Mitochondrial DNA-based genetic evaluation of autochthonous cattle breeds in Croatia

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ABSTRACT: Genetic diversity and phylogenetic relationship of three Croatian autochthonous cattle breeds was analyzed using a sequence of the mtDNA D-loop region. Among Busha, Istrian, and Slavonian Syrmian Podolian cattle 146 unrelated animals were tested. The sequencing of 780 base pairs of the mtDNA D-loop region revealed 39 polymorphic sites representing 28 different haplotypes. The highest numbers of haplotypes were observed in the Busha population and the lowest in the population of Slavonian Syrmian Podolian cattle, while the highest level of sequence diversity within a population was observed in the Istrian cattle. Our results indicate a high level of mtDNA diversity in the populations of Busha and Istrian cattle and a low level of preserved diversity in the population of Slavonian Syrmian Podolian cattle. The sequence analysis showed substantial subdivision between the breeds (F_{ST} = 0.1434), and a large fraction of variation within the breeds. Although the dominant haplotypes are classified as the T3 haplogroup, some of the haplotypes are classified as the rarer T2 and T5 haplogroups. Genetic information based on mtDNA typing has a great importance for the future conservation management and preservation of genetic diversity in autochthonous cattle breeds.

Keywords: mtDNA; genetic diversity; *Bos taurus*; phylogeny; conservation

INTRODUCTION

Cattle breeds are recognized as an important part of biodiversity and genetic heritage. According to FAO (2009), out of the 1351 cattle breeds worldwide 14.8% are extinct, with European and Caucasian breeds accounting for 20.4% of the extinct breeds. Therefore, it is very important to preserve the genetic diversity of the remaining breeds, mostly captured in nonselected autochthonous breeds (Medugorac et al. 2009), and genetic characterization is the first step in choosing an efficient conservation strategy. The importance of AnGR conservation, as one of the four Strategic Priority Areas of the Global Plan of Action for AnGR, was recognized by FAO (2007).

Croatia has three autochthonous cattle breeds that arise from the intersection of the migratory routes: Busha cattle (BU), Istrian cattle (IC), and Slavonian Syrmian Podolian cattle (SP). BU is small, shorthorn (brachyceros) type of cattle that spread from the Near East across the Balkan Peninsula. IC and SP belong to the Podolian group, which arrived to Pannonian Croatia from Eastern Europe by the Danubian migration route. Thus a Podolian group of breeds from the south of Italy influenced the population of IC (Maretto et al. 2012). These breeds were developed using different breeding approaches. BU is a very small breed with withers height of 90–112 cm and body mass of 150–250 kg, used for milk and beef production. It is resistant and well-adapted to severe environmental conditions (high and low temperatures and lack of pasture) in the mountainous (Lika) and Mediterranean Croatia (Dalmatia). Although today's population counts approximately 450 breeding animals, it is still critically endangered. IC is a well-adapted breed with moderate frame

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size (average withers height 134 cm, body mass 550 kg, light to dark grey body colour) (Ivankovic et al. 2007) which came to Istria more than 2500 years ago (Smalcelj et al. 1958). At the end of the 18th century, a smaller part of the population was cross-bred with Podolian bulls from Italy to improve meat production characteristics, food utilization, weight gain, and precocity (Ogrizek 1957). Current population comprises more than 1000 breeding animals, and belongs to the group of endangered breeds. SP cattle are a longhorn, resilient breed with a moderate-sized frame (withers height 125–140 cm, body mass 400–600 kg) and a light to dark grey body colour. It has been speculated that they originate from the steppes of Russia and Ukraine, Podolia and Volhinia, from where they migrated to the regions of Central and South Europe (the Danubian route). Until the 19th century, SP had been primarily used for field work and draught power, and to a smaller extent for milk and meat production. Considering the population size (243 breeding animals), SP cattle are a critically endangered breed.

Although a study of the natural genetic variation among Croatian autochthonous cattle breeds by microsatellites was reported by Ramljak et al. (2011), a large part of molecular variation remains unknown. The efforts to better understand the genetics of these three breeds for conservation purposes also include an analysis of mtDNA. Thus we report the first study undertaken to assess the genetic diversity and the phylogenetic relationship using the D-loop sequence.

MATERIAL AND METHODS

Blood samples were collected as follows: 50 samples from IC, 47 from SP, and 49 from BU cattle. The animals were selected from a broad breeding area, sampling 4–5 individuals per herd at several locations (8–12 herds per breed; Figure 1). Mitochondrial DNA was isolated from blood samples as described by White and Densmore (1992). The proximal part of the D-loop region was PCR amplified using primers P28 (5'-GTAAAACGACGGCCAGTCTCACCATCAACCCCCAAAGC-3') and HF (5'-GCCCCATGCATATAAGCAAG-3') amplifying a fragment of the D-loop region, between the $tRNA_{Pro}$ gene and the central conserved sequence block (GenBank Accession No. BRS V00645.1, nt 15 920–16 338, 1-361) (Anderson et al. 1982). The PCR reaction mixture (20 µl) contained template of DNA (50 ng), 10 pmol of each primer, reaction buffer (10mM Tris-HCl pH 8.3; 50mM KCl; 5mM $MgCl_2$; 20µM dNTPs), and 0.4 U Taq Polymerase (PE Applied Biosystems, Foster City, USA). The PCR reaction was performed on a PTC-100 thermal cycler (MJ Research, Waltham, USA) starting with initial denaturation at 95°C for 5 min, followed by 32 cycles of 94°C (60 s), 52°C (30 s), 72°C (60 s) and final extension at 72°C (5 min). PCR fragments were sequenced using ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM® 310 Genetic Analyzer (PE Applied Biosystems). Multiple alignment of mtDNA sequences was performed using Clustal-W program (Version 1.82, 2001) and used for further

