GENETIC CHARACTERIZATION OF ALPINE SHEEP BREEDS

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ABSTRACT

The aim of this study was to characterize from the genetic point of view eight alpine sheep breeds reared in Italy (Bergamasca, Biellese, Schwarzbraunes Bergschaf and Tiroler Bergschaf), Germany (Brillenschaf and Weisses Bergschaf) and Slovenia (Bovška and Jezerzko-Solčavska) through the use of microsatellite molecular markers. Allelic richness was rather high in each breed highlighting a considerable genetic diversity. However, the study evidenced a significant departure from Hardy-Weinberg equilibrium in all analyzed breeds caused by a heterozygote deficiency. Such lack seems to be caused by a rather high level of inbreeding. The genetic differentiation among breeds was rather low ($F_{ST} = 0.064$) but significant. The neighbour-joining tree obtained from Reynolds' genetic distance estimates, showed the presence of three groups formed by the three Bergschaf breeds, the Italian Bergamasca and Biellese and the two Slovenian breeds together with the German Brillenschaf. Such grouping is in accordance with the breeds' region of origin and with their known history. Concluding, microsatellite resulted to be a useful tool to investigate breed variability and to characterize alpine sheep breeds. Obtained findings suggest the need to set up a conservation plan aiming to safeguard and increase the genetic variability of the studied breeds compromised by the high level of inbreeding. Microsatellites genotyping could help to monitor breed variability and to organize matings.

Key words: sheep / breeds / microsatellites / genetic characterization

GENETSKA KARAKTERIZACIJA ALPSKIH PASEM OVC

IZVLEČEK

Namen študije je bil opisati osem pasem ovac, rejenih v Italiji (bergamaška, bieleška, črno-rjava gorska in tirolska gorska ovca), Nemčiji (brillenschaff in bela gorska ovca) in v Sloveniji (bovška in jezersko-solčavska) z mikrosatelitskimi markerji. Visoko raznovrstnost alelov smo našli v vseh pasmah, kar kaže na pomembno genetsko raznovrstnost. V študiji je evidentiran značilen odstop od Hardy-Weinbergovega ravnovesja v vseh analiziranih pasmah kot posledica heterozigotnega primanjkljaja. Le-ta naj bi bil posledica visoke stopnje parjenja v sorodstvu. Genetsko razlikovanje med pasmami je bilo dokaj nizko ($F_{ST} = 0,064$) vendar značilno. Sosedsko povezovalno drevo, povzeto po Reynoldsovi oceni genetske razdalje, je pokazalo prisotnost treh skupin iz treh pasem gorskih ovc, italijanske bergamaške in bieleške in dveh slovenskih pasem skupaj z nemško brillenscahaff. Takšno grupiranje je v skladu z rejskimi izvornimi področji posameznih pasem in njihovim znanim poreklom. Mikrosateliti so torej primerno orodje za preučevanje raznolikosti pasem in za opredelitev alpskih pasem ovc. Dobljeni rezultati kažejo, da je potrebno narediti ohranitveni načrt za zavarovanje in povečanje genetske raznolikosti preučevanih pasem, ki so že tako ogrožene zaradi visoke stopnje parjenja v sorodstvu. Genotipizacija mikrosatelitov lahko pripomore pri spremljanju raznolikosti pasem in organiziranju parjenja.

Ključne besede: ovce / pasme / mikrosateliti / genetska karakterizacija

INTRODUCTION

Alpinet Gheep – Alpine network for sheep and goat promotion for a sustainable territory development – is a project developed by fifteen partners from Italy, Austria, Germany and Slovenia within the European Union cooperation programme Interreg IIIB Alpine Space. The project realised a specific survey (Feldmann *et al.*, 2005) on the consistency and diffusion of the main sheep and goat breeds in the Alpine area. The study revealed the presence of 60 different sheep breeds. A considerable number of them is under conservation programs while a not well defined number has already died out. Sheep and goat breeding in the Alpine area, has been decreasing continuously since about fifty years although it represents important economic, environmental and sociological issues. Sheep and goat production is frequently the only possible enterprise in marginal areas and play an important environmental role (Rancourt *et al.*, 2006). Moreover, in these less favourite areas, many typical products, especially cheeses, have been developed from sheep and goat milk and, they are often linked to one autochthonous breed (Scintu and Piredda, 2007). Considering the importance of sheep breeding in marginal areas, and the general level of endangerment of local populations, this work aimed to genetically characterize some Alpine sheep breeds located in three European countries (Italy, Germany and Slovenia). The study was carried out investigating nineteen microsatellite molecular markers. Microsatellites are particularly suitable for this kind of studies and have already been used in many different species (Jordana *et al.*, 2001; Dalvit *et al.*, 2008; Glowatzki-Mullis *et al.*, 2008). A deeper knowledge of the genetic variability and diversity of the analyzed populations will help to estimate their possible degree of endangerment and to suggest possible solutions for their conservation.

