Lamb meat traceability: The case of Sambucana sheep

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Abstract

Genetic traceability has a key role in the product certification, but it is rarely implemented in sheep so far, especially in the fresh meat sector. In this study, the case of the Sambucana sheep is analysed with the aim of developing a genetic system able to certify the origin of its traditional product, the Sambucano lamb, protected by a registered trademark. A set of 14 microsatellite markers was identified as an efficient tool to genetically discriminate the Sambucana sheep from other breeds potentially involved in mislabelling and to allow for an effective allocation test of meat cuts labelled as 'Guaranteed Sambucano lamb'. The paternity test proved to be an additional means to improve the reliability of the control. The traceability system here described is easy to implement in local minor sheep breeds and is recommended in the framework of meat certification.

Key words: genetic traceability, meat, sheep, Sambucana breed

1. Introduction

The conservation of valuable local breeds is a worldwide recognized imperative to contrast the loss of genetic resources in livestock species, which are of vital importance to agriculture, food production, rural development and the environment (FAO, 2007). Of the several actions suggested to preserve the existing biodiversity (FAO, 2013), the valorization and protection of typical products derived from local minor breeds is now playing a prominent role, for increasing consumer awareness of food nutritional properties and safety. Also socio-cultural and ethical implications lead the consumers to choose products derived from traditional breeds, because they are linked to a specific area of origin, and so representative of historical and geographical identity (Montossi et al., 2013). The increased interest in the origin of lamb meat for the consumer decisions has been demonstrated by several studies, especially in Europe (Font i Furnols et al., 2011; Hersleth et al., 2012). Therefore, the link 'breed-product' can become an effective means to satisfy the consumer's expectations, which in turn can contribute to improve the self-sustainability of the breed.

Italy has a wide variety of local breeds, from which an extraordinary richness of typical products are derived. In order to protect their peculiarity, many of them have obtained the EC labels (Protected Designation of Origin, PDO, or Protected Geographical Indication, PGI), and many others are recognized by registered trademarks. Most of them concern dairy products or meat-derived products, while very few concern fresh meat.

One interesting example in this sector is represented by the Sambucana sheep, an autochthonous breed traditionally reared in Cuneo province (Piedmont Region, Italy), mainly for meat production, with the commercialization of lambs weighing between 18 to 20 kg. The massive crossing with the Biellese breed in the middle of the last century caused a drastic decline of the Sambucana, with 1400-1600 heads and about 60 purebred rams estimated at the end of the '70s (Brooke and Ryder, 1978). To avoid its definitive loss, a conservation programme was started in 1985, which included the foundation of the Breeders' Association in 1988 and the establishment of the Centre for the ram selection, as well as many other supporting activities. As a result the breed size increased, with 126 rams and 2733 ewes registered in 2014, distributed in 49 herds in the Piedmont Region (Asso.Na.Pa., 2014). The conservation of the breed took great advantage from the valorization of the meat production, resulting in the inclusion of the Sambucano lamb in the Traditional Farming Products of the Piedmont Region (Regione Piemonte, 2016) and in the Slow Food Presidia (Slow Food Foundation, 2016). In 1992, the 'Guaranteed Sambucano lamb' trademark was registered at the Chamber of Commerce of Cuneo, and managed by a farmers' cooperative framework dealing with marketing the product.

In this context, the certification of the breed of origin becomes necessary for the sake of product protection, because the high commercial value of the labelled product could bring about the risk of fraudulent mislabeling. In the case of fresh meat, unintentional errors can also occur along the production chain, from the birth of the animal to the butcher's shop, passing through several steps where the animal identification may be replaced by another identification.

Among the different tools for breed traceability, the DNA-based methods are widely recognized as the most powerful (Dalvit et al., 2007; Scarano and Rao, 2014; Sentandreu and Sentandreu, 2014), because they can be applied at any stage of the production chain to assign a given product to a given breed on the basis of their genetic similarity using a set of markers. So far, breed allocation based on molecular markers has been profitably applied for meat traceability in different animal species, including cattle (Dalvit et al., 2008; Rogberg-Muñoz et al., 2014) and pigs (Garcia et al., 2006; Oh et al., 2014), while in sheep molecular traceability systems are still difficult to implement for the high cost of genotyping relative to

the economic value of the single animal, especially in minor breeds, which usually suffer from lack of funds.

