

## GENETIC CHARACTERIZATION OF AUTOCHTHONOUS CATTLE BREEDS, CIKA AND BUSHA, USING MICROSATELLITES

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### ABSTRACT

Slovenian autochthonous Cika and Croatian autochthonous Busha cattle were characterized by using nine microsatellites. For each locus, allelic frequencies and heterozygosity were computed. The number of alleles identified for studied polymorphic loci varied from six to ten in Cika population and from four to eight in Busha population. The mean number of alleles per locus was eight (Cika) and five (Busha), respectively. The average expected heterozygosity ( $H_{exp}$ ) for the nine loci ranged between 0.7733 (Cika) and 0.7199 (Busha). The highest  $H_{exp}$  was computed for locus *BM2113* (0.8521) in Cika and 0.8099 in Busha, suggesting that this locus could be the most informative for both populations. Observed heterozygosity ranged from 0.6316 to 0.825 in Cika and from 0.6667 to 1.0 in Busha indicating a high level of polymorphism for this set of markers. The average observed heterozygosity was slightly lower in Cika (0.7403) compared to Busha population (0.7775). Both breeds were in genetic equilibrium according to the Hardy-Weinberg test. Nei's standard genetic distance  $D$  (Nei, 1978) was 0.065. The genetic difference between the two breeds could be in accordance with their brachycerous origin. The used set of markers proved informative and robust for genotyping in both Cika and Busha breeds. This set of markers can now be applied for screening higher numbers of animals to analyze possible sub-population structures, improve our knowledge of origin and phylogenetic relationships to other breeds and help to construct efficient preservation plans for these two endangered breeds.

Key words: Cika / Busha / cattle / genetic relationship / microsatellite

## GENETSKA KARAKTERIZACIJA AVTOHTONIH PASEM GOVEDI CIKE IN BUŠE Z UPORABO MIKROSATELITOV

### IZVLEČEK

Karakterizirali smo slovensko avtohtono pasmo ciko in hrvaško avtohtono pasmo bušo z uporabo devetih mikrosatelitov. Za vsak lokus smo izračunali frekvence alelov in heterozigotnost. Določili smo šest do deset alelov na polimorfni lokusih v populaciji cike in štiri do osem v populaciji buše. Povprečno število alel na lokus je bilo osem (cika) oziroma pet (buša). Povprečna pričakovana heterozigotnost za devet lokusov je bila med 0,7733 (cika) in 0,7199 (buša). Najvišjo pričakovano heterozigotnost smo izračunali za lokus *BM2113*, in sicer 0,8521 pri ciki in 0,8099 pri buši. Ta lokus bi tako lahko bil najbolj informativen pri analiziranju obeh populacij. Povprečna opazovana heterozigotnost je bila med 0,6316 in 0,825 pri ciki oziroma med 0,6667 in 1,0 pri buši, kar potrjuje visoko informativnost izbranega niza genetskih označevalcev. Pri obeh pasmah smo opazili visoko stopnjo polimorfnosti na vseh devetih lokusih. Povprečna opažena heterozigotnost je bila manjša (0,7403) pri ciki kot pri buši (0,7775). Obe pasmi sta bili v genetskem ravnotežju v skladu s Hardy-Weinbergovim testom. Standardna genetska distanca po Neiju (Nei, 1978) je znašala 0,065, kar je v skladu z njunim brahicerim izvorom. Izbrani niz označevalcev je torej informativen in robusten za genotipiziranje obeh pasem. Ta niz bi v prihodnje lahko uporabili za podrobnejše analize

genetskih odnosov do drugih pasem, iskanje možnih pod-populacij znotraj teh pasem ter za razvoj učinkovitega načrta ohranjanja obeh ogroženih pasem.

Ključne besede: cika / buša / govedo / genetsko sorodstvo / mikrosateliti

## INTRODUCTION

Local breeds are important to preserve as they are well adapted to local climate, food supply and other local environmental factors, which often shows in their superior robustness. The use of new breeding techniques and technologies, introduction of highly productive breeds, demographic pressure and support of industrial agriculture contributed to the loss of valuable traits or decrease in numbers of animals of local breeds. Animals that co-evolved over hundreds of years with their local environment and rearing system are now being replaced with more productive breeds. Selection for more productive breeds in controlled conditions has often resulted in elimination of animals from their natural environment.

