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Effectiveness of a 95 SNP panel for the screening of breed label fraud in the Chinese meat market

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ABSTRACT

Breed assignment has proved to be useful to control meat trade and protect the value of special productions. Meat-related frauds have been detected in China; therefore, 95 SNPs selected from the ISAG *core* panel were evaluated to develop an automated and technologically updated tool to screen breed label fraud in the Chinese meat market. A total of 271 animals from four Chinese yellow cattle (CYC) populations, six *Bos taurus* breeds, two *Bos indicus* and one composite were used. The allocation test distinguished European, Japanese and Zebu breeds, and two Chinese genetic components. It correctly allocated Japanese Black, Zebu and British breeds in 100, 90 and 89% of samples, respectively. CYC evidenced the Zebu, Holstein and Limousin introgression. The test did not detect CYC components in any of the 25 samples from Argentinean butchers. The method could be useful to certify Angus, Hereford and Japanese Black meat, but a modification in the panel would be needed to differentiate other breeds.

1. Introduction

The demand of consumers for food safety and quality results in a number of certifications and labels applied to food, such as genetically modified organism (GMO)-free, organic food and USDA certificate. Therefore, the control of possible labeling frauds became necessary (Loureiro & Umberger, 2007). In the case of animal products, individual identification has been useful for safety controls, while breed and species discrimination has been used to protect the value of special productions, such as particular raw materials and geographical indications.

DNA is present as a tracing marker in raw meat and its products even after several processing steps, so that it can be used throughout the supply chain. Molecular traceability has been proposed and tested (Dalvit, De Marchi, Dal Zotto, Gervaso, Meuwissen, & Cassandro, 2008; Dalvit, Marchi, & Cassandro, 2007; Felmer, Sagredo, Chávez, Iraira, Folch, Parra, et al., 2008; Negrini, Nicoloso, Crepaldi, Milanesi, Marino, Perini, et al., 2008; Rodríguez-Ramírez, Arana, Alfonso, González-Córdova, Torrescano, Guerrero Legarreta, & Vallejo-Cordoba, 2011) with markers that are mainly used for animal identification, namely, microsatellites (STRs) and single nucleotide polymorphisms (SNPs) (Allen, Taylor, McKeown, Curry, Lavery, Mitchell, et al., 2010; Negrini, Nicoloso, Crepaldi, Milanesi, Colli, Chegdani, et al., 2009). Recent advances in high-throughput DNA genotyping and bioinformatics maximize the advantages of SNPs, which have become popular because of their simpler nomenclature and suitability for automated analysis (Rincon, Weber, Van Eenennaam, Golden, & Medrano, 2011). Simultaneously, since millions of SNPs became accessible, the selection of markers for each particular case became a problem.

Laboratories around the world have been asked to standardize their procedures for animal DNA testing and forensics (Budowle, Garofano, Hellman, Ketchum, Kanthaswamy, Parson, et al., 2005). In this sense, the use of a single panel for different forensic cases and applications has been an issue for many of the scientific societies. The selection of SNPs for each forensic situation has also been discussed (Budowle & van Daal, 2008); the differential information content between breeds and SNPs has been demonstrated even using large SNP panels (Bradbury, Hubert, Higgins, Bowman, Paterson, Snelgrove, et al., 2011; Hozé, Fouilloux, Venot, Guillaume, Dassonneville, Fritz, et al., 2013); and methods for the selection of markers for breed assignment have also been proposed (Dimauro, Cellesi, Steri, Gaspa, & Sorbolini, 2013; Ramos, Megens, & Crooijmans, 2011). In order to reach an international standardization of DNA laboratories, the International Society for

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Animal Genetics (ISAG) has suggested a panel of 100 SNPs for animal identification, but their usefulness for many breeds and situations is still under consideration.