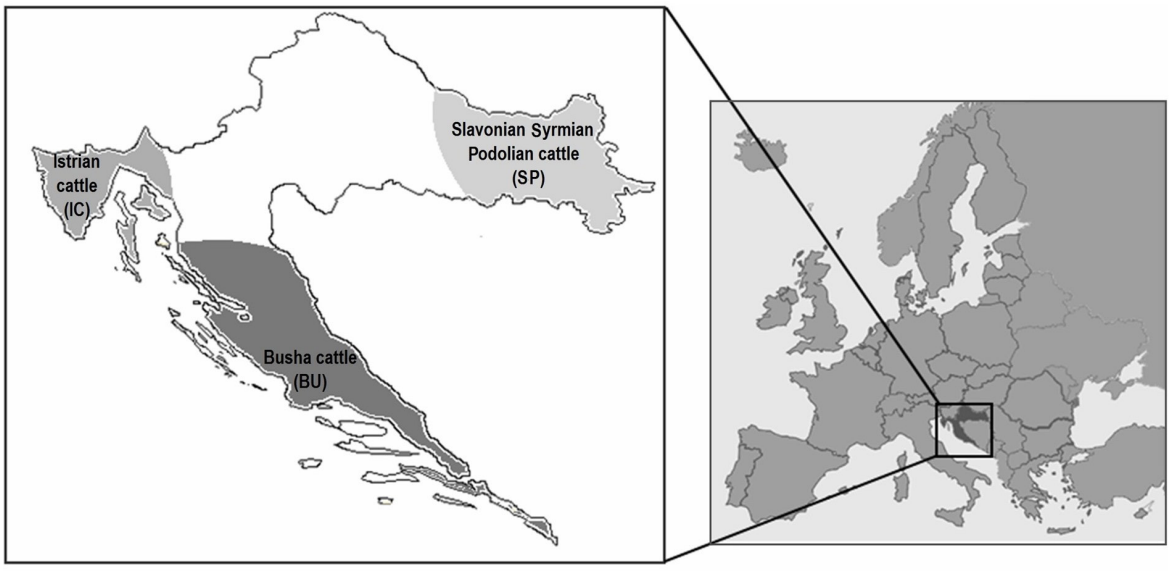

Figure 1. Geographical locations of three sampled autochthonous Croatian cattle breeds

analysis by the Molecular Evolutionary Genetics Analysis (MEGA) software (Version 5, 2011) and Phylogeny Inference Package (PHYLIP) (Version 3.5c, 1993).

Partitioning of genetic diversity using the analysis of molecular variance (AMOVA) and linearized F_{ST} values (Slatkin 1995) was performed with Arlequin software package (Version 3.0, 2005). Genetic distances among different mtDNA haplotypes were calculated by the two-parameter method of Kimura (Kimura 1980) and an unrooted tree was drawn using the neighbour-joining method (Saitou and Nei 1987). Sequences were analyzed based on the position of polymorphic SNPs and haplogroups were determined according to Achilli et al. (2009). Bootstrap analyses (1000 data sets) were used to assess confidence in the branching order and values higher than 40 were entered into the dendrogram. To estimate the inter- and intra-species sequence divergence in the mtDNA control region, the data from this study were compared with 121 sequences of the mtDNA control region from the genus *Bos*. GenBank accession numbers for the data presented in this study are as follows: JQ437310–JQ437356 (http://www.ncbi.nlm.nih.gov/GenBank).

