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Traceability of four European Protected Geographic Indication (PGI) beef products using Single Nucleotide Polymorphisms (SNP) and Bayesian statistics

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ABSTRACT

The use of SNPs in combination with Bayesian statistics for the geographic traceability of cattle was evaluated using a dataset comprising 24 breeds from Italy, France, Spain, Denmark, the Netherlands, Switzerland and UK genotyped with 90 polymorphic markers.
The percentage of correct assignment of the individuals to their Country of origin was 90%, with an average assignment probability of 93% and an average specificity of 92%. The higher value was observed for UK breeds (97% of correct assignment) while Swiss animals were the most difficult to allocate (77% of correct assignment).
Tracing of Protected Geographic Indication (PGI) products, the approach correctly assigned 100% of Guaranteed Pure Highland Beef; 97% of "Vitellone dell'Appennino Centrale" breeds; 84% of Ternera de Navarra, and 80% of Boeuf de Chalosse.
Methods to verify Products of Designated Origin (PDO) and Protected Geographic Indication (PGI) products will help to protect regional foods and promote the economic growth of marginal rural areas by encouraging the production of high quality niche market foods.

1. Introduction

By the late 1990s, a new geographical diversity of agro-food emerged. While the globalization of trade in foodstuffs continues apace, Europe has experienced an increasing interest in foods with local and regional identities. Local agri-food production systems have indeed been characterized by various strategies to promote local/regional food products (Murdoch, 2000; Goodman, 2004; Marsden, Banks, & Bristow, 2002; Ilbery & Maye, 2005). This trend has led to legislation in Europe to provide legal protection to regional foods, through the 'PGI' (Protected Geographic Indication) and 'PDO' (Protected Designation of Origin) labels (European Union Regulation (EEU) 2081/92). The former are products produced, processed and prepared in a specific geographic area using defined materials and methodology. The latter are products in which at least one stage of production is covered by the geographical protection. In both cases the geographic component is the key aspect of the protection. The aims of this legislation were: to support diversity in agricultural production, to protect consumers by giving them information on the specific characteristic of the product and to protect product names against fraud and imitation (Ilbery & Kneafsey, 2000; Parrott, Wilson, & Murdoch, 2002). Legislation of appropriate methods to ensure "traceability" is indeed essential and plays a key role in any modern food safety control and verification system for products. An effective traceability system contributes to prevent frauds, provides an effective method for the assessment and management of food risk, facilitates disease control procedures and contributes to consumer confidence in product safety.

DNA-based methods offer the possibility to identify animals and animal derived foodstuffs at different taxonomic levels, from single individuals, to breeds or population, species and higher taxon, along the food chain from the farm to consumption. Therefore, they can provide a way to verify the accuracy of traditional identification methods such as ear tagging animals and product labelling. DNA-based approaches have also several further advantages over systems based on paper audits: (i) DNA is a relatively stable molecule even when treated at high temperature (up to 120 °C); (ii) the results of a DNA assay is independent of age and sex; (iii) testing can be carried out starting from a wide variety of biological

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materials; (iv) the results are highly repeatable and can be easily standardized and automated (Lenstra, 2003).

Recent advances in high-throughput DNA sequencing and bioinformatics have facilitated the identification and validation of large collections of Single Nucleotide Polymorphisms (SNPs) in a number of species (Hall, 2007). SNPs are the fundamental unit of genetic variation and are very useful molecular markers due to their abundance (Heaton et al., 2005), genetic stability (Markovtsova, Marjoram, & Tavaré, 2000) and suitability to automated analysis (Lindblad-Toh et al., 2000). SNPs have been successfully employed in association analysis as markers for a wide range of traits (Chen & Abecasis, 2007; Wollstein et al., 2007), to estimate linkage disequilibrium (Angius et al., 2008) and to identify genes by linkage studies (Hamada et al., 2005).