The Sambucana breed is an interesting case-study also because the traceability system could benefit from the distinctive organization of the supply chain, as the production cycle occurs in a well-defined geographic area, data are recorded along the whole chain and the slaughtered lambs derive from a limited number of rams. In this situation, the establishment of a database with the genetic profiles of the used rams could be useful to implement a paternity test in order to verify if the profile of a meat cut is compatible with one of the Sambucana rams, hence confirming the assignment to the breed.

Based on all these considerations, the aim of our study was to develop a genetic traceability system for fresh sheep meat applied to the control of the 'Guaranteed Sambucano lamb' supply chain, by investigating the different aspects concerned: i) resolving power of a set of microsatellite markers; ii) genetic differentiation of the Sambucana breed from other Italian sheep breeds, including those potentially involved in the traceability system; iii) breed assignment and paternity test of meat cuts purchased in different shops and labelled as 'Guaranteed Sambucano lamb'.

2. Material and methods

2.1. Samples and DNA extraction

Blood samples were collected from Sambucana subjects (n=58) and from other nine Italian sheep breeds purposely chosen: Biellese (n=57), reared in the same Region and often used for crossing, potentially mislabeled as Sambucana; Sarda (n=20), also potentially involved in mislabeling, being the most widespread Italian breed and marketed in the whole country; Frabosana (n=22), Saltasassi (n=13) and Savoiarda (n=20), minor Piedmontese breeds, for which possibility of crosses with breeds reared in the same area exists; Bergamasca (n=23), often used in the past for crossbreeding to improve the Sambucana for meat production; Appenninica (n=25) and Merinizzata (n=22), meat breeds reared in a different area; Comisana (n=30), as representative of a dairy breed. Individuals as little related as possible were sampled. In addition, blood samples were collected from 186 Sambucana rams, representing most of the males used for reproduction in the production area, and meat cuts labelled as 'Guaranteed Sambucano lamb' (n=49) were anonymously purchased from butcher shops located in the Piedmont region.

Genomic DNA was extracted from blood and meat samples using respectively the NucleoSpin Blood and the NucleoSpin Tissue kits (Macheray-Nagel, Düren, Germany), according to the manufacturer's instructions.

2.2. Markers and genotyping

Fifteen microsatellite markers, included in the panels suggested by the International Society for Animal Genetics (ISAG) and/or FAO, were amplified: OarCP049, OarFCB304, CSRD247, INRA063, HSC, MAF214, McM527, OarFCB020, D5S2, MAF065, INRA023, TGLA53, ETH10, ETH225, BM1824 (FAO/ISAG, 2004). Fragments were resolved in a DNA sequencer ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The allele size was assigned using the Genemapper 4.0 software (Applied Biosystems, Foster City, CA) and the allele nomenclature was standardized using reference samples from the Comparison Tests organized by the ISAG.

2.3.Statistical analysis

The marker informativeness was evaluated across breeds by computing the number of observed and effective alleles using the Popgene software version 1.32 (Yeh et al., 1999). The FSTAT 2.9.3.2 software (Goudet, 2002) was used to analyse the single-locus F_{ST} and the

linkage disequilibrium between loci. The Polymorphism Information Content (PIC) of each marker was also calculated (Nagy et al., 2012). The within-population variability was estimated by the allele frequencies, observed number of alleles per locus, allelic richness, as well as observed and expected heterozygosity, using the FSTAT 2.9.3.2 software (Goudet, 2002).