Chinese meat consumption per capita has grown in the last years; beef imports, for instance, increased four times in 2013 compared to 2012 (USDA, 2014). The native Chinese cattle is Chinese yellow cattle (CYC); it comprises *Bos taurus* in the northern agricultural region, *Bos indicus* in the south and southwest agricultural regions, and mixed origin in the central agricultural region (Jia, Chen, Zhang, Wang, Lei, Yao, & Han, 2007; Lei, Chen, Zhang, Cai, Liu, Luo, et al., 2006). For many years, several European cattle breeds such as Holstein, Limousin, Charolais and Simmental have been introduced to improve dairy and beef production (Longworth, Brown, & Waldron, 2001). In this sense, the superior meat quality of crossbreeds between CYC and foreign breeds has been evaluated (Li, Zhu, Wang, He, & Cao, 2014; Zhou, Liu, Xiu, Jian, Wang, Sun, & Tong, 2001). Furthermore, the price of imported beef (Argentinean or Australian) at international brand supermarkets is higher than that of local cattle beef.

It is possible to detect labeling errors (Capoferri, Bongioni, Galli, & Aleandri, 2006), particularly meat-related frauds in China (Chen, Liu, & Yao, 2010), with DNA methods. Due to the growing Chinese meat imports, it is necessary to ensure the origin of meat and its quality. Thus, the "Argentine–Chinese Center in Food Science and Technology" (CCAFST) was established to develop and implement appropriate tools for these commitments. Research performed with STRs to discriminate foreign breed meat from CYC meat and with candidate gene SNPs for traceability have been recently published (Ripoli, Wei, Rogberg-Muñoz, Guo, Goszczynski, Fernandez, et al., 2013; Rogberg-Muñoz, Wei, Guo, Carino, Castillo, et al., 2014). The present work evaluates the effectiveness of a 95 SNP panel selected from the ISAG *core* panel and proposed for animal identification and paternity testing to develop an automated screening method to detect fraudulent labeling of CYC beef as imported beef.

2. Materials and methods

2.1. Samples:

Two groups of samples were used in this study, a reference data set from known breed or population animals (Reference Sample), and a test data set from unknown breed animals (Trial Sample). The Reference Sample included meat samples collected in four Chinese commercial slaughterhouses (Supplementary Figure SF1) from 80 individuals classified as CYC. One slaughterhouse was located in the Chinese North region (Ch2) and the other three were in the Central Agricultural region (Ch1, Ch3 and Ch4). Samples were collected from animals with a typical phenotype of the local cattle, so that each sampling group represented the cattle in the area of influence of that slaughterhouse and therein a population. In addition, blood samples were collected from 191 animals belonging to six *B. taurus* breeds: 25 Angus (ANG), 21 Hereford (HER), 25 Holstein (HOL), 20 Limousin (LIM), 21 Japanese Black (JBL), and four Japanese Brown (JBR); two *Bos indicus* breeds: 25 Brahman (BRH) and 25 Nelore (NEL); and one composite breed of 25 Brangus (BRN). These represent the most common breeds raised in beef exporter countries. The Trial Sample consisted of a group of 25 samples collected in Argentina, 15 meat samples collected randomly in butcher shops (therefore breed was unknown), and 10 samples collected in a commercial slaughterhouse that differentiates the breed of the animal by phenotypic observation (then the alleged breed was considered).

2.2. DNA extraction and genotyping

DNA was extracted from blood samples using the Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA) and from meat samples according to the methods previously reported by Giovambattista, Lirón, Villegas-Castagnasso, Peral-García, and Lojo (2001). Genotyping was performed using the Sequenom platform (www.sequenom.com), Neogen genotyping service, USA (www.neogen.com). At the moment of the assay (year 2013), a 95 SNP panel (Supplementary Table ST1) was selected by the company from the 100 *core* SNP panel recommended by ISAG (2012) to be used for individual identification and paternity testing.

2.3. Genetic variability

Allele frequencies, Nei's observed (H_o) and unbiased expected heterozigosity (H_e) over all loci were estimated. The level of genetic differentiation was described through population pairwise F_{ST} index. All data were calculated using the GENEPOP 4.0 software (Rousset, 2008).