The FAO has proposed an integrated program for the global management of genetic resources using microsatellite methodology for breed characterization. For the analysis of genetic diversity of European cattle breeds, a list of 30 microsatellite markers have been proposed. Molecular and genetic analyses of various cattle populations could help to identify breeds with a common genetic origin as well as define their differences, which is information necessary for efficient management of genetic diversity conservation.

There has been a very few molecular genetic studies on Cika and Busha populations with the first and seminal study published by Medjugorac *et al.* (1994). Both breeds have brachycerous origin and belong to the shorthorn group of cattle. The aim of this study was to check nine genetic markers (microsatellites) for their informativeness and perform a preliminary characterization of genetic structure and relationship of the two endangered populations of Cika and Busha cattle.

### Cika cattle

Cika cattle or Cika is the only Slovenian autochthonous cattle breed, which has been preserved to the present day. In the second half of the 19<sup>th</sup> century Cika cattle arose from the crossbreeding of light-red, red or brown-red coloured Bohinj cattle with Mœlthaler and Pinzgauer cattle. The breed proved its superiority in that particular local environment exhibiting a relatively high milk yield and excellent adaptability to rearing in mountainous regions. In the 1950s there were still approximately 80 000 Cika cattle, but their numbers decreased rapidly with crossbreeding and replacing with other breeds (Čepon *et al.*, 1999).

Nowadays (data from 1.6.2007), the population of Cika cattle numbered 1 625 animals (Sector for Identification and Registration at the Ministry of Agriculture, Forests and Food). In accordance to the Regulation on conservation of farm animal genetic resources (Pravilnik o ohranjanju..., 2004), based on the number of purebred females (927), Cika cattle is classified in the class "at risk". Unfortunately, the population of Cika is very heterogeneous, because of animals with a high genetic contribution of Pinzgauer breed from historic mating and artificial insemination programs. It is estimated from morphological assessments, that there are only around 185 Cika females exhibiting the autochthonous, original Cika breed phenotype favoured by the current breeding programme. From this point of view, the Cika population in this particular phenotype is therefore in the "endangered" class. So, special care is still needed for the preservation of Cika cattle in the original phenotype.

Cika cattle are perfectly adapted to grazing in difficult mountain environment, such as rough climatic conditions, meagre food resources and long distances to summer pastures over 1000 m and often above 2 000 m above sea level. The breeding goal for Cika cattle is dual purpose with

emphasis on milk production. However, Cika cattle are mostly reared in the cow-calf system for beef production. To lesser extent Cika cattle is still used for milk production in traditional regions for Alpine dairy-farming, especially on Bohinj and Kamnik mountain pastures. Some breeders have preserved the traditional way of rearing this cattle breed. During the vegetation period, herds are moved from lowland farms to mountain pastures where they process the milk into cheese and other milk products.

The head of Cika is small and their eyes are wide set. The coat colour of the animals is red to reddish with a typical white stripe on top of back and white stripe spread from breast to the belly. The border between the red and white colour could be toothed. The horns are short, dark and turned forward. The mucous membrane is rose in colour, and the hoofs are dark.

Cika cattle are widespread in their traditional areas in Slovenia; Bohinj, Kamnik, Kobarid, Solčava and their surroundings. The preservation program for Cika cattle was established in 1991 by the Ministry of agriculture, forestry and food. Since 2001 breeders are included in the Association for the preservation of Cika cattle in Slovenia. Since 2005 Cika cattle have been accepted into a breeding program.

### **Busha cattle**

The Busha is a Croatian autochthonous and protected cattle breed which was, and still is bred in extensive conditions in the mountainous areas of Croatia, that is in the Lika and Dalmatia region. The Busha has a crude constitution and reaches maturity at the age of two years (Buša, 2008). Fertility is good, and the average birth weight of calves is around 15 kg. The coat colour varies in different shades of brown or grey with a characteristic lighter stripe along the back. Lighter hair around their darkly pigmented snout (called also a venison snout), darkly coloured horns and hooves are characteristics of the breed. The chest is narrow, but broad and deep, while the back is narrow, short and straight. The average cow's height in withers is around 100 to 115 cm, while weight is around 180–250 kg. Bull's weigh is around 300 kg.