Using the same software the between-breed diversity was evaluated by the pairwise fixation index (F_{ST}) and the global F_{ST}. The sequential Bonferroni correction (Rice, 1989) was applied to correct for the effects of multiple tests. To analyse in more details the population differentiation the Structure 2.3.4 software (Pritchard et al., 2000) was also employed, conducting the analysis with the admixture model, Locprior option (considering the sampling location, basically the Region where the breed is reared), correlated allele frequencies, burn-in 200,000 and MCM iteration of 500,000. The results were graphically displayed using the DISTRUCT program (Rosenberg, 2004). The same procedure was used for the assignment of declared 'Guaranteed Sambucana lamb' meat samples, but in this case only the Piedmontese breeds as well as Bergamasca and Sarda were included in the test. In addition, the Cervus 3.0.3 software (Kalinowski et al., 2007) was employed to calculate for each meat sample the likelihood of paternity assignment to one of the tested rams.

3. Results and discussion

3.1. Marker statistics

The number of observed and effective alleles, together with the Polymorphism Information Content and the Fixation index of the 15 markers used are reported in Table 1. A total of 198 alleles were observed, with a mean of 13.2/locus, ranging from 4 (ETH10) to 21 (CSRD247 and INRA063). The number of the effective alleles was in general much lower (1.19 ÷ 7.83, mean value 4.60), for the very low frequency of many alleles. Fourteen loci had PIC values exceeding 0.5 (0.59 ÷ 0.86), which was indicated as the limit for considering a marker highly informative (Botstein et al., 1980), while ETH10 had a value of 0.17, so poorly contributing to the characterization of the within-breed variation, as shown for other sheep breeds also (Lasagna et al., 2011). Moreover, the F_{ST} value for ETH10 locus (Table 1) was also low, indicating a limited variability of this locus across-breed. Therefore, due to the low discriminating power, ETH10 was excluded from subsequent analyses. The other loci had F_{ST} values ranging between 0.040 (INRA023) and 0.108 (MAF214).

The test for linkage disequilibrium indicated that the considered loci are not linked, so suitable for downstream applications.

3.2. Within-breed variability

The population statistics are shown in Table 2. The mean number of observed alleles showed a wide range, between 3.33 in Saltasassi and 9.27 in Biellese, which also had the lowest (0) and the highest (12) number of private alleles, respectively. As these data can indicate true differences in the allele distribution, but they are influenced by unequal number of sampled individuals for each population, the allele richness was computed. In this case less variable results among breeds were obtained. The low number of alleles in Savoiarda and Saltasassi was confirmed. Coherently with the history of the breeds, the lowest degree of heterozygosity, taken as a measure of genetic variability, was observed in Sarda (0.53), which is the most intensively selected among the considered breeds, and in Saltasassi (0.49) and Savoiarda (0.55), which have the lowest breed size (Asso.Na.Pa., 2014).

3.3. Between-breed variability

The overall F_{ST} value was 0.082 (P = 0.001), which means that about 8% of the total genetic variability is due to between-breed differences and the remaining part is attributed to

differences between individuals. According to Balloux and Lugon-Moulin (2002), the observed value should be interpreted as indicative of a moderate between-breed variation. However, a survey on 57 sheep breeds with a wide geographic distribution – so potentially depositary of high genetic diversity – reported an F_{ST} of 0.057 (Peter et al., 2007). Therefore, the level of genetic differentiation found among the Italian breeds investigated in this study can be regarded as quite high.

All the pairwise F_{ST} values were also significant (P = 0.001), confirming the considerable level of genetic differentiation among these breeds (Table 3). The lowest value (0.026) was observed for the comparison between Sambucana and Biellese, consistently with the past crossing programs. The highest values (> 0.10) were found for comparisons involving Saltasassi and Savoiarda breeds: the low genetic effective size, that induced the reduction of the within-breed variability highlighted in this study, contributed to enhance their differentiation from the other breeds.