2.4. Genetic structure and assignment tests

Structure 2.3.4 (Pritchard, Stephens, & Donnelly, 2000) was used to cluster Reference Samples and allocate Trial Samples. Tests were run using the admixture model with correlated allele frequencies, and a burn-in of 100,000 iterations followed by 1,000,000 Marcov Monte Carlo iterations. Two tests were performed:

2.4.1. Test 1 (Population differentiation)

It was performed to evaluate the ability of the SNP set to differentiate foreign breeds and Chinese populations using the Reference Sample set (9 foreign breeds and 4 Chinese populations). The number of clusters to be simulated (K) was set from 2 to 8.

2.4.2. Test 2 (Allocation of Trial Samples)

It was carried out to evaluate the effectiveness of the method to allocate foreign samples into clusters. This test included all foreign breeds and Chinese populations (Reference Sample) and the Trial Sample ($n = 25$; $K = 8$).

3. Results and discussion

3.1. Genetic variability

The number of SNPs with a minor allele frequency (MAF) < 0.05, H_e and H_o for all breeds and populations is presented in Supplementary Table ST2 (S2). Five breeds (NEL, BRH, JBL, CH4, and HER) had SNPs with MAF < 0.05. Since the original ISAG *core* panel was developed from European *B. taurus* breeds, it was expected that breeds genetically distant from the European ones (NEL, BRH and JBL) presented low variation in some SNPs; thus, the use of this panel for identification, paternity test or individual traceability should be carefully evaluated for those breeds. Nevertheless, fixed (monomorphic) markers could be highly informative in breed assignment (Orrú, Napolitano, Catillo, & Moioli, 2006).

The F_{ST} index that evaluated the degree of information for breeds used this SNP panel (Supplementary Table ST3). Pairwise F_{ST} was highest (0.497) between JBL and NEL, and lowest (0.013) between CH1 and CH3. As expected, the greatest values for pairwise F_{ST} were found between *B. taurus* and *Bos indicus* breeds (from 0.268 to 0.497), since they represent the two main domestication centers. Within the Chinese populations, CH4 pairwise F_{ST} value was greater than that for the rest of the populations, probably due to the higher Zebu introgression in this area (southern). The remaining Chinese pairwise F_{ST} were low (between 0.013 and 0.039), evidencing the lower degree of differentiation among them.

3.2. Genetic structure and assignment test

3.2.1. Test 1 (Population differentiation)

The included breeds represent most of the major breeds raised in the principal beef exporter countries. Fig. 1 shows the structure bar plots for $K = 2$ to 8. All foreign breeds were clustered separately or could be differentiated by their origin at $K = 6$, at which Japanese breeds were

