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Application of a panel of microsatellite markers for the genetic traceability of bovine origin products

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INTRODUCTION

Traceability: what and why

Traceability is defined as the ability to maintain a credible custody of identification for animals or animal products through various steps within the food chain from the farm to the retailer (McKean, 2001). In particular, this term was defined by the EC regulation 178/2002 as "the ability to trace and follow a food, feed, food producing animal or ingredients, through all stages of production and distribution". In the last few years traceability has become important for consumers that nowadays are very careful concerning their nutrition. This consumers' lack of confidence, in particular towards food of animal origin, is due to several reasons including both food safety and socio-economical changes. The B.S.E. (Bovine Spongiform Encephalopathy) has certainly been the most serious food scandal of the last years causing a drastic reduction of beef consumption in all Europe, it was then followed by the dioxin crisis and avian influenza in the poultry sector (Ciampolini *et al*., 2000, Goffaux *et al*., 2005), in addition, also the incidence of food borne diseases due to microbial contamination of processed food has increased in the last decades (Opara & Mazaud, 2001). Besides these "food scandals" also socio-economical reasons have contributed to increase people interest in what they eat and in how and where it is produced. At present, consumers are more aware than before of ecological and environmental matters and the demand for organic food and for products obtained in an eco-sustainable way has increased (Opara & Mazaud*,* 2001), nevertheless the industrialization processes, as well as the market globalization, has made impossible people direct control of food making (Milanesi *et al*., 2003). All these reasons contribute to the need of finding a system to trace food products. Traceability is the answer to the consumers' demand of transparency and it is becoming synonymous of safe and quality food.

The European legislation on traceability sector

The European Community (E.U.) has always paid great attention to food safety problems. One of the reasons is that the agro-alimentary sector is very important for European economy. The E.U. is the biggest producer of food products and beverages in the world (White Book, 2000) with a food and beverages industries production of 15% of the total E.U. manufacturing output (corresponding to 600 billions Euro). The second reason can be found in the Roma Treaty (1957) which instituted the E.U., stating that one of its aims is the "achievement of a high level of health protection" and "the strengthen of consumers' protection". So, food safety measures have always been present in the E.U. legislation but, in the last years, in particular

after the first B.S.E. outspread in 1997, the legislation had been implemented in order to be faithful to its aims regarding health protection and to gain again consumers' trust.

The three most important E.U. documents regarding food safety are the Green Paper (1997), the White Paper (2000) and the European Regulation 178/2002 (applied from 1st January 2005); in particular with the latter a traceability system has been introduced in the food sector and it is now mandatory, even if for the beef sector such system already existed thanks to the Regulations 1760/2000 and 1825/2000 established soon after the B.S.E. crisis.

It is important to notice that not only E.U. has such a strict legislation regarding traceability of food products, in particular Japan as well has strict rules, above all for the beef sector, traceability is mandatory, at least for exported beef, also in Brazil, Australia, Argentina and Canada even if with different depth and systems while in the U.S.A. traceability is still on a voluntary basis (Smith *et al*., 2005).

Conventional and geographical traceability

Traceability systems are mandatory in all the E.U member countries and, as described before, it is particularly important for livestock and animal origin products. Anyway there are several types of traceability depending on how it is obtained and on which information it gives. The so-called conventional traceability consists on the labelling system such as in the beef sector and on the management of processed food by batches. It is extremely useful for keeping the individual information of each animal and it is less expensive and easier to achieve than other methods but, being based on tags, passports and papery documents (see the beef sector), it could be counterfeit (Cunningham & Meghen, 2001). Geographic traceability instead has another purpose, in fact it does not aim to identify an individual but to identify the geographic origin of a product through the study of "track elements" such as volatile compounds or microbial flora (Bailoni *et al*., 2000, Pillomel *et al*., 2003, Mauriello *et al*., 2003, Franke *et al*., 2005); it is particular interesting in products as cheeses and beef produced in typical, often mountain area.

Genetic traceability

Genetic traceability is based on the identification of both animals and their products through the study of DNA, it permits different level of identification: individual, breed and species.