During lactation, which lasts around 8 months, Busha produce about 800 l of milk with 4 to 6% of milk fat. Their muscles are not well developed, dressing percentage is around 55%, and so good beef production potentials cannot be expected. Busha shows high disease resistance and adaptation to deficient nutritional and other environmental conditions.

According to the Annual report of the Croatian livestock centre for the year 2007, 31 Busha breeders rearing 125 cows under the reproduction control and 55 female offspring were registered in Croatia (Hrvatski stočarski centar, 2008). In respect to the number of cows ( $n = 125$ ) and bulls ( $n = 16$ ), effective population size ( $N_e$ ) was 50.74, which puts this breed, into "in danger" class (Posavi, 2002). For the purpose of breed preservation from extinction, an Organization of Busha's breeders in the city of Gospić was founded in year 2005. Breeding history, exterior and productive characteristics as well as the current state of population size were reported by Posavi *et al.* (2002). Busha's phenotypic and genetic characteristics were researched by Konjačić *et al.* (2004) as well as by Ivanković *et al.* (2004). The genetic characterization of Busha based on polymorphisms of blood proteins indicate that Busha is phylogenetically most distant from Slavonian-syrmian podolian cattle, and that it is closer to the Istrian and Grey Dalmatian cattle breeds (Ivanković *et al.*, 2004).

## MATERIAL AND METHODS

### Animals

A total of 40 Cika cattle and 12 Busha cattle have been analyzed. Blood samples have been taken from the tail vein (*Vena coccigica*) in Winter and Spring months of 2008. Animals were unrelated according to the known pedigree data although pedigrees from some animals were unavailable. The samples of Cika cattle included in this study represented animals in the original autochthonous phenotype as detailed in the Cika breeding programme.

### Microsatellites markers

A total of nine microsatellites were studied (*ETH225*, *BM2113*, *INRA23*, *TGLA227*, *TGLA126*, *TGLA122*, *BM1824*, *SPS115*, *ETH10*). These markers were selected from a set of microsatellites recommended by the Food and Agriculture Organisation program MoDAD for global management of genetic diversity and conservation of resources ([http://lprdad.fao.org/cgi-bin/getblob.cgi?sid=2ac355fa086bac6e215ced38c3f518a3\\_50005883](http://lprdad.fao.org/cgi-bin/getblob.cgi?sid=2ac355fa086bac6e215ced38c3f518a3_50005883)). DNA was extracted from blood cells using commercial kit QIA DNA Blood kit (Qiagen, Hilden Germany). PCR amplifications were performed in multiplex reactions using 20 µl reaction volumes with 2.0 µl buffer (10x*Taq* buffer with KCl; Fermentas), 0.8 µl dNTP mix, 0.6 µl of each primer, 1.2 µl MgCl<sub>2</sub> (25 mM), 13.7 µl H<sub>2</sub>O and 0.1 µl *Taq* polymerase. To this mixture, 2.0 µl DNA sample was added and 1 droplet of mineral oil. PCR program conditions were as follows: 94 °C denaturation for 3 min, followed by 35 cycles of 94 °C for 30 sec, 60 °C annealing for 20 sec, 72 °C extension for 1 min. For markers *ETH225*, *BM2113*, *INRA23*, annealing temperature was at 55 °C.

### Statistical analyses

Microsatellite allele frequencies, observed ( $H_{obs}$ ), and expected ( $H_{exp}$ ) heterozygosity were calculated using the GENETIX (Belkhir *et al.*, 1996–98) software package. Also, test of deviation from the Hardy-Weinberg equilibrium and Nei's standard genetic distance with correction for sample size (Nei, 1978) between breeds was calculated from allele frequencies using the GENETIX program.

