

PAPER

Temporal variation in genetic diversity and population structure of Burlina cattle breed

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Abstract

We analysed the temporal variation of inbreeding, genetic variability and population structure in the Burlina (BUR) cattle breed. A total of 279 individuals were chosen for the analysis representing a period of 19 years (1991-2010) and analysed using 24 microsatellite markers. A total of 235 alleles were detected in the population with a mean of 9.79 ± 3.91 alleles per locus. In the 19-year period, a stable pattern in the mean number of alleles was found. The mean observed heterozygosity was 0.63 and it was slightly lower than the expected in all birth year groups. Neither an increase nor a decrease in heterozygosity and inbreeding estimates were detected over the years, with the exception of the F_{IS} index which was close to zero in two birth year groups: 2001-2002 and 2006. Absence of bottleneck events was proved and structure analysis revealed an increase in breed complexity over the years and a clear differentiation with the Italian Holstein Friesian cattle breed. Molecular markers were successfully applied in the monitoring of the genetic variability of BUR thus enabling the planning and the application of strategies for the *in situ* conservation of genetic resources and the improving of breed identity.

Introduction

The implementation of conservation strategies to preserve and maintain genetic diversity in local endangered breeds requires the understanding of population genetic characteristics (Hall and Bradley, 1995). Molecular genetic analysis already provided information on population origins, diversity and relationships in numerous livestock breeds (Groeneveld *et al.*, 2010; Lenstra *et al.*, 2012), but the understanding of population dynamics is becoming more important to guide conservation of local

endangered breeds and maintain their genetic resources for the future (Bruford *et al.*, 2003).

The Burlina breed (BUR) is an Italian dairy cattle breed reared in northeastern Italy; it is a small size local breed well adapted to mountains and marginal areas. After a fast decrease in population numbers from the 15,000 heads in 1930 to 214 in early 1990s the present population account for around 400 cows mainly reared in Treviso and Vicenza provinces (AIA, 2008). In recent years, many efforts have been placed towards the safeguard and the promotion of this local breed from both management and economical point of view. A regional conservation programme was set up since 1980s aiming to contrast the reduction of population size and the increasing of inbreeding (Bittante *et al.*, 1992). More recently, Pretto *et al.* (2009) compared milk production parameters of BUR to that of Italian Holstein Friesian (IHF) breed; Penasa *et al.* (2010) analysed genetic parameters of production and quality traits of BUR and Pacini *et al.* (2008) studied the k-casein variants using polymerase chain reaction-temperature gradient gel electrophoresis (PCR-TTGE) for the same breed. The importance of BUR, from an economical point of view, is related to the production of a typical cheese called *Morlacco* and, from a rearing point of view, to its frugal characteristic that along with its longevity and better functional performances are of particular interest especially in marginal areas (Pretto *et al.*, 2009). Such characteristics and the importance of maintaining and safeguarding genetic resources for biodiversity conservation evidenced the need for a molecular characterisation of BUR initially exploited by Dalvit *et al.* (2008); the authors evidenced the low inbreeding coefficient (F_{IS}) and the high variability present in the population but also the close relationship with the IHF breed. Despite the gain in knowledge on the molecular characterisation of the breed, there is currently a lack of information on the over-time variation of the genetic variability in BUR. This kind of analysis was successfully applied in local Italian chicken breeds by Zanetti *et al.* (2011) and in the Basque Pottoka pony population (Rendo *et al.*, 2012) but, to our knowledge, little is known regarding the temporal variation of genetic variability in local cattle breeds. Moreover, for limited and local livestock populations, it is very important to implement a correct breeding programme that improve production and quality traits while minimising inbreeding and the loss of genetic variability.

Here we describe a temporal analysis of microsatellite allele frequencies at twenty four microsatellite markers in a small local cattle

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breed (Burlina) over a period of 19 years (1991-2010). The objectives of this study are: i) to evaluate changes in genetic diversity and inbreeding over time; ii) to assess the mutation drift equilibrium and to detect the occurrence of recent genetic bottleneck event in this population; iii) to analyse genetic structure of the population over time; iv) to compare the genetic diversity of BUR with the close related IHF breed over time. We discuss the findings and compare molecular data with pedigree information available for the BUR population.

Materials and methods

Sample collection and microsatellite amplification

A total of 279 animals belonging to the Burlina cattle breed were collected to represent a twenty-year period from 10 different herds. Care was taken to include unrelated animals according to pedigree information. In particular, blood samples or frozen semen were collected for 32 animals born between 1991 and 2000, 35 animals born between 2001 and 2002, 23 born in 2003, 35 born in 2004, 52 born in 2005, 34 born in 2006, 41 born in 2007 and 27 born between in 2008 and 2010. For comparison purposes, 36 unrelated individuals

belonging to Italian Holstein Friesian cattle breed were also included in the analysis.