Recently, computational methods have been developed specifically to assign individuals to populations (Falush, Stephens, & Pritchard, 2007). The first tests were based on frequency statistics and calculated the probability of drawing a specific single multilocus genotype from each potential source group (e.g. a number of populations) by matching genotypes with the observed allele frequencies at each locus in each group (Paetkau, Calvert, Stirling, & Strobeck, 1995). Subsequently, Rannala and Mountain (1997) pioneered the use of Bayesian statistics developing a partially Bayesian assignment approach to estimate population allele frequencies, and a frequency approach to compute the statistical significance of individual assignments. Now, several different methods and software are available for clustering populations and assigning individuals to likely populations of origin (Manel, Gaggiotti, & Waples, 2005).

The aims of this study were to evaluate SNP markers for their ability to allocate cattle individuals to their Country of origin and to trace individuals belonging to breeds with special beef certification (PGI) namely: "Vitellone dell'Appennino Centrale", Italy, "Ternera de Navarra", Spain, "Boeuf de Chalosse", France and the "Guaranteed Pure Highland Beef", UK. To address these issues we applied Rannala & Mountain Bayesian allocation algorithm to a SNP dataset comprising 24 beef, dairy and double-purpose breeds from Italy, France, Spain, Denmark and UK genotyped with 90 SNP markers.

2. Materials and methods

2.1. Collection of samples

A dataset of 1047 minimally related animals belonging to 24 European breeds (Table 1) was used. All animals were genotyped with 90 independent SNP markers (GeneBank Accession No. from ss77831721 to ss77831810) located in 72 genes that potentially to affect meat quality and production traits. The animals came from two sources: 249 animals belonging to 13 cattle breeds were sampled by the authors and genotyped by KBiosciences using their proprietary KASPUR system (www.Kbioscience.com), while the remaining animals where sampled and genotyped within the EU project GemQual (QLRT-1999-30147). The average percentage of missing data in the dataset was 0.03%.

The breeds sampled included those used in the production of four European PGI products, specifically:

i) *Vitellone Bianco dell'Appennino Centrale*: An Italian PGI product for which only Chianina, Marchigiana and Romagnola breeds are allowed.
ii) *Ternera de Navarra*: A Spanish PGI. Pirenaica, Blonde d'Aquitaine, Pardo Alpina (Brown Swiss cattle), Charolais and their crosses can be commercialised under this certification.
iii) *Boeuf de Chalosse*: Only Limousin, Blonde d'Aquitaine, Bazadaise (not sampled) are admitted to this brand.
iv) *Guaranteed Pure Highland Beef*: Only fresh beef products from the Highlands are backed up by a Certificate from the Highland Cattle Society approved by the Trade Mark Examiner.

Table 1
Number of individuals sampled, country of origin and selection purpose of the breeds analysed

Country of origin	Country of sampling	Breed	Acronym	No. of individuals	Selection purpose
DK	DK	Red Cattle	RED	57	Dairy
CH	DK	Simmental	SIM	19	Beef
ES	ES	Asturiana de la montaña	CAS	55	Dairy
ES	ES	Asturiana de los Valles	RAV	56	Beef/Dairy
ES	ES	Avilena	AVI	53	Beef
ES	ES	Pirenaica	PIR	71	Beef
FR	FR	Blonde d'Aquitaine	BLO	19	Beef
FR	FR	Charolais	CHA	82	Beef
FR	FR	Limousin	LIM	96	Beef
FR	FR	Maine Anjou	MDA	19	Beef/Dairy
FR	FR	Parthenaise	PAR	14	Dairy
FR	FR	Salers	SAL	20	Beef/Dairy
IT	IT	Chianina	CHI	19	Beef
CH	IT	Brown	ITB	21	Dairy
NL	IT	Holstein Friesian	ITH	77	Dairy
IT	IT	Italian Red Pied	PRI	23	Beef/Dairy
IT	IT	Marchigiana	MCG	55	Beef
IT	IT	Maremmana	MMA	22	Beef
IT	IT	Piemontese	PIM	85	Beef
IT	IT	Romagnola	ROM	19	Beef
UK	UK	Aberdeen Angus	ABA	38	Beef
UK	UK	Highland	HIG	46	Beef
UK	UK	Jersey	JER	46	Dairy
UK	UK	South Devon	SOD	35	Beef
		Total		1047	

For our purpose we use the animals of the correct breeds already in our dataset as "illustrations" of animals that could be part of the PGI scheme.

