



Mitochondrial DNA as a tool for identification of genetic diversity among domestic animals

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ABSTRACT

Mitochondrial DNA (mtDNA) is genetic marker that is often used in population and evolutionary biology. The best preserved part of the D-loop is its central part while other segments are subject to change. Busha is autochthonous breed of cattle that is bred extensively on the territory of Republic Croatia. During the last century there has been significant reduction in her size which has led to an endangerment of the breed. Busha is the part of cultural heritage and valuable gene source, which is the one of the reasons for its preservations. Research was conducted on 15 samples of Busha. Samples were collected on five different locations on territory of Croatia. Research was carried out on the most variable part of mtDNA (D-loop). The results of the research revealed the variability in the mtDNA D-loop sequences. The most common substitutions within the D-loop were C/T and A/G substitutions. Degree of genetic divergence within population is nearly 27%. The aim of this paper was to examine efficiency of mtDNA as a molecular marker in the analysis of genetic diversity among animal population. In this research we have used population of busha.

(Keywords: Buša, genetic diversity, molecular marker, mitochondrial DNA (mtDNA))

INTRODUCTION

Marker systems are developing depending on the required type of DNA identification, reliability, specifics and analysis speed (Ivanković, 2005). Molecular markers are identified DNA sequences that can be found on the specific locations in the genome and are transferred according to standard inheritance laws, from one generation to the next (Guimaraes *et al.*, 2007). Mitochondrial DNA is an important marker in determining genetic diversity among animals. There are several reasons why mtDNA is used as a molecular marker; those are the absence of recombinations, simple organization, maternal inheritance and a high degree of mutation in relation to nuclear DNA (Ballard and Rand, 2005). It is an important marker for determination the genetic diversity among animals and also can be used for creating tree that follows movement of mothers' line and leads to the first mtDNA. Galtier *et al.* (2009) state that the paternal mtDNA is removed earlier, during and after fertilization. With this method it is ensured that the organism contains mtDNA from only one parent and it is possible to follow the line to the first female unit. Mitochondrial genes represent a string of genetic information all the way back to the first female unit or a group of female units (Ballard and Rand, 2005). It is a highly conserved and congealed circular molecule and located in the mitochondrial matrix. The size of an average molecule is 16.5kb, but it varies and is dependent on the species (Kukat *et al.*, 2011). In cattle it is 16 338 base pairs long, in horses 16 660 base

pairs, and in chickens 16 782 base pairs. Analysis of mtDNA contributes to the evidence of domestication places. The presence of nucleotide substitution in this region is 2.8 to 5.0 times larger than in other mtDNA regions (Ivanković, 2005; Soares *et al.*, 2013). The control region, the D-loop, represents the most variable part of mitochondrial DNA. It is one of the reasons for its use in genetic research. The D-loop is the main control region for mitochondrial DNA expression. MtDNA also gives information at the intercontinental level (Lenstra *et al.*, 2014). Determining the mtDNA sequence variations in the control region can be used as a very useful tool for clarification of the species and diversification of cattle breed.