RESULTS

The 780-bp fragment of the mtDNA D-loop region was sequenced (15 920–16 338; 0–361). The mean diversity within all breeds was 0.0033 ± 0.0007. Out of the 39 polymorphic sites, 32 were caused by transitions, four by transversions, and three were the consequence of a deletion (Table 1). The share of polymorphic positions in relation to the whole sequence was 5.0%, out of which transitions were represented by 4.1%. Deletions were observed in three haplotypes (Hap 07, Hap 09, and Hap 28). The average nucleotide sequence diversity among all haplotypes was 3.63 (from 0.13 to 1.41%) and the average distance among haplotypes was 0.0047 (from 0.0013 to 0.0143). The average sequence diversity among SP cattle is smaller (2.20; 0.28%) than among BU (3.76; 0.48%) and IC (4.42; 0.59%). In the whole sample, 28 different haplotypes were observed, as shown in the neighbour-joining tree (Figure 2). Only one observed haplotype is shared by all three breeds (Hap 09), another one is shared by BU and IC (Hap 06), and another one by BU and SP (Hap 14), indicating some intercrossing over the past century. Although IC and SP belong to the same Podolian cattle group, they did not share a single haplotype in this study. In the BU population, 14 haplotypes (Hap 01–14) were observed whose sequence differed in 1–8 nucleotide mutations (from 0.13 to 1.03%; Tables 1 and 2), with the most common haplotype being Hap 14 (22.4%). The average mean group distance within the BU population was 0.0033 ± 0.001. In the population of IC, thirteen haplotypes were observed (Hap 06, Hap 09, Hap 15–25), with sequences differing in 1–11 nucleotide mutations (from 0.13 to 1.41%; Tables 1 and 2). The most abundant was Hap 25 (18%), while two haplotypes (Hap 15, Hap 16) had a frequency of 10%, and other eight haplotypes had frequencies lower than 8% (Table 1). The population of IC was the only one found to have T2 haplogroup (Hap 20), its frequency being 6.0%. The largest genetic distance was observed between the haplotypes Hap 17 and Hap 20 (0.0143). The average mean group distance among IC (0.0042 ± 0.0010) was higher than among the other two investigated cattle breeds. The lowest number of haplotypes, with 1–4 nucleotide differences in the sequences (0.13–0.51%; Tables 1 and 2), was observed in SP (Hap 26–Hap 28, Hap 14, Hap 09).

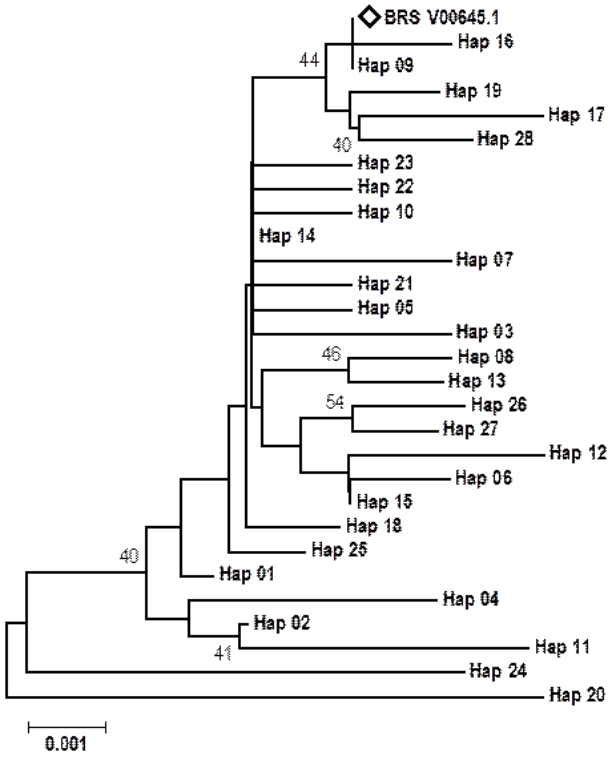

Figure 2. Neighbour-joining tree of cattle haplotypes based on the D-loop region mtDNA (nt 15 920–16 338; 1-361). The values on the branch are bootstrap support based on 1000 replications

