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BREED ASSIGNMENT TEST OF SLOVENIAN CATTLE BREEDS USING MICROSATELLITES

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ABSTRACT

A population analysis of 1,474 non-related animals (563 Holstein, 548 Simmental, 168 Brown cattle and 195 crossbreeds) was carried out using 12 microsatellite markers. The reallocation of known individuals to their breeds of origin and the assignment of unknown individuals was tested. A STRUCTURE analysis of all individuals revealed three genetic clusters corresponding to three breeds while crossbreeds mostly clustered with Simmentals and to a lesser extent to Holstein. The assignment and Migrants Detection test was carried out using GeneClass. The results from the assignment test revealed that in purebreds >98% animals were assigned to the correct breed. The crossbreeds were assigned to Simmental (85%) and Holstein (15%) as expected. Individual assignment tests provided excellent assignment success in all three highly differentiated cattle breeds (F_{ST} between 0.076 and 0.095) and could prove very useful for numerous forensic applications.

Key words: cattle / genetics / microsatellite markers / assignment test / Slovenia

1 INTRODUCTION

The assignment tests use genetic information to uncover the population membership of individuals, providing different methods of determining the population of origin of unknown individuals. The interest in applying the assignment methods has come from the population genetic investigations, and have practical applications, such as parentage analysis and tracing animals as well as animal products back to their breed of origin (Shackell *et al.*, 2001; Dalvit *et al.*, 2008a). The last application has been promoted by the increasing market demand for comprehensive and integrated food safety policies.

The use of DNA molecular markers, especially microsatellites but also single nucleotide polymorphisms (SNPs), for parentage testing (Koskinen, 2003; Kathiravan *et al.*, 2012) has been extensively investigated but only a few studies have explored their practical applications for tracing meat or dairy products at the breed level. DNA methods have already been proposed for such tracing (Ciampolini *et al.*, 2006; Dalvit *et al.*, 2008b). The use of multiallelic microsatellite markers is quick, simple and a relatively low number of markers need to be analysed to provide high assignment success.

In this study, the efficiency where a set of 12 microsatellites was able to allocate individual cattle to their original breed was evaluated. The performance was compared with different Bayesian and frequency-based allocation methods implemented in GeneClass and STRUCTURE programmes when applied to the data of 1,474 calves in Slovenia.

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2 MATERIAL AND METHODS

2.1 SAMPLES AND DNA ISOLATION

Hair root samples of 1,474 calves from the Slovenian cattle population were collected between 1 January 2010 and 1 January 2012. Calves were randomly selected from the whole newborn cattle population to provide non-related individuals. The animals belonged to three breeds: 563 Holstein, 548 Simmental, and 168 Brown while the remaining 195 animals were crossbreeds between Simmental and Holstein.

Genomic DNA was extracted using a commercial kit (NucleoMag 96 Tissue; MACHEREY-NAGEL GmbH & Co.) on MagMax (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions.

2.2 GENOTYPING

Twelve microsatellite loci (SPS115, TGLA122, INRA23, TGLA53, TGLA227, BM1824, ETH10, TGLA126, BM2113, ETH3, ETH225 and BM1818) have been used and described by van de Goor *et al.* (2009). The loci were genotyped using the Bovine Genotypes™ Panel 1.2 (Thermo Scientific). The commercial kit was used according to the manufacturer's instructions.

Fragment analysis of PCR products was performed on an ABI3130xl Genetic Analyzer. Alleles were called using a GeneMapper (version 4.0) program (Applied Biosystems, Foster City, CA, USA), and named according to ISAG nomenclature.

2.3 PARENTAGE VERIFICATION

Parentage was checked using a self-developed program in Oracle, and detected deviations between genotypes and pedigrees were checked manually. Mismatched samples were re-isolated, and re-analysed, in some cases individual loci PCRs were performed. This also increased the reliability of the analysis and excluded the possibility of detected mutations.