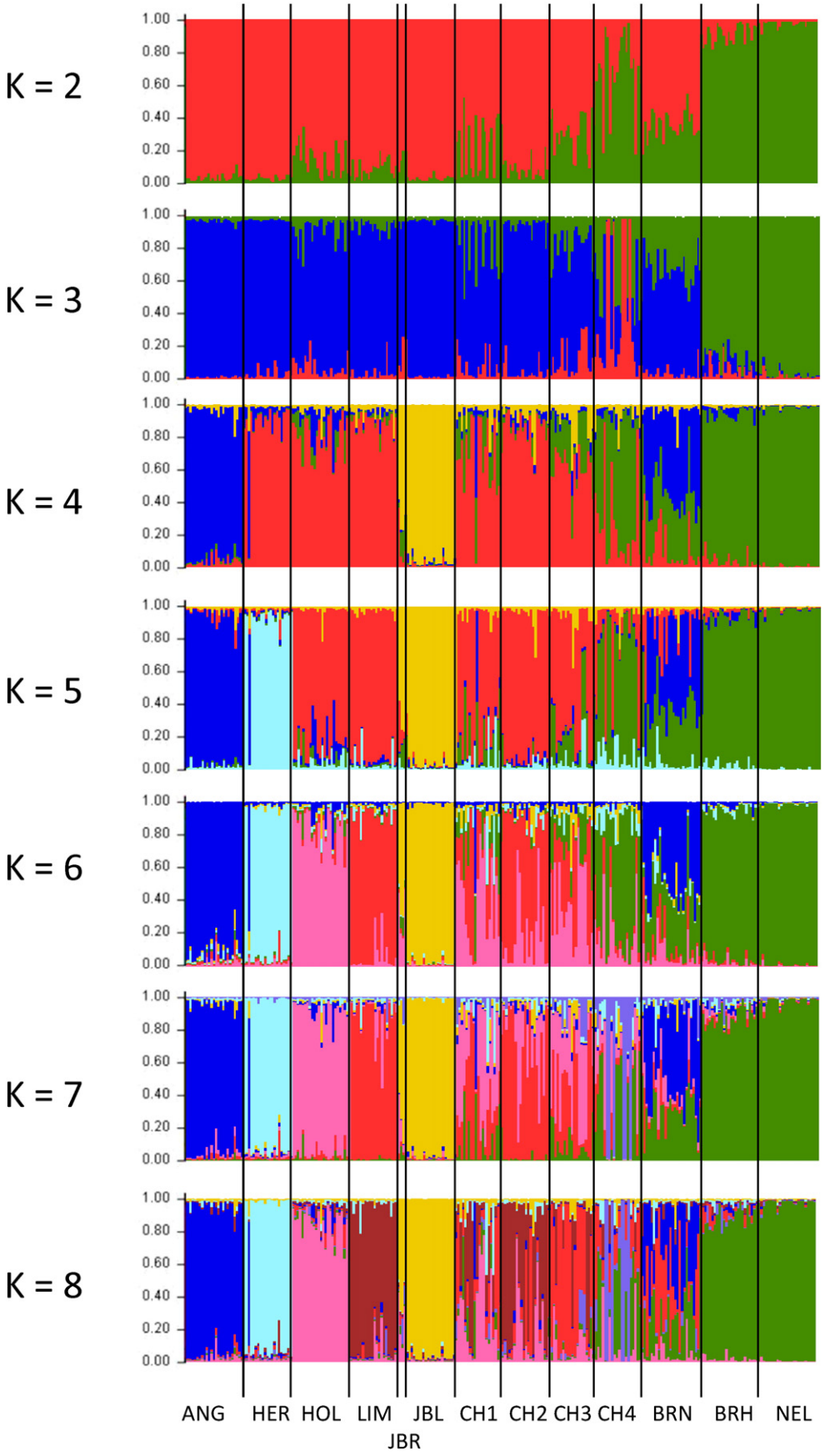

Fig. 1. Structure plot for clustering results considering all studied breeds and populations. K is the parameter that sets the number of clusters to be simulated. Angus (ANG), Hereford (HER), Holstein (HOL), Limousin (LIM), Japanese Brown (JBR), Japanese Black (JBL), Brangus (BRN), Brhaman (BRH), Nelore (NEL), Chinese Populations (CH1, CH2, CH3, CH4).

