

MITOCHONDRIAL DNA D-LOOP SEQUENCE ANALYSIS OF BUSHA CATTLE

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Abstract

Maintaining the genetic diversity of autochthonous breeds is very important and should be included in animal breeding strategies and conservation plans with the aim to preserve animals, adapted to variety of husbandry systems and different environmental conditions. Mitochondrial DNA (mtDNA) variation studies were particularly useful in establishing relationship and variability between and within domestic species populations. The mtDNA D-loop sequencing was used to determine the genetic status of 41 Busha cattle from different parts of Croatia along with 21 Busha mtDNA sequences from Genbank, and assessing Busha cattle phylogenetic relationship to the other Croatian autochthonous cattle breeds, as well as to the commercial cattle breeds and main described mtDNA haplotypes. The analysis of Busha cattle mtDNA D-loop sequences revealed presence of 26 different haplotypes, which confirms a high genetic variability of the preserved maternal components. Comparison with major cattle haplogroups sequences revealed that majority of Busha cattle were in the T3 haplogroup (44%), while other haplotypes were present in lesser extent (8% T1, 7% T2, 13% T4 and 5% T6). Haplotype H2 (23% of all Busha cattle sequences) didn't cluster with any of the T haplogroups. Employment of more powerful genetic markers should reveal the correct status of the new haplotype H2 and better understanding of genetic variation and relationships among Busha cattle and entire brachicerous cattle population.

Keywords: autochthonous cattle breeds, Busha cattle, mitochondrial DNA, phylogeny, cattle mitochondrial DNA haplogroups

INTRODUCTION

Maintaining the genetic diversity, i.e. the extent of genetic variation within and among breeds, is very important and should be included in animal breeding strategies and conservation plans of domestic animals, especially in autochthonous breeds, with the aim to preserve animals, adapted to variety of husbandry systems and different environmental conditions.

One of the most commonly used genetic markers is mitochondrial DNA (mtDNA) sequence. Generally, it is believed that mtDNA exhibits a maternal inheritance. Recently, biparental inheritance of mtDNA in humans was proved in three unrelated multigeneration families, showing high levels of

mtDNA heteroplasmy (Luo *et al.*, 2018). Nevertheless, identification of mtDNA sequence haplotypes is widely used in genetic variability studies, since mtDNA has a high level of variability and can often be traced to pre-domestic populations (Lenstra *et al.*, 2012). Most studies were performed on highly polymorphic displacement loop (D-loop) region, in the last decade also on the whole mtDNA genome sequences, which was helpful in understanding the origin of mitochondrial haplogroups.

mtDNA variation studies were particularly useful in establishing relationship between domestic species and their wild relatives (Bruford *et al.*, 2003), in identification of domestication sites (Naderi *et al.*, 2008; Hristov *et al.*, 2015a), to trace the

maternal origin of population back to ancient times (Upadhyay *et al.*, 2017), and also for the variability studies within and between populations (Ivanković *et al.*, 2014).

The ancient mtDNA sequencing projects on aurochs (*Bos primigenius*) suggest that modern cattle might result from two domestication events in southwest Asia, which gave rise to taurine (*Bos taurus*) and zebuine (*Bos indicus*) cattle, respectively (Troy *et al.*, 2001). Independent domestication in Africa and East Asia has also been postulated (Mannen *et al.*, 2004). Recently, it was shown that the oldest European cattle breed is the Balkan brachicerous cattle (Hristov *et al.*, 2015b), which belongs to the Busha cattle (*Bos primigenius f. Taurus*).

Cattle mtDNA data analysis reveals several basic haplogroups with different local geographical distributions and frequencies (Lenstra *et al.*, 2014). The macro-haplogroup T includes five haplogroups T1–T5, which are found in all modern cattle populations (Magee *et al.*, 2014). T3 haplogroup is probably of Middle East origin and is predominant among European taurine populations. The T5 haplogroup was reported only in Italy (Bonfiglio *et al.*, 2010) and Croatia (Ivanković *et al.*, 2014). Middle East origin of other basic haplogroups T1 and T2 have also been pointed out (Lenstra *et al.*, 2014; Troy *et al.*, 2001). The T1 haplogroup is disseminated predominantly in Africa and South Europe (Bonfiglio *et al.*, 2012). The T2 has been found only in few South European and Asian cattle populations (Achilli *et al.*, 2008; Ivanković *et al.*, 2014) and the T4 haplogroup has its origins probably in North and East Asia (Mannen *et al.*, 2004). A newly defined haplogroup T6 is Balkan-specific, found in 14 out of 17 analyzed ancient samples (8 – *B. primigenius*; 7 – *B. taurus* and 2 – *B. taurus/B. primigenius*) from Bulgaria (Hristov *et al.*, 2015b). This haplogroup significantly prevailed in ancient samples (75%) and surprisingly also in the present day Shorthorn Rhodopean cattle population (20%), a Busha cattle from Bulgaria.