2.2. Statistical analysis

Summary statistics were calculated using PowerMarker V3.0 (URL: http://www.powermarker.net) with the default setting, these included Reynolds, Weir, and Cockerham (1983) genetic distance. Not considering the terms related to the population size, it can be simplified as:

$$\theta_w = \frac{\sum_{j=1}^{m}\sum_{i=1}^{aj}(p_{ij}-q_{ij})^2}{2\sum_{j=1}^{m}\left(1-\sum_{i=1}^{aj}p_{ij}q_{ij}\right)} \quad (1)$$

where p_{ij} and q_{ij} are the frequencies of ith allele at the jth locus in populations X and Y, respectively, while a_j is the number of alleles at the jth locus, and m is the number of loci examined. Not considering mutation, this distance measure is expected to rise in a linear way depending on amount of genetic drift and therefore is appropriated to study cattle breeds that have diverged recently.

The distance matrix was graphically visualised by Multi-Dimensional Scaling techniques [MDS] using the software Statistica ver. 7.0 (StatSoft Inc. http://statsoft.com).

The relationships at individual level were assessed by the Correspondence Analysis using the "AFC sur populations" option available in the program GENETIX (Belkhir, Borsa, Chikhi, Raufaste, & Bonhomme, 2004).

Finally the variance associated to the differentiation among clusters of populations (here Country or PGI) was estimated by AMOVA using the freely available Arlequin software (Excoffier, Smouse, & Quattro, 1992; http://lgb.unige.ch/arlequin/).

2.3. Allocation test

The allocation of an anonymous animal to a predefined category (Country or PGI) within a set was performed by the Bayesian method developed by Rannala and Mountain (1997).

All the allocation tests were performed using Geneclass2 (http://www.montpellier.inra.fr/URLB/geneclass/geneclass.html).

The probability of assignment was performed by simulating 1000 individuals by MC re-sampling procedure and setting the "Type I" error to 0.05 (Assignment threshold of score = 0.05; Piry et al., 2004). Five independent runs were compared.

To evaluate the results of the allocation tests three indexes were used: (i) sensitivity, or Rate of Correct Assignment, calculated as number of correct allocation to a category "*j*"/number of animals sampled from category "*j*"; (ii) overall average assignment probability, as the average of the probability of any correct assignment calculated per category; (iii) specificity, calculated as the number of correct assignment to category "*j*"/total (correct + incorrect) assignment to category "*j*".

3. Results and discussion

DNA-based techniques can be directly used for both of the two key requirements of the traceability: (i) tracking, or the ability to follow a product through the supply chain from the farm to the consumer and (ii) tracing, or the ability to identify the origins of an item upstream in the supply chain.

We focused on bovine meat and meat products and used data from a 90 SNP panel together with a Bayesian statistics framework to test the assignment of individual cattle to their Country of origin: Italy, France, Spain, and UK. Using the same approach, the accuracy of identification of individuals belonging to breeds covered by the PGI labels Ternera de Navarra, Beouf de Chalosse and Vitellone dell'Appennino Centrale; and to discriminate beef product from Highland breed protected by a special certified of origin and tracking system was investigated.

As the power of the assignment depends closely on the level of genetic heterogeneity between breeds, the principal population genetics parameters per country and per PGI (Table 2) were estimated and used. Only in the UK breeds was a statistical difference between Observed and Expected heterozygosity and a Fis value statistically different from 0 seen: this occurs when significant deficit of heterozygotes is expected.

Pair-wise Reynolds genetic distances were calculated between Countries and between PGIs and the distance matrices were graphically represented by Multidimensional Scaling multivariate techniques (Fig. 1a, 1b). At the Country level, breeds from Italy, France and Spain clustered close together, while breeds from UK and Switzerland were separated from all others. Therefore when the PGI brands were compared the Highland most clearly separated from Italian, French and Spanish PGI brands.