The DNA purification was performed from 50 µL of whole blood or one unit of extended frozen semen using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) with minor modifications. All individuals were genotyped at twenty four microsatellite markers. Markers were mainly selected from the Food and Agriculture Organization of the United Nations/International Society for Animal Genetics (FAO/ISAG) panel to maintain the option for combining this data set with other studies using the same complement of markers (FAO, 2011). Microsatellites markers (*TGLA57*, *TGLA126*, *INRA016*, *ILST008*, *BM203*, *BM1818*, *INRA006*, *CSSM14*, *ETH152*, *RM12*, *TGLA122*, *ETH185*, *TGLA227*, *BM2113*, *TGLA53*, *ETH10*, *MM12*, *INRA64*, *INRA023*, *SPS115*, *ETH3*, *ETH225*, *BM1824*, *BLA2*) were amplified in multiplex PCRs using fluorescence-labeled primers in a total volume of 12.5 µL (Appendix Table 1). Polymerase chain reactions were performed using the Type-IT Microsatellite PCR Kit (Qiagen) starting from 60 ng of purified DNA. Compatible mutliplexes were pooled prior to sizing by capillary electrophoresis on a CEQ 8000 BeckmanCoulter (Brea, CA, USA) instrument. Allele sizing was performed using Genetic Analysis Software v9.0 (Beckman Coulter).

Genetic diversity within populations

Genotypes were checked for errors and null alleles using MSA v4.05 (Dieringer and Schlötterer, 2003) and MICROCHECKER 2.2 (Van Oosterhooft *et al.*, 2004). The MSA v4.05 software was used to calculate allelic frequencies, number of alleles per locus, allelic richness (AR), observed (H_o) and expected (H_e) heterozygosity and F -statistic for each birth year group and for the whole population. We tested for linkage disequilibrium and Hardy-Weinberg equilibrium (HWE) using the programme GENEPOP v4.0 (Rousset, 2008) and

false discovery rate was used to correct for multiple comparisons. Molecular coancestry (f_{ij}) indexes were calculated using MolKin v3.0 (Gutiérrez *et al.*, 2005). Allelic richness and private alleles per population were calculated using rarefaction method (N_{AR} and P_{AR} , respectively) to adjust for different group sizes with Allelic Diversity AnalyZer (ADZE) (Szpiech *et al.*, 2008). We used the programme GENHET (Coulon, 2010) to calculate the internal relatedness (IR), an estimate of parental relatedness, and statistical significance of differences among birth year groups was determined using general linear models in R (R Development Core Team, 2012). Within-breed significant differences of MNA, H_o , H_e , f_{ij} and IR were calculated between birth year groups using Holm correction in R. The variation between allelic diversity and heterozygosity was exploited as the basis for statistical tests to evaluate the mutation drift equilibrium with the software BOTTLENECK (Cornuet and Luikart, 1996). Estimates of expected heterozygosity at mutation-drift equilibrium were calculated using the stepwise mutation model (SMM) and two-phase model (TPM), the variance of TPM assumed in the present study was 12, the TPM model was composed of 95% SMM and 5% infinite allele model as suggested by the programme authors (Piry *et al.*, 1999). The significance of any deviations from mutation-drift equilibrium was based on the sign test, we also used the mode-shift test to determine if the allele frequency distribution has been shifted towards more common alleles with fewer low frequency alleles as would be expected in the case of a bottleneck.

Population structure

Genetic structure, breed assignment percentages and the degree of admixture, if any, for the whole BUR population and for each birth year group, using the same IHF animals as an out-group, were investigated using the Bayesian clustering approach implemented in

STRUCTURE v2.2.3 (Pritchard *et al.*, 2000). The most likely number of populations (K) given the observed genotypic data was estimated by running 50 independent runs for each K ($1 \leq K \leq 18$) for the whole BUR dataset and 50 independent runs for each K ($1 \leq K \leq 4$) for each birth year group analysis. The admixture model with correlated allele frequencies was used with a burn-in length of 100,000 followed by 500,000 MCMC iterations for data collection. The most likely number of K clusters fitting the observed data was established by plotting the $\ln Pr(GIK)$ values obtained in the 50 independent runs for each K, as suggested by Pritchard *et al.* (2000), and by estimating delta K (ΔK) statistics, as proposed by Evanno *et al.* (2005). The output obtained from STRUCTURE was used directly as input by the cluster visualisation programme DISTRUCT (Rosenberg, 2004).