The results of the population structure are reported in Fig. 1, where the bar plots for *K* values ranging from 2 to 9 are shown. Each animal is represented by a vertical line, divided into segments whose size and color indicate the relative proportion of the animal genome corresponding to a particular cluster. With *K* = 2 to 4 the Piedmontese breeds were allocated in a single cluster, clearly separated from the other more heterogeneous breeds. The origin from the same ancestral population and the sharing of the same geographical area with possible past admixture events could be the reasons for their clustering together. However, with increasing *K*, clusters including Savoiarda, Frabosana and Saltasassi were sequentially separated. The genetic drift, consequence of their low population size (Asso.Na.Pa., 2014), could have contributed to increase the genetic differentiation between these breeds. Only with *K* = 9, the Sambucana was separated from Biellese, even if a degree of admixture with this breed was present in several samples. As for the other breeds, a mixed status was still evident for Appenninica and Merinizzata, which experienced frequent crossing with the same breeds, including Berrichon du Cher, Ile de France and Bergamasca to a lesser extent (Bigi, 2008).

3.4. Meat sample assignment

To evaluate the reliability of the meat sample assignment only the breeds possibly involved in mislabeling were included in the test with Sambucana, that is the other four Piedmontese breeds as well as Bergamasca and Sarda.

The results obtained with the Structure software for *K* values from 2 to 7 (Fig. 2) showed that, from *K* = 5, Sambucana and meat samples were included in the same cluster and differentiated from all the other breeds. The test indicated that about 82% of the meat samples were included in this cluster. The remaining samples were allocated to the Biellese (10%), Bergamasca (4%) and Sarda (2%) clusters, while 2.0% of the samples were not assigned to any cluster. Another result seems worth underlying for its practical implications: no individuals belonging to other breeds were assigned to the Sambucana cluster. As a whole, these results imply a certain risk of excluding a ‘true’ Sambucana meat cut as derived from the breed, but a negligible risk of accepting a ‘false’ Sambucana meat cut as ‘true’.

With the paternity test, the genotype of each meat cut was compared against the genotypes of the candidate fathers to find the most likely ‘true’ parent: 32 meat samples were assigned to one of the 186 tested rams at 95% confidence and 13 at 80% confidence, while four samples remained unassigned (two of them were not assigned to any breed with the Structure test also).

As for the samples unassigned with the paternity test, different hypotheses can be put forward. First, the meat samples are really not derived from the Sambucana breed, due to unintentional errors or voluntary mislabeling. As detailed data are recorded all along the supply chain of the ‘Guaranteed Sambucana lamb’ (e.g., flock of origin of the lambs, flocks

where the rams were used, butcher shops where the carcasses were delivered), additional information could be useful to verify this hypothesis and identify the possible critical points. Second, the meat sample might be derived from a Sambucana lamb whose father was not present in the group of the tested rams. The development of a complete and continuously updated database with the genetic profile of the used rams could avoid this situation. Of course, the probability of finding the true father could be increased if the mother's genotype were known. However, at present the extra-costs to genotype the ewes prevent its implementation in a minor breed such as Sambucana. Third, the effectiveness of the set of markers might not be high enough to allow for the identification of the likely true father. In this respect it must be taken into account that the discriminatory power certainly is an intrinsic feature of the markers, but it is also depending on the inbreeding level of the breed, which decreases the genetic variability, hence increasing the similarities among individuals (Fernández et al., 2013). So the presence of related subjects among the putative fathers could negatively affect the discriminatory power of the markers used. This can be the case for the Sambucana breed, where the animals now existing emerged after a severe reduction of the population size. Therefore, even if the microsatellites used for this study showed a quite high degree of variability, the inclusion of additional markers might contribute to improve the results of the paternity test.

Another aspect to be evaluated is the possible role of the private alleles for the authentication of the product. In this respect it should be considered that an allele is useful for a highly reliable allocation/exclusion test when it is private *and* fixed, which means to be present in the homozygous state in all the individuals of one breed and absent in all the individuals of the other breeds. The private alleles found in the present study lack the latter characteristic, having frequencies ranging from 0.009 to 0.087, so they are of little use for traceability purposes.