MATERIAL AND METHODS

Animal sampling and microsatellite analysis

A total of 306 individual blood samples were collected from the following populations: Biellese (BIE, 44), Bergamasca (BER, 45), Schwarzbraunes Bergschaf (SBE, 41), Tiroler Bergschaf (TBE, 30), Bovška (BOV, 40), Jezerzko-Solčavska (JSO, 49), Brillenschaf (BRI, 28) and Weisses Bergschaf (WBE, 29). Analyzed animals can be considered as a representative sample of the breed of origin as they were collected from different flocks trying to avoid closely related individuals; the number of sampled farms varied from 4 to 17. Blood samples were collected from each animal in 5 ml vacutainer tubes containing sodium citrate as anticoagulant, and stored at $-20\,°C$ until analyses were performed. DNA extraction was carried out employing the "Gentra System PUREGENE DNA purification kit" (Gentra System, Minneapolis, Minnesota, USA) starting from 300 µl of whole blood. DNA samples were then amplified by PCR in correspondence of the following 19 loci: OarAE54; OarFCB20, URB58, McM527, *INRA23*, TGLA53, MAF65, OarCP49, MAF214, HSC, INRA63, McM42, OarAE119, OarAE129, ILSTS087, OarFCB304, OarCP34, OarCP20 and CSRD247. The investigated loci were chosen, according to ISAG/FAO Standing Committee Recommendations (2004), and consulting a previous study on Austrian sheep breeds (Baumung *et al.*, 2006), aiming to analyze high polymorphic markers located all over the genome. Details about the protocol used for microsatellite amplification are available upon request. Allele size was determined with a CEQ^{TM} 8000 Genetic Analysis System (Beckman Coulter, Fullerton, California, USA).

Statistical analysis

Number of alleles per locus, allelic frequencies and observed and expected heterozygosity were calculated using Genetix version 4.05.2 (Belkhir *et al.*, 1996–2004). Exact tests for deviation from Hardy–Weinberg equilibrium (HWE) (Guo and Thompson, 1992) were applied using the Markov Chain Monte Carlo simulation (100 batches, 5 000 iterations per batch, and a dememorization number of 10 000) as implemented in GENEPOP version 3.4 (Raymond and Rousset, 1995). GENEPOP 3.4 was used also to test for population differentiation, for each locus an unbiased estimate of the Fisher's exact test was computed to verify if the allelic distribution was different between pair of breeds. The Fstat 2.9.3 software (Goudet, 1995) was employed in calculations of allelic richness (an estimation of mean number of alleles per locus corrected by sample size), gene diversity (Nei, 1987), and estimation of Wright's fixation index (Weir and Cockerham, 1984). Genetic differentiation among breeds was estimated through Reynolds' genetic distances. Reynolds' genetic distances were calculated by mean of PHYLIP package (Felsenstein, 1993–2002), they are the most suited distances for relatively closely populations like breeds in Europe which diverged during short times; in fact, in this case, the amount of mutations is negligible and the main factor to describe genetic variability is random drift (Eding and Laval, 1999). A neighbour-joining consensus tree was reconstructed and tree robustness was evaluated by bootstrapping over loci (1 000 replicates) using PHYLIP package (Felsenstein, 1993–2002), the dendrogram was depicted using the software package TreeVieew version 1.6.6 (Page, 2001).