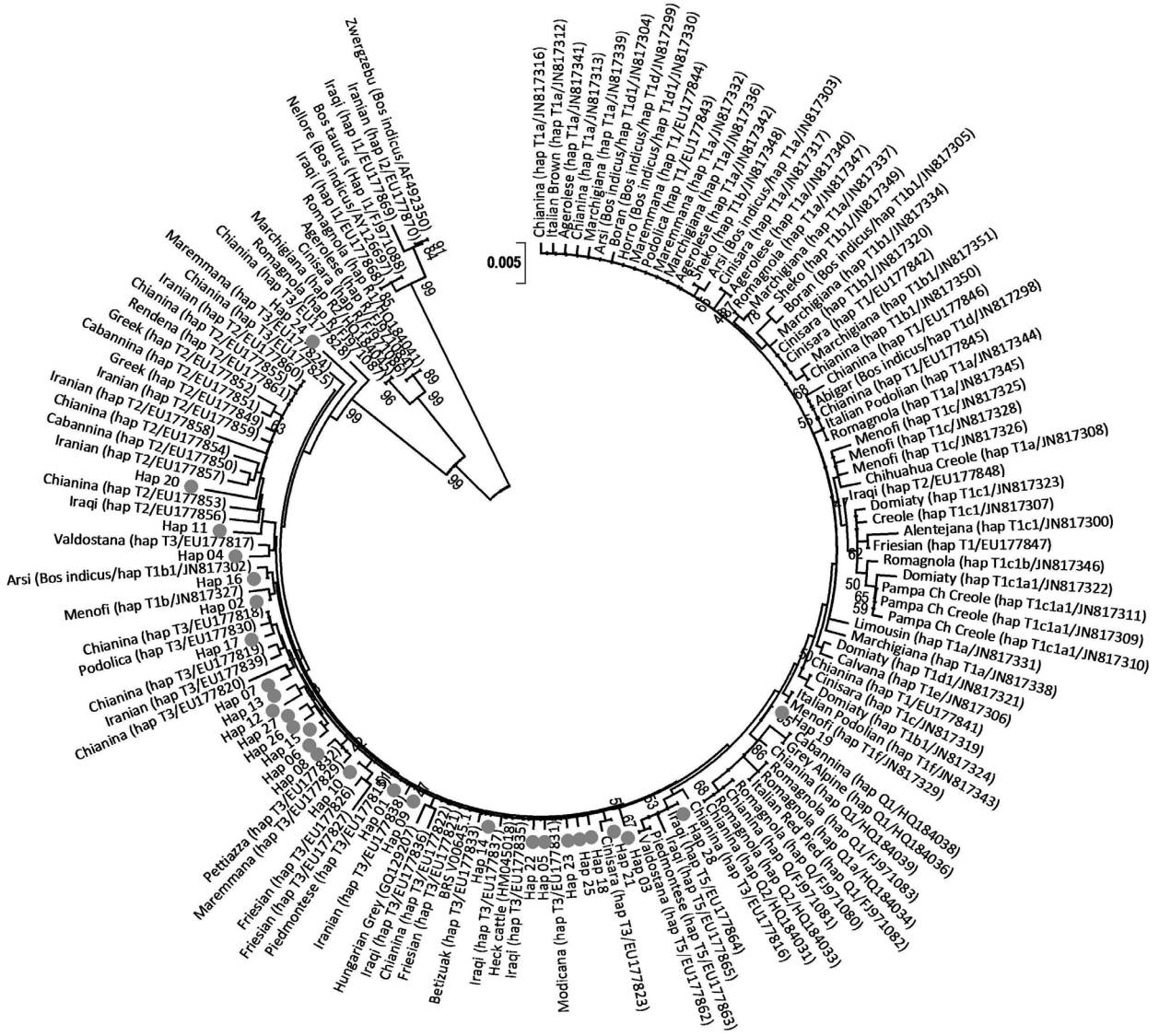

Figure 3. Neighbour-joining tree showing the relationships among 149 partial mtDNA sequences from members of the genus *Bos* including haplotypes of the Croatian autochthonous cattle breeds with their sequence (●)

The two dominant haplotypes were Hap 28 (36.2%) and Hap 27 (29.8%) and they were not shared by the other two autochthonous cattle breeds. The average mean group distance among SP (0.0023 ± 0.0011) was smaller than among the IC and BU cattle, which is expected in the view of the total size and the geographical distribution of the breed.

The AMOVA analysis showed substantial subdivision among the breeds (F_{ST} = 0.1434, P < 0.001), but with a large fraction of variation found within the populations. The partitioning levels of genetic diversity within and among the populations revealed that 85.66% of the total genetic variance existed within populations, and 1.23% between BU, IC, and SP cattle (Table 3). As for the fact that 15.57% of the total genetic variance was present between the Podolian group (consisting of IC and SP) on the one hand and BU cattle on the other, the analysis of molecular variance strongly supported the division between theese two groups.

The mean genetic distance between IC and BU cattle was the largest (0.0051), followed by the distance between IC and SP (0.0042), while the mean genetic distance between BU and SP cattle was the smallest (0.0039). The mean within-group distance was larger in the population of IC (0.0054) than in the populations of BU (0.0045) or SP (0.0028).

As expected for European cattle, the T3 haplogroup comprises the vast majority of mtDNA (84.25%) considering all three breeds. At the breed

Table 1. Haplotypes, haplogroups, and nucleotide substitutions observed in autochthonous cattle breeds in Croatia relative to the *Bos taurus* reference sequence (GenBank BRS V00645.1)