4. Conclusions

The genetic traceability can profitably complement the conventional traceability of lamb meat in order to certify the origin of the traditional products. The set of markers used was able to genetically discriminate the Sambucana sheep from other breeds potentially involved in mislabelling and to allow for an effective allocation test in order to protect the labelled 'Guaranteed Sambucana lamb'. Moreover, the genotyping of the Sambucana rams used for reproduction made it possible the implementation of the paternity test, which proved to be an additional means to improve the reliability of the control system. Therefore, the procedure here described is recommended in the framework of the product certification, at least for random controls aimed at discouraging fraud.

In general, the study suggests a genetic traceability system easy to implement in local minor sheep breeds. Of course, financial efforts are needed to support such activities, but an economic return in the medium-term can be expected. In fact, a reliable certification of origin, together with an appropriate communication, will certainly contribute to the valorisation of traditional breeds, giving them a competitive advantage, with positive economic and social effects.

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Table 1
Marker characteristics: chromosomal location (Chr), number of observed alleles (Na), number of effective alleles (Ne), polymorphism information content (PIC), fixation index (F_{ST}).

Locus	Chr	Na	Ne	PIC	F_{ST}
OarCP049	17	18	4,60	0.75	0.050
OarFCB304	19	17	3,64	0.67	0.061
CSRD247	14	21	5,90	0.80	0.096
INRA063	14	21	6,12	0.81	0.070
HSC	20	17	7,11	0.85	0.051
MAF214	16	17	3,05	0.60	0.108
McM527	5	11	4,01	0.71	0.079
OarFCB020	2	13	5,57	0.79	0.081
D5S2	5	8	3,47	0.66	0.050
MAF065	15	9	4,05	0.70	0.100
INRA023	1	14	7,83	0.86	0.040
TGLA53	12	13	7,15	0.84	0.099
ETH10	5	4	1,19	0.17	0.039
ETH225	9	10	2,67	0.59	0.089
BM1824	1	5	2,94	0.60	0.066

Table 2
Breed statistics: mean number of observed alleles (Na), allele richness (AR), number of private alleles (PA), expected heterozygosity (He), observed heterozygosity (Ho).

Breed	Na	AR	PA	He	Ho
Sambucana	8.73	4.43	7	0.68	0.62
Biellese	9.27	4.77	12	0.71	0.65
Frabosana	6.47	4.50	5	0.70	0.65
Saltasassi	3.33	2.97	0	0.51	0.49
Savoiarda	4.93	3.53	2	0.56	0.55
Bergamasca	6.47	4.58	5	0.71	0.68
Appenninica	6.80	4.41	2	0.68	0.63
Merinizzata	6.67	4.75	2	0.73	0.68
Comisana	7.07	4.71	8	0.71	0.64
Sarda	6.07	4.14	5	0.66	0.53

Table 3
Pairwise and global fixation index (F_{ST}).

Breed*	SAM	BIE	FRA	SAL	SAV	BER	APP	MER	COM	SAR
SAM	0.000									
BIE	0.026	0.000								
FRA	0.069	0.053	0.000							
SAL	0.109	0.100	0.149	0.000						
SAV	0.095	0.118	0.128	0.155	0.000					
BER	0.060	0.035	0.071	0.156	0.126	0.000				
APP	0.087	0.053	0.105	0.180	0.168	0.067	0.000			
MER	0.048	0.032	0.047	0.126	0.119	0.037	0.063	0.000		
COM	0.074	0.056	0.068	0.144	0.163	0.071	0.079	0.045	0.000	
SAR	0.048	0.045	0.067	0.130	0.135	0.070	0.097	0.049	0.059	0.000
Global F_{ST} = 0.082 (P = 0.001)										

* SAM, Sambucana; BIE, Biellese; FRA, Frabosana; SAL, Saltasassi; SAV, Savoiarda; BER, Bergamasca; APP, Appenninica; MER, Merinizzata Italiana; COM, Comisana; SAR, Sarda