Busha is an autochthonous Croatian breed of cattle. It is extensive breeding in the areas of Lika and Dalmatia (Konjačić *et al.*, 2004; Simčić *et al.*, 2008). Busha is not present exclusively in the area of Lika, it can also be found in the regions of Papuk, Psunj, Žumberak and the Krka National Park. Međugorac *et al.* (2008) state that there are several subpopulations of the Busha that inhabit areas of Croatia, Bosnia and Herzegovina, Montenegro, Serbia, Kosovo, Albania, Macedonia, Romania, Bulgaria, Greece, Turkey and countries of the Near East. Their morphology has been determined in several studies: Adametz, 1895; Frangeš, 1903; Ogrizek, 1930; Ogrizek, 1941; Rako, 1943; Rako 1947; Šmalcelj and Rako, 1955; Šmalcelj, 1956; Puškaš, 1983; Šic *et al.*, 1994; Konjačić *et al.*, 2004 (Bulić *et al.*, 2007). Busha is a breed with crude constitution and small physical frame. The color of the hairs is single color brown, red to black with a stripe on the back which is in contrast with the basic color. "Doe snout" is a characteristic of the breed, i.e. dark pigment in the mucous skin with a white hairy ring around it. Ridge height is 100 to 110 cm, body mass of cows is 250 kg, while the bulls can weigh up to 300 kg. Horns are short with a light coating around the base and black tips (Čačić *et al.*, 2012). The genetics of the Busha was determined, on the DNA sequence and blood protein polymorphism level, by Ivanković *et al.*, 2004; Konjačić *et al.*, 2005. Development of modern cattle production is based on neglecting the animal genetic limits. The consequence of that is full usage of useful genes to the degree that it represents a danger for the animal health. Autochthonous breeds has a high economic significance, as a gene banks they are indispensable tool in attempts to repair and improve genetic status of modern and high productive breeds of cattle.

MATERIAL AND METHODS

The research was conducted on 15 cattle of the Busha breed. The blood was collected by puncturing the jugular vein and placed in tubes with an EDTA coagulant. The blood samples were frozen within 3 hours after collection and kept at a temperature of 4 °C up to that point. Molecular and genetic analysis of the collected samples was conducted at the biological research lab of the Faculty of Agriculture in Osijek.

DNA isolation was conducted from 200 µl of homogenized blood using the phenol-chloroform extraction method (25:24:1) (Ausubel *et al.*, 2000). A TE buffer was used for blood plasma washing and leukocyte separation (Tris-EDTA, pH 8.3). Lysis buffer and proteinase K were used for destroying leukocyte and FOR protein removal. DNA washing was done twice using phenol-chloroform-isoamyl alcohol (PCI, 25:24:1) and chloroform-isoamyl alcohol (CI, 24:1). The isolated DNA was dissolved in 96% alcohol and then washed with 70% alcohol. The DNA acquired this way was dissolved in 25 µl of deionized filtered H₂O. Isolation check was done using electrophoresis on 2% agarose gel.

Figure 1.

Areas where were collected samples for analysis



The PCR conditions were 5 min of initial denaturation at 95 °C, followed by 35 cycles of elongation at 95°C within 50 sec, 61 °C within 50 sec and 72 °C within 50 sec, final extension at 72 °C during the 6 min. PCR reaction was prepared in 20 µl of mixture consisting the following: 12.1 µl ddH₂O, 2.0 µl buffer, 1.2 µl MgCl₂, 1.0 µl dNTP, 1.0 µl F primers, 1.0 µl R primers, 0.2 µl Taq polymerase and 1.5 µl DNA. Two primer pairs were used to amplify the D-loop region MITb1 - (59-CTGCAGTCTCACCATCAACC-39) and MITb2 - (59-CTCCTCGGACAAGATATTAG-39).

PCR products were sent to South Korea for sequencing (Macrogen inc.). Computer program ClustaW was used for sequence alignment (*Thompson et al.*, 1994). Phylogenetic connection between units within a population was determined using a neighbor-joining (NJ) algorithm (*Tamura and Nei*, 1993). Bootstrop levels within the tree were determined with 1,000 repetitions. All the stated analyses were made using the computer program MEGA, version 3.1 (*Kumar et al.*, 2004). Analyses of molecular variance (AMOVA), F_{ST} values, as well as nucleotide differences (*Nei*, 1978) are done using the program Arlequin ver. 3.01 (*Excoffier et al.*, 2006).

RESULTS AND DISCUSSION

Conducted research has revealed the variability in the mtDNA D-loop sequences. Genetic structure within the studied population was examined using the AMOVA method. The method was made based on the allele content of different haplotypes, as well as their frequency. It is widely used tool for quantifying the various levels of population structure to patterns of genetic variation. First research which has included comparison of mtDNA different cattle breeds was conducted by *Bradley et al.* (1996). Research which was conducted till now showed that genetic variability declines with increasing distance from the domestication sites (*Lenstra et al.*, 2014). Cattle MtDNA D-loop sequences are divided into five mtDNA haplogroups: T, T1, T2, T3 and T4. T3 haplogroup is the most frequent group and it presents the dominant haplotype group.