Results and discussion

Estimates of genetic diversity

A total of 235 alleles were detected in the whole population across the 24 investigated microsatellite markers. The number of alleles ranged from 2 (*ILST008*) to 19 (*TGLA122*) with a mean of 9.79 ± 3.91 alleles per locus (Appendix Table 1); the *ILST008* marker accounted for only 2 alleles and hence it was not used in subsequent analysis. Average H_o was 0.63 ± 0.18 (range 0.14 to 0.94) and average H_e was 0.69 ± 0.14 (range 0.13 to 0.87) across loci. No evidences for genotyping errors or presence of null alleles were detected. Significant departure from HWE, after Bonferroni correction ($P < 0.05$), was detected within IHF population (1 locus) and in four birth year groups inside the BUR population (1 to 3 loci, Table 1); AR was moderate with an average of 7.45 ± 2.54 .

The global values of F_{IS} , inbreeding coeffi-

Table 1. Genetic diversity measures for each birth year group of Burlina cattle breed and populations.

BY	N	MNA	$N_{AR}(24)$	$P_{AR}(24)$	H_o (SD)	H_e (SD)	f_{ij} (SD)	IR	F_{IS}^*	HWE*
1991-2000	32	6.35	5.24	0.07	0.62 (0.15)	0.68 (0.14)	0.32 (0.03)	0.10	0.087	-
2001-2002	35	6.43	5.38	0.08	0.66 (0.17)	0.67 (0.15)	0.32 (0.03)	0.04	0.014	-
2003	23	6.09	5.52	0.14	0.65 (0.18)	0.70 (0.14)	0.30 (0.03)	0.07	0.070	-
2004	35	6.48	5.42	0.14	0.63 (0.18)	0.67 (0.15)	0.32 (0.03)	0.08	0.064	2
2005	52	6.83	5.17	0.08	0.59 (0.17)	0.66 (0.16)	0.33 (0.03)	0.13	0.108	3
2006	34	6.39	5.31	0.10	0.68 (0.16)	0.68 (0.14)	0.32 (0.03)	0.02	0.006	-
2007	41	6.65	5.38	0.10	0.62 (0.16)	0.69 (0.12)	0.31 (0.04)	0.09	0.095	1
2008-2010	27	6.48	5.31	0.19	0.61 (0.16)	0.68 (0.13)	0.31 (0.03)	0.11	0.102	1
IHF	36	6.35	4.91	0.97	0.57 (0.24)	0.63 (0.22)	0.28 (0.03)	0.23	0.094	1

BY, birth year; N, sampled individuals; MNA, mean number of alleles, N_{AR} , allelic richness obtained with rarefaction method, sample size is given in brackets; P_{AR} , private alleles, sample size is given in brackets; H_o , observed heterozygosity; H_e , expected heterozygosity; SD, standard deviation; f_{ij} , molecular coancestry index; IR, internal relatedness; F_{IS} , Wright's fixation index; HWE, Hardy-Weinberg equilibrium; IHF, Italian Holstein Friesian. * $P < 0.05$.

cient of an individual relative to the total (F_{IT}) and effect of subpopulations compared to the total population (F_{ST}) were 0.073, 0.106 and 0.036 ($P < 0.001$) indicating that the departure from the HWE is mainly due to an excess of homozygotes within the population. Gene diversities values were similar among birth year groups for the BUR population and larger compared to that obtained for the IHF. The mean MNA value found in BUR (6.46) is comparable to that of IHF (6.35), N_{AR} values (allelic richness calculated using the rarefaction method) were uniformly moderate with an average of 5.29 ± 0.11 alleles for the BUR breed and significantly lower for the IHF (4.91) while the P_{AR} was close to zero in BUR and close to one for IHF. H_o was always lower than H_e in both BUR and IHF with a slightly larger number of homozygotes in IHF ($H_o = 0.567$) respect to BUR ($H_o = 0.633$). The F_{IS} index was slightly positive, on average, confirming a relatively small heterozygote deficiency. Molecular coancestry is another method by which within-group diversity can be measured; the average f_{ij} value among BUR groups was uniformly limited (0.32). The internal related-

ness was significantly lower within BUR individuals [0.08 (95% CI 0.06 to 0.10)] compared to IHF individuals [0.23 (95% CI 0.18 to 0.28)]. Heterozygosity tests revealed a significant heterozygosity deficit in almost all BUR groups under the SMM mutation model (Table 2); in contrast, gene diversity under the TPM mutation model for birth years 2003, 2006 and 2007 were at mutation-drift equilibrium. All time

periods showed a normal L-shaped allele frequency distribution. The temporal variation of genetic diversity was quantified in terms of MNA, N_{AR} heterozygosity, F_{IS} , f_{ij} and population structure. During the ~20 years period no significant loss of alleles occurred at the microsatellite analysed, the MNA slightly fluctuated among the years without any significant loss or gain of alleles (Table 2, Figure 1a), it

Table 2. Mutation drift equilibrium tests for each population (performed in BOTTLENECK).