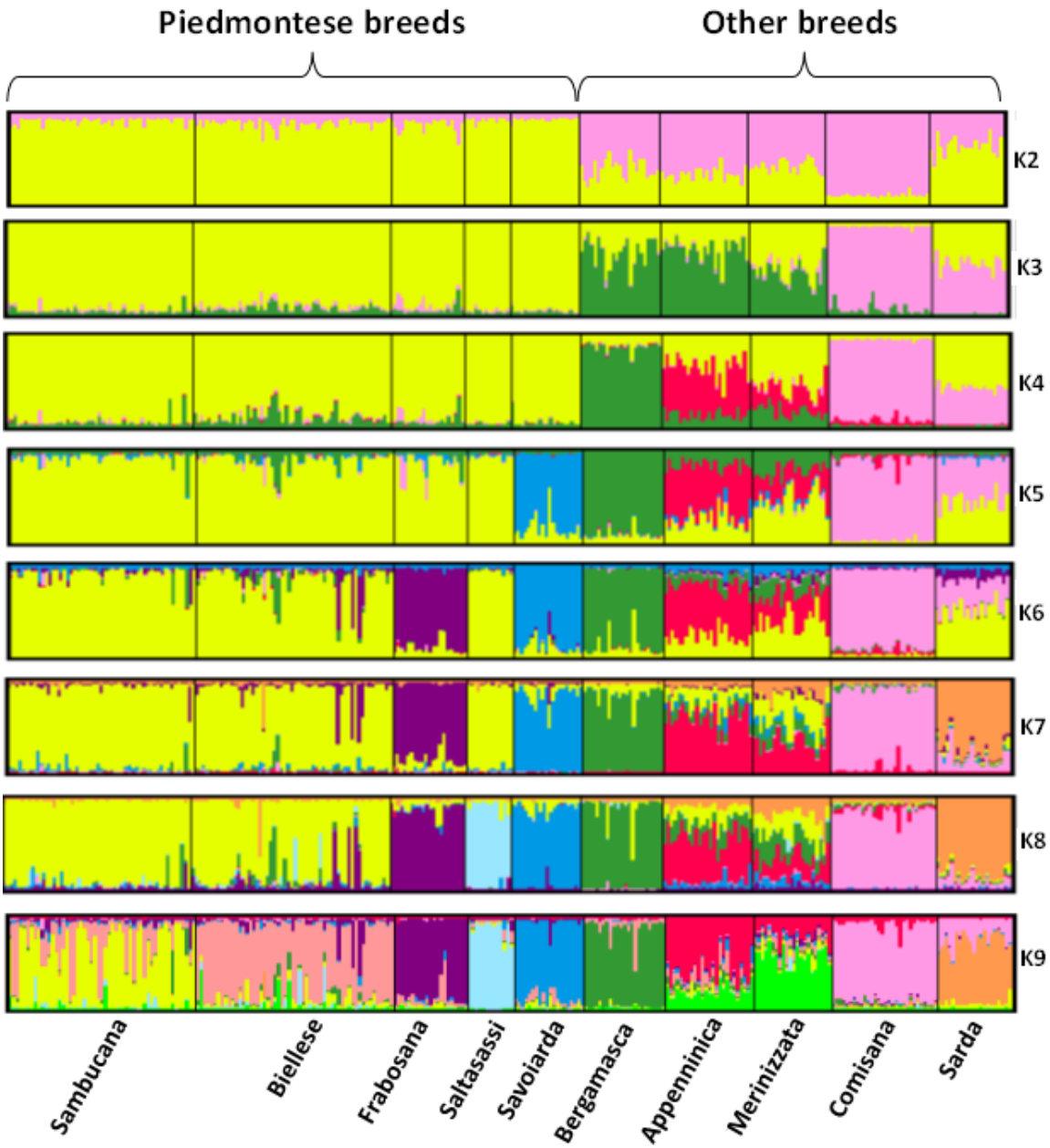

Fig. 1. Structure plot for clustering analysis of the 10 Italian sheep breeds studied. K = number of simulated clusters.