Genetic traceability is based on some DNA characteristic (Mackie *et al*., 1999, Cunningham & Meghen, 2001):

- DNA is inalterable during the all animal life
- DNA is stable to the different treatments of processed food
- DNA is present in every cell of the organism

Once the DNA is extracted from the chosen matrix (it can either be animal tissue, blood, muscle, hair, sperm, faeces or even a processed food such as cheese) it is analysed by molecular markers; since the introduction of PCR (Polymerase Chain Reaction) many different markers have been discovered and studied but at present the most widely used are microsatellites and SNP (Single Nucleotide Polymorphism) (Mariani *et al*., 2005).

Individual genetic traceability

Traceability on an individual level, found its best application on the beef sector. Indeed, the European Regulation 1760/2000 assure beef traceability until the animal is alive but, at the slaughter-houses, there could be mistakes both voluntary and involuntary especially when the origin of different anatomic cuts must be maintained. Several studies were conducted on this topic, all of them aimed to analyse the DNA fingerprinting of a sample of animals, once obtained the fingerprinting the "match probability" (M.P.) was calculated. M.P. is the probability to find two individuals sharing, by chance, the same allelic profile at the studied loci (Weir *et al*., 1996). The most widely used markers are microstaellites (Peelman *et al*., 1998, Sancristobal-Gaudy *et al*., 2000, Arana *et al*., 2002, Fernando-Vazquez *et al*., 2004, Herraeza *et al*., 2005, Orrù *et al*., 2006) and SNP (Heaton *et al*., 2002, Heaton *et al*., 2005, Herraeza *et al*., 2005). All these researches revealed the efficacy of both markers for individual traceability with different results depending on the type, number of chosen markers and level of polymorphism, anyway M.P was always inferior to one over one million. As molecular analyses are quite expensive is indeed necessary to find few highly polymorphic markers able to discriminate well the individuals in order to reduce the costs (Orrù *et al*., 2006); besides costs the other problem that must be solved for implementing such system is the organization of the beef chain, in fact it will be necessary to create "banks" conserving a sample (e.g. hairs) of each animal to analyse in case of problems.

Breed genetic traceability

Breed genetic traceability permits to assign or exclude the breed of origin to a product. Such ability is of great importance as today there are many typical products, some protected by the European label PDO or PGI, that are made by one breed only or that cannot be made with some breeds. Some examples are the PDO Parmigiano Reggiano "Vacche Rosse" Italian cheese produced only with milk obtained by the Reggiana dairy cows (Gandini and Oldenbroek, 1999), the PDO Fontina Italian cheese made only with Valdostana breed. There are also examples regarding the meat industry such as the Cinta Senese ham and lard obtained only with this typical Italian pig breed or the Spanish Jamon Serrano made only with Iberian pig breeds (Garcìa *et al*., 2006). The list could be long and it is essentially made up of typical products of Mediterranean countries such as France, Italy and Spain (Pancaldi *et al*., 2005), for this reason most of the studies are performed in such nations. It is important to underline that these products are usually very ancient and their preservation becomes as well the protection of old traditions and cultures, moreover the utilized breeds are often typical and endangered and their only chance to survive is their use for the production of typical and high quality products. So researches regarding breed genetic traceability are often linked with studies about breed characterization (Ovilo *et al*., 2000, Ciampolini *et al*., 2000, Maudet *et al*., 2002, Carriòn *et al*., 2003, De Marchi *et al.*, 2003) and, sometimes, also conservation through the use of molecular markers methods (Alderson *et al*., 2004).