Population	SMM			TPM, 10%		
	n_{def}	n_{exc}	P	n_{def}	n_{exc}	P
1991-2000	14*	9*	0.040*	14*	9*	0.040*
2001-2002	15*	8*	0.016*	14*	9*	0.041*
2003	15*	8*	0.014*	13	10	0.093
2004	16*	7*	0.005*	15*	8*	0.016*
2005	17*	6*	0.001*	14*	9*	0.042*
2006	13	10	0.088	11	12	0.311
2007	14*	9*	0.044*	12	11	0.186
2008-2010	16*	7*	0.005*	16*	7*	0.005*

SMM, stepwise mutation model; TPM, two-phased mutation model; n_{def} , number of loci with heterozygosity deficiency; n_{exc} , number of loci with heterozygosity excess; P, probability of departure from mutation-drift equilibrium using a sign test. * $P < 0.05$.

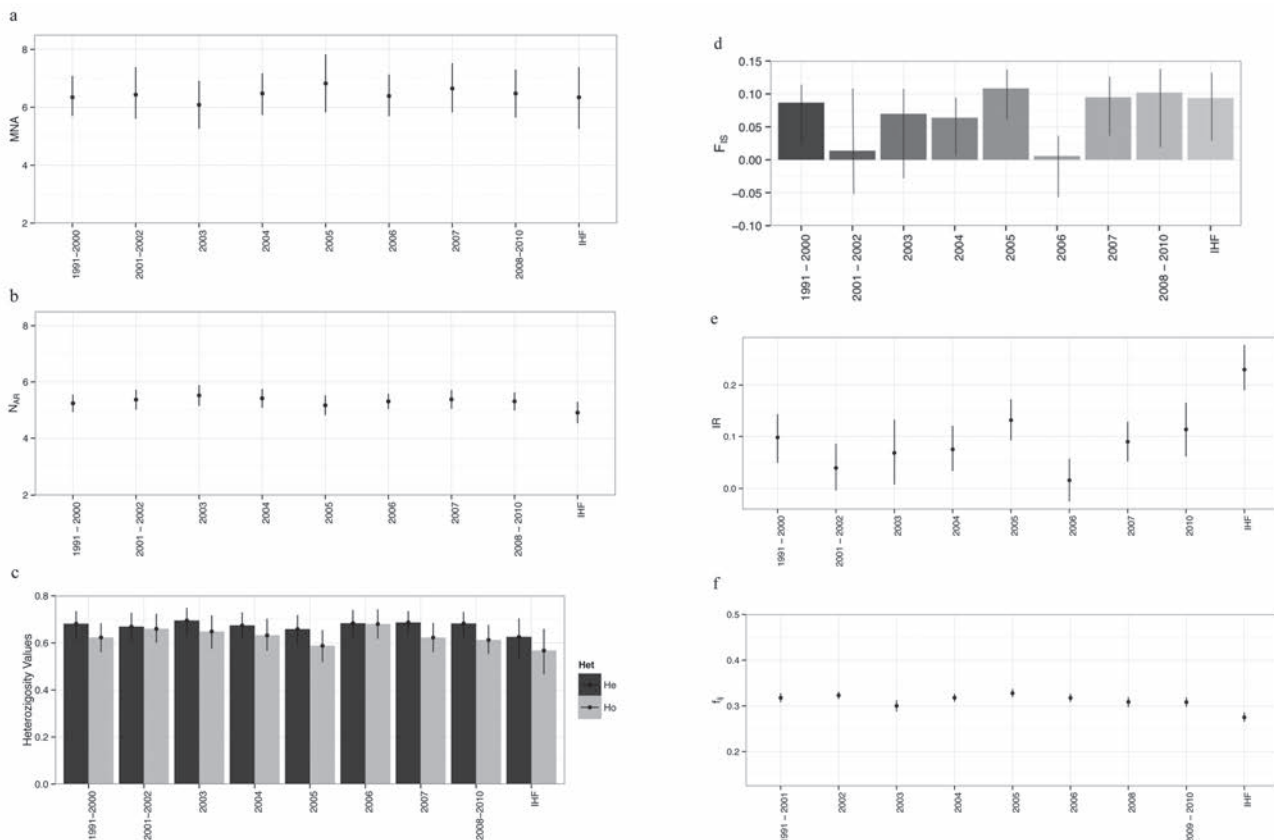


Figure 1. Averages with standard deviation of a) mean number of alleles (MNA), b) allelic richness obtained with rarefaction method (N_{AR}), c) observed (H_o) and expected (H_e) heterozygosity, d) inbreeding coefficient (F_{IS}), e) internal relatedness (IR), and f) molecular coancestry coefficients (f_{ij}) for each birth year group. IHF, Italian Holstein Friesian.