Table 2
Summary statistics calculated per Country and per PGI

	Major.Allele	No. Ind.	Av.	Het. Exp.	Het. Obs.	Fis
Country						
France	0.7803	250	0.9772	0.3006	0.2869	0.0478
Italy	0.7822	224	0.9804	0.3026	0.2835	0.0744
Spain	0.7784	235	0.9667	0.3045	0.2884	0.0549
UK	0.7765	165	0.947	0.3121*	0.2679*	0.1451**
Swiss	0.7958	40	0.9808	0.2808	0.2763	0.0360
PGI						
PGI_SPAIN	0.7748	153	0.9651	0.302	0.2947	0.0312
PGI_FRANCE	0.7895	115	0.9832	0.2889	0.2863	0.0177
PGI_ITALY	0.7798	94	0.9774	0.3041	0.2927	0.0482
HIGHLAND	0.8366	46	0.9486	0.2233	0.2197	0.0282

Av. = Availability defined as 1−Obs/n, where Obs is the number of observations and n is the number of individuals sampled. Het. Exp. = Heterozygosity expected following Weir (1996); Het. Obs. = Heterozygosity Observed; Fis = inbreeding-like effects within populations.

* *t*-Test significant thresholds $p < 0.05$

** Statistically different from 0.

Analysis of Molecular Variance a method of estimating population differentiation directly from molecular data and testing hypotheses about such differentiation, showed that 10.7% of the total variation is explained by the between breeds component and that less than 3% accounts for the between countries component or between PGI.

The relationships between individuals were also estimated by the Factorial Correspondence Analysis (AFC), a multivariate canonical analysis particularly suitable for the treatment of qualitative data. The results showed an overall weak genetic differentiation between breeds (data not shown). From the traceability point this reduces the possibility to unequivocally assign an individual correctly to the population of origin, as the power of the assignment is directly correlated with the genetic distance between the populations (Latch, Dharmarajan, Glaubitz, & Rhodes, 2006). Nevertheless, molecular approaches, in combination with specific statistical methods have been successfully used to identify the source breed of cattle individuals (Ciampolini et al., 2006; Dalvit et al., 2008). The recent availability of high-throughput molecular methods to genotype large SNP panels in combination with algorithms, using Bayesian statistics, are promising tools for the secure assignment of individuals to populations.

To test these methods for identifying the Country of origin of an animal, and to trace PGI meat products a panel of 90 SNPs identified within genes potentially affecting meat quality were used. The SNP markers were tested in 24 breeds including breeds that have undergone intensive genetic selection for milk production, beef production, or for dual purposes (dairy and beef). This wide range of breeds was examined considering that a significant source of meat in the European market is represented by young bulls belonging to dairy breeds – mainly Holstein Friesian – that are sold at significantly lower price compared to high quality meat from PGI.

The choice of the SNPs in genes that were candidates for meat quality could potentially result in a ascertainment bias, – as the variability in these genes in dairy breeds may be higher than in beef breeds as the dairy breeds have not been under selection for meat quality. To exclude this potential bias, the samples were labelled according to their use (beef, dairy or dual purpose) and the efficiency of assigning individuals to breed was tested and compared between these classifications. The average percentage of correct assignment was comparable between dairy and beef cattle and higher than 90% in both cases. The double purpose animals had a lower percentage of correct assignment (however still about 80%), possibly because selection has been less intense and hence these breeds are more genetically diverse. These results indicate that the SNP panel used was not biased between the genetic selection categories.

The panel was therefore used to test how well the Countries of origin could be assigned exploiting the geographic component of the genetic variance in cattle (Cymbron, Freeman, Isabel Malheiro, Vigne, & Bradley, 2005; Troy et al., 2001). The results are reported in Table 3. The overall rate of correct assignment was 90%, the average assignment probability 93% and the average specificity 88%.