To assign an individual or a product to a breed two approaches are possible:

- Deterministic: it consists on finding molecular markers with different allelic variants fixed in different breeds; in this way it will be possible to develop simple analyses protocols without the need of statistical inference (Milanesi *et al*., 2003). That is the case of several studies regarding the MC1R gene, responsible of coat colour in many species (Klungland *et al*., 1995, Rouzaud *et al*., 2000, Kriegesmann *et al*., 2001, Maudet *et al*., 2002, Carriòn *et al*., 2003, Crepaldi *et al*., 2003, Russo *et al*., 2004, Crepaldi *et al*., 2005), and the research of breed specific AFLP markers (Alves et al., 2001, Negrini et al., 2003).
- Probabilistic: it consists on utilizing set of markers with typical allelic frequencies in different breeds (Milanesi *et al*., 2003). The breed assignment is obtained by statistical methods based on maximum likelihood functions (Paetkau *et al*., 1995), Bayesian methods (Rannala *et al*., 1997) and genetic distances methods (Cornuet *et al*., 1999).

The researchers' role in this field should be the reduction of analyses cost even if molecular methods should be used not as standard control but as "super control", anyway the Parmigiano Reggiano "Vacche Rosse" example shows that the product-breed link is able to improve the breed economic profitability (Gandini and Oldenbroek, 1999).

Species genetic traceability

Studies regarding species genetic traceability are particularly important for the dairy sector to certify the milk and, mainly, the cheese origin; in fact several reasons may lead to fraudulent use of cows' milk in goat, ewe or buffalo cheese, these motives could be the higher prices or the greater seasonal fluctuations in yield in species either than cows milk (Maudet & Taberlet, 2002). The EU, with the Regulation 1086/1996, established a reference method for the detection of cows' milk based on isoelectric focusing of γ-casein, however, protein-based methods for species identification may fail after excessive proteolysis or heat-induced denaturation of the indicator proteins; DNA methods could be the solution for such problem as this molecule persist in ripened cheese (Plath *et al*., 1997). Several studies were performed using different methods based on the study of the β-casein gene (Plath *et al*., 1997) or on different region of the mitochondrial DNA (Bania *et al*., 2001, Maudet & Taberlet, 2002).

Species genetic traceability is promising also for the fishery industry, in fact, as stated by the EU Regulation 2065/2001 the fish must be labelled with the species name even if it is processed. The majority of the researches concern the study of mitochondrial DNA as it is smaller than the genomic and it could be analyzed faster, moreover it is present in the cells in more copies (Mackie *et al*., 1999). Most of the studies utilize the gene coding for the cytocrome b in tuna and salmon species (Bartelett *et al*., 1991, Unseld *et al*., 1995, Quintero *et al*., 1998, Russel *et al*., 2000, Rehbein *et al*., 2005).

Application of a genetic traceability method: a field trial on beef and dairy cattle breeds

At the Department of Animal Science of Padova University was conducted a study to test the efficacy of a panel of twelve microsatellite markers for the genetic traceability of bovine products (milk and beef). As it was explained, in the bovine sector, both individual and genetic traceability are important; the first one can guarantee beef safety responding to the consumers' confidence crisis, while the second could become a useful instrument for the valorisation of typical breeds and their products. Two dairy breeds: Holstein Friesian (HF) and Brown Swiss (BS) (cosmopolitan dairy cow) and four Italian beef breeds: Chianina (CHI), Marchigiana (MAR) and Romagnola (ROM) (typical breeds of the centre of Italy, all derived from *Bos primigenius)* and Piemontese (PIE) (typical breed of the Piedmont region in the north-west of Italy derived from *Bos brachyceros)* have been analysed.

Different kind of matrixes were chosen for DNA extraction (blood, milk and muscles) to verify: a-the possibility to genotype even starting from a small quantity of DNA (milk); b-to check for some mistakes along the beef chain as in the case of beef where blood and muscles were collected separately; c-the efficacy of the microsatellites panel for individual traceability (defined by M.P.) and for breed traceability using two methods one based on maximum likelihood and the other based on a Bayesian statistical approach.

MATERIALS AND METHODS

Population sampling

Six cattle breeds were analysed, two dairy breeds: HF (n=41) and BS (n=53) and four typical Italian beef breeds: CHI (n=24), MAR (n=23), ROM (n=22) and PIE (n=24). For all animal blood samples were collected in 5 ml vacutainer tubes containing sodium citrate. In addition for dairy and beef breeds milk and muscle samples were respectively collected; the final dataset consisted on 374 samples that were stored at –20°C until the analyses were performed.