Busha cattle (*Bos primigenius f. taurus*) is one of three Croatian autochthonous cattle breeds and is existent throughout the Balkan Peninsula. The breed is represented with a different name, depending on the geographical locations. The genetic profiling of Busha populations from Bulgaria (Hristov *et al.*, 2015a; Hristov *et al.*, 2017), Serbia (Kantanen *et al.*, 2009), and Croatia (Ramljak *et al.*, 2011; Ivanković *et al.*, 2014) showed higher diversity in Busha populations as compared with other surrounded autochthonous cattle breeds. All of those breeds show a small height (ranged from 90 to 115 cm), a body weight of adult animals ranging between 150 and 250 kg, and animals mostly located in the mountains. As other local cattle breeds, they are well adapted to the very harsh feeding and housing conditions, resistant to diseases and have a long

production life. Today's Croatian population counts 1309 cows and 114 bulls.

The aim of present work was to determine the genetic status of 41 Busha cattle from different parts of Croatia based on sequencing of mtDNA D-loop, along with 21 Busha mtDNA sequences from Genbank, and assessing Busha cattle phylogenetic relationship to the other Croatian autochthonous cattle breeds, as well as to the commercial cattle breeds and main described mtDNA haplotypes.

MATERIALS AND METHODS

Tissue samples were collected from 41 individuals of Busha cattle from different parts of Croatia. Genomic DNA was extracted from ear clips using DNeasy Blood & Tissue Kit (Qiagen GmbH, Germany). The D-loop region in the length of 994bp was amplified by using forward primer 5'-CAGTCTCACCATCAACCC-3' and reverse primer 5'-TCAGTGCCTTGCTTGGGT-3' amplifying a fragment between the *tRNA_{Pro}* gene and the central conserved sequence block (nt positions 15741-16338; 1-396) of the *Bos taurus* mitochondrion complete genome NCBI Reference Sequence (NC_006853.1). The same primer pair was used for sequencing of the PCR products, which were extracted from agarose gel with GeneJET Gel Extraction Kit (Thermo Scientific). Sequences were opened and analyzed in FinchTV 1.4.0 (Geospiza). All sequences were trimmed to the equal length of 703bp (between nt positions 15791 and 280 of the NCBI reference sequence).

Additional 60 sequences of the same length were retrieved from the Genbank; accession numbers are available in the Supplemental data file. Complete alignments were performed in ClustalX 2.1 with the default parameters and iteration of each alignment step.

Alignments were exported in the Nexus format and further analyzed with PopArt software (Leigh and Bryant, 2015). The software was used to infer haplotype network from the alignment data using the integer Neighbor Joining Network method. This method begins with a Neighbor-Joining tree, but sets the branch lengths to integer values so that they represent the number of mutations between sequences. To differentiate between different breeds, the breed names were imported as traits for each analyzed sequence. PopArt was also used to calculate basic statistics, such as number of identical sequences, nucleotide diversity, number of segregating sites, number of parsimony-informative sites, Tajima's D statistic (Tajima, 1999) and AMOVA (Excoffier *et al.*, 1992), for which significance is assessed using a parametric distribution (a beta distribution for Tajima's D, and an F distribution for AMOVA). Measuring of molecular variation among demes (AMOVA) used groups defined by traits (i.e. breeds). Different Busha cattle haplotypes were determined based on the identical sequences data.

