolded) (Achilli et al. 2009)
[c]polymorphic site specific for the T3c sub-haplogroup
dots show the same sequence as the reference

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Despite that the mtDNA analysis includes only 20 sequences, it allows for the definition of various haplotypes and haplogroups (Table 1). The haplogroups T1 and T2 were presented by one sequence with frequencies of 5%. As it was expected, the majority of the remaining sequences belong to the T3 haplogroup (90%). The group was dichotomically divided into two branches T3a and T3b according to SNP at position 169 A/G (Figure 2B). In the SRC population, 173 G site is predominant (50% from T3 haplogroup, 9 animals). These haplotypes belong to the T3c sub-haplogroup and are presented with frequency of 45% for the entire SRC population set (Table 1). In the T3c sub-haplogroup the haplotype SRC-4.2 was of the highest frequency (20%).

The overall mean of genetic distance between the haplotypes in the BGC population was lower (0.003 ± 0.002) than that in SRC population (0.006 ± 0.001). The coefficient of differentiation in the entire population was 0.005 (Kimura 1980; Tamura et al. 2011).

In the BGC population T3 haplogroup showed a dichotomic phylogeny, with two major haplotypes BGC-4.1 and BGC-4.2 (Figure 2A), while the SRC breed's phylogenetic structure was formed by T3a, T3b, and T3c sub-haplogroups with various haplotypes (Figure 2B).

DISCUSSION

The Balkan region is a crucial point of European civilizations development and a historically important crossroad for the spread of livestock. In the present study two ancient cattle populations were characterized, attempting to compare the Bulgarian Grey and the Shorthorn Rhodopean Cattle and to establish their state among other Balkan cattle populations. The results from the D-loop sequence analyses showed that these breeds have mitotypes similar to other Balkan and European cattle breeds. Both of them possess three basic haplogroups – T1, T2, and the predominant T3 (82% for BGC and 90% for SRC). All the three haplogroups were defined with domestication origin from Fertile Crescent (Bonfiglio et al. 2010) and were spread with almost equal frequencies in the Near East (Achilli et al. 2009; Kantanen et al. 2009). The T1 haplogroup is the most frequent among African cattle while in Europe it was found with less than 10%, mainly in the Mediterranean region (Cortes et al. 2008). The Middle Eastern T2 haplogroup showed similar distribution in Europe as the T1 haplogroup (Troy et al. 2001).

T1 and T2 haplogroups diversity. The present research results showed a low frequency of the T1 haplogroup in BGC (2/39) and SRC (1/20) populations (5%) (Table 1, Figure 2). T1 haplogroup was presented by about 5% among the Italian Peninsula cattle population mtDNA set (Bonfiglio et al. 2010). A comparison with mtDNA data sets from different Balkan (Greek, Bulgarian, and Croatian) cattle breeds both for Brachicerous (Busha) and Podolian types was also performed (Supplementary Tables S1 and S2). It showed that the T1 haplogroup was found only within sequence sets from the present research represented by one SRC haplotype and one BGC haplotype (Table 1).

The T2 haplogroup among investigated population samples was presented by 13% (5/39) for BGC and 5% (1/20) for SRC (Table 1, Figure 2). This haplogroup was found in Greek Brachykeratiki (Beja-Pereira et al. 2006), and Bulgarian Grey (Bonfiglio et al. 2010), and nowhere in the Northwestern Balkans except a single sample among the Istrian cattle breed (Ivankovic et al. 2012). Among the available Balkan sequence data sets, the T2 haplogroup was represented by similar frequencies – about 10% for both Brachicerous and Podolian cattle (Supplementary Tables S1 and S2).

The above-mentioned data for T1 and T2 haplogroups can be explained by the later and limited livestock introduction from Anatolia. This dissemination pattern is similar to the spreading of both haplogroups in the Iberian (Cortes et al. 2008) and Italian (Bonfiglio et al. 2010) Peninsulas.

T3 haplogroup diversity. As expected, the T3 haplogroup is the most frequent showing quite different mitotypes in the current research data (Figure 2). This haplogroup is presented with 4 haplotypes for BGC and 10 haplotypes for SRC (Table 1).