DNA extraction and PCR amplification

The DNA extraction from blood was carried out utilizing the "Gentra System PUREGENE DNA purification kit" starting from 300 µl of whole blood, while extraction from muscles was carried out utilizing the "Gentra Systems PUREGENE DNA purification kit" for solid tissue. DNA extraction from milk was obtained as well utilizing a modification of the "Gentra System PUREGENE DNA purification kit" starting from 200 µl of milk; in particular milk was added to a tube containing 800 µl of Cell Lysis Solution (50 mM Tris HCl Ph 8, 20 mM EDTA, 2% SDS) and, to obtain maximum yield 6 µl of Proteinase k (20 mg/ml) were added. A RNase treatment was performed adding 6 µl of RNase solution (final concentration 0,04 µg/µl) and incubating at 37°C for one hour. To obtain protein precipitation 400 µl of Protein Precipitation Solution (Ammonium Acetate 10 M) were used and, after centrifugation, 800 µl of 100% Isopropanol were added to the supernatant in order to precipitate the DNA that then was washed with 300

µl of 70% Ethanol and resuspended in 40 µl of sterile water. DNA quantification was obtained through electrophoresis on agarose gel.

Once obtained and diluted, the DNA was amplified by PCR in correspondence of the 12 microsatellite loci studied. The studied loci were chosen in accordance to FAO recommendations: 8 loci out of 12 are part of the FAO list for cattle (FAO, 2004) and consulting the biblyography (Table 1) in order to have high polymorphic markers spread all over the genome. For the amplification 3 µl of DNA (about 25 ng), were added to a reaction mix containing: 1 pmol/µl of primer forward and reverse, 1X PCR buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris HCl pH 8,8, 0,01% Tween 20), 0,26 mM of every dNTPs, 2,5 mM of MgCl2 and 0,04 U/µl of *Taq* DNA polymerase, in a final volume of 25 µl. For each locus the forward primer was labelled with a fluorocrome in order to analyse the obtained PCR products by capillary electrophoresis. The 12 microsatellites were individually analysed by a PX2 Thermohybaid thermal cycle at the following conditions, the X temperature is the annealing t° of each primer:

Number of cycles	**Phase**	**Temperature**	**Time**
1	Initial denaturation	95°C	5′
	Denaturation	94°C	30′′
40	Annealing	X°C	1′
	Extension	72°C	1′
1	Final extension	72°C	10′

Amplicons were diluted to better achieve a quantity of 10 ng necessary for the capillary electrophoresis, to optimise the cost of analyses four multiplex were constituted. For the analyses the sequencer ABI 3730XL (Applied Biosystem) was used and the electrophoretic profiles were read through the use of the Genescan and Genotyper software (Applied Biosystem).

Statistical analyses

Genetic variability of markers and populations were studied; observed (Ho) and expected (Het) heterozigosity, Polymorphism Information Content (PIC), mean number of allele per locus were calculated for each locus and over all loci for each breed utilizing the POPGENE (Yeh and Boyle, 1997) and the CERVUS (Marshall, 1998) software. Exact test for Hardy-Weinberg equilibrium and for population differentiation were performed through the use of the GENEPOP 3.3 (Raymond and Rousset, 1995) software, while F-statistics were calculated with the FSTAT 2.9.3 (Goudet, 2001) software.

Individual identification through the use of match probability formula was calculated according to Weir *et al*., (1996). Two different approaches were implemented for breed assignment: one was based on a maximum likelihood method and performed by the WHICHRUN 3.2 (Banks and Eichert, 2000) software while the second was based on a Bayesian approach implemented in the STRUCTURE 2.1 (Pritchard, 2000) software.

Table 1 Microsatellite loci utilized for the analyses and their characteristics.

