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## Analysis of genetic and cultural conservation value of three indigenous Croatian cattle breeds in a local and global context

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### Keywords

Cattle; conservation; genetic diversity; genetic structure; microsatellites; sustainable breeding.

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### Summary

It is widely accepted that autochthonous cattle breeds can be important genetic resources for unforeseeable environmental conditions in the future. Apart from that, they often represent local culture and tradition and thus assist in the awareness of ethnic identity of a country. In Croatia, there are only three indigenous cattle breeds, Croatian Buša, Slavonian Syrmian Podolian and Istrian Cattle. All of them are threatened but specialized in a particular habitat and production system. We analysed 93 microsatellites in 51 animals of each breed to get thorough information about genetic diversity and population structure. We further set them within an existing frame of additional 16 breeds that have been genotyped for the same marker set and cover a geographical area from the domestication centre near Anatolia, through the Balkan and alpine regions, to the north-west of Europe. The cultural value was evaluated regarding the role in landscape, gastronomy, folklore and handicraft. The overall results recognize Croatian Buša being partly admixed but harbouring an enormous genetic diversity comparable with other traditional unselected Buša breeds in the Anatolian and Balkan areas. The Podolian cattle showed the lowest genetic diversity at the highest genetic distance to all remaining breeds but are playing an important role as part of the cultural landscape and thus contribute to the tourist industry. The genetic diversity of the Istrian cattle was found in the middle range of this study. It is already included in the tourist industry as a local food speciality. Current and future conservation strategies are discussed.

### Introduction

Indigenous cattle breeds are considered as precious genetic resources that tend to disappear because of the new economic and agricultural demands (e.g. Taberlet *et al.* 2008). In lack of the wild ancestor, local breeds, which are not subject to strict artificial selection, often conserve a considerable amount of genetic diversity in comparison with highly selected commercial breeds (Medugorac *et al.* 2009). The evaluation of genetic variability, its distribution among the population and the possible detection of

rare alleles, as an indicator of populations with unique genetic variants, are nowadays a prerequisite for breed conservation because resources for conservation are limited (Reist-Marti *et al.* 2006). Apart from that, local indigenous breeds represent not only possible genetic resources but also cultural and traditional values of the respective country and contribute to ethnic identity (Gandini & Villa 2003).

In the early twenties of the 19th century, almost all cattle production in Croatia was based on three local cattle breeds, Croatian Buša, Slavonian Syrmian

Podolian Cattle and Istrian Cattle. With the rapid development of the economy and the gradual increase in people's living standards, the production performance of the native breeds did not fully meet the current market demand anymore. As a consequence and comparable to other countries, alien highly selected and more productive breeds have been introduced to upgrade native breeds or to simply replace them. As a result, the population sizes of indigenous breeds have dramatically declined, and all of the three native Croatian breeds are now classified as endangered and included in a National Cattle Breeding Program launched in 2007.

This study addresses the circumstance described earlier by firstly characterizing the genetic diversity and structure between and within the three autochthonous Croatian cattle breeds. Second, the relationships with southern neighbours represented by five Buša breeds of Bosnia and Herzegovina, Macedonia, Kosovo and Albania as well as with northern neighbours represented by seven alpine breeds and further to breeds from the north-west of Europe and from Anatolia close to the domestication centre of cattle are examined. All these breeds except the Croatian breeds have been analysed under the same conditions, and the results have been published in Medugorac *et al.* (2009). Third, the information of within-breed diversity of each Croatian autochthonous cattle breed will be discussed within the framework of further breeding programmes and strategies including the aspects of the cultural value of each breed. Forth, we will discuss our material as a representative example for conservation inferences in analogue countries.

## Material and methods

### Breeds

The three autochthonous cattle breeds that have been analysed in this study are Croatian Buša (HRB), Istrian Cattle (HRI) and Slavonian Syrmian Podolian Cattle (HRP). There is no additional native cattle breed in Croatia. Of each breed, 51 individuals were sampled during 2006. Characteristic for all three breeds is a very low nominal population size (Table 1). Therefore, the sample of 51 preferably unrelated animals represents the total population very well. Table 1 lists the abbreviations, breeding purpose, sample size, sex ratio within sample, nominal and effective population size ( $N_{e_{CAA}}$ ) as revealed by the Croatian Agricultural Agency (CAA).  $N_{e_{CAA}}$  is calculated as  $N_{e_{CAA}} = 4N_mN_f/(N_m + N_f)$  where  $N_m$

**Table 1** Breed name and code, main breeding objective (Purpose), and sample size (N), sex ratio female/ male (F/M), nominal population size (NN), effective population size estimated from numbers of breeding males and females in the population ( $N_{e_{CAA}}$ )

| Breed             | Code | Purpose           | N  | F/M   | NN  | $N_{e_{CAA}}$ |
|-------------------|------|-------------------|----|-------|-----|---------------|
| Croatian Buša     | HRB  | Dairy-beef-(work) | 51 | 40/11 | 269 | 59            |
| Slavonian Syrmian |      |                   |    |       |     |               |
| Podolian Cattle   | HRP  | Work-beef         | 51 | 46/5  | 171 | 33            |
| Istrian Cattle    | HRI  | Work-beef-(dairy) | 51 | 45/5  | 789 | 80            |

The sporadic and/or secondary purpose of the breed is in parentheses.

and  $N_f$  are the numbers of males and females in the population (Falconer & Mackay 1996).

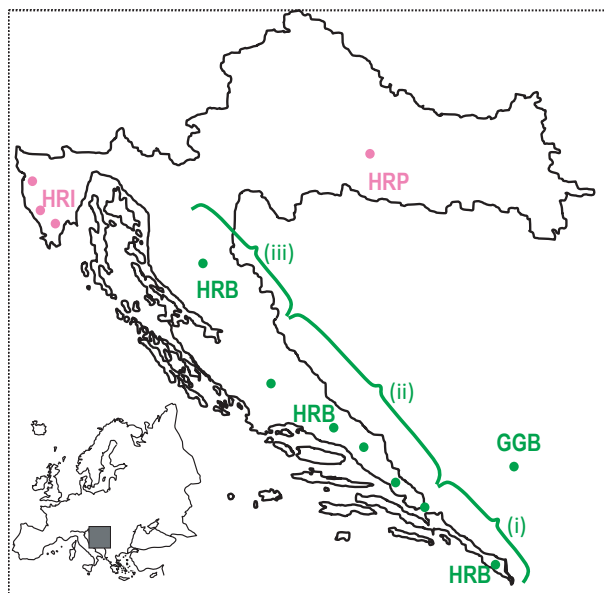
### Croatian Buša

Buša (*Busha* or *Buscha*) has been and currently is bred over the large mountainous and rocky areas of Lika and Dalmatia, which contributed to a well adaptation to difficult environmental conditions. It can be aligned to other Buša types previously described in Medugorac *et al.* (2009). It has a small body frame, the withers' height from 90 to 112 cm. A detailed description of HRB is given in Supplementary Figure S1. Comparable to Buša breeds or types in the neighbour countries, HRB was used for milk (production is around 700–1300 litres with 4–6% milk fat), beef and only sporadically for work. It is a long-living breed.