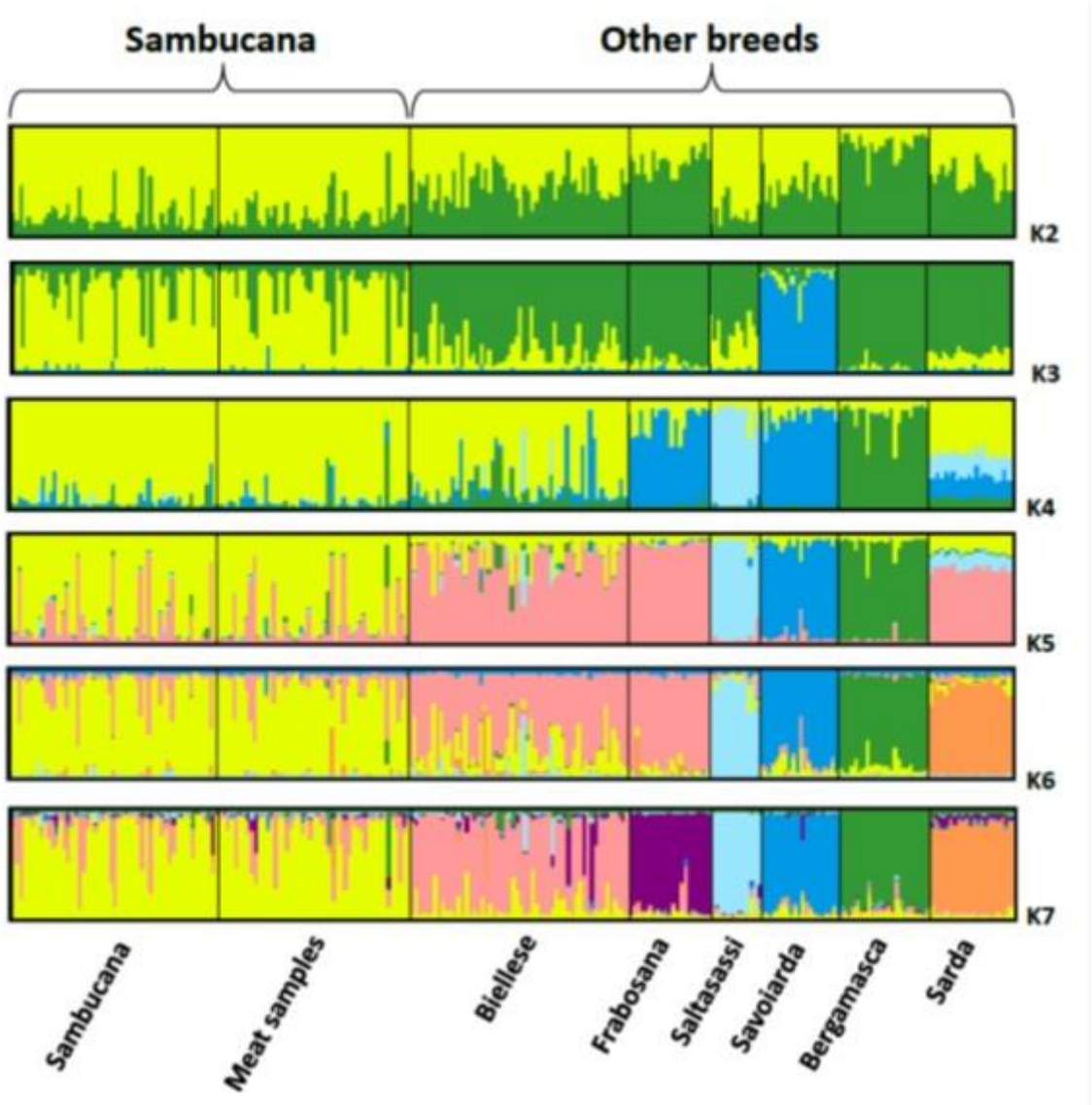

Fig. 2. Structure plot for clustering analysis of 'Guaranteed Sambucano lamb' meat cuts and 7 sheep breeds. K = number of simulated clusters.