The highest value was observed for the UK breeds with 97% of individuals correctly allocated with an average assignment probability of 98.4%. Swiss animals were the most difficult to allocate in spite of their high genetic distances (Fig. 1). However, the presence in the dataset of the Danish Red Cattle and Italian Red Pied, which are genetically closely related to Swiss Simmental, decreased both

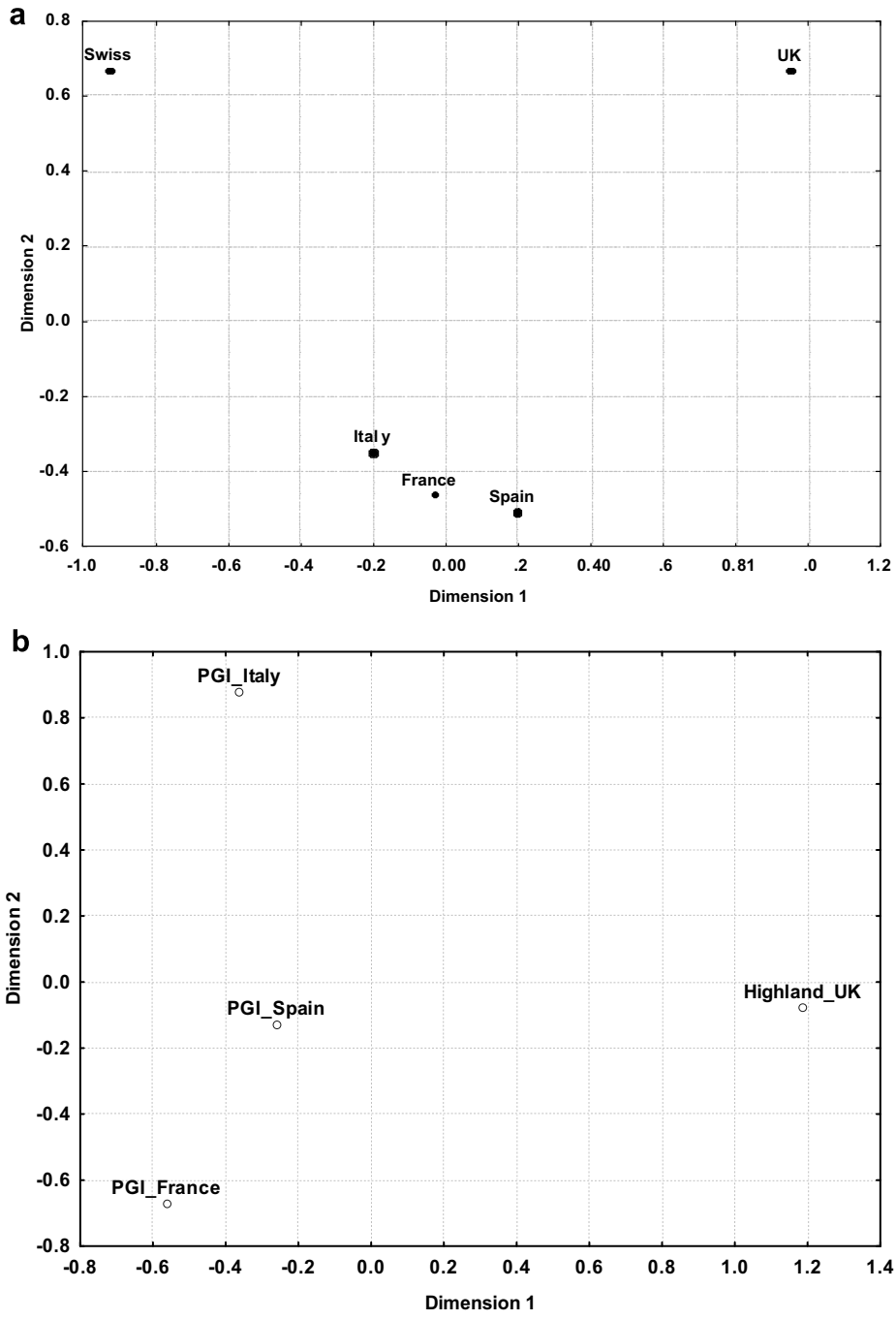

Fig. 1. (a and b) Multi-dimensional scaling representation of the Reynolds genetic distances calculated between Countries (a) and PGI (b).