2.4 GENETIC STRUCTURE AND ASSIGNMENT TEST

Null alleles and their effect on F_{ST} values were estimated using the FreeNA program with 1,000 replications (Chapuis and Estoup, 2007). The basic population parameters were calculated in FSTAT (Goudet, 2001). The genetic structure of the analysed samples was determined by STRUCTURE that identifies clusters without a priori knowledge of the population structure. The program runs Markov chain Monte Carlo (MCMC) simulations to partition individuals into K clusters. The basic assignment criteria are the minimization of Hardy-Weinberg and linkage disequilibria (Pritchard *et al.*, 2000). For runs estimating ln Pr(X|K) under a certain K, runs with 100,000 steps (preceded by 20,000 burn-in steps) were repeated seven times for each K.

The accuracy of assigning individuals to the breed of origin based on genotype data was studied using individual assignment tests, implemented in the program GeneClass (Piry *et al.*, 2004). The program includes several assignment methods, but only the Bayesian statistical approach was applied due to its known efficacy with a threshold value for a successful individual assignment set at 0.99 (Cornuet *et al.*, 1999).

3 RESULTS AND DISCUSSION

3.1 POPULATION ANALYSIS AND NULL ALLELE ESTIMATION

All breeds were in Hardy-Weinberg equilibrium (HWE) ($P < 0.001$), and the pairwise F_{ST} values (Weir and Cockerham, 1984) were all significant ($P < 0.01$). The average F_{ST} value was 0.084 (0.064 including crossbreeds). The observed heterozygosity was highest in crossbreeds and Holsteins (0.73), compared to slightly lower values in other breeds (Table 1). The observed heterozygosity in the Holstein breed is similar (0.73 vs 0.72) to that found in the study by van de Goor *et al.* (2011) in a Holstein population. They estimated population parameters using the same set of microsatellite loci. The observed heterozygosity in other breeds ranged between 0.55 and 0.75. We found no null alleles with an estimated frequency above the 0.05 threshold.

3.2 GENETIC STRUCTURE

The dataset of 1,747 calves and 12 markers was examined using the STRUCTURE program under different assumptions of the number of population clusters (K from 1 to 10) without any pre-assignment of the population affiliation. The calculation of ln Pr(X|K) from the output, as described by Evanno *et al.* (2005), produced a modal value of the statistic at K = 3, that matches with the 3 main breeds: Holstein, Simmental and Brown (Fig. 1).

STRUCTURE infers the proportion of ancestry (q) of every cluster for every individual. While most individuals had a major (defined as $q > 0.5$) contributor cluster

Table 1: *Genetic diversity indices of microsatellite marker data*

Breed	N	H_E	H_O	A	F_{IS}
Holstein	563	0.73	0.73	9.5	0.002
Simmental	548	0.72	0.73	9.6	−0.007
Brown	168	0.70	0.71	7.2	−0.002
Crossbreed	195	0.73	0.73	9.2	0.005

N: number of individuals; H_E: expected heterozygosity in the population; H_O: observed heterozygosity; A: average number of alleles per loci; F_{IS}: values showed no statistically significant deviations from HWE ($P < 0.001$)

corresponding to a known breed, some deviations were detected. In the Holstein breed, 11 individuals were identified as Simmental and 3 as Brown (in total 98% identified with a corresponding breed), of the Simmental breed, 4 were identified as Holstein and 1 as Brown (in total 99% identified with a corresponding breed). All individuals of the Brown breed were identified as the Brown breed. The main contributors to the crossbreeds were Simmental (165 individuals), Holstein (23) and Brown (1), while 6 crossbreed individuals had any contributing genetic cluster with $q > 0.5$.

The results of the STRUCTURE program are comparable with the findings obtained by Negrini *et al.* (2007) and Dalvit *et al.* (2008a) in studies on breed assignment in Italian Cattle breeds and slightly lower than what was found by Koskinen (2003) in dog breeds.

3.3 ASSIGNMENT TEST

Using 12 microsatellite markers, the individual assignment tests provided 98.9% assignment success in the Holstein breed and 99.1% in the Simmental breed. The best assignment success (100%) was in the Brown breed. Crossbreeds were assigned to the Simmental (85%) and the Holstein (15%) breeds as expected.

Maudet *et al.* (2002) and Dalvit *et al.* (2008a) were able to assign a breed designation to unknown individuals with an assignment success between 67% and 100% using STR data from 6 to 23 microsatellites from 4 to 7 cattle breeds. The assignment method and the confidence of the assignment test greatly influenced the assignment success.