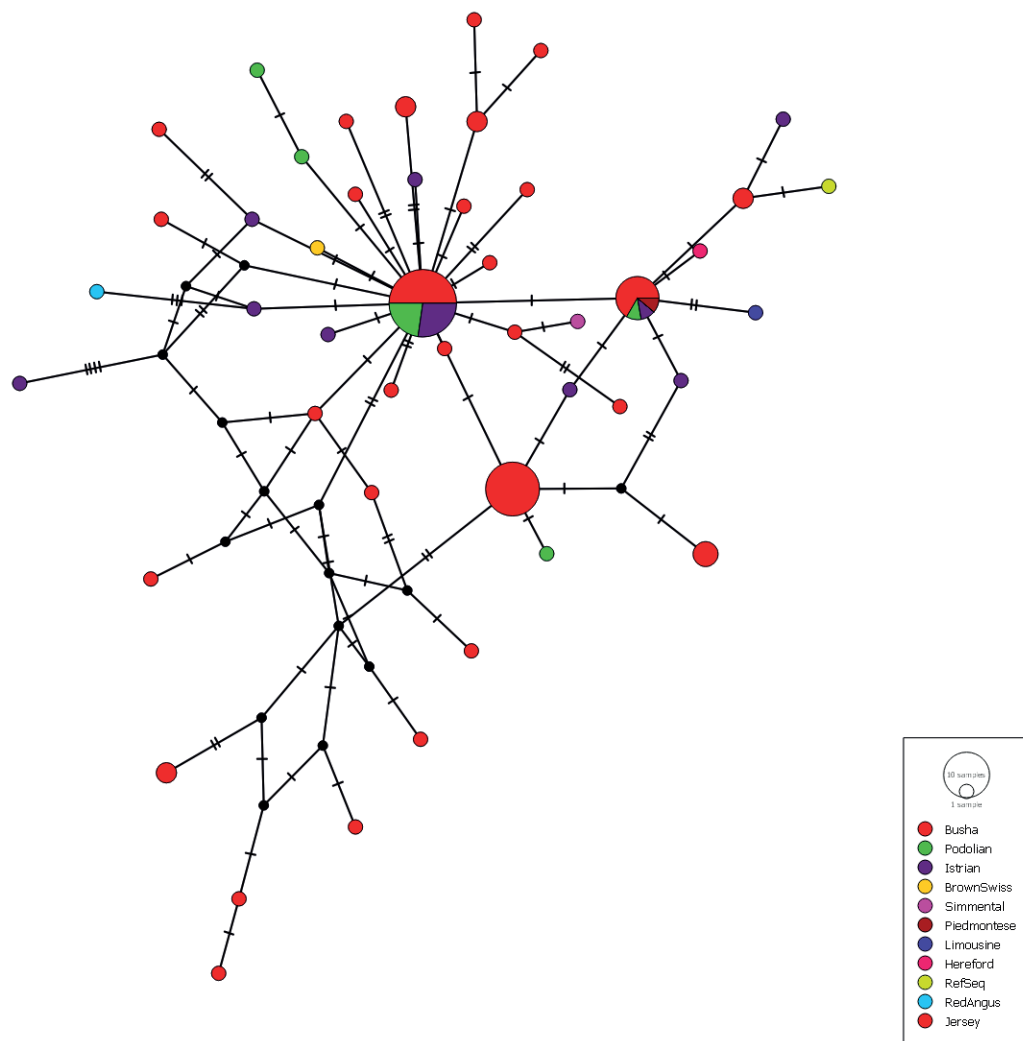
Alignment of determined Busha cattle haplotypes and Genbank sequences of main cattle mtDNA haplotypes (T1–T6, Q, P, I1, I2), as well as NCBI reference sequence and sequences of *B. indicus* and *Bison bison* was conducted in MEGA X software (Kumar *et al.*, 2018). The analyses performed in MEGA X included calculation of overall mean and pairwise distances between haplotypes, the nucleotide frequencies, number of variable and parsimony informative sites. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004). Analysis involved 39 nucleotide sequences. All ambiguous positions were removed for each sequence pair and there were a total of 735 positions in the final dataset.

A NeighborNet network from aligned haplotypes was constructed in SplitsTree4 program (Huson and Bryant, 2006), version 4.14.8.

RESULTS

The statistical analysis of aligned 122 sequences, 62 of them Busha cattle, of mtDNA D-loop region (703 sites) revealed 52 segregating sites and 24 parsimony-informative sites. The Tajima's D value was 173.784, meaning that there are more haplotypes (more average heterozygosity) than number of segregating sites and that rare alleles are present at low frequency. The π value of nucleotide diversity was 0.806489.

The AMOVA analysis didn't show any statistical significant subdivision among the breeds ($P < 0.001$). The partitioning levels of genetic diversity within and among the populations revealed that 83% of the total genetic variance existed within populations, and 10% between different groups of cattle.