Table 3
Assignment statistics calculated grouping individuals according to the Country of origin

	No. Ind.	Not Ass.	Sens.	Spec.	Av. Prob.
Italy	200	22	0.89	0.89	0.92
France	250	30	0.88	0.89	0.9
UK	165	5	0.97	0.99	0.98
Spain	235	24	0.90	0.91	0.93
Swiss	40	9	0.78	0.72	0.92
Overall	890	90	0.90	0.88	0.93

No. Ind. = number of animal sampled per Country; Not Ass = number of animal not assigned; Sens. = percentage of correct allocation with probability higher than 50%; Spec = specificity; Av. Prob. = average probability of the correct assignments.

the rate of correct assignment and the specificity (78% of correct assignment and 72% of specificity).

In addition to assigning individuals to breeds it is commercially useful to verify the origin of meat at Country level. Kapferer (1992), observed that marketing food products using a geographic indication of origin as part of the label permits consumers' associations with the region and provides the product with a positive image. Moreover such geographic traceability increases transparency, and consumer confidence in the face of recent food scares, e.g. Bovine Spongiform Encephalopathy, the dioxin and the poultry avian influenza crisis (Lloyd, McCorriston, Morgan, & Rayner, 2006; Puntaric, Smit, Bosnir, & Topolovec, 2000; Peiris, de Jong, & Guan, 2007). The approaches described identify genetic variations, which

are often linked to specific geographic distributions, such as indigenous and local breeds. However using genetic data to assign individuals to breeds is probabilistic and therefore does not give a definitive assignment, and the interpretation of the data is dependent on the thresholds set. Nevertheless, the confidence level obtained in terms of specificity and sensitivity is very high, for some breeds.

The approach was also evaluated for the identification of particular PGI or PDO products linked to specific breeds.

The present study focused on four PGI products linked to specific breeds and therefore are suitable for a DNA based traceability: Vitellone Bianco dell'Appennino Centrale (Chianina, Marchigiana and Romagnola breeds); Ternera de Navarra (Pirenaica, Blonde d'Aquitaine, Brown Swiss cattle, Charolais and their crosses); Boeuf de Chalosse (Limousin, Blonde d'Aquitaine) and Guaranteed Pure Highland Beef (beef products from Highland). The analyses were performed either considering each of these specific PGI products individually or testing the four PGI products simultaneously to evaluate if they could be distinguished. Although for some PGI products – e.g. Ternera de Navarra-cross-bred individuals are accepted; only pure animals were considered in this study. The results are reported in Table 4.

Table 4
Assignment statistics calculated grouping the breed allowed for the production of PGI

	No. Ind.	Not Ass.	% Correct	Spec.	Av. Prob.
Vitellone bianco	93	3	0.97	0.96	0.97
Boeuf de Chalosse	115	22	0.80	0.88	0.81
Ternera de Navarra	193	31	0.84	0.93	0.81
Hghland	96	0	1.00	1	1
Overall	497	56	0.90	0.94	0.90

No. Sampled = number of animal sampled per PGI. Not Ass. = number of animal not assigned. % Correct = % Correct = percentage of correct allocation with probability higher than 50%. Spec. = specificity. Av. Prob. = average probability of the correct assignments.

The panel of SNPs allocated correctly 97% of animals belonging to Vitellone dell'Appennino Centrale with an average assignment probability of 97%. Only 22% (11 out of 93) of correct allocations had a probability of being correctly assigned of less than 90%. For Boeuf de Chalosse 80% of the samples were correctly assigned with an average probability of 88% and sensitivity of 81%. Samples from the Ternera de Navarra PGI were correctly assigned in 84% of cases with an average probability of 93% and a specificity of 91%. Finally, Highland samples were assigned completely.

Of course decreasing the assignment threshold will result in more animals being correctly assigned but also in a loss of specificity, although with a slow rate (Fig. 2).

Alternatively to use all the available data, carrying out this type of verification analysis in a commercial setting can be limited to the identification of the most likely contamination or source of fraud. In this case the cluster can be carried out against potential fraud breeds increasing the percentage of correct allocation.