In 2008, the total population size was approximately 300 living animals settled in the area of Lika and Dalmatia (Figure 1). HRB was classified endangered with  $N_{e_{CAA}} = 59$  (CAA 2009), and a protection programme was launched by the government in 2003. During 2005, an Association of Croatian Breeders of Buša has been established.

### Slavonian Syrmian Podolian cattle

HRP is representative of a formerly large Podolian cattle population of the Pannonian Plain and now found in Slavonia, the Croatian part of the Pannonian Plain. During the past, the Podolian cattle not only spread throughout a large part of Pannonia but was also found adjacent to the Mediterranean Sea in southern Europe and northern Africa. The animals are of moderate-sized frame and were primarily used for agricultural work and beef. They were sold as trek ox into mountainous and rocky areas usually populated by Buša because of their superior working ability. A detailed description of the HRP breed and



**Figure 1** Origin of sampled cattle breeds. Breed abbreviations are in Table 1. The three areas where HRB was sampled were (i) south of the river Neretva (i.e. south Dalmatia), (ii) central Dalmatia and (iii) Lika.

breed history with the known genetic bottleneck is given in Supplementary Figure S2. The current population consists of nine bulls, 107 cows and 55 female calves and heifers (CAA 2009). Because it was found on the edge of survival, in 1997 a programme of systematic protection of the remaining population was conducted. HRP is still classified as critically endangered with  $N_{e_{CAA}} = 33$ .

### Istrian cattle

HRI is a Mediterranean member of the Podolian cattle group mentioned earlier. HRI is bred on the Istrian rocky peninsula and the island of Krk. The long-living HRI animals of moderate frame size (withers' height from 135 to 150 cm) were used for work, beef and only sporadically for milk production (Ivanković *et al.* 2006). A detailed description of the HRI is given in Supporting information Figure S3. Care about HRI was taken by the Association of Istrian Cattle founded in 1990. HRI is classified as potentially endangered ( $N_{e_{CAA}} = 80$ ).

### Microsatellite analyses

A total of 93 microsatellites was used, 30 of them are recommended by the International Society for Animal Genetics and the Food and Agriculture Organization of the United Nations (FAO; <http://dad.fao.org/cgi-bin/getblob.cgi?sid=bc17043966092ec>

31a207ca1efb8e1fe,50005882) for evaluating cattle diversity (listed in Table S1, Supporting information). To validate allele scores, all samples were genotyped twice in two independent courses. DNA extraction, genotyping procedure and exclusion of outlier have been profoundly described in Medugorac *et al.* (2009).

### Genetic variability

To integrate the limited information of the three breeds into a broader genetic context, the analyses of genetic variability have been carried out in comparison with 16 previous analysed breeds listed in Table 2 (Medugorac *et al.* 2009). Estimates of genetic variability, observed ( $H_O$ ) and expected heterozygosity ( $H_E$ ) (Nei 1987), allelic richness ( $AR$ ) and  $F$ -statistics ( $F_{IT}$ ,  $F_{IS}$  and  $G_{ST}$ ) (Weir & Cockerham 1984) for each locus including population pairwise  $F_{ST}$  were determined using FSTAT v.2.9.3. (Goudet 2001). Significance values for  $F_{IS}$  and pairwise comparisons of population differentiation with Bonferroni-corrected to account for multiple comparisons were calculated in ARLEQUIN v3.1 (Excoffier *et al.* 2005).

Fisher's exact test was used to determine the deviation from Hardy–Weinberg equilibrium (HWE) using GENEPOP v.4.0 software package (Raymond & Rousset 1995). Unbiased estimates of exact p-values were obtained by the Markov Chain Monte Carlo (MCMC) algorithm (10 000 dememorizations, 1000 batches and 8000 iterations per batch).

The alleles were classified in three levels according to their frequency: *common alleles* (observed in all 19

**Table 2** Breed name and code of 16 European breeds analysed under the same conditions in Medugorac *et al.* (2009) as reference network for the three Croatian breeds

| Breed                  | Code | Origin             |
|------------------------|------|--------------------|
| Anatolian Black        | ABB  | Turkey (Anatolia)  |
| Macedonian Buša        | MBU  | Macedonia          |
| Illyrian Mountain Buša | IMB  | Albania            |
| Illyrian Lowland Buša  | ILB  | Albania            |
| Red Metohian Buša      | RMB  | Kosovo             |
| Gray Gacko Buša        | GGB  | Bosnia-Herzegovina |
| Tyrolean Grauvieh      | TGV  | Austria            |
| Original Braunvieh     | OBV  | Germany            |
| Murnau-Werdenfelser    | MWF  | Germany            |
| Austrian Murbodner     | AMB  | Austria            |
| Franken Gelbvieh       | FGV  | Germany            |
| Fleckvieh              | FV   | Germany            |
| Tarentaise             | TAR  | France             |
| Red Holstein           | RH   | Germany            |
| Blanc-Bleu Belge       | BBB  | Belgium            |
| Galloway               | GLW  | Germany (Scotland) |

subpopulations), *private alleles* (alleles observed in one subpopulation) and *rare alleles* (non-private alleles with a frequency <0.01 over the whole population).

#### Effective population size based on LD ( $N_{eLD}$ )

In this study, the marker densities were highest for the chromosomes 1, 2, 3 and 6. We estimated  $N_{eLD}$  on the basis of the marker-marker LD ( $\chi^2_{df}$ ) by weighted least squares regression, which takes into account the heterogeneity of the LD variances (Zhao *et al.* 2005). This was carried out for all marker pairs on these four chromosomes for the three considered breeds with the same procedure as described in Medugorac *et al.* (2009).  $N_{eLD}$  does not directly represent the true effective population size ( $N_e$ ) but it is well related (Zhao *et al.* 2005). Therefore,  $N_{eLD}$  can be understood as an estimator of haplotype diversity as well as a relative value of  $N_e$ .