reached the maximum value in 2005 (6.83) and the minimum value in 2003 (6.09). To analyse if the observed differences were attributable to variations in the number of genotyped animals, the N_{AR} was preferred for trend analysis. N_{AR} values did not show any significant variation among the years (Figure 1b), the maximum N_{AR} value was detected in 2003 (5.52) and the minimum in 2005 (5.17), thus supporting the evidence of the absence of clear changes in the number of alleles over time. During the analysed period, no significant increase or decrease in both H_o or H_e was detected (Figure 1c). Values of H_o ranged from 0.588 in 2005 to 0.680 in 2006 and values of H_e ranged from 0.658 in 2005 to 0.695 in 2003. The F_{IS} estimate showed a similar temporal trend with positive values between 6 and 10%, indicating a slight excess of homozygotes, with the exception of 2001-2002 and 2006 where F_{IS} was close to zero; all values were inside the 95% CI (Table 1, Figure 1d). Significant pairwise differences using Holm correction ($P < 0.05$) were detected in BUR for f_{ij} estimates between 2003 (0.300 ± 0.030) and 2005 (0.328 ± 0.032), and between each BUR birth year group and IHF (0.275 ± 0.030 , Figure 1f). Also IR estimates were significantly different between 2005 [0.132 (95% CI 0.094 to 0.169)] and 2006 [0.016 (95% CI -0.030 to 0.062)], and between each BUR birth year group and IHF [0.230 (95% CI 0.185 to 0.275)] (Figure 1e). Despite significant pairwise differences among birth year groups, IR estimates seem to increase from 2001 to 2005 then, in 2006, IR reaches the minimum value and it subsequently increased until 2010 (Figure 1e).

The population structure and its variation over time was investigated using the Bayesian approach implemented in the software STRUCTURE v.2.3.3. The cluster analysis performed without prior information on breed or birth year groups evidenced a meaningful pattern of mean $\ln \Pr(G|K)$ values from $K=2$ to $K=9$ (Figure 2a). According to delta K (ΔK) statistics, following Evanno *et al.* (2005), a mean peak at $K=2$ and secondary peaks at $K=3$, $K=6$, $K=9$ were found (Figure 2b). At $K=2$ the BUR population clearly separated from IHF with proportion of memberships in the two predefined clusters larger than 0.970 and 0.995 for BUR and IHF individuals, respectively. With the increase in the number of K clusters the differentiation between IHF and BUR was maintained and a complex pattern of cluster membership distributions was detected in the BUR population (Figure 3). To further investigate temporal variation of cluster memberships in BUR and to detect any temporal variation in proportion of memberships or admix-

ture between BUR and IHF individuals we performed STRUCTURE analysis for each birth year group separately using the same IHF population as out-group. The most likely number of clusters was two for 1991 to 2000, 2001 to 2002, 2003, 2004 and 2007, three in 2006 and four in 2005 and 2009 to 2010 (Appendix Figure 1). When the most likely number of cluster detected was larger than two it was always due to subdivisions in BUR groups rather than to the presence of admixed individuals between BUR and IHF (data not shown). Italian Holstein Friesian always grouped as a separate cluster with proportion of memberships larger than 0.972 (Appendix Figure 2). For $K=3$, the temporal variation in proportion of cluster assignment percentages was investigated. A subtle modification in cluster assignment proportions in BUR was evidenced by plotting the proportion of membership as a function of birth year. In 1991 to 2000 the pro-

portion of membership to cluster A was 0.392 (0.599 to cluster B) and it shifted to 0.670 in 2008 to 2010 (0.313 cluster B; Appendix Figure 3). The third cluster was composed by IHF individuals. These results further evidenced that in recent times the population structure of BUR increased in complexity.

General remarks

Currently, the BUR risk status according to FAO-Domestic Animal Diversity Information System (DAD-IS) is critical, therefore the monitoring of genetic diversity analysis over time is mandatory to maintain this local breed and its genetic resources. Based on historical records, a large decline in BUR population occurred firstly during the beginning of 19th century (First World War) and, secondly, until 1980s (breed substitution). However, we do not know if this decline occurred as a rapid series of bottlenecks and if the decline in genetic diversity is still ongoing in the breed-