T3a and T3b sub-haplogroups. For the BGC population only the T3a sub-haplogroup, based on 169 G nucleotide position, was found (Figure 2). Its representative haplotype is BGC-4.1 (33%, 13/39). This haplotype was also prevalent in other Podolian cattle breeds with 20% (Supplementary Table S1). Regarding mtDNA data set from the Italian Peninsula (Bonfiglio et al. 2010), that haplotype was proved to be predominant (about 12%). Among the T3a haplotypes for BGC the

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predominant one is BGC-4.2 (41%, 16/39) presented with motif 15965 C/169 G. This haplotype was observed with 15% among Balkan Podolian cattle but only found in the BGC population. In particular 15965 C/169 G motif was observed only with about 2% (2/125) in Italian Red Pied cattle (Bonfiglio et al. 2010). Polymorphic site 15965 C was found rarely in databank sequences (GenBank Accession Nos. FN562665, FN557411 – Israel; FJ815984 – USA; J815971 – Argentina) and with less than 1% in the Italian data set (Bonfiglio et al. 2010). The sporadic occurrence of 15965 SNP with no geographic correlation gives a reason to suggest that there is no phylogenetic significance of its presence but rather it is a random variable site. The other 15 Balkan haplotypes containing 169 G are only locally distributed which can be explained in a similar manner as 15965 SNP (Supplementary Table S1).

Apart from this the T3b sub-haplogroup (169 A, BRS) was not found in the BGC population but among the Balkan data sets it was represented by 5 haplotypes and observed with only about 13% from T3 haplogroup (Supplementary Table S1). Data shown here allows for speculating that the T3b sub-haplogroup is not typical for the Balkan Podolian cattle.

The autochthonous Brachicerous SRC population formed 10 different haplotypes in the T3 haplogroup. Some of them (SRC-4.1, SRC-4.2, and SRC-5.1) were found to be similar to other Brachicerous Busha cattle (Supplementary Table S2). Among the T3 haplotypes for SRC, these containing 169 A and 169 G SNPs are with equal frequencies (50%, Table 1). On the other hand, T3a haplotypes from Busha cattle are presented with expectedly higher frequencies (about 85%, Supplementary Table S2). The variable position 169 is the basic SNP that determines the dichotomical division of the T3 haplogroup to T3a/b sub-haplogroups (Achilli et al. 2009). Among available Gene Bank mtDNA sequences the frequency of 169 A site is about 34% (747/2580). Regarding the Balkan and Italian Peninsulas population sets this frequency is slightly lower – about 16% (Bonfiglio et al. 2010; Ivankovic et al. 2012).

T3c sub-haplogroup. In the D-loop highly variable control region the other predominant polymorphic site is at position 173. In the Gene Bank mtDNA data sets, 173 G SNP is present with about 8% (141/2580). With regard to Italian D-loop data (Bonfiglio et al. 2010, not submitted in Gene Bank), the percentage is a bit higher – 13% (330/1784). A similar frequency of 173 G was also observed among Balkan data sets but with differences in particular populations. The frequency of 173 G in the SRC population is 50% and that gives the reason to group the haplotypes with that SNP in a T3c sub-haplogroup. That newly named sub-haplogroup was defined not based on particular phylogenetic correlations, but due to the high variability of position 173 similar to that of the 169 polymorphic site. T3c sub-haplogroup contains mitotypes with motifs 169 G/173 G and 169 A/173 G. Different proportions of the two motifs were observed within the sequences. Gene Bank mtDNA sequences assay showed similar presence of the motifs (3% for 169 G/173 G and 5% for 169 A/173 G). In Bonfiglio et al. (2010) prevalence of 169 A/173 G motif (~10%) over 169 G/173 G motif (~3%) was detected. Almost all sequence data of Chinese cattle breeds (Lai et al. 2006) showed the presence of 173 G associated with the position 169 A. In contrast, the investigation on the South American cattle breeds descendent from the Portugal cattle breed showed prevalence of 173 G associated with 169 G (Mirol et al. 2003). Information about both the above-mentioned motifs showed clear interrelations between 173 G and 169 A/G transition. This could be explained by an augmentation of instability of the 169 variable position as a result of the presence of closely located 173 G. Additional proof for this statement are the multiple haplotypes sharing identical polymorphisms in combination with the 169 G/173 G and 169 A/173 G motifs (data not shown).

The newly named T3c sub-haplogroup (173 G) showed different dissemination among the Balkan cattle populations. It is the prevalent T3 sub-haplogroup for Brachicerous type cattle (about 20%). Contrary, its presence in Podolian cattle is only about 8%.

The present study allows for the presumption that Shorthorn Rhodopean Cattle as a part of the Balkan Busha cattle is an ancient breed with appreciable genetic diversity and may represent the earliest European cattle. This suggestion is further supported by data for basic phenotypic characteristics of livestock from the Neolithic age to the period of the Roman Empire and, in particular, by the dramatic body size decrease (Russell 1998; Ajmone-Marsan et al. 2010; Orton 2012).

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CONCLUSION

The present study showed various frequencies of the T1, T2, and T3 haplogroups in the Balkan cattle populations. In particular, the T3 haplogroup predominates in both Brachicerous and Podolian groups with different haplotype frequencies. The T2 haplogroup has appreciable frequencies among Brachicerous and Podolian data sets. The T1 haplogroup, almost exclusively describing African mtDNA, is found at low frequencies in both breeds.

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