#### Clustering analyses based on genetic distances

To infer the relationships between breeds and individuals,  $D_A$ -distances (Nei *et al.* 1983) between breeds and individual distances based on the proportion of shared alleles  $D_{PS} = -\ln(PS)$  (Bowcock *et al.* 1994) were calculated. A neighbour network was constructed and plotted with the program SPLITSTREE4 (Huson & Bryant 2006). To present the  $D_{PS}$ -distance matrix in the two-dimensional (2D) space, we used a heuristic approach as described in Veit-Kensch *et al.* (2007). This analysis was again performed in comparison with 16 previously published breeds to show where the three local breeds stand in relation to other European breeds.

#### Clustering and assignment analyses based on multilocus genotypes

An assignment test was carried out with the program GENECLASS (Piry *et al.* 2004) based on multilocus genotypes and the method first described by Paetkau *et al.* (1995). STRUCTURE 2.2 (Pritchard *et al.* 2000; Falush *et al.* 2003) was implemented to determine the most likely number of clusters ( $K$ ) in the dataset of 19 breeds, independent of breed affiliation. To get more information about the wider European context, ten independent runs of  $K = 1$  to 19 were carried out. To determine the most likely hierarchical structure, we used the log probability of data  $\ln P(D)$  and estimated the delta  $K$  ( $\Delta K$ ) statistics as explained in Evanno *et al.* (2005). The STRUCTURE results were plotted with

Distruct (Rosenberg 2004). For the genetic structure analyses, we sorted the HRB animals along the sampling area from south-east (south of Dubrovnik) to north-west (north of Zadar, i.e. Lika) and built three provisional groups: animals sampled (i) south of the river Neretva (i.e. south Dalmatia), (ii) from central Dalmatia and (iii) from Lika (Figure 1).

#### The conservation strategy

We used two neutral conservation strategies: (i) Neutral diversity presented by the total number of alleles ( $N$ ) in the respective subpopulations and (ii) Neutral diversity with principle of complementarity, presented by the cumulative number of neutral alleles ( $NC$ ) of the combination of the first  $x$  subpopulations (Bonin *et al.* 2007). Both strategies were applied for  $x = 1-19$ .

#### Analysis of cultural value

To assess the cultural value of the three local breeds of Croatia, we followed a methodology outlined in Gandini & Villa (2003). They applied the concept of cultural property to local breeds. The premise is that local cattle breeds represent cultural property of a country because they were and still can be a point of reference in ancient local traditions. Two aspects have been elaborated, the value as historical witness based on seven parameters (antiquity, agricultural systems, role in landscape, role in gastronomy, role in folklore, role in handicrafts, presence in forms of higher artistic expression) and subsequently the value as custodian of local traditions based on four parameters (role in maintaining landscape, gastronomy, folklore and handicrafts) (Gandini & Villa 2003). So far, this method relies on a qualitative basis.

## Results

#### Microsatellite variability

In the Table S1, the total number of alleles ( $n_A$ ) per locus,  $H_E$  and  $H_O$ ,  $PIC$ ,  $G_{ST}$  and  $N_m$  values are presented. The mean number of alleles per locus was 12.38. The average  $H_O$  for all loci was 0.629, while the average  $H_E$  for all loci was 0.718.

#### Neutral genetic diversity among breeds

In Table 3,  $tA$ ,  $pA$ ,  $rA$ ,  $mA$ ,  $AR$ ,  $H_E$ ,  $H_O$  and  $F_{IS}$  and  $N_{eLD}$  are presented in comparison with the other 16 breeds.



**Table 3** Summary statistics of neutral genetic diversity of 19 cattle breeds

| Breed | tA  | pA   | rA    | mA   | AR    | H <sub>E</sub> | H <sub>O</sub> | F <sub>IS</sub> | Ne <sub>LD</sub> |
|-------|-----|------|-------|------|-------|----------------|----------------|-----------------|------------------|
| ABB   | 906 | 36   | 200   | 9.74 | 8.439 | 0.778          | 0.711          | 0.047***        | 543.9            |
| MBU   | 715 | 9    | 87    | 7.69 | 7.417 | 0.744          | 0.694          | -0.039          | 302.6            |
| IMB   | 760 | 10   | 94    | 8.17 | 7.254 | 0.726          | 0.726          | 0.005           | 358.4            |
| ILB   | 704 | 6    | 66    | 7.57 | 7.329 | 0.726          | 0.667          | 0.028           | 306.4            |
| RMB   | 815 | 15   | 130   | 8.76 | 7.806 | 0.747          | 0.721          | 0.013           | 521.9            |
| GGB   | 708 | 3    | 70    | 7.61 | 6.901 | 0.717          | 0.667          | 0.028**         | 405.2            |
| HRB   | 792 | 10   | 118   | 8.52 | 7.393 | 0.730          | 0.648          | 0.064***        | 560.9            |
| HRP   | 451 | 2    | 14    | 4.85 | 4.455 | 0.583          | 0.593          | -0.040          | 117.0            |
| HRI   | 634 | 3    | 46    | 6.82 | 6.008 | 0.677          | 0.635          | 0.010           | 255.1            |
| TGV   | 554 | 5    | 31    | 5.96 | 5.444 | 0.663          | 0.652          | -0.020          | 199.6            |
| OBV   | 605 | 2    | 49    | 6.51 | 5.877 | 0.678          | 0.66           | -0.028          | 287.2            |
| MWF   | 529 | 4    | 29    | 5.69 | 5.219 | 0.661          | 0.657          | -0.032          | 149.2            |
| AMB   | 574 | 2    | 38    | 6.17 | 5.672 | 0.665          | 0.661          | -0.051          | 200.8            |
| FGV   | 562 | 3    | 35    | 6.04 | 5.538 | 0.643          | 0.625          | -0.026          | 274.6            |
| FV    | 629 | 3    | 47    | 6.76 | 5.922 | 0.667          | 0.660          | -0.015          | 409.9            |
| TAR   | 544 | 0    | 22    | 5.85 | 5.500 | 0.654          | 0.630          | -0.004          | 249.7            |
| RH    | 577 | 4    | 36    | 6.20 | 5.608 | 0.663          | 0.642          | 0.001           | 272.9            |
| BBB   | 584 | 3    | 39    | 6.28 | 5.721 | 0.661          | 0.619          | -0.007          | 231.8            |
| GLW   | 511 | 2    | 22    | 5.49 | 5.000 | 0.616          | 0.565          | 0.056***        | 241.2            |
| Mean  | 640 | 6.42 | 61.74 | 6.88 | 7.497 | 0.684          | 0.654          | -0.001          | 309.9            |

Total number of alleles (tA); number of private alleles (pA); number of rare alleles (rA); mean number of alleles (mA); unbiased expected heterozygosity ( $H_E$ ); observed heterozygosity ( $H_O$ ); fixation index ( $F_{IS}$ ) and its significance level  $p$ ; effective population size ( $Ne_{LD}$ ) based on LD. The Croatian breeds are marked grey.