Locus		Primer sequence	Cromosome	T° annealing (°C)	Fragment lenght (bp)
BM1818	FW	AGCTGGGAATATAACCAAAGG	23	58	257-279
	RW	AGTGCTTTCAAGGTCCATGC			
MM 12	FW	CAAGACAGGTGTTTCAATCT	9	58	110-134
	RW	ATCGACTCTGGGGATGATGT			
ETH185	FW	TGCATGGACAGAGCAGCCTGGC	17	64	216-242
	RW	GCACCCCAACGAAAGCTCCCAG			
TGLA126	FW	CTAATTTAGAATGAGAGAGGCTTCT	20	58	116-130
	RW	TTGGTCTCTATTCTCTGAATATTCC			
ILST0008	FW	GAATCATGGATTTTCTGGGG	14	60	173-178
	RW	TAGCAGTGAGTGAGGTTGGC			
SPS115	FW	AAAGTGACACAACAGCTTCTCCAG	15	64	247-261
	RW	AACGAGTGTCCTAGTTTGGCTGTG			
RM12	FW	CTGAGCTCAGGGGTTTTTGCT	7	58	103-107
	RW	ACTGGGAACCAAGGACTGTCA			
TGLA053	FW	GCTTTCAGAAATAGTTTGCATTCA	16	62	151-183
	RW	ATCTTCACATGATATTACAGCAGA			
BL42	FW	CAAGGTCAAGTCCAAATGCC	13	62	231-237
	RW	GCATTTTTGTGTTAATTTCATGC			
ETH3	FW	GAACCTGCCTCTCCTGCATTGG	19	62	98-126
	RW	ACTCTGCCTGTGGCCAAGTAGG			
BM203	FW	GGGTGTGACATTTTGTTCCC	27	58	207-237
	RW	CTGCTCGCCACTAGTCCTTC			
TGLA122	FW	CCCTCCTCCAGGTAAATCAGC	21	58	136-182
	RW	AATCACATGGCAAATAAGTACATAC			

RESULTS AND DISCUSSION

Correspondence of blood-milk and blood muscles profiles

The genotypic profiles obtained from the different matrixes of the same animal were checked to verify if there was a correspondence between them. For the dairy breeds animals a sample of blood and one of milk were analysed separately and the profiles were compared. It resulted that the 7.4% of the samples presented different profiles at three or more loci, while the 15.9% presented different profiles at one or two loci. Regarding the correspondence between blood and muscles collected from beef breeds, 9.7% of samples presented different profiles at three or more loci while the 8.6% of profiles differed at one or two loci. It was assumed that samples differing at more than three loci were probably mismatched during the collection and they were discarded from the statistical analyses. Probably, in samples differing at one or two loci a mismatch occurred during the PCR preparation or mistakes were made during the allele assignment, it was decided to keep them anyway for further analyses but the differing loci were not considered. It must be said that collection of milk or muscles is preferable than blood, in fact there is no need for a veterinary and it is less stressing for the cows but much more care must be taken, though, collecting samples during milking or at the slaughter-houses could be confusing and at high risk of contamination. Moreover these results proved that there were genotyping errors suggesting that this aspect must be taken into account when proceeding with the statistical analyses.

Genetic variability

In the six analyzed breeds a total of 115 alleles were detected with an average of 9.6 allele per locus, the most polymorphic locus was TGLA122 with 18 detected alleles while RM12 was the least with only 3. The average Ho and Het were equal to 0.62 and 0.68, respectively, ranging from 0.34 (RM12) to 0.74 (MM10) the former and 0.38 (RM12) to 0.88 (TGLA53) the latter. The PIC over all loci was 0.63 ranging from 0.31 (RM12) to 0.86 (TGLA53). In Table 2 are shown the genetic variability results per breed. The mean number of alleles varied between 5.2±2.3 in the ROM and 6.2±3.1 in the HF breed; Ho varied from a minimum of 0.56±0.21 in the BS breed to a maximum of 0.67±0.19 in the PIE breed. The PIE breed showed a higher level of heterozigosity compared to CHI and HF as reported by Orrù *et al.,* (2006). Results are also comparable to that obtained by Canon *et al.,* (2001) in a study concerning Mediterranean beef cattle breeds performed with 16 microsatellite markers.

Table 2 Observed (Ho) and expected (Het) heterozigosity, mean number of alleles and their standard deviation (SD) for all breeds.