clustered in one group and *Bos indicus* in another, whereas results for BRN evidenced a composite origin (ANG × BRH). Chinese breeds appeared as a mixture of breeds including LIM, HOL and *Bos indicus*, in agreement with the reported geographical gradient of Zebu genes from south to north (Lei, Chen, Zhang, Cai, Liu, Luo, et al., 2006; Zhang, Wang, Chen, Wu, Han, Chang, et al., 2007) and the historical European genetic introgression with HOL and LIM to improve milk and meat production, respectively (Longworth, Brown, & Waldron, 2001). In this sense, the lack of structure of CYC could be the consequence of the genetic sharing between populations, as they are all considered CYC. Nevertheless, some breeds have been selected in China, such as Qinchuan, Yanbian, Nanyang, Jinnan and Luxi cattle (Qiu, Zhiyong, & Zhijie, 1993). Despite all this, two Chinese exclusive components were detected, one in CH4 (K = 7) and the other in CH3 (K = 8). According to the estimated frequencies for each cluster (K = 8), the component detected in CH4 would be mainly influenced by markers DQ786757 (rs29019900), AY761135 (rs29003723), DQ674265 (rs29011266), AY849380 (no rs) and AY842473 (rs29001956). Those markers could be considered highly informative for that Chinese component as the difference of frequency with all other components was greater than 0.4. On the other hand, the component detected in CH3 would be mainly influenced by markers DQ470475 (no rs), DQ404153 (no rs), DQ837645 (rs29015870), DQ650636 (rs29024525) and DQ489377 (rs29026932). Finally, K = 8 was selected to perform the allocation test.

The specific breed clustering results and the percentage of correct or incorrect assignment are presented in Table 1. JBL and NEL resulted in 100% of samples correctly assigned, while results for ANG and HER were 88 and 90.5% of samples assigned correctly, respectively. In these two last breeds, the rest of the samples were assigned as European *B. taurus*, except for one ANG sample that was assigned as *B. taurus* with no Chinese component detected. These results suggest a possible use of this methodology to identify (and certify) samples from JBL, NEL, ANG and HER. When exploring the results for LIM, HOL and BRH, the percentage of correct assignment was between 76 and 80%, but the remaining samples were not assigned to any group. In the specific case of HOL, 12% were assigned as European *B. taurus*. For these breeds, it would be necessary to improve the panel to identify a higher percentage of samples. Finally, Chinese samples and BRN resulted in a high percentage of incorrect assignments or were not assigned to any cluster, probably due to their admixture condition. Only 19% of the Chinese samples were assigned as Chinese, 45% of them were not assigned and the rest were wrongly assigned; among them, 15% were assigned as LIM. This is in agreement with the already mentioned LIM introgression into China and implies the need for more and different SNPs to differentiate LIM from CYC.

As the method is proposed for screening a possible fraud of labeling CYC meat as "imported beef", the fraction of Chinese genetic component detected was evaluated (see Supplementary Table ST4). The Chinese component in JBL, NEL, ANG and HER was not higher than 0.15, reflecting the lack of genomic sharing among these breeds and the *B. taurus* component of Chinese cattle. In the case of LIM and HOL, 93% of samples did not present a Chinese component higher than 0.15, which was between 0.15 and 0.50 only in 7% of them. However, different results were observed in Chinese samples: while 60% of CH3 and CH4 samples had a Chinese component higher than 0.50, only a few CH1 and CH2 samples (15%) presented a Chinese component higher than 0.50, showing the higher influence of European breeds, particularly LIM, on those last two populations, and the need for more and/or different markers to correctly differentiate them.

Results of the clustering study suggest that this method could clearly differentiate ANG, HER and JBL from CYC, while the other breeds studied could be mistaken in an allocation test because of the genetic influence of *Bos indicus*, LIM and HOL in CYC. In this sense, imported beef into China during 2013 came mainly from Australia (163,995 t), Uruguay (79,630 t), New Zealand (37,040 t), Canada (24,387 t) and Argentina (9220 t) (USMEF, 2014). Considering that British breeds are the main breeds raised in all these countries, the method described could screen for fraudulent labeling of CYC meat as imported from those countries. Furthermore, it could determine the reliability of meat labeled as ANG, HER or JBL, either imported or locally produced. These results agree with those recently obtained with STR in the same samples (Rogberg-Muñoz, Wei, Guo, Carino, Castillo, et al., 2014), even though more and/or different SNP are needed to reach the same discrimination power of the 22 STR panel used.