\*\*\* $p < 0.001$ , \*\* $p < 0.01$ .

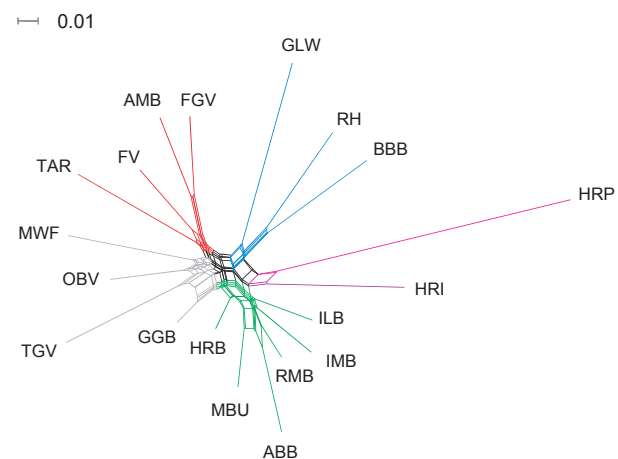
The mean number of alleles ( $mA$ ) ranged from 4.85 (HRP) to 9.74 (ABB). HRB displayed a number of alleles (8.52) higher than the average among the Buša breeds. Looking at each of the Podolian breed itself, HRP showed the minimum number of alleles per marker (4.85), while HRI was about the average number (6.82) of the other breeds. As shown in Table 3, similar relations are expressed by  $AR$ ,  $pA$  and  $rA$ . HRB showed a high  $pA$  (10) and  $rA$  (118). HRP showed the lowest  $pA$  and  $rA$  (2 and 14, respectively). HRI is comparable to dual-purpose breeds like FV and OBV with  $pA = 3$  and  $rA = 46$ . Only nine private alleles with frequencies higher than 0.05 were found (4 in ABB, 2 in MWF, 2 in TGV and 1 in HRP). HRP showed the lowest diversity, not only in comparison with Croatian breeds but also in comparison with all investigated breeds ( $H_O = 0.593$ ,  $H_E = 0.583$ ,  $mA = 4.85$ ,  $rA = 14$ ). Among all 19 breeds, only in HRP  $H_E$  was lower than  $H_O$ . Between the groups of breeds, Buša showed  $H_E$  above 0.72, and the other groups reached values below 0.68. The fixation coefficient ( $F_{IS}$ ) was in the range from -0.051 in AMB to 0.064 in HRB. There are four significant  $F_{IS}$  values associated with heterozygote deficiency ( $p < 0.001$  in ABB, HRB, GLW and  $p < 0.01$

in GGB; Table 3). There is no significant heterozygote excess. Regarding the  $F_{IS}$  value across 93 neutral loci, two Croatian breeds HRB and HRP well represent two extremes (0.064 and -0.040 respectively), and HRI is in the middle position between both (0.010).  $Ne_{LD}$  as estimated here represents the haplotype diversity of 58 markers from chromosome 1 ( $n = 25$  maker), 2 ( $n = 7$ ), 3 ( $n = 9$ ) and 6 ( $n = 17$ ), which is well related to the true  $Ne$ . According to the aforementioned diversity parameter, HRB showed the maximum  $Ne_{LD}$  (561) and HRP the minimal  $Ne_{LD}$  (117), while HRI showed more than the double  $Ne_{LD}$  (255) compared to HRP. Nevertheless, it is below the average of all breeds (Table 3).

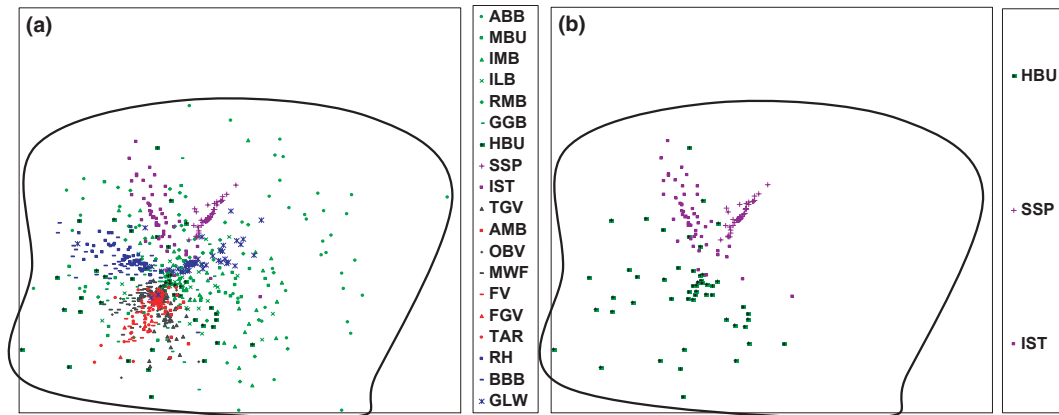
### Breed relationships based on genetic distances

Nei's  $D_A$ -distance was plotted as a neighbour network in Figure 2. The two podolian breeds split from the Buša cluster and shortly afterwards from themselves; HRP was found at the end of the longest branch of the network. HRB was included into the Buša cluster with a very short branch but closer to the root and interwoven to GGB that was found in one of the two alpine clusters. The three northern breeds were also found in a discrete cluster.

Figure 3(a,b) display the  $D_{PS}$  distances in the two-dimensional presentation (2DD). The *diversity space* represents the same background for each subfigure.



**Figure 2** Phylogenetic network of Nei's  $D_A$ -distance with the program SPLITSTREE4 (Huson & Bryant 2006). The Croatian breeds are shown within a framework of 16 European breeds (abbreviations in Table 2). Blue cluster: north-western breeds. Red and grey cluster: Alpine breeds. Green cluster: Buša breeds. Violett cluster: Podolian breeds.