## RESULTS AND DISCUSSION

The number of alleles identified in the polymorphic loci varied from six to ten in Cika population and four to eight in Busha population. A possible explanation for the observed lower number of alleles in Busha could be a smaller sample size of Busha compared to Cika. The mean number of alleles per locus (Table 1) was eight in Cika and five in Busha. The smallest number of alleles and low heterozygosity scores were observed for locus *TGLA122* in Busha. On the other hand, relatively high heterozygosity, and the largest number of alleles was observed for the same marker in Cika (Table 1). At each locus there were one and up to six alleles specific for one population only (Fig. 1). Although most of them are present at relatively low frequencies, these alleles could be potentially considered as so-called private alleles for Cika and Busha population. The highest numbers of alleles (10) were found for loci *INRA23* and *TGLA122* in Cika population, while the lowest (4) for loci *SPS115* and *TGLA122* in Busha population. The locus *TGLA122* was most different in respect to the number of alleles between populations (Fig. 1) and could therefore poses most power to differentiate the two breeds.

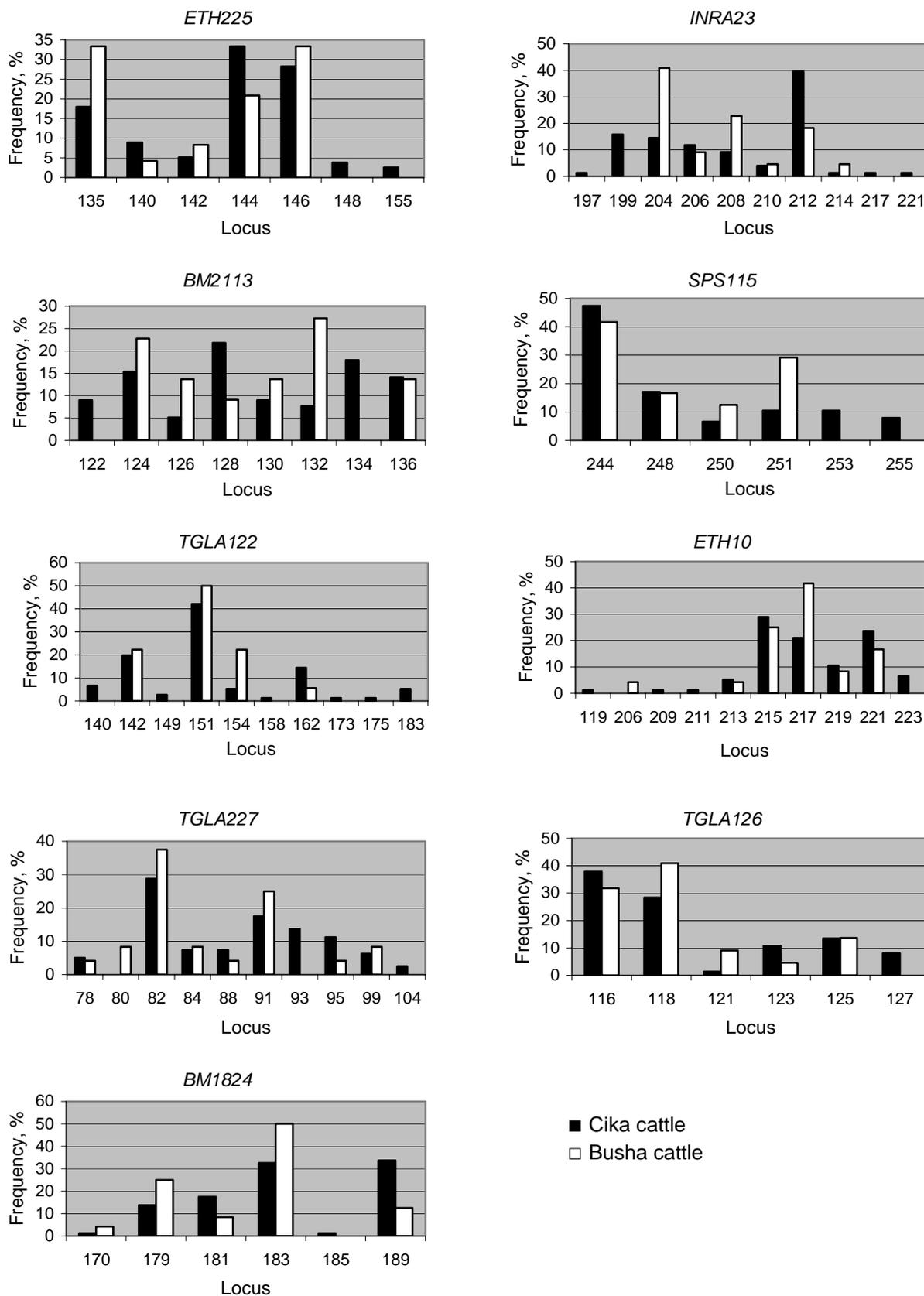


Figure 1. Distribution of alleles for Cika and Busha cattle.

Statistical analysis of microsatellite data was performed for each individual population. The observed ( $H_{obs}$ ) and expected ( $H_{exp}$ ) heterozygosity are shown in Table 1. The average expected heterozygosity for the nine loci ranged between 0.7733 (Cika) and 0.7199 (Busha). The highest  $H_{exp}$  was calculated for locus *BM2113* (0.8521) in Cika population while the lowest  $H_{exp}$  had locus *TGLA122* (0.6481) in Busha population. Locus *BM2113* exhibited the highest level of heterozygosity and was the most informative locus for both populations. Most loci were found to be highly polymorphic and informative with expected heterozygosity over 70%; below this level were only three markers (*SPS115*, *TGLA122*, *BM1824*) in Busha population only. For all loci in both breeds, no significant deviation from the Hardy-Weinberg equilibrium was detected. Calculated genetic distance between Cika and Busha (Nei, 1978) was 0.065.