Haplotype	No. of haplotype observation	Haplotype frequencies in entire populations	Haplogroup	Control-region motifs	Nucleotide differences from the *Bos taurus* reference sequence (BRS V00645.1)																																						
					15959	15964	16012	16022	16042	16050	16051	16057	16067	16069	16104	16110	16113	16119	16122	16125	16133	16135	16138	16141	16154	16164	16185	16200	16202	16226	16231	16247	16255	00008	00052	00106	00163	00169	00173	00177	00215	00231	00272
				BRS V00645.1	G	T	A	G	T	C	T	G	A	A	C	C	T	T	T	A	T	T	T	T	A	T	G	G	G	A	C	C	T	G	G	T	A	A	A	T	T	A	T
Hap 01	5	0.034	T3	8, 169	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.
Hap 02	4	0.027	T3	8, 106, 169	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.	G	.	.	.	.	.
Hap 03	4	0.027	T3	16050, 16051, 169	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
Hap 04	4	0.027	T3	16057C, 16135, 16141, 8, 169	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	C	.	C	.	.	.	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.
Hap 05	4	0.027	T3	16069, 169	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
Hap 06	6	0.041	T3	16125, 169, 173	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.
Hap 07	3	0.021	T3	15959, 15964, -, 169	A	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	G	.	.	.	.	.
Hap 08	3	0.021	T3	16133, 16164, 169	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
Hap 09	17	0.116	T3	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.
Hap 10	2	0.014	T3	16231, 169	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	G	.	.	.	.	.
Hap 11	1	0.007	T3	16012, 16057C, 16185, 8, 106, 169	.	.	C	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	A	.	C	.	G	.	.	.	.	.
Hap 12	1	0.007	T3	16067, 16119, 169, 173	.	.	.	.	.	.	.	.	G	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.
Hap 13	1	0.007	T3	16119, 16164, 169	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
Hap 14	17	0.116	T3	169	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
Hap 15	5	0.034	T3	169, 173	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.
Hap 16	5	0.034	T3	16113	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 17	4	0.027	T3	16042, 163	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
Hap 18	3	0.021	T3	16247, 169	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.
Hap 19	3	0.021	T	16255	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.
Hap 20	3	0.021	T2	16022, 16057C, 16110, 16138, 16154, 16185, 16255, 169, 215	.	.	.	A	.	.	.	C	.	.	.	G	.	.	.	.	.	.	C	.	G	.	A	.	.	.	.	.	C	.	.	.	.	G	.	.	C	.	.
Hap 21	3	0.021	T3	16200, 169	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
Hap 22	2	0.014	T3	169, 177	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	.
Hap 23	2	0.014	T3	169, 272	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	C
Hap 24	2	0.014	T3	16057C, 16122, 16138, 16154, 16164, 16247, 8, 169	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	C	.	G	C	.	.	.	.	.	T	.	A	.	.	.	G	.	.	.	.	.
Hap 25	9	0.062	T3	16138, 169	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.
Hap 26	2	0.014	T3	16104, 16202, 169	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.	.	.	.	.
Hap 27	14	0.096	T3	16104, 169, 173	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	G	.	.	.	.
Hap 28	17	0.116	T5	16255, 163, 169, –	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	G	G	.	.	.	-	.

Table 2. Number of nucleotide differences (above diagonal) and Kimura two-parameter distances (below diagonal) among the twenty-eight mtDNA haplotypes (the highest value in bold)

	Hap 01	Hap 02	Hap 03	Hap 04	Hap 05	Hap 06	Hap 07	Hap 08	Hap 09	Hap 10	Hap 11	Hap 12	Hap 13	Hap 14
Hap 01	–	1	3	3	2	3	3	3	2	2	4	4	3	1
Hap 02	0.0013	–	4	4	3	4	4	4	3	3	3	5	4	2
Hap 03	0.0039	0.0052	–	6	3	4	4	4	3	3	7	5	4	2
Hap 04	0.0039	0.0052	0.0078	–	5	6	6	6	5	5	5	7	6	4
Hap 05	0.0026	0.0039	0.0039	0.0065	–	3	3	3	2	2	6	4	3	1
Hap 06	0.0039	0.0052	0.0052	0.0078	0.0039	–	4	4	3	3	7	3	4	2
Hap 07	0.0039	0.0052	0.0052	0.0078	0.0039	0.0052	–	4	3	3	7	5	4	2
Hap 08	0.0039	0.0052	0.0052	0.0078	0.0039	0.0052	0.0052	–	3	3	7	5	2	2
Hap 09	0.0026	0.0039	0.0039	0.0065	0.0026	0.0039	0.0039	0.0039	–	2	6	4	3	1
Hap 10	0.0026	0.0039	0.0039	0.0065	0.0026	0.0039	0.0039	0.0039	0.0026	–	6	4	3	1
Hap 11	0.0052	0.0039	0.0091	0.0065	0.0078	0.0091	0.0091	0.0091	0.0078	0.0078	–	8	7	5
Hap 12	0.0052	0.0065	0.0065	0.0091	0.0052	0.0039	0.0065	0.0065	0.0052	0.0052	0.0104	–	3	3
Hap 13	0.0039	0.0052	0.0052	0.0078	0.0039	0.0052	0.0052	0.0026	0.0039	0.0039	0.0091	0.0039	–	2
Hap 14	0.0013	0.0026	0.0026	0.0052	0.0013	0.0026	0.0026	0.0026	0.0013	0.0013	0.0065	0.0039	0.0026	–
Hap 15	0.0026	0.0039	0.0039	0.0065	0.0026	0.0013	0.0039	0.0039	0.0026	0.0026	0.0078	0.0026	0.0039	0.0013
Hap 16	0.0039	0.0052	0.0052	0.0078	0.0039	0.0052	0.0052	0.0052	0.0013	0.0039	0.0091	0.0065	0.0052	0.0026
Hap 17	0.0052	0.0065	0.0065	0.0091	0.0052	0.0065	0.0065	0.0065	0.0026	0.0052	0.0104	0.0078	0.0065	0.0039
Hap 18	0.0026	0.0039	0.0039	0.0065	0.0026	0.0039	0.0039	0.0039	0.0026	0.0026	0.0078	0.0052	0.0039	0.0013
Hap 19	0.0039	0.0052	0.0052	0.0078	0.0039	0.0052	0.0052	0.0052	0.0013	0.0039	0.0091	0.0065	0.0052	0.0026
Hap 20	0.0117	0.0130	0.0130	0.0130	0.0117	0.0130	0.0130	0.0130	0.0117	0.0117	0.0117	**0.0143**	0.0130	0.0104
Hap 21	0.0026	0.0039	0.0039	0.0065	0.0026	0.0039	0.0039	0.0039	0.0026	0.0026	0.0078	0.0052	0.0039	0.0013
Hap 22	0.0026	0.0039	0.0039	0.0065	0.0026	0.0039	0.0039	0.0039	0.0026	0.0026	0.0078	0.0052	0.0039	0.0013
Hap 23	0.0026	0.0039	0.0039	0.0065	0.0026	0.0039	0.0039	0.0039	0.0026	0.0026	0.0078	0.0052	0.0039	0.0013
Hap 24	0.0078	0.0091	0.0117	0.0104	0.0104	0.0117	0.0117	0.0091	0.0104	0.0104	0.0117	0.0130	0.0091	0.0091
Hap 25	0.0026	0.0039	0.0039	0.0065	0.0026	0.0039	0.0039	0.0039	0.0026	0.0026	0.0078	0.0052	0.0039	0.0013
Hap 26	0.0039	0.0052	0.0052	0.0078	0.0039	0.0052	0.0052	0.0052	0.0039	0.0039	0.0091	0.0065	0.0052	0.0026
Hap 27	0.0039	0.0052	0.0052	0.0078	0.0039	0.0026	0.0052	0.0052	0.0039	0.0039	0.0091	0.0039	0.0052	0.0026
Hap 28	0.0039	0.0052	0.0052	0.0078	0.0039	0.0052	0.0052	0.0052	0.0039	0.0039	0.0091	0.0065	0.0052	0.0026