**Figure 3** Individual  $D_{P5}$ -distances in two-dimensional space. Each dot resembles an individual according to the respective legend. The black surrounding is defined here as *diversity space*. (a) All individuals of 19 European breeds including Croatian breeds. Blue: north-western breeds. Red and grey: Alpine breeds. Green: Buša breeds. Violet: Podolian breeds. (b) Croatian autochthonous breeds.

In Figure 3(a), the distribution of all 872 individuals of the 19 breeds within the *diversity space* is shown. The seven alpine breeds (red) cluster together, and the three north-western breeds (blue) build two separate clusters, [GLW] and [RH,BBB]. The Buša breeds or types (green) are scattered over the whole background of all breeds, ABB is covering even areas of the diversity space, where no other breed is found. The two podolian breeds form two adjacent clusters. Figure 3(b) shows the three Croatian breeds. The considerable genetic diversity of HRB is illustrated by the space that is covered by the individuals in comparison with the concise clouds of HRI and HRP.

#### Population structure and admixture level

For the whole data set, the assignment test correctly assigned 89% (772/872) of individuals in the self-classification analysis. More than 87% of the mismatches were found within the Buša group (GGB included). The highest number of incorrectly assigned individuals belonged to HRB (21) and GGB (16) with most frequent exchange of individuals between these two breeds. Mismatches from other breeds were predominantly found in HRB ( $n = 19$ ) and GGB ( $n = 17$ ). Only four HRI and one HRP individual were mismatches. In the alpine and north-western European breeds that are well monitored, only 2% of individuals were allocated to other populations.

The results of the STRUCTURE analysis (Figure 4) and corresponding  $LnP(D)$  and  $\Delta K$  analysis (Figure 5) resulted in  $\Delta K$  peaks at  $K = 2$ ,  $K = 3$ ,  $K = 5$  and  $K = 10$ . The results of the most representative runs

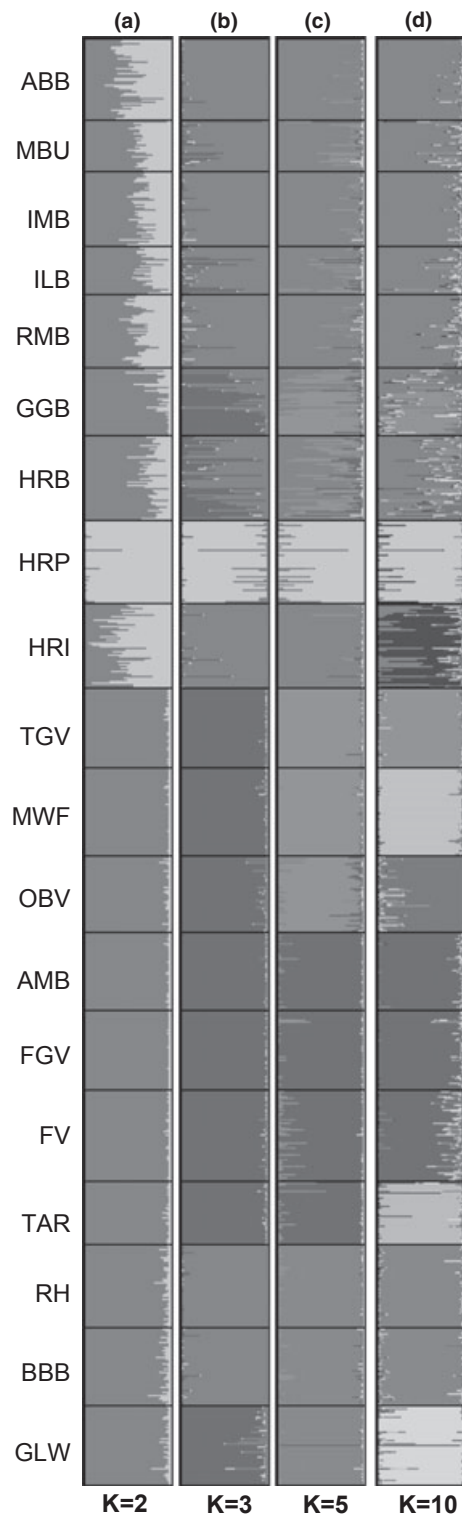
for these  $K$ 's are presented in the following sections (Figure 4a–d). Taking into account all results so far including that  $\Delta K$  is strongly dependent on the variability between the runs of each  $K$ , the most possible true  $K$  can be located at  $K = 10$ .

At  $K = 2$ , all breeds except HRP were found in one cluster during 10 runs (Figure 4a). The group of Buša breeds (GGB included) showed an intermixed structure, with an average membership index of 0.311 to HRP. HRI individuals were found in an intermediate position, slightly closer to HRP but with a low average membership index of 0.689.

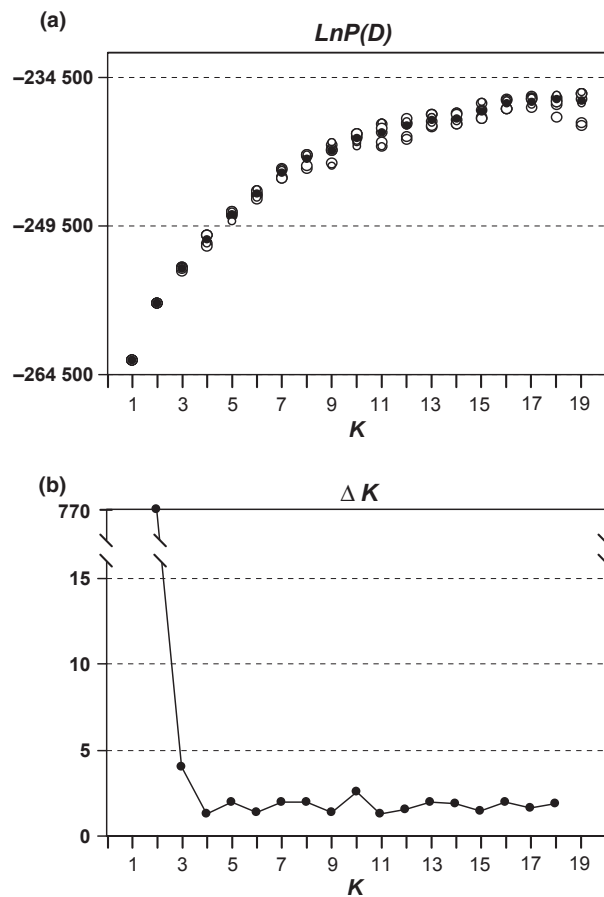
At  $K = 3$ , four of ten runs showed the pattern in Figure 4(b). HRP was found in its own cluster during all ten runs. The alpine breeds shaped up into one cluster, while in the remaining cluster Buša breeds and north-western breeds were found. GLW was found within the alpine cluster, but in the other six runs it joined the cluster of the other two north-western breeds.