RESULTS AND DISCUSSION

Firstly, it is worth mentioning that most of the breeds analyzed in this study have never been genetically characterized before, so comparison of results with previous literature is often difficult. In the analyzed breeds a total of 333 alleles were detected across the 19 investigated loci and all markers were found to be polymorphic in each of the 8 populations. MAF214 showed the highest number of alleles per locus (31) while OarCP34 the lowest (10); the mean gene diversity across loci was 0.820 evidencing the good level of information of the chosen microsatellite set.

Table 1.   Sample size, observed and expected heterozygosity, allelic richness and average F_{IS} in Biellese (BIE), Bergamasca (BER), Schwarzbraunes Bergschaf (SBE), Tiroler Bergschaf (TBE), Bovška (BOV), Jezerzko-Solčavska (JSO), Brillenschaf (BRI) and Weisses Bergscahf (WBE). A significant deviation from Hardy-Weinberg equilibrium was detected in each breed.

Breed	Sample size	H. exp. ± S.D.	H. obs. ± S.D.	Allelic richness	F_{IS}
BIE	44	0.794 ± 0.085	0.717 ± 0.159***	9.0	0.098
BER	45	0.782 ± 0.090	0.722 ± 0.149***	8.5	0.078
SBE	41	0.793 ± 0.071	0.728 ± 0.148***	8.9	0.083
TBE	30	0.765 ± 0.073	0.707 ± 0.177***	7.4	0.077
BOV	40	0.741 ± 0.113	0.682 ± 0.168***	7.7	0.081
JSO	49	0.765 ± 0.070	0.690 ± 0.132***	8.0	0.098
BRI	28	0.769 ± 0.084	0.672 ± 0.183***	8.4	0.128
WBE	29	0.731 ± 0.114	0.578 ± 0.151***	8.2	0.213

The allelic richness, the average number of alleles per locus corrected by sample size, ranged from 8.9 in BIE to 7.3 in TBE with an average of 8.2 alleles per breed (Table 1). Though, the

sheep breeds analyzed evidenced a considerable diversity; allelic richness was slightly higher than what observed by Peter *et al.* (2007) in a study on 57 European sheep breeds which included BER and WBE and by Tapio *et al.* (2005) in Northern European sheep breeds. Estimates of observed heterozygosity confirm the remarkable level of diversity evidenced in the alpine breeds; average observed heterozygosity varied from a maximum of 0.728 in SBE to a minimum of 0.578 in WBE. Overall heterozygosity estimates are comparable with what found in Swiss sheep breeds by Stahlberger-Saitbekova *et al.* (2001) and in Austrian sheep breeds by Baumung *et al.* (2006), while they are slightly higher compared to Spanish breeds analyzed by Alvarez *et al.* (2004). In each of the studied population a highly significant (P < 0.001) departure from HWE was detected, as shown in Table 1. This disequilibrium was caused by a significant heterozygote deficiency in each breed which was particularly high in WBE. In the overall population the homozygote excess (F_{IT}) was 0.159 ± 0.025, it was due in part to the genetic differentiation among breeds ($F_{ST} = 0.064 \pm 0.004$) and, to a bigger extent, to a significant homozygote excess within breeds ($F_{IS} = 0.111 \pm 0.006$). Positive F_{IS} estimates indicate either the presence of inbreeding and/or a Wahlund effect (presence of population substructure within breed) as observed by Pariset *et al.* (2003) and by Peter *et al.* (2007). As samples were collected from many flocks, presence of a hidden substructure cannot be excluded; to support this hypothesis, it is worth mentioning that WBE samples, showing the highest F_{IS} (0.213), were collected in 17 farms. A significant homozygote excess was observed also in other studies on sheep breeds (Pariset *et al.*, 2003; Mukesh *et al.*, 2006; Sodhi *et al.*, 2006). Authors agree that the management of flocks is the main region of this high level of inbreeding; in fact, rams are reared together with ewes allowing the mating with close relatives as daughters. A high inbreeding is risky as it could lead to genetic diseases and, moreover, it can strongly reduce animals fitness (Meszaros *et al.*, 1998). Exchanging rams among farms rearing the same breed could help to avoid this problem, as long as rams show a different genetic pool.
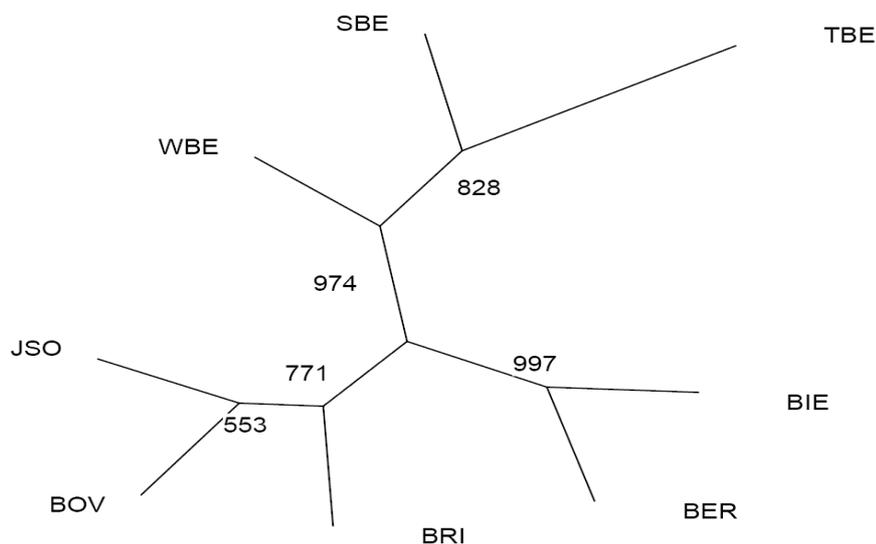