Breed	Number of samples	Ho± SD	Het ± SD	Mean number of alleles ± SD
Brown Swiss	51	0.56 ± 0,21	0.58 ± 0,22	5.5 ± 2.5
Holstein Friesian	36	0.65 ± 0,18	0.61 ± 0.17	6.2 ± 3.1
Chianina	23	0.65 ± 0.14	0.66 ± 0.13	5.7 ± 2.6
Marchigiana	21	0.66 ± 0.15	0.67 ± 0.13	5.7 ± 2.6
Romagnola	19	0.60 ± 0.19	0.61 ± 0.18	5.2 ± 2.3
Piemontese	21	0.67 ± 0.19	0.68 ± 0.15	5.9 ± 2.6
Total	171	0.62 ± 0.14	0.68 ± 0.15	9.6 ± 4.7

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Test for Hardy-Weinberg equilibrium revealed that for any locus a homozygote excess was found and the Fis inbreeding coefficient for the entire population was equal to 0.02. Anyway only for TGLA126 a significant heterozygote excess ($P<0.05$) was found, in particular in the HF breed ($P<0.01$). Analysing the results by breed it was also observed that in the ROM and BS breed three loci (BM1818 and TGLA126 for ROM and TGLA122 for BS) showed a significant heterozygote deficit ($P<0.05$) despite of these results no deviation from Hardy-Weinberg equilibrium was observed in any of the studied breeds (data not shown). Data seem to be in contrast with what obtained by Ciampolini *et al*. (1995) who found a particular situation of unbalance in the PIE, probably due to two markers that were not used in this study.

Private alleles were found in each breed (Table 3) in particular almost 22% of detected alleles were found in one breed only; HF showed the most of them (8) in particular alleles 162 and 170 of TGLA122 had a high frequency (12.5% each) and were probably the responsible of the heterozygote excess. The MAR breed showed only one present with a low frequency (2.5%) while allele 156 of TGLA122 was present only in the PIE breed and it was the private allele with the highest frequency (21.4%).

On the bases of allelic frequencies and differences in the fixed alleles the Fst index (Weir and Cockerham, 1984) was calculated. The Fst index is a measure of population subdivision and

resulted equal to 0.094 meaning that almost 10% of the total genetic variability can be attributed to differences among breeds while the remaining 90% was due to individual variability within breed. Such results are in accordance with what found in literature for European cattle breeds (Canon *et al*., 2001; Maudet *et al*., 2002, Ciampolini *et al*., 2006) where Fst values ranged from 0.7 to 0.9. The significance of breed differences tested using the exact test for population differentiation based on allele frequencies were highly significant ($P<0.001$) between all pair of breeds.

Table 3 Private alleles found in the six breeds.

Locus/Breed[1]	BS	HF	CHI	MAR	ROM	PIE
BM1818	**279** (0,02)					
ETH185		**216** (0,01)		**226** (0,03)		
		235 (0,10)				
		237 (0,01)				
BM203	**237** (0,09)		**213** (0,09)			
	229 (0,02)					
TGLA122	**154** (0,05)	**162** (0,13)	**168** (0,07)		**158** (0,03)	**156** (0,21)
		170 (0,13)	**174** (0,02)			**180** (0,02)
		182 (0,04)				
ILST0008					**178** (0,03)	
					173 (0,03	
SPS115					**261** (0,03)	
ETH3			**106** (0,07)			**98** (0,03)
						261 (0,03)
TGLA53		**155** (0,06)				
		183 (0,06)				

[1]BS = Brown Swiss, HF = Holstein Friesian, CHI = Chianina, MAR = Marchigiana, ROM = Romagnola, PIE = Piemontese. Bold numbers are the names of the alleles expressed in base pair and the numbers in brackets are their frequencies.