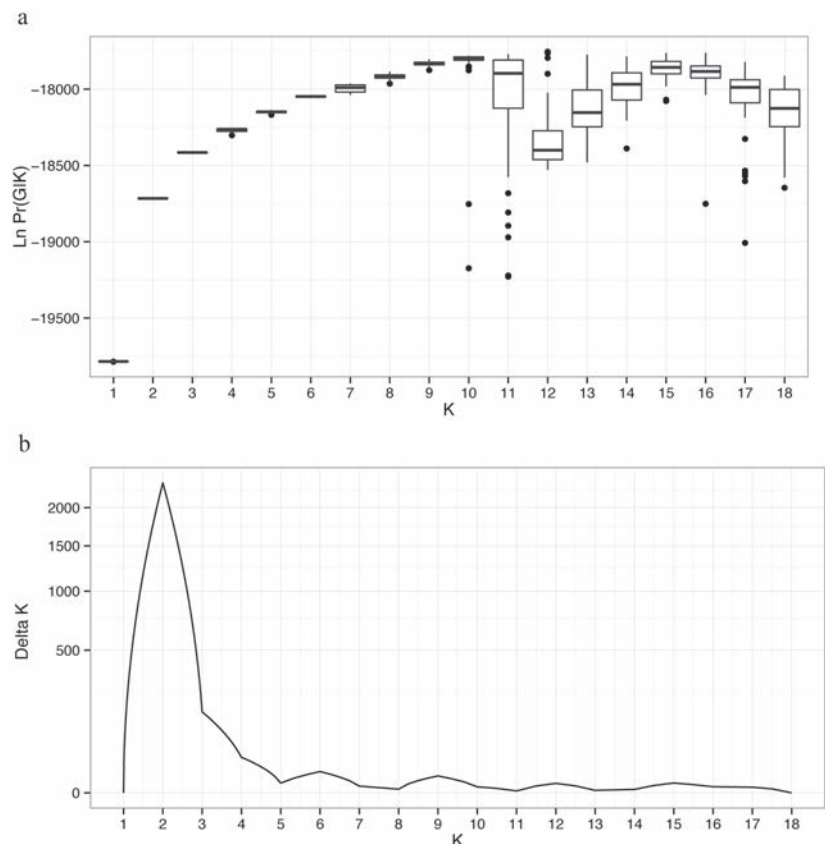


Figure 2. Estimated posterior probabilities of $\ln \Pr(G|K)$. a) $\ln \Pr(G|K)$ values are presented as a function of the number of clusters among 50 runs; b) ΔK values calculated following Evanno *et al.* (2005).

ing population. Battagin *et al.* (2010) recently analysed pedigree information of BUR available since 1980. In the last ~30 years the number of recorded individuals increased significantly together with the number of inbred animals; at the beginning of the conservation programme, bulls of the local breed were mated to pure and crossbred Burlina cows and then backcross to Burlina was practiced (Bittante *et al.*, 1992). Presence of inbred animals from pedigree records started in 1990s and reached about the 81% of female and 88% of male calves in 2009. The first effort to include molecular information for the conservation and management of BUR population beside the implementation of a pedigree register was performed by Del Bo *et al.* (2001) and Dalvit *et al.* (2008). Dalvit *et al.* (2008) investigated the genetic variability of BUR and its genetic distinctness with Brown Swiss (BRU) and IHF by using 12 microsatellite markers as a prerequisite for a conservation programme aimed at increasing reared animals, monitoring breed identity and limiting inbreeding. These preliminary research was fundamental in describing genetic diversity estimates in BUR, but provided only limited and static information on how breed variability and distinctness were developing. Our results spanned a 20-year period including animals from 1991 to 2010 overlapping those recorded by pedigree information (Battagin *et al.*, 2010) and previous molecular studies (Del Bo *et al.*, 2001; Dalvit *et al.*, 2008). The mean MNA value found in BUR (6.46) is comparable to that previously obtained by Dalvit *et al.* (2008) for the same breed (6.70) and to that of IHF (6.35), and larger than the value reported by Del Bo *et al.* (2001) (5.59). Despite its very small population size, the mean values of H_o and H_e in BUR were relatively high (0.634 ± 0.165 and 0.678 ± 0.141 , respectively). Estimates were closer to values recently obtained for other local/native breeds (Medugorac *et al.*, 2011; Delgado *et al.*, 2011; Acosta *et al.*, 2012; Bozzi *et al.*, 2012) rather than to those obtained for more widespread commercial populations (Maretto *et al.*, 2012). These results could be explained by the fact that BUR breed has always been reared by small breeders following their own separated breeding schemes with the use of their own sires and to its late enrollment in the Italian Herd Book, therefore maintaining sufficient diversity parameters. Recently, Simčič *et al.* (2013) investigated the current genetic diversity status of Cika cattle. This breed seems to share a similar history to that of BUR in terms of rapid population reduction (closed to extinction in 1991), admixture with another more productive breed

