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Foreign meat identification by DNA breed assignment for the Chinese market

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ABSTRACT

Methods for individual identification are usually employed for traceability, whereas breed identification is useful to detect commercial frauds. In this study, Chinese Yellow Cattle (CYC) samples plus data from six *Bos taurus* breeds, two *Bos indicus* breeds, and one composite breed were used to develop an allocation test based on 22 microsatellites. The test allowed discriminating all foreign breeds from the CYC, although some CYC individuals were wrongly allocated as Limousin or Holstein, probably due to the recent introduction of these breeds into China. In addition, CYC evidenced a previously reported Zebu cline (south–north) and a possible structure within the *B. taurus* component that should be confirmed. An independent test performed with meat samples of unknown breed origin from Argentina allocated 92% of them to either Angus, Hereford, or their crossbreed, but none was identified as CYC. We conclude that the test is a suitable tool to certify meat of foreign breed origin and to detect adulterations of CYC beef labeled as imported meat.

1. Introduction

The outbreak of epidemics in livestock populations (*e.g.* bovine spongiform encephalopathy — BSE — and avian influenza) and striking cases of chemical contaminations related to feedstuff (*e.g.* dioxin crisis and food-borne diseases) led to an increasing demand for food safety in the last two decades. As a consequence, and in order to safeguard the authenticity of food products, there is a great interest to develop methods for food traceability at both population and individual levels (Dalvit, De Marchi, & Cassandro, 2007). In general, methods for individual identification are employed for traceability, whereas discrimination of breeds and species is particularly useful to detect commercial frauds and to protect the value of local productions (García et al., 2006; Negrini et al., 2008).

Several individual identification methods have been employed to develop traceability systems, including ear tags, ruminal bolus, retinal analysis, DNA markers, and tracking devices (Dalvit et al., 2007; Lehr, 2013; Li, Wei, Pan, & Guo, 2009). Among them, DNA markers have the advantage of being ubiquitous in animal tissues and remain in many products of animal origin even after heat treatment or other processing steps. Hence, DNA-based traceability systems can be applied at any stage of the food chain. Two kinds of markers are mainly used for animal identification: microsatellites — STRs, and single nucleotide polymorphisms — SNPs (Allen et al., 2010; Negrini et al., 2008). Microsatellites are characterized by a large number of alleles at each locus (Dalvit, De Marchi, Targhetta, Gervaso, & Cassandro, 2008; Vignal, Milan, San Cristobal, & Eggen, 2002) and, thus, are highly informative. For this reason, they are widely used at present in traceability systems (Baldo et al., 2010; Dalvit et al., 2008; Orrù, Napolitano, Catillo, & Moioli, 2006).

World meat trade is expected to grow by 2021 due to the demand of the large economies in Asia, Latin America and the oil exporting countries (OECD-FAO, 2012). In this scenario, China will play a major role. By 2013, China was the third-largest beef consumer and producer in the world (USDA, 2014). In turn, it has quadruplicated its beef imports by 2013 (400,000 tons) relative to 2012 (100,000 tons), and those imports will keep growing for the next years (USDA, 2014). Meat production in China is mainly based on native cattle breeds that show a very particular geographical distribution in connection to their origin: they are found to be *Bos taurus* in the northern agricultural region, *Bos indicus* in the south and southwest agricultural regions, and mainly admixed in the central agricultural region (Jia et al., 2007; Sun, Chen, Lei, Lei, & Zhang, 2008). This native cattle is collectively called "Chinese Yellow Cattle" (CYC) and, although certain breeds such as Qinchuan cattle,

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Nanyang cattle, Luxi cattle, Yanbian cattle, or Jinnan cattle have been established, interchange of genetic material across breeds and regions is the norm. Additionally, several European cattle breeds have been introduced to improve dairy and beef production during the last years, particularly Holstein, Limousin, Charolais, and Simmental (Longworth, Brown, & Waldron, 2001). Notwithstanding, China has not yet established a beef quality grading system or standards of slaughtering and