	Hap 15	Hap 16	Hap 17	Hap 18	Hap 19	Hap 20	Hap 21	Hap 22	Hap 23	Hap 24	Hap 25	Hap 26	Hap 27	Hap 28
Hap 01	2	3	4	2	3	11	2	2	2	7	2	3	3	3
Hap 02	3	4	5	3	4	12	3	3	3	8	3	4	4	4
Hap 03	3	4	5	3	4	12	3	3	3	8	3	4	4	4
Hap 04	5	6	7	5	6	12	5	5	5	9	5	6	6	6
Hap 05	2	3	4	2	3	11	2	2	2	8	2	3	3	3
Hap 06	3	4	5	3	4	12	3	3	3	8	3	4	4	4
Hap 07	3	4	5	3	4	12	3	3	3	8	3	4	4	4
Hap 08	3	4	5	3	4	12	3	3	3	8	3	4	4	4
Hap 09	2	3	4	2	3	11	2	2	2	7	2	3	3	3
Hap 10	2	3	4	2	3	11	2	2	2	7	2	3	3	3
Hap 11	6	7	8	6	7	12	6	6	6	9	6	7	7	7
Hap 12	3	3	3	3	3	12	3	3	3	9	3	3	3	3
Hap 13	2	3	4	2	3	11	2	2	2	8	2	3	3	3
Hap 14	2	3	4	2	3	11	2	2	2	7	2	3	3	3
Hap 15	–	3	4	2	3	11	2	2	2	8	2	3	3	3
Hap 16	0.0039	–	5	3	4	12	3	3	3	8	3	4	4	4
Hap 17	0.0052	0.0065	–	4	5	12	4	4	4	9	4	5	5	5
Hap 18	0.0026	0.0039	0.0052	–	3	11	2	2	2	8	2	3	3	3
Hap 19	0.0039	0.0052	0.0065	0.0039	–	12	3	3	3	8	3	4	4	4
Hap 20	0.0117	0.0130	0.0130	0.0117	0.0130	–	11	11	11	12	11	12	12	12
Hap 21	0.0026	0.0039	0.0052	0.0026	0.0039	0.0117	–	2	2	8	2	3	3	3
Hap 22	0.0026	0.0039	0.0052	0.0026	0.0039	0.0117	0.0026	–	2	8	2	3	3	3
Hap 23	0.0026	0.0039	0.0052	0.0026	0.0039	0.0117	0.0026	0.0026	–	8	2	3	3	3
Hap 24	0.0078	0.0091	0.0117	0.0091	0.0104	0.0130	0.0078	0.0078	0.0078	–	8	9	9	9
Hap 25	0.0026	0.0039	0.0052	0.0026	0.0039	0.0117	0.0026	0.0026	0.0026	0.0078	–	3	3	3
Hap 26	0.0039	0.0052	0.0065	0.0039	0.0052	0.0130	0.0039	0.0039	0.0039	0.0091	0.0039	–	4	4
Hap 27	0.0039	0.0052	0.0065	0.0039	0.0052	0.0130	0.0039	0.0039	0.0039	0.0091	0.0039	0.0052	–	4
Hap 28	0.0039	0.0052	0.0065	0.0039	0.0052	0.0130	0.0039	0.0039	0.0039	0.0091	0.0039	0.0052	0.0052	–