The average observed heterozygosity was lower (0.7403) in Cika compared to (0.7775) in Busha population. The highest  $H_{obs}$  had loci *ETH225* and *TGLA126* (1.000) in Busha population. Concerning the two breeds, they were in genetic equilibrium according to Hardy-Weinberg test. Allele frequencies were used to estimate genetic distance between Cika and Busha. Nei's standard genetic distance  $D$  (Nei, 1978) was 0.065. The genetic difference between those two breeds could be in accordance with their brachycerous origin.

Table 1. Parameters of genetic information content of nine microsatellite loci

Locus	No. of alleles		$H_{exp}$		$H_{obs}$	
	Cika	Busha	Cika	Busha	Cika	Busha
<i>ETH225</i>	7	5	0.7643	0.7257	0.6667	1.0000
<i>INRA23</i>	10	6	0.7735	0.7355	0.7105	0.8182
<i>BM2113</i>	8	6	0.8521	0.8099	0.8205	0.8182
<i>SPS115</i>	6	4	0.7136	0.6979	0.8158	0.7500
<i>TGLA122</i>	10	4	0.7517	0.6481	0.6316	0.1111
<i>ETH10</i>	9	6	0.7971	0.7257	0.7105	0.9167
<i>TGLA227</i>	9	8	0.8369	0.7708	0.8250	0.9167
<i>TGLA126</i>	6	5	0.7396	0.7025	0.7568	1.0000
<i>BM1824</i>	6	5	0.7306	0.6632	0.7250	0.6667
Average	8	5	0.7733	0.7199	0.7403	0.7775

## CONCLUSIONS

One of the important steps in development of efficient autochthonous breed protection programs is characterization of population genetic variability and to assess the genetic structure and relatedness to other breeds. We used microsatellite markers to analyze genetic variation within Cika and Busha breeds and compared the frequency distributions within each locus and breed. The chosen set of nine microsatellites proved informative since most of the markers showed high levels of expected heterozygosity with the average ranging from 0.7199 to 0.7733. The Nei's genetic distance (0.065) between the Cika and Busha population showed relatedness between those two populations. The used set of markers can be applied in more detailed studies in the future by analyzing more breeds, larger numbers of animals per breed. This should allow detection of possible sub-population structures, improve our knowledge of origin and phylogenetic relationships to other breeds and provide a basis for preservation of the breed in the original phenotype favoured by the current selection schemes and breeding programmes.

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