1: Integer Neighbor Joining Network of Busha cattle mtDNA D-loop aligned sequences in relation to other Croatian autochthonous cattle breeds (Podolian, Istrian) and other cattle breeds, showing 3 main haplogroups, one specific only for the Busha cattle

Integer Neighbor Joining Network of Busha cattle, other two Croatian autochthonous cattle breeds (Podolian, Istrian) and other cattle breeds (Brown Swiss, Simmental, Piedmontese, Limousine, Hereford, Red Angus, Jersey, and NCBI reference sequence) showed 3 main haplogroups (Fig. 1). The largest haplogroup consisted of 23 sequences belonging to Croatian autochthonous breeds (12 Busha cattle – 50%, 5 Podolian cattle – 22.7% and 6 Istrian cattle – 27.3%). The second was characteristic only for Busha cattle (14 representatives, i.e. 23% of all Busha cattle analyzed sequences) and the third contained 9 sequences (6 Busha cattle – 66.7%, 1 Podolian cattle – 11.1%, 1 Istrian cattle – 11.1% and 1 Piedmontese cattle – 11.1%). Sequences of 31 Busha cattle (50.8%) were found in this three haplogroups. Altogether 26 haplotypes were observed in Busha cattle population, 17 of them represented just by one sequence.

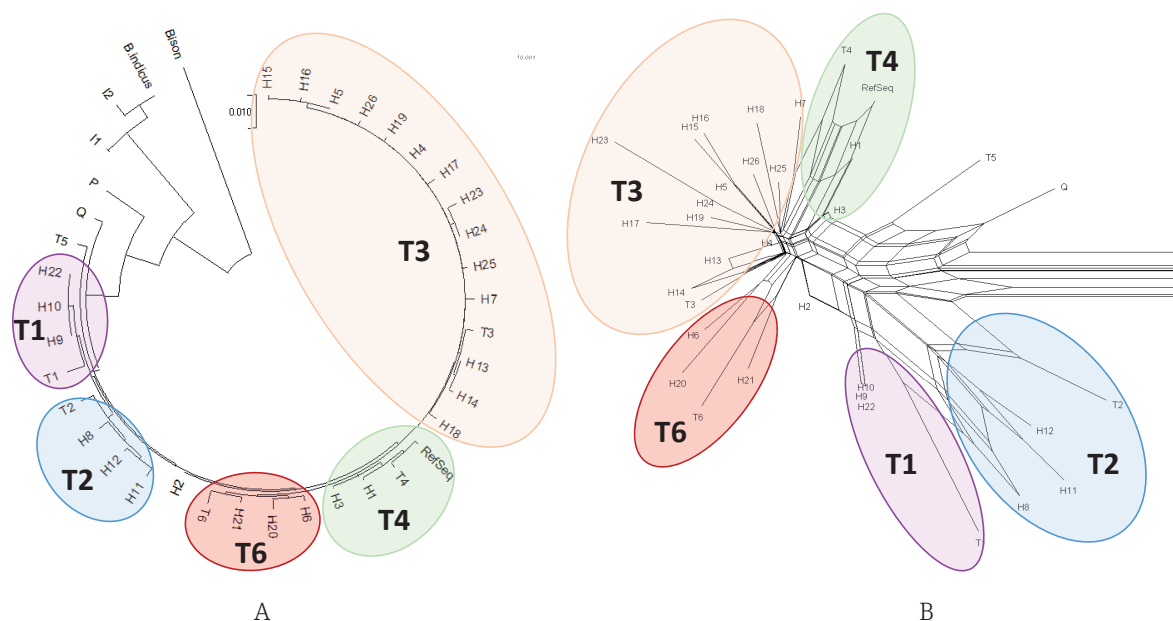
Alignment of determined 26 Busha cattle haplotypes and Genbank sequences of main cattle mtDNA haplotypes (T1–T6, Q, P, I1, I2), as well as NCBI *B. taurus* reference sequence and sequences of *B. indicus* and *Bison bison* was used for construction of Neighbor-joining tree (Fig. 2A) and NeighborNet network (Fig. 2B) of cattle haplotypes based on the mtDNA D-loop region sequences (nt 15791-16338; 1-280). The Neighbor-joining tree and NeighborNet network showed clustering of Busha cattle haplotypes with 5 main cattle mtDNA haplotypes of the macro-haplogroup T. The majority of samples were in the T3 haplogroup (44%), while other haplotypes were present in lesser extent (8% T1, 7% T2, 13% T4 and 5% T6). Interestingly,

haplotype H2 (23% of all Busha cattle sequences) didn't cluster with any of the T haplogroups neither in the Neighbor-joining tree, neither in NeighborNet network, but was placed close to the T6 clade in both cases.