Animal identification

The probability to find, by chance, two individuals sharing the same genotype is called match probability (MP), it was calculated for each locus and for different set of markers and is illustrated in Table 4. To calculate MP the population was divided into two groups, dairy breeds (HF and BS) and beef breeds (CHI, MAR, ROM and PIE). In fact differences in the number of allele per locus and on their frequencies led to different informative content per locus in the two groups. For dairy breeds the most informative microsatellite was ETH185 while for beef breeds it was TGLA53. MP was also calculated for the two groups considering different set of markers built using microsatellites which showed the best MP. It is possible to observe that choosing the 5 most informative markers the probability to find two identical individuals was of about 1E-06 for both groups (one over one million) and about 6E-07 for the entire population. The MP calculated considering the all population was always lower than that calculated for the two distinct groups probably due to the genetic structure of the sample; in fact it was demonstrated that Fst index influences significantly the power of assignment of markers; the more the breeds are differentiated the less loci are needed for a correct assignment (Bjørnstad

and Røed, 2002); in this case the Fst index of the entire studied population was higher than that obtained for the two subgroups evidencing a lower differentiation within dairy breeds and beef breeds (data not shown), in accordance to Bjørnstad and Røed (2002).

Table 4 Match probability calculated for each locus for dairy breeds, beef breeds and for the all population (left side) and match probability calculated with different panel of markers in dairy, beef and for the all population (right side).

Locus	Dairy breeds	Beef breeds	All breeds	N° loci	Dairy breeds	Beef breeds	All breeds
BM1818	0.1633	0.1075	0.1252	12	1.5686E-10	1.88877E-11	1.12722E-11
ETH185	**0.0529**	0.0477	0.0400	11	2.49506E-10	4.91925E-11	2.47139E-11
MM12	0.0911	0.1049	0.0879	10	6.13664E-10	1.36071E-10	6.66096E-11
TGLA126	0.2229	0.0756	0.1187	9	1.56438E-09	3.79171E-10	1.91698E-10
BM203	0.0587	0.1699	0.0895	8	7.01777E-09	2.23132E-09	1.27474E-09
TGLA122	0.0544	0.0957	0.0701	7	3.38073E-08	1.58918E-08	8.99612E-09
RM12	0.6287	0.3840	0.4561	6	1.85992E-07	1.41637E-07	7.18402E-08
ILST008	0.4066	0.3589	0.3710	5	1.13877E-06	1.31791E-06	6.05454E-07
SPS115	0.1818	0.1404	0.1417	4	1.24999E-05	1.25603E-05	6.76202E-06
ETH3	0.2076	0.1122	0.1504	3	1.68993E-04	1.31223E-04	7.69058E-05
TGLA53	0.0740	**0.0364**	**0.0274**	2	2.87952E-03	1.73663E-03	1.09779E-03
BL42	0.3923	0.3615	0.3475	1	5.29143E-02	3.63701E-02	2.74268E-02

Breed assignment

Results on breed assignment obtained implementing a "maximum likelihood method" are shown in Table 5. Results are grouped considering different error percentage, choosing a percentage of error lower than 1% only the 43% of samples are correctly assigned to their breed of origin. The 82% of BS breed samples had a correct assignment while only the 14% of MAR animals were assigned correctly; on the other hand no incorrect assignments were found. Choosing a higher error percentage more individuals were assigned correctly but the numbers of samples incorrectly assigned also increased. In particular considering an error rate lower than 10%, the 5% of MAR and PIE the 10% of ROM animals were assigned to another breed.

Table 5 Proportion of individuals assigned to their breed or to another through a probabilistic approach based on a "maximum likelihood" function in the six breeds:

	Correct assignment			Incorrect assignment		
Error %	<1%	<10%	no threshold	<1%	<10%	no threshold
BS	0.82	0.92	0.98	0.00	0.02	0.00
HF	0.56	0.80	0.94	0.00	0.00	0.06
CHI	0.30	0.52	0.87	0.00	0.00	0.13
MAR	0.14	0.33	0.62	0.00	0.05	0.33
ROM	0.53	0.84	0.84	0.00	0.10	0.05
PIE	0.24	0.52	0.86	0.00	0.05	0.09
TOTAL	0.43	0.65	0.85	0.00	0.04	0.11

BS = Brown Swiss, HF = Holstein Friesian, CHI = Chianina, MAR = Marchigiana, ROM = Romagnola, PIE = Piemontese.