Kantanen *et al.* (2009) has investigated geographical patterns of mtDNA diversity of Eurasian taurine cattle (*Bos taurus*).

Table 1.

Types of nucleotide substitution in the D-loop region of the investigated cattle

<i>Nucleotide substitution</i>	<i>Number</i>	<i>Percentage (%)</i>
C/T	211	55.2
A/G	148	38.7
A/C	10	2.6
G/T	2	0.5
C/G	7	1.8
A/T	4	1.0
Total	382	100.00

The *Table 1.* shows that the largest numbers of nucleotide substitutions in the D-loop region are C/T and A/G substitutions. There was 211 C/T substitution which represents 55.2%, and there are 148 A/G substitutions which are 38.7%. These two substitutions are the most common in D-loop. This substitution is also a characteristic of some other native breeds of cattle in the region (Cika), so it indicates a phylogenetic connection and the possibility of uncontrolled cross breeding in the very early stages of domestication. *Lenstra et al.* (2014) has shown that geographically differentiation of cattle mtDNA is stronger than in other domestic animals. *Cai et al.* (2014) conducted research which has included the European and Near Eastern domestic cattle and show the haplogroup distribution pattern of Chinese domestic cattle. Research which has conducted by *Ludwing et al.* (2013) included the investigations of six novel mitochondrial genes from the White Park Cattle. *Ivanković et al.* (2010) has conducted similar research. They investigate levels of genetic variability between Istrian cattle and Slavonian – Syrmian podolian cattle using microsatellites and D-loop as genetic markers. They have analyzed proximal part of the D-loop region. *Ivanković et al.* (2014) observed phylogenetic relationship of Croatian autochthonous cattle breeds. They have analyzed D-loop region of Busha, Istrian, and Slavonian - Syrmian podolian cattle populations. Results of their research have show that there is high level of mtDNA diversity in Busha population.

Table 2.

Genetic variation within the investigation Busha population

	B14	B15	B21	B18
B14	-	***	***	***
B15	0.0293	-	n.s.	n.s.
B21	0.0265	0.0287	-	n.s.
B18	0.0264	0.0278	0.0259	-

***P <0.001

The samples were grouped regarding the collection area. There were 4 samples collected from North part of Croatia (B14), 6 samples from Dalmatia region (B15), 1 sample from

Istria region (B18) and 4 samples from Lika region (B21). Data were analysed using MEGA version 3.1., while FST values and analysis of molecular variance were made using Arlequin version 3.01. A statistically significant difference was detected within the population ($P < 0.001$) concerning genetic variability between unit B14 and the other units in the population. Other units display a comparable level of genetic variability (average of 0.27%), which indicates approximate homogeneity and genetic stability of the population. The stated results point to a conclusion that unit B14 is not a Busha, it is instead a crossbreed which is similar to the Busha externally, but genetically it belongs to another breed, most probably a cross between several breeds. The reason for making that conclusion is the occurrence of statistically significant genetic difference which was determined between unit B14 and other individuals within the Busha population.

CONCLUSIONS

The largest numbers of nucleotide substitutions in the D-loop region are C/T and A/G substitutions. A statistically significant difference was detected within the population ($P < 0.001$) concerning genetic variability between unit B14 and all the other units in the population. Other units display a comparable level of genetic variability (average of 0.27%), which indicates approximate homogeneity and genetic stability of the population. Conservation of genetic resources today is set as the imperative in livestock production. Busha represents a valuable genetic resource that must be protected and preserved. Information from mtDNA has great importance for conservation genetics in autochthonous cattle breeds.

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