Table 2 to be continued

	Hap15	Hap16	Hap 17	Hap 18	Hap 19	Hap 20	Hap 21	Hap 22	Hap 23	Hap 24	Hap 25	Hap 26	Hap 27	Hap 28
Hap 01	2	3	4	2	3	9	2	2	2	6	2	3	3	3
Hap 02	3	4	5	3	4	10	3	3	3	7	3	4	4	4
Hap 03	3	4	5	3	4	10	3	3	3	9	3	4	4	4
Hap 04	5	6	7	5	6	10	5	5	5	8	5	6	6	6
Hap 05	2	3	4	2	3	9	2	2	2	8	2	3	3	3
Hap 06	1	4	5	3	4	10	3	3	3	9	3	4	2	4
Hap 07	3	4	5	3	4	10	3	3	3	9	3	4	4	4
Hap 08	3	4	5	3	4	10	3	3	3	7	3	4	4	4
Hap 09	2	1	2	2	1	9	2	2	2	8	2	3	3	3
Hap 10	2	3	4	2	3	9	2	2	2	8	2	3	3	3
Hap 11	6	7	8	6	7	9	6	6	6	9	6	7	7	7
Hap 12	2	5	6	4	5	**11**	4	4	4	10	4	5	3	5
Hap 13	3	4	5	3	4	10	3	3	3	7	3	4	4	4
Hap 14	1	2	3	1	2	8	1	1	1	7	1	2	2	2
Hap 15	–	3	4	2	3	9	2	2	2	8	2	3	1	3
Hap 16	0.0039	–	3	3	2	10	3	3	3	9	3	4	4	4
Hap 17	0.0052	0.0039	–	4	3	**11**	4	4	4	10	4	5	5	3
Hap 18	0.0026	0.0039	0.0052	–	3	9	2	2	2	6	2	3	3	3
Hap 19	0.0039	0.0026	0.0039	0.0039	–	8	3	3	3	9	3	4	4	2
Hap 20	0.0117	0.0130	**0.0143**	0.0117	0.0104	–	9	9	9	10	7	10	10	8
Hap 21	0.0026	0.0039	0.0052	0.0026	0.0039	0.0117	–	2	2	8	2	3	3	3
Hap 22	0.0026	0.0039	0.0052	0.0026	0.0039	0.0117	0.0026	–	2	8	2	3	3	3
Hap 23	0.0026	0.0039	0.0052	0.0026	0.0039	0.0117	0.0026	0.0026	–	8	2	3	3	3
Hap 24	0.0104	0.0117	0.0130	0.0078	0.0117	0.0130	0.0104	0.0104	0.0104	–	6	9	9	9
Hap 25	0.0026	0.0039	0.0052	0.0026	0.0039	0.0091	0.0026	0.0026	0.0026	0.0078	–	3	3	3
Hap 26	0.0039	0.0052	0.0065	0.0039	0.0052	0.0130	0.0039	0.0039	0.0039	0.0117	0.0039	–	2	4
Hap 27	0.0013	0.0052	0.0065	0.0039	0.0052	0.0130	0.0039	0.0039	0.0039	0.0117	0.0039	0.0026	–	4
Hap 28	0.0039	0.0052	0.0039	0.0039	0.0026	0.0104	0.0039	0.0039	0.0039	0.0117	0.0039	0.0052	0.0052	–

Table 3. Hierarchical analysis of molecular variance (AMOVA)

Variance component	Estimate	Variance components	Percentage of variation	*F*-statistics
Among populations	σ_a^2	–0.0217	–1.23	F_{CT} = –0.0123
Among groups (IC and SP vs BU)*	σ_b^2	0.2748	15.57	F_{SC} = 0.1538
Within populations	σ_c^2	1.5120	85.66	F_{ST} = 0.1434

*Istrian (IC) and Slavonian Syrmian Podolian (SP) cattle represent one group, while Busha (BU) cattle represent the second group

level, haplogroup T2 is found only in IC (6.0%) and haplogroup T5 in SP cattle (36.17%), which represents the Podolian group.

The haplotype sequence data from this study were compared with 121 of published sequences covering 780-bp fragment of the mtDNA D-loop region (15 920-16 338; 1-361) available in the GenBank database. In order to determine inter- and intra-breed relationships within the three native breeds and the breeds that are phylogenetically more similar to the native ones, we selected sequences mainly from the Podolian and brachiceros type of cattle (Figure 3). In the analyzed sequences, 124 polymorphic sites were found.