3.2.2. Test 2 (Allocation of Trial Samples)

Results concerning the ability of the method to allocate the 25 Trial Samples collected in Argentina are presented in Table 2. As expected, 60% of the unknown breed samples were allocated to ANG or ANG crossbreeds, because Argentine cattle is mainly Angus (IPCVA, 2014); the rest were HER and HOL crossbreed, LIM crossbreed or *B. taurus* and, consistent with the clustering study, no Chinese genetic component was detected in any of them. Regarding known breed samples, nine were correctly allocated and one ANG alleged sample was misallocated. Considering that the first test clearly differentiated ANG from the rest of the breeds, and that the ANG genetic component was

Table 1
Results of assignment test 1.

Breed	N	ANG	HER	HOL	LIM	JBL	CHI	BRN	ZEB	EUR	BTNC	NA	% Correct Assigned	% Incorrect Assigned	% EUR	% BTNC	% NA
ANG	25	22	–	–	–	–	–	–	–	2	1	–	88	–	8	4	–
HER	21	–	19	–	–	–	–	–	–	2	–	–	90,5	–	9,5	–	–
HOL	25	–	–	19	–	–	–	–	–	3	–	3	76	–	12	–	12
LIM	20	–	–	–	16	–	–	–	–	4	–	–	80	–	20	–	–
JBR	4	–	–	–	–	–	–	–	–	–	3	1	–	–	–	75	25
JBL	21	–	–	–	–	21	–	–	–	–	–	–	100	–	–	–	–
CH1	20	–	–	–	2	–	1	–	–	7	–	10	5	10	35	–	50
CH2	20	–	–	–	9	–	–	–	–	8	1	2	–	45	40	5	10
CH3	20	–	–	–	1	–	8	–	–	–	–	11	40	5	–	–	55
CH4	20	–	–	–	–	–	6	–	–	1	–	13	30		5	–	65
BRN	25	–	–	–	–	–	2	8	–	–	–	15	32	8	–	–	60
BRH	25	–	–	–	–	–	–	–	20	–	–	5	80	–	–	–	20
NEL	25	–	–	–	–	–	–	–	25	–	–	–	100	–	–	–	–

Breeds: Angus (ANG), Hereford (HER), Holstein (HOL), Limousin (LIM), Japanese Brown (JBR), Japanese Black (JBL), Brangus (BRN), Brahman (BRH), Nellore (NEL), Chinese Populations (CH1, CH2, CH3, CH4). Genetic components: Chinese (CHI), European *B. taurus* (EUR), *Bos indicus* (ZEB), *B. taurus* not Chinese (BTNC), Not Assigned (NA). An animal was accepted as belonging to a particular breed if the proportion of membership to an individual cluster was greater than 0.8. If the proportion for each individual cluster was lower than 0.8, the sum of the four European breeds (European component) was taken into account, and a sample was considered European *B. taurus* (EUR) if that value was greater than 0.8. If the sample was not clustered into European, the sum of the proportions of all *B. taurus* breeds not Chinese was considered. In this case, the limit value to consider the breed origin as *B. taurus* not Chinese (BTNC) was 0.85.

Table 2
Results of the breed allocation test for the Trial Samples.*

	Unknown breed		Known breed		
Breed Assigned*	Number	Percentage	Number	Correct	Percentage
ANG	8	53%	3	2	67%
HER	1	7%	–	–	–
HOL	–	–	2	2	100%
LIM	–	–	1	1	100%
BRN	–	–	2	2	100%
BRH	–	–	2	2	100%
EUR ANG crossbreed	1	7%	–	–	–
EUR HOL crossbreed	1	7%	–	–	–
EUR LIM crossbreed	3	20%	–	–	–
B.taurus not Chinese	1	7%	–	–	–
TOTAL	15	100%	10	9	90%