Given the high assignment success, the number of loci could be reduced. Ciampolini *et al.* (2006) showed that a given predetermined level of statistical significance (e.g. < 1%) can be obtained with a number of microsatellite loci <15 for a substantial share of the animals. This means that a sequential test based on the consecutive typing of several sets of markers (say, 5 markers per set) could decrease the number of total typed genotypes substantially, while keeping constant the value of statistical significance.

A chosen set of markers can be applied in agriculture for the traceability of animals or animal products (e.g. assignment of a carcass, an embryo, sperm to a breed, or a milk sample). According to the EU legislation (regulations EC 1760/2000 and 1825/2000), every beef cut must show a label carrying the following information: an identification code referring to an animal or to a group of animals, and the country where the animal was born, fattened, slaughtered, and sectioned (Dalvit *et al.*, 2008b). An assignment test could be useful when a more powerful and secure identification is required, for example, in the recall of all animal cuts in the event of health risks. The use of microsatellites for paternity testing has been extensively investigated (Baron *et al.*, 2002; Koskinen, 2003) and the impact of misidentification on

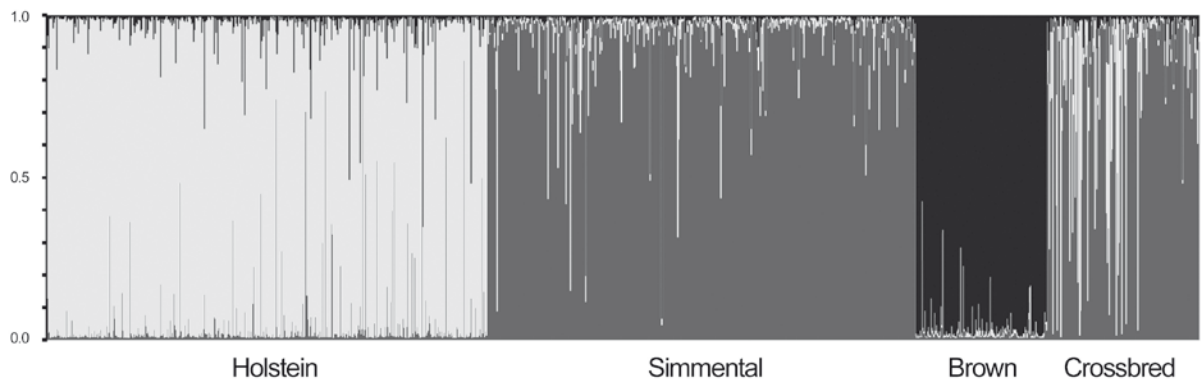

Figure 1: *Estimated population structure as inferred by the STRUCTURE analysis of the microsatellite markers DNA data. The most probable number of clusters (K = 3) is based on ΔK the method (Evanno et al., 2005). A different colour represents different genetic groups or clusters. Each column represents an individual and the amount of a given colour in a column reflects the relative contribution (q) of a given cluster to its genetic makeup.*

selection was demonstrated (Baron *et al.*, 2002; Visscher *et al.*, 2002).

Breed conformation is of special value for meat and dairy products coming from more than one individual. For example, milk from several cows is used to produce cheese. Allele frequencies from a pooled DNA sample can be calculated (Schnack *et al.*, 2004) and compared to known breed allele frequencies. Such a test, currently under development, would be of special interest to monitor the origin of traditional or geographically protected food products.

4 CONCLUSIONS

The presence of null alleles in the microsatellite set used in this study was recorded previously (van de Goor, 2011), but none was confirmed in the breeds examined in this study.

The individual assignment tests provided excellent assignment success in all three highly differentiated cattle breeds. Different studies suggest it could prove very useful for numerous forensic applications. For example, they could be applied for determining the breed of origin of individuals (or tissues), as has been attempted in some earlier studies (Dalvit *et al.*, 2008b; Ciampolini *et al.*, 2006).

The results also indicate that STRUCTURE software and GeneClass can be complementary tools to assess breed integrity and assignment. Before practical implication the optimization of the cost: benefit of the assignment test should be optimized.

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