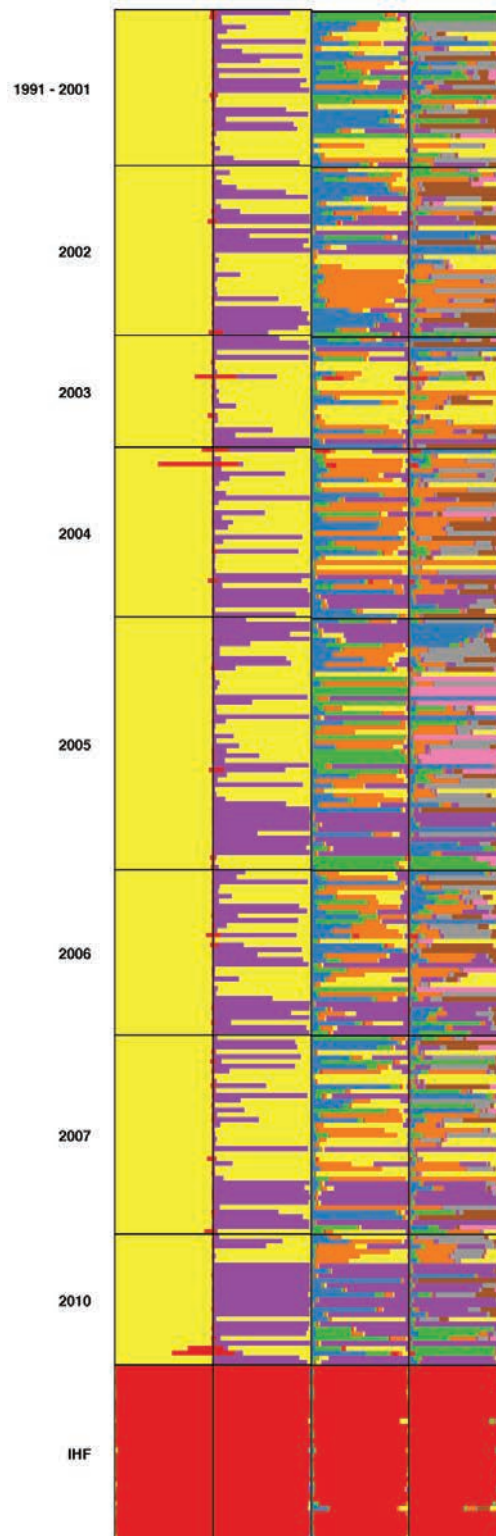


Figure 3 Estimated group assignment percentages for the whole BUR population divided in birth year groups based on a Bayesian clustering approach as implemented in STRUCTURE v.2.2.3. Values of runs with the largest $\ln \Pr(G|K)$ for $K=2$, $K=3$, $K=6$ and $K=9$, among the 50 independent runs, are shown. Each individual is represented by a single vertical line broken into K colour segments, with lengths proportional to the estimated membership to the inferred cluster.

(Pinzgauer) and the recent set up of a conservation breeding scheme. Despite these similarities, the Cika breed showed larger H_e and H_o values compared to BUR, which is partly explained by larger variability present in Balkan breeds (Medugorac *et al.*, 2009) and the presence of admixture with the Pinzgauer breed. In BUR, the MNA was always larger than 6.09 during the 20-year time frame and also individual heterozygosity was not significantly different between time periods (Table 1, Figure 1 a,c). The loss in allelic diversity compared to heterozygosity variation is common in bottlenecked population (Maruyama and Fuerst, 1985). To investigate the presence of bottleneck events we tested for deviations from mutation drift equilibrium using BOTTLENECK (Cornuet and Luikart, 1996). Results showed in Table 2 evidenced that the majority of the investigated loci exhibited a heterozygosity deficiency under both SMM and TPM models. The test for mode shift in frequency distribution of different alleles did not reveal any mode shift from the normal L-shaped distribution for each birth year group in BUR and for the whole BUR population (data not shown), therefore rejecting the bottleneck hypothesis. The absence of a bottleneck signal during the first period of observation and in the following time periods could indicate that the decline in gene diversity occurred before the observed period or that it is occurring in a slow deterministic manner. Similarly, Bray *et al.* (2009), Ganapathi *et al.* (2012) and Sanz *et al.* (2013) investigated small local cattle breed using microsatellite markers and tested for demographic bottlenecks. Strong evidences of demographic bottlenecks were found in Bargur cattle (Ganapathi *et al.*, 2012) and in the Woodmagic Dexter breed which originated by only five individuals (Bray *et al.*, 2009) but no evidence of bottleneck events was found in the Casta Navarra fighting bull population probably due to gene flow occurrences across herds (Sanz *et al.*, 2013). Despite the limited number of BUR individuals recorded during early 1980s owing to breed replacements attempts with the more productive Brown Swiss and Holstein Friesian, we did not detect any genetic signature of demographic bottleneck. The heterozygosity deficiency observed might be caused by a reproductive isolation imposed by breeders with the aim of maintaining BUR features.