* Angus (ANG), Hereford (HER), Holstein (HOL), Limousin (LIM), European *B. taurus* (EUR).An animal was accepted as belonging to a particular breed if the proportion of membership to an individual cluster was greater than 0.8.If the proportion for each individual cluster was lower than 0.8, the sum of the four European breeds (European component) was taken into account, and a sample was considered "European crossbreed" if that value was greater than 0.8. If a sample was included in the "European crossbreed" cluster, then if the proportion of a particular breed was greater than 0.4 (and lower than 0.8), the specific breed was also made explicit. If the sample was not clustered into European, the sum of the proportions of all *B. taurus* breeds not Chinese was considered. In this case, the limit value to consider the breed origin as "*B. taurus* not Chinese" was 0.85.

one of the first detected (K = 4), the wrongly allocated sample could be due to a mistake at the slaughterhouse, when labeling the sample, or at any of the laboratory processing steps

As already mentioned, this research was performed within the frame of the "Argentine–Chinese Center in Food Science and Technology" to develop and implement appropriate tools to ensure the origin and quality of meat. Previous research with 22 STRs (Rogberg-Muñoz, Wei, Guo, Carino, Castillo, et al., 2014) and 6 SNPs located in candidate genes for meat quality (Ripoli, Wei, Rogberg-Muñoz, Guo, Goszczynski, Fernandez, et al., 2013) proved to be useful. The performance of the 6 SNPs for the allocation of samples greatly improved with the use of the 95 SNPs panel. As in that previous research, the correct allocation of ANG, HER and JBL was between 75 and 82%, and lower for the rest of the breeds. Herein, higher correct assignment percentages were obtained for those breeds (above 88%), whereas those for HOL and LIM were 76% and 80%, respectively. However, the set of 95 SNPs tested here was less informative than the 22 STRs to discriminate CYC cattle from influencing continental breeds but, as already mentioned, it could differentiate CYC from British breeds (HER and ANG) and JBL. Since ANG and HER are the main breeds raised for meat production in the principal exporter countries to China, this method would be useful to certify those breeds in the Chinese market.

Finally, the continuous technological development had great advantages for SNPs detection, especially in terms of automaticity and repeatability when genotyped into array technologies. However, STRs still have numerous advantages: i) they are more polymorphic (and hence more informative as markers); ii) their diversity has been more recently generated due to their higher mutation rate, iii) they can detect new polymorphisms within a marker, and iv) most importantly, they have been used for more than two decades (Butler, Coble, & Vallone, 2007). Consequently, SNPs should "prove" to be as useful as STRs, and a process of marker selection and standardization among laboratories has to be (and is being) done in most species and forensic situations (Børsting, Mikkelsen, & Morling, 2012; Fernández, Goszczynski, Lirón, Villegas-Castagnasso, Carino, et al., 2013; Fernández, Rogberg-Muñoz, Lirón, Goszczynski, Ripoli, Carino, et al., 2014; Glover, Hansen, Lien, Als, Høyheim, & Skaala, 2010; Hansen, Beacham, McIntosh, & Wallace, 2010; Krjutskov, Viltrop, Palta, Metspalu, Tamm, Suvi, et al., 2009; Ogden, 2011; Yu, Selvaraj, Liang-Chu, Aghajani, Busse, Yuan, et al., 2015). This research presents useful information to continue with that migration in cattle.

4. Conclusions

The method used in this study could correctly discriminate the British breeds ANG and HER and JBL from CYC, but several CYC could be wrongly allocated as LIM or HOL crossbreeds, especially CYC samples obtained in the central and northern areas (CH1 and CH2). The test executed with unknown breed samples from Argentina did not detect the CYC component in any of them, supporting the possibility of using SNPs to detect foreign breed meat in the Chinese market. In this sense, frauds could include CYC meat labeled as imported meat or CYC meat labeled with a foreign breed denomination. As a result of the increased use of SNPs, the information content of the different panels should be tested for each special situation or breed. As proposed, this SNP panel would be useful to certify ANG, HER or JBL meat (either imported or locally produced) in the Chinese market, but more markers should be added and/or different markers should be selected to differentiate CYC from LIM or HOL.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meatsci.2015.08.014.

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