DISCUSSION

The numbers of identified haplotypes indicate a high genetic variability of the maternal component of BU and IC, and a relatively low level of variability in SP cattle. In the BU population, fourteen mtDNA haplotypes were observed, indicating a high-level genetic diversity of the preserved maternal components. We assume that this is the result of a larger number of females included in several nucleus herds for the purpose of a conservation programme spread over a wide geographic area. The large genetic diversity preserved in the BU population was also confirmed by Medugorac et al. (2011) and Ramljak et al. (2011), by microsatellite analysis. Medugorac et al. (2011) observed several "metapopulations" of shorthorn cattle (*B. brachiceros*) related to Croatian Busha within the Balkan migration routes. Such related populations are valuable for conservation programs, especially in order to maintain a minimum level of inbreeding, i.e. a maximum level of neutral genetic diversity. The observed low level of conserved genetic variability of the maternal components in SP (five haplotypes) is the result of the government policy and the economic situation (the emphasis is on commercial breeds, whose production levels could not be matched by the native breeds) during the second half of the 20th century, resulting in a genetic bottleneck. SP cattle were traditionally used as draft cattle and, after the intensification of agricultural production, they lost their primary function but were insufficiently competitive as a dairy or beef breed. Although Croatia made a compilation of autochthonous cattle breeds after the War of Independence in the 1990s, only a single remaining herd with twenty breeding animals was found which then served as the basis for revitalization of the breed. Therefore, we assume that a significant portion of genetic diversity of maternal components of SP cattle had been lost before the start of the systematic conservation program. With the aim of preserving the breed and avoiding higher levels of inbreeding, a limited amount of semen of Hungarian and Serbian Podolian bulls was used, but the maternal component was not imported. In the population of IC, a considerable level of the maternal component variability has been preserved, as confirmed by the 13 observed mtDNA haplotypes. A high amount of conserved genetic variation in the population of IC was detected by Ramljak et al. (2011) and Maretto et al. (2012) using microsatellites, compared to a lower level in the BU population. The above claim is explained by IC having a more restricted breeding area, as well as by the centuries-old informal principles of breeding in Istria which favoured the local breed, very well adapted to the climate. The breeders of IC and other domestic animals in Istria persistently resisted to the introduction of allochthonous breeds because the descendants had much poorer traits than the native breeds in terms of resistance and working ability. Therefore, there are very few traces of the use of non-native bulls (Podolian or brachyceros type) in Istria, and occasional introductions were limited and conducted with a great care. This is supported by Maretto et al. (2012), who observed a closer positioning of IC with respect to the geographically more distant southern Italian breeds (Maremmana and Podolica), than to the geographically closer beef type breeds (Chianina,

Marchigiana, and Romagnola), suggesting a well-managed and tended ancient trade in animals across the Adriatic Sea. In the three autochthonous cattle populations, although the dominant haplogroup was T3 (84.25%), some of the observed haplotypes belonged to rarer haplogroups, T5 (11.64%) and T2 (2.05%). In the BU population, the dominant and the most abundant haplotypes belong to the haplogroup T3, which is dominant in the European cattle breeds. Although BU showed the greatest genetic diversity, the highest number of haplotypes (four) was found in the IC. Bonfiglio et al. (2010) reported the presence of haplogroups T1, T2, and Q in the Italian cattle breeds, presuming that these originated from Africa or the Middle East. The gene flow of maternal lineages between Italian breeds and Istrian cattle, and the geographical proximity of Istria and Italy, contributed to the appearance of haplotype T2 in the population of IC (6%). Haplogroup T5 is present only in the SP population (36.17%). A similar haplogroup was observed in the Piedmontese, Valdostana, and Iraqui breeds, suggesting some influence of the Mediterranean migration routes on the shaping of the haplotype of CP cattle (Figure 3). A higher proportion of T5 haplotypes in SP cattle can be partly explained by the small size of the initial nucleus herd (only 20 individuals) and the effect of the bottleneck in the population during the last two decades (Ramljak et al. 2011). Also, there is a high chance that, within herds, female individuals were related (mother–daughter) since the herd was rescued during the war and no pedigree data exist.

An analysis of the inter- and intra-genetic diversity of mtDNA indicates significant differences between the components of maternal breeds (F_{ST} = 0.1434), which justifies their differential conservation and breeding approach.

The results of the diversity of maternal components show that in order to preserve the uniqueness of the genetic components of BU, IC, and SP cattle, exchange of individuals (crossbreeding) between these breeds should be avoided. As IC and SP cattle are very similar in terms of the exterior (both belong to the Podolian cattle group), justifiability of their crossbreeding was examined, but the idea was abandoned since the analyses showed that genetically they were two different populations. The results of the study of maternal components in the three autochthonous breeds helped clarifying the process of their formation, and provided an insight into the current status of the maternal genetic structure of the breeds. These results will be helpful for the further process of conservation and protection of the remaining genetic diversity and economic reaffirmation, which is crucial for the sustainability of endangered autochthonous breeds.

CONCLUSION

Our results show that the three Croatian autochthonous cattle breeds studied, especially IC and BU cattle, have preserved high levels of mtDNA diversity. The structure of haplotypes and haplogroups supports previous research results about the presence and spreading of maternal components in the European cattle breeds and migration routes. The conclusions based on the examination of mtDNA diversity support the opinion that the three indigenous cattle breeds under this survey are genetically separate entities, which in terms of conservation should be managed independently. The implemented breeding strategy should emphasize the need for maximum preservation of the inter- and intra-population genetic diversity of maternal (and paternal) components. In addition to the previously conducted research of exterior and production traits, the presented observations on the structure of mtDNA haplotypes are very useful for the preservation and management of genetic diversity of autochthonous cattle breeds.

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