At  $K = 5$ , half of the runs resulted in the pattern presented in Figure 4(c) and showed a clustering in the group of Buša (including HRI), in alpine group 1 [TGV-MWF-OBV], alpine group 2 [AMB-FGV-FV-TAR], the three north-western breeds and [HRP]. The pattern in the remaining five runs changed according to the status of GLW, which was then found in an own cluster. Thus, either the alpine groups were reunited in one cluster, or BBB and RH were found within the Buša group.

At  $K = 10$ , the most frequent pattern (4 of 10) is shown in Figure 4(d). HRI was found in an own cluster during all 10 runs, the same applies to GLW and OBV. During seven runs, MWF was found in an own cluster; in the three remaining runs, it clustered



**Figure 4** Clustering assignment of 19 cattle breeds with STRUCTURE. For each  $K = 2$  to 19, 10 runs were performed. The representative run of  $K = 2$ (a),  $K = 3$ (b),  $K = 5$ (c) and  $K = 10$ (d) is shown.



**Figure 5** Uppermost hierarchical structure based on  $\Delta K$ . (a) Estimated likelihood,  $LnP(D)$  for values of  $K$  ranging from 1 to 19. The mean  $LnP(D)$  for each  $K$  over 10 runs is represented by solid dots. (b)  $\Delta K$  calculated as according to Evanno *et al.* (2005). The modal value of this distribution corresponds to the true  $K^*$  or the uppermost level of structure.

together with TGV. The group of AMB, FGV and FV was constant during nine runs.

### Conservation strategies

In Table 4, both neutral diversity (N) and neutral diversity with the principle of complementarity (NC) are shown for 19 breeds. In both strategies, the same three breeds are prioritized: ABB, RMB and HRB. If these three breeds ( $x = 3$ ) would be preserved, more than 90% of neutral diversity would be caught. Both strategies suggest a similar conservation priority until  $x = 7$ . Six breeds were from the Buša group, with the exception of GGB in the N strategy. In the NC strategy, FV replaced GGB (Table 4). These seven breeds preserve more than 95% of the neutral genetic diversity within the whole dataset of this

**Table 4** Population ranking under two different conservation strategies

| Neutral diversity |            | Neutral diversity with principle of complementarity |             |              |
|-------------------|------------|---|-------------|--------------|
| Pop               | N          | Pop   | NC          | NC (%)       |
| ABB               | 906        | ABB   | 906         | 0.786        |
| RMB               | 815        | RMB   | 993         | 0.861        |
| <b>HRB</b>        | <b>792</b> | <b>HRB</b>  | <b>1041</b> | <b>0.903</b> |
| IMB               | 760        | MBU   | 1066        | 0.925        |
| MBU               | 715        | ILB   | 1082        | 0.938        |
| GGB               | 708        | IMB   | 1094        | 0.949        |
| ILB               | 704        | FV  | 1104        | 0.958        |
| <b>HRI</b>        | <b>634</b> | AMB   | 1112        | 0.964        |
| FV                | 629        | OBV   | 1119        | 0.971        |
| OBV               | 605        | TGV   | 1124        | 0.975        |
| BBB               | 584        | RH  | 1129        | 0.979        |
| RH                | 577        | <b>HRI</b>  | <b>1134</b> | <b>0.984</b> |
| AMB               | 574        | MWF   | 1138        | 0.987        |
| FGV               | 562        | FGV   | 1142        | 0.990        |
| TGV               | 554        | BBB   | 1146        | 0.994        |
| TAR               | 544        | GGB   | 1147        | 0.995        |
| MWF               | 529        | <b>HRP</b>  | <b>1151</b> | <b>0.998</b> |
| GLW               | 511        | GLW   | 1153        | 1.000        |
| <b>HRP</b>        | <b>451</b> | TAR   | 1153        | 1.000        |
| Total             | 1153       | Total   | 1153        |              |

(1) Neutral diversity presented by the total number of alleles (N) in the respective subpopulations. (2) Neutral diversity with principle of complementarity, presented by the cumulative number of neutral alleles (NC) when the first  $x$  subpopulations are combined, NC(%) is the percentage of neutral alleles that would be conserved with the respective  $x$  breeds. Both tables are sorted according to the conservation priority in each of the strategy. The Croatian breeds are marked grey.

study. If additional seven breeds are added, conservation of more than 99% of the neutral genetic diversity is ensured. According to N and NC strategies, neither HRI (NC  $x = 12$ ) nor HRP (NC  $x = 17$ ) showed a very high conservation priority.

### Cultural value of local breeds

All three breeds were dominant in their farming areas until the middle of the 20th century and then gradually decreased. Owing to extensive farming system, all breeds had a role in shaping landscape. In the past, people were using everything, from skin to horns. HRB is not very suitable for meat use because the muscle fibres are very rough with little intramuscular fat. Rich feeding leads to fatty trunk and carcass and fat deposition around internal organs without increase in gain (Rako 1947). While HRP is suitable for meat production, HRI is already implemented in local tradition as a food speciality. HRI is included in folklore and traditions and HRB and

**Table 5** Analysis of cattle breeds (a) historical witnesses (b) custodians of local traditions (role in the presence)

| Breed | Landscape | Gastronomy | Folklore | Handicrafts |
|-------|-----------|------------|----------|-------------|
| (a)   |           |            |          |             |
| HRB   | ++        | –          | +        | +           |
| HRP   | ++        | +          | ++       | +           |
| HRI   | ++        | ++         | ++       | ++          |
| (b)   |           |            |          |             |
| HRB   | ++        | –          | +        | –           |
| HRP   | ++        | +          | +        | –           |
| HRI   | ++        | ++         | ++       | +           |

– negligible value, + limited value, ++ noticeable value.

HRP only in cattle market events. For HRI, there are two main cultural events, Jakovlje (exhibition of oxen) and Mandalenjina (exhibition of breeding animals), in Istria. HRI is involved in events like fairs (not HRB and HRP), weeding parade, tandem pull and pageant manifestation (not HRB and HRP).

Considering handicrafts, there are sculptures from wood or stone and paintings of HRI, not that much for HRB and HRP. From HRB and HRP, there exist some old photographs. The prominent horns from HRP and HRI have been used, e.g. for engraving and in hunting. The results of the analysis are shown in Table 5a and b.