It is interesting to notice that beef animals were never assigned incorrectly to dairy breeds while the only BS individual incorrectly assigned (P<10%) was assigned to the PIE breed.

Percentage of correct assignment revealed to be quite low in particular for beef breeds; this result may be due to the fact that such breeds are not very differentiated and have similar characteristics, in particular the MAR breed had been crossed in the past with the CHI and the ROM breeds (Ciampolini *et al*., 1995).

In order to obtain better breed assignment a Bayesian statistical approach was performed. The STRUCTURE 2.1 software allows, first of all, the determination of the most probable number of populations in the whole dataset and then it calculates the proportion of membership of each individual to the inferred clusters; in this way one can assign the animal to the population for which such proportion is higher. Four models based on different assumptions were evaluated; only one model assumed as the most probable number of population six (admixture model with independent allelic frequencies) while all the other assumed five as the most probable number of populations (data not shown). The models giving the better assignment are those assuming that there was no admixture among the populations meaning that individuals had not a mixed ancestry but came purely from one population. The same model was chosen also by Milanesi *et al.* (2003) in a similar study concerning different dairy and beef breeds.

In Table 6 the proportion of individuals correctly assigned to their breed of origin is shown; an average of 62% of animals was correctly assigned ranging from 29% in the PIE breed to the 92% of the BS considering an error rate of 1%. Comparison of these results with the ones obtained with the maximum likelihood method revealed that the Bayesian approach seemed to be better evidencing correct attribution always higher than the first approach. Also in this case the higher percentages of correct attribution were obtained for dairy breeds confirming a lower differentiation among beef breeds. The higher power of attribution was already proved by Cornuet *et al.,* (1999) in a study comparing the power of discrimination of different approaches.

Table 6 Proportions of individuals correctly assigned to their breed of origin or to another breed through a Bayesian approach in the six breeds:

	Correct assignment			Incorrect assignment		
Threshold	>99%	>90%	no threshold	>99%	>90%	no threshold
BS	0.92	0.96	0.98	0.00	0.00	0.02
HF	0.81	0.94	0.94	0.00	0.00	0.06
CHI	0.87	0.96	1.00	0.00	0.00	0.00
MAR	0.43	0.67	0.90	0.00	0.00	0.10
PIE	0.29	0.57	0.90	0.00	0.05	0.05
ROM	0.42	0.74	0.84	0.05	0.05	0.05
TOTAL	0.62	0.81	0.93	0.01	0.02	0.05

BS = Brown Swiss, HF = Holstein Friesian, CHI = Chianina, MAR = Marchigiana, ROM = Romagnola, PIE = Piemontese.

CONCLUSIONS

The first results of this study evidenced the possibility to use milk or beef samples to organize a genetic traceability system; anyway it was pointed out that genotyping errors were present

suggesting to be careful when proceeding with statistical analysis. Mismatch errors were also found underling the importance of a particular care while collecting samples.

In conclusion this study demonstrated the efficacy of the chosen microsatellite set for individual traceability while the discriminating power of such set for breed assignment should be improved. Regarding individual identification four markers (TGLA53, ETH185, TGLA122 and MM12) could be used to achieve a probability of finding two identical individuals equal to six over one million if beef and dairy breeds are considered together. This study evidenced as well the importance of population differentiation for individual identification as it was already pointed out by other researches (Bjørnstad and Røed, 2002). The Bayesian approach considering populations having no common ancestors and independent allele frequencies was the best one for breed assignment, nevertheless if a percentage of error of 1% is chosen the correct assignment are not very satisfactory (62% on average) in particular for beef breeds. This result can be explained by the fact that assignment method seemed to be influenced by differentiation among populations, number of studied loci and number of analysed samples (Cornuet *et al*., 1999 and Bjørnstad and Røed 2002) suggesting to add more samples per breed and to prove more loci. In particular it could be interesting to change the least informative loci (RM12 and ILST008) with more polymorphic ones in order to had informative power without increasing the cost of analysis.

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