Figure 1. Neighbour-Joining tree obtained with 1000 bootstraps on Reynolds' genetic distances among breeds: Biellese (BIE) Bergamsca (BER), Tiroler Bergschaf (TBE), Schwarzbraunes Bergschaf (SBE), Bovška (BOV), Jezerzko-Solčavska (JSO), Brillenschaf (BRI), Weisses Bergscahf (WBE).

Dalvit, C. *et al.* Genetic characterization of alpine sheep breeds.

83

According to F_{ST} genetic distance estimates the closest breeds were BIE and BER (0.016) while the most differentiated were BOV and WBE (0.103) (data not shown). A significant (P < 0.001) differentiation between allelic distribution of all pairs of breeds was detected. On the whole, it is possible to say that the studied breeds showed a low but significant genetic differentiation; such results are in accordance with other studies on European and Middle-Eastern (Peter *et al.*, 2007) and Spanish (Alvarez *et al.*, 2004) sheep breeds. Baumung *et al.* (2006) obtained a higher F_{ST} value (0.08) among Austrian sheep breeds while Gizaw *et al.* (2007) evidenced a lower genetic differentiation ($F_{ST} = 0.046$) among Ethiopian sheep populations.

Fig. 1 shows the neighbour-joining consensus tree of Reynolds' genetic distance. Three clusters evidencing high bootstrap value were detected, one including the three Bergschaf breeds (Tiroler, Schwarzbraunes and Weisses), one including BRI, BOV and JSO, and one composed by BIE and BER. This clustering confirm previous knowledge on these breeds, in fact, SBE, TBE and WBE are very similar breeds, they were obtained crossing the Steinschaf sheep breed with the Bergamasca one but they fixed different wool colour (Pastore, personal communication). The high similarity between BIE and BER seem to be caused by the close geographic area in which these breeds are reared, moreover, it is known that crosses between them took place in the past (Pastore, personal communication).The third group is composed by the two Slovenian breeds, BOV and JSO and the German breed, BRI, with a bootstrap value of 90.8%. This grouping as well is in accordance with breed origins. In fact BRI, originated in the 18th century in formerly southern Carinthia (today Slovenia) from a local Steinschaf population, BOV and JSO had origins in the same area and BOV was obtained as well from crosses with the local Steinschaf population (Feldmann *et al.*, 2005).

CONCLUSIONS

In conclusion, obtained results suggest that the situation of analyzed breeds is risky as their variability is compromised by a high level of inbreeding, and some of them are already classified as endangered. An exchange of rams, owing different genetic pool, among farms rearing the same breed is advisable to increase the breed genetic variance. Molecular markers methods could be used as a tool to establish the genetic difference among rams in order to organise matings.

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