The correct assignment was highest for Vitellone del Appennino Centrale and Highland. This is for a number of reasons, firstly these products use only pure breeds and these breeds are unique to the respective PGI. The Ternera de Navarra and Boeuf de Chalosse products have a breed in common (Blonde d'Aquitaine) making an overlap in the definition of these products inevitable and hence lowering both assignment and specificity of the tests. The efficiency to identify cross-bred individuals using this marker set and method for analyses remains to be investigated.

4. Conclusion

Recent market research has shown that consumers are concerned with the provenance of the food they buy and therefore it is important to develop simple tests to control the origin of a product. In this view, DNA-based trace-back systems are able to identify the source of meat products and trace them through the supply chain. Availability of such test data is of potential benefit for con-

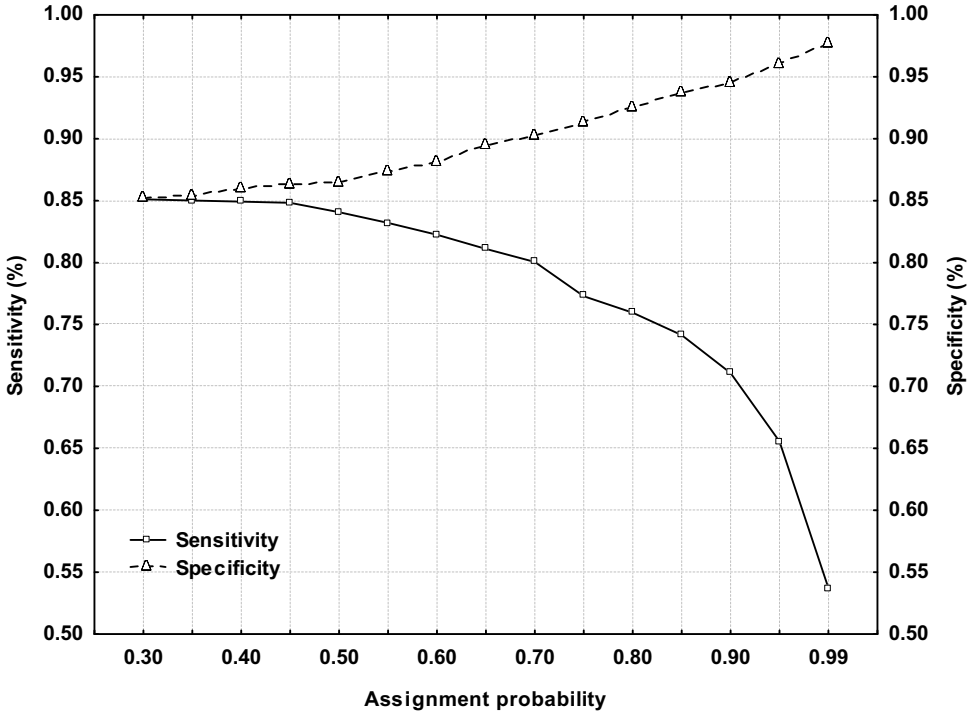

Fig. 2. Line plot of %sensitivity and %specificity against the % of the probability. As expected increasing the assignment threshold results in a higher specificity but also in a loss of sensitivity.

sumers, while it also enables processors and retailers to ensure the identity of products.

SNP and Bayesian statistics have the potential to be implemented for geographic traceability, and to test the authenticity of DOP and PGI products linked to a specific breed. Considering the unequal geographical distribution of DOP and PGI registered regional foodstuffs, with more than 75% of the products registered in five southern EU states (France, Italy, Greece, Portugal and Spain) and considering also that most these products come from rural areas, a DNA-based effective methods of brand protection – through the verification of authenticity of the products – may also provide tools for sustaining the viability of small farming and rural communities improving the Economy of marginal areas.

The main barrier to implementation of these control methods is the cost of DNA based tests, which is still high. In addition the choice of markers could be better optimised to increase the assignment rate, which is mainly correlated with the allele frequencies of markers used. In the future both these constraints may be removed with the availability of a larger number of SNP coming from the Bovine whole genome sequencing and HapMap projects, and by the implementation of novel high-throughput typing technologies that will significantly reduce test costs.

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