### Discussion

This study is the first report on the population structure and extent of genetic variability using microsatellite genotype data in the three Croatian autochthonous breeds (HRP, HRI and HRB). Prior to this study, there was a controversial discussion on (i) admixture of alpine genes into HRB and the adequacy of the conservation support of the government, (ii) the degree of genetic relationship between Pannonian and Mediterranean podolian breeds in this small country, (iii) the extent of the historical known genetic bottleneck in HRP, (iv) the necessity or the possible sense of a cooperation between neighbour countries breeding highly related cattle breeds or strains. These considerations are analogous for a variety of breeds under conservation and their respective countries.

### HRB

#### *Genetic variability and structure*

In HRB, the Croatian representative of the Buša group, we found a  $H_E$  value of 0.73, with a significant deficit of heterozygotes of 6.4%. This is



probably due to the existence of diverse gene pools caused by current habitat fragmentation (Wahlund effect) and/or different degree of admixture of alpine genes along the HRB dispersal area. HRB exists as small fragments of an originally large population. Isolated farms keep only a couple of mainly old cows and partly their offspring. To minimize the relationship and maximize the breed purity, we sampled only one animal per farm, and as rule of thumb, this was the old cow, i.e. lower probability to be upgraded by alpine breeds. We sampled this population from five main locations with a distance from 90 to 430 km surrounded by rocky and mountainous areas. Artificial insemination is not practiced to overcome geographical distances, and genetic exchange between fragments of the gene pool does not happen. This could be an explanation for the absence of some allele combinations and for the *prima facie* controversial deficit of heterozygotes in a highly diverse population. This also explains the results of STRUCTURE (Figure 3d) and the assignment test, where influences from alpine breeds are notable and result in a closer relation to GGB. There is a gradual increase in the proportion of alpine genes from south (29%) over central Dalmatia (47%) to Lika (62%). The difference between south Dalmatia and Lika is significant ( $p = 0.0032$ ) and between south and central Dalmatia indicative ( $p = 0.077$ ). Animals with a proportion of membership to the Buša cluster over 0.5 were 74, 55 and 33% in south Dalmatia, central Dalmatia and Lika, respectively. Considering only these HRB animals, the average membership in the Buša cluster is at 75%. As HRB appears as a heterogeneous population, our results can be used in addition to the phenotype to differentiate between pure Buša animals and cross-bred animals, which simulate Buša morphology and size because of poor husbandry.

#### Conservation value

From a global point of view, the conservation value of HRB is remarkable despite the admixture level of alpine genes. It was set on the third place in the conservation priorities regarding neutral diversity (Table 4). The high haplotype diversity ( $Ne_{LD}$ ) and the number of rare and private alleles in Buša can only partly be explained by the introduction of genetics of a well-managed and selected population with an essential lower diversity, like TGV or OBV. Furthermore, there already exists an efficient local conservation programme, which resulted in approximately 25% nominal increase in the still threatened population during the last 5 years (Croatian Livestock Center (CLC) 2004; CAA 2009). This was

mainly carried out with governmental subvention. According to our knowledge, most current Buša populations of south-east Europe are vanishing without concrete financial support and conservation programmes in the respective countries. The population of the Illyrian Lowland Buša sampled in 1998 vanished in a social and financial collapse of Albania immediately after sampling. Large parts of the Illyrian Mountain Buša disappear, too. Gray Gacko Buša and Macedonian Buša also shared a common destiny in the years of political turbulence on Balkan during the beginning of 1990. This increases the global value of an effective Buša conservation programme in Croatia where the infrastructure is already implemented. As soon as the presently existing neighbour Buša subpopulations will be organized in breeding or conservation programmes, a controlled exchange of animals or semen or embryos is desirable and will on all participating sides reduce the loss of genetic diversity in the highly fractured Buša population. These results can underpin the efforts of further HRB conservation especially in south and central Dalmatia. They further animate the regulated exchange of selected animals with neighbour countries without loss of breed identity.

Between the parameters  $Ne_{CAA}$  (Table 1) and  $Ne_{LD}$  (Table 3), we observed a large discrepancy for all three breeds, especially for HRB. The high correlation (0.906) between the nominal population size and  $Ne_{CAA}$  (Table 1) was remarkable and suggests that the relationship data should be used for future estimations of  $Ne$  in the annual reports of the CAA. We prefer  $Ne_{LD}$  as the relative estimator of  $Ne$ .

#### Cultural value

Comparable to other Buša populations of Balkan, HRB played an important role in the daily life of people. This was the abundant breed that covered the utilization of milk, meat and work in extensive farming systems through all seasons of the year and thus resembles the typical agricultural landscape of mountainous and coastal areas. Although milk and beef production does not seem to be suitable on a broader economic basis, it represents the basic nutrition of local predominantly farming people. Furthermore, as shown by Gandini *et al.* (2007), longer expected total and productive lives of local breeds can partly compensate the reduced yield in comparison with more commercial breeds. In the case of Buša, the environmental conditions are extremely challenging because of scarce and mountainous areas and poor food quality. Taken this into account, Buša seems to be the breed of choice for a sustainable

niche agriculture in combination with landscape conservation and tourism in marginal lands.

## HRP

### *Genetic variability and structure*

In HRP, a low genetic diversity was observed. The mean number of alleles and gene diversity was 4.85 and 0.583, respectively. The long branch of the phylogenetic network (Figure 2) together with the small and limited *diversity space* that is covered by HRP (Figure 3b) indicates a strong genetic drift within this population. This is further supported from historical data of a bottleneck and the resulting low  $N_{e_{CAA}}$  and  $N_{e_{LD}}$ . In the middle of the 20th century, the number of autochthonous HRP animals has been drastically reduced by systematic upgrading with more productive European breeds. At the end of the 20th century, breeding of HRP in Croatia was based on four bulls and 16 cows from one herd (P. Caput, personal communication). The HRP population increased to 40 individuals and was then located in a confined and isolated area (Krizevacki Lemes) without the possibility of gene flow. With this background, the 51 sampled animals represent 44% of the total adult population descending from 18 founders. The sampled animals are related but their average relationship is lower than the total population. Therefore, the allele frequency estimates are not biased but appear more precise than they actually are (Chakraborty 1991). The same applies to HRB and HRI. Individual distances within breed, based on identity by state and not allele frequency, were low ( $D_{PS}$ , Figure 3), and the structure analysis showed an own cluster from  $K = 2$  onwards (Figure 4). The random drift through the strong genetic bottleneck dramatically reduced  $tA$  and haplotype diversity ( $N_{e_{LD}}$ ) in HRP (Table 3). This resulted in the outstanding highest  $\Delta K$  peak at  $K = 2$  (Figure 5b) and biased the result of the STRUCTURE analysis (Figure 4). According to overall objective information and our experience,  $\Delta K$  peaks at  $K = 2$  is an artefact because the method of Evanno *et al.* (2005) is sensitive to the bottleneck and standard deviation of  $LnP(D)$ . Alternative methods to  $\Delta K$  are needed.