The statistical analyses performed in MEGA X included calculation of overall mean and pairwise distances between haplotypes, the nucleotide frequencies, number of variable and parsimony informative sites. Pairwise distances between Busha cattle haplotypes ranged from 0.00142 to 0.01149 and the mean overall distance among all haplotypes was 0.02. The between group distance between Busha cattle and Istrian cattle was 0.00477, between Busha and Podolian cattle 0.00351 and between Istrian and Podolian cattle 0.00338, respectively. The number of variable sites among all haplotypes was 111 and dropped to 42 (Busha cattle and T haplogroups) and to 34 sites when considering only Busha cattle haplotypes. Among 42 variable sites between Busha cattle and T macro-haplogroup haplotypes, 19 were parsimony informative. The average nucleotide composition was 29.8% T, 23.9% C, 30.4% A and 16% G, respectively.

DISCUSSION

The analysis of 62 Busha cattle mtDNA D-loop sequences (nt 15791-16338; 1-280) revealed presence of 26 different haplotypes, which confirms a high genetic variability of the preserved maternal components as already described by other authors (Kantanen *et al.*, 2009; Ramljak *et al.*, 2011; Ivanković *et al.*, 2014; Hristov *et al.*, 2015a). Contrary to Ivanković *et al.* (2014) describing the majority



2: Neighbor-joining tree (A) and NeighborNet network (B) of cattle haplotypes based on the mtDNA D-loop region sequences (nt 15791-16338; 1-280) including 26 haplotypes observed in Busha cattle and Genbank sequences of main cattle mtDNA haplotypes (T1–T6, Q, P, I1, I2), as well as NCBI *B. taurus* reference sequence and sequences of *B. indicus* and *Bison bison*

of haplotypes (over 80%) in the T3 haplogroup and in line with findings of Hristov *et al.* (2015a) with 57% of haplotypes in the haplogroup T3, the results of present research revealed clustering of 44% of Busha cattle sequences with T3 haplotype sequence. Similar to Hristov *et al.* (2015a), T1 and T2 haplotypes were observed in much lower frequencies (8% T1 and 7% T2). 5% of Busha cattle haplotypes clustered together with newly described T6 haplogroup, which is less than in the results of Hristov *et al.* (2015a) where 20% of Shorthorn Rodopean cattle population belong to this haplogroup. Nevertheless, 14 sequences (23%) of H2 Busha cattle haplotype (majority of them from Imotski region in southern Dalmatia) didn't cluster with described major T haplogroups, but were close to the T6 haplogroup. Detailed insight into the sequences of H2 haplotype and comparison with higher number of T6 haplogroup sequences should reveal the correct status of this haplotype (haplogroup T6, its new subtype, or even a new haplogroup).

The statistical analysis of the results suggests high variability among Busha cattle breed, since 34 out of 42 variable sites in analyzed Busha cattle, Istrian and Podolian cattle and major T haplotypes were present in Busha cattle population. The between group distance between Busha cattle and other two Croatian autochthonous breeds confirmed findings of Ivanković *et al.* (2014) with the largest mean genetic distance between Istrian cattle and Busha cattle, while the genetic distance between other two groups is smaller. Also the partitioning levels of genetic diversity within and among the populations was in line with the previous results (Ivanković *et al.*, 2014) showing that majority (83%) of the total genetic variance existed within populations, and only 10% between different groups of cattle. The Tajima's D results suggests that there are more haplotypes (more average heterozygosity) than number of segregating sites and that rare alleles are present at low frequency, which was also observed on the haplotype level with high number of haplotypes represented just by one sequence.

CONCLUSION

The analysis of 62 Busha cattle mtDNA D-loop sequences (nt 15791-16338; 1-280) revealed high variability among Busha cattle breed with 26 different haplotypes, but with large number of haplotypes present at low frequencies. Comparison with major cattle haplogroups sequences revealed that majority of Busha cattle belongs to the T3 haplogroup (44%), while other haplotypes were present in lesser extent (8% T1, 7% T2, 13% T4 and 5% T6). Interestingly, haplotype H2 (23% of all Busha cattle sequences) didn't cluster with any of the T haplogroups neither in the Neighbor-joining tree, neither in NeighborNet network, but was placed close to the T6 clade in both cases. Detailed insight into the sequences of H2 haplotype, comparison with higher number of T6 haplogroup sequences and employment of more powerful genetic markers such as SNPs and whole genome sequencing should reveal the correct status of this haplotype and better understanding of genetic variation and relationships among Busha cattle and entire brachicerous cattle population.

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