According to Battagin *et al.* (2010) the F_{IS} in BUR started to increase from 1992 and reached the 4.81% in 2009 but the absence of pedigree records, before 1980s, limits the analysis of inbreeding using only pedigree information. According to our results, using microsatellite markers, F_{IS} was limited in the range of 0.006

(2006) to 0.108 (2005) without any temporal variation trend (Figure 1d). Molecular coancestry, which is another method to calculate within-group diversity, was 0.32 (Table 1), on average, which is limited if compared to that obtained in other local breeds (Bozzi *et al.*, 2012). Also for f_{ij} we could not identify any particular trend (Figure 1f); significant differences were detectable only between IHF and all birth year groups ($P < 0.05$). The IR estimate seem to constantly increase from 1991 to 2005 and from 2006 to 2010; significant pairwise differences ($P < 0.05$) were found between years 2005 and 2006 and between IHF and all BUR groups (Figure 1e). Higher values of internal relatedness suggest that the parents of a particular individual are more closely related than another individual with a lower internal relatedness. The high values of IR found in 2005 and 2010 could be an effect of the larger clusterisation and fragmentation of animals born in these two years as evidenced in STRUCTURE analysis (Appendix Figure 1) or of the use of particular closely related sires. In 2005 and 2010 we identified the two largest F_{IS} values and, interestingly, Battagin *et al.* (2010) also evidenced the two largest peaks of inbreeding according to pedigree information for the same years. Values of molecular coancestry, limited and stable over the years, together with F_{IS} , N_{AR} and H_e estimates are therefore a clear indication of the high diversity and variability present and conserved during the last two decades in the BUR breed. To further investigate the relationship of BUR with IHF in terms of presence of admixture and to detect changes in cluster assignment percentages over the analysed period we performed STRUCTURE analysis without any prior information on breed or birth year group. In a previous study, Dalvit *et al.* (2008) used 12 microsatellite markers to analyse genetic diversity in BUR and its relationship with IHF and Italian BRU breeds. The authors reported a pairwise F_{ST} distance between BUR and IHF of 0.047 and of 0.103 between BUR and BRU; moreover, the proportion of membership of BUR individuals was only 66% to the BUR cluster with a 30% of individuals assigned to the IHF cluster. In the present study, we did not detect any admixture with IHF breed as evidenced in Figure 3. At $K=2$ the two breeds clearly differentiated with proportion of memberships larger than 0.972 and, with the increase in the number of K clusters, we detected a more complex pattern only in BUR but the differentiation with IHF was maintained. The F_{ST} distance calculated between BUR and IHF was 0.116 ($P < 0.001$), therefore supporting the clear genetic differentiation among them. Moreover Nei's genetic distance estimate was 0.245 in the present study which

is similar to that obtained by Del Bo *et al.* (2001) using seventeen microsatellite markers (0.272). The differences obtained with the study from Dalvit *et al.* (2008) could be mainly explained by the larger number of loci used to characterise the two populations as well as by the larger number of individuals genotyped and farm sampled. STRUCTURE analysis was also implemented to evaluate possible changes in breed assignment percentages over the years. Each birth year group of animals was tested using the same IHF group as out-group to evaluate the presence, if any, of particular clusterisations of BUR animals. Results illustrated in Appendix Figure 1 shows that only for animals born in 2005, 2006 and 2008-2010 the more likely number of cluster describing the data was higher than two. The increase in the number of clusters only affected BUR sub-groups and no admixed individuals were found. The increase in the presence of substructures inside recent sub groups of BUR animals and the absence of admixture could be the result of genetic drift rather than to ancestral differences before the set up of the conservation scheme. These new findings clearly suggest that if crossbreeding with IHF happened between the Second World War and 1970s a nucleus of original alleles has been maintained over the years probably thanks to those small breeders which followed their own breeding scheme with the use of their own BUR sires therefore preserving over time different rare alleles. Overall, our results indicate that the management of BUR worked properly in the recent years. Paternity tests, the maintenance of herds with only purebred individuals also supported by public incentives together with the programme of restocking of reproductive males selected by breed experts for use in artificial insemination have avoided, in the last 19 years, the loss of genetic diversity in BUR.

Conclusions

Molecular markers were successfully used for monitoring the genetic variability inside the BUR cattle breed. To date, the conservation and the breeding scheme was mainly based on Herd Book information and no data were available to evaluate genetic variability and population structure and their variation over time. Our results indicate that, despite its limited diffusion, a high genetic variability and a low inbreeding are still present in the BUR breed and, more importantly, these values were maintained during the last two decades. These trends are positive indicators of the good planning and management of the conservation pro-

gramme and could be the basis for a systematic approach aimed at the improvement of the selection scheme, preferably with a higher number of markers, for the increasing of the value of this local breed while maintaining its genetic resources and, possibly, improving its breed identity.

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