### *Conservation value*

HRP can be understood as a fragment of a formerly large Podolian population of the Pannonian plain. The other most relevant and larger fragments of the same populations are in the neighbour countries Serbia and Hungary. HRP should be highly related to both subpopulations according to the known recent

history (Figure S2). HRP shows distinctive phenotypic features (Figure S2) and is well adapted to the typical Pannonian landscape. Owing to a severe bottleneck effect, HRP lost a large fraction of the neutral diversity and the effective population size is very small (Table 2), i.e. 31% alleles less than in the remaining cattle populations. We observed 1300 alleles in total, but only two of these are private for HRP and one of these has a relatively high frequency (0.059 in HRP). Thus, this breed was of minor ranking in the conservation strategy regarding neutral diversity (Table 4). A comparable situation was only found in GLW, while GLW is under high artificial selection pressure and HRP is under a strong stochastic drift. Genetic drift affects all loci in the same way, but neutral and artificial selection favours different alleles in different breeds or locations (e.g. Slatkin 1987).

Our results demonstrate a massive loss of genetic diversity in HRP and significant differentiation to HRI ( $F_{ST} = 0.132$ ,  $p < 0.05$ ). This argues against reciprocal genetic exchange between HRP and HRI but favours immigration of Pannonian Podolian animals (Hungarian and Serbian) or gametes (semen) to reduce inbreeding in HRP for today and in the future. Genetic data on the other two populations are lacking. But there is the possibility and the necessity of co-operation for a successful *in situ* conservation. If the Hungarian and/or Serbian subpopulations of the Pannonian Podolian Cattle are larger and contain more than all the diversity present in HRP, there are still long- and short-term aspects of *in situ* conservation of HRP. One long-term aspect is that a conservation action is better protected if it takes place in two independent locations, e.g. to shield from epidemics.

### *Cultural value*

Through its role as a historical witness of local traditions, HRP possesses an economic value in the tourist industry in the last consequence, which is an important part of the Croatian economic system. A current example is the nature park 'Lonjsko polje', an alluvial area along the river Sava marked by traditional grazing practices in which native breeds, such as the Posavina horse and HRP, are preserved (<http://www.vjesnik.hr/pdf/2007/05/28/18A18.PDF>). The sight of the large gray Podolian cattle with long and lyra-curved horns in an *in situ* Pannonian landscape as current custodians of local traditions has a specific historic value. Such historic and aesthetic values reflect cultural heritage and are the essential components of quality of life for local people and for tourists.

## HRI

### *Genetic variability and structure*

HRI is found in the middle between HRB and HRP with  $H_E = 0.677$  and  $MA = 6.82$  (Table 3). These values are still higher than the results from the alpine group but lower than from the Buša group. Although both HRI and HRP represent the Podolian cattle group, these two breeds are genetically well differentiated,  $F_{ST} = 0.132$ . This is even larger than the average  $F_{ST}$  value between HRP and seven southern Buša neighbours (0.112). According to the haplotype diversity ( $Ne_{LD}$ , Table 3) and the longest branch in the phylogenetic network (Figure 2), this strong genetic differentiation between the two Podolian breeds resulted from the strong genetic bottleneck in HRP.

### *Conservation value*

As demonstrated in the Results section, HRI is found in the median position between two extremes represented by HRB and HRP. The diversity of HRI is larger or comparable to other non-Buša breeds (Table 3). Although the genetic diversity in HRI is substantially higher than in HRP, the observed population structure is more compact and much more stable than in HRB. Therefore, we look at HRI as a distinct population that can and should progress without any immigration of neighbour gametes.

### *Cultural value*

Although the conservation strategies applied (NC) do not prioritize HRI on a global level (twelfth of 19, Table 4), this breed is a historical witness of the culture and history of the country and further serves as a custodian of local traditions through food specialties. There exists organized meat production and successful marketing of branded goods (bacon, sausage, fresh meat), which are highly appreciated by consumers and reach a higher price, up to 20% higher than the meat products from commercial breeds. There are other examples of utilization of local cattle breeds in producing recognizable products such as Mirandesa cattle in Portugal, Piemontese, Chanina, Merchigiana, Reggiana and Ramangola cattle in Italy or Hinterwälder cattle in Germany (FAO, 1999). HRI is furthermore embedded in popular cultural events.

## Conclusion

The analysis of the three autochthonous breeds of Croatia covered both genetic and cultural aspects. Each breed played an important role in the cultural history of the country and still represents a historical

witness. The current involvement in the tourist industry resembles their value as custodians of local traditions and attributes a market value to the cultural aspect. From a long-term genetic view, the conservation of HRB is of global interest with the remarkable genetic diversity within a European context. On the other hand, the conservation programmes of both Podolian breeds, especially HRP, are justified by cultural reasons rather than by reasons of neutral genetic diversity. As already outlined by Gandini *et al.* (2007), it appears reasonable to develop a cultural index for local breeds to be able to compare this value not only on a qualitative basis. According to a common past breeding area, current population admixture and habitat fragmentation of HRB, we suggest and encourage controlled exchange with neighbour pure Buša populations. According to relatively high governmental support per breeding animal and with respect of the complete population census, it is advisable to close the herdbooks of these breeds now and introduce paternity control. The next step includes the definition of genetic breed identity and thus common rules of target exchange. Owing to opposite reasons (pure but compact and effectively very small population), we suggest an appropriate cooperation between Pannonian Podolian subpopulations of HRP type, but not HRI. In our opinion, this study can serve as an example for other small countries where agriculture is starting to compete on a global level. As long as the local breeds still exist, their global and/or local conservation value should be thoroughly evaluated in a global and local context.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Detailed description of Croatian Buša (HRB).

**Figure S2** Detailed description of Slavonian Syrmian Podolian Cattle (HRP).

**Figure S3** Detailed description of Istrian Cattle (HRI, recognizable synonym is 'boškarin').

**Table S1** This table summarizes information about all markers and all populations the study refers to (19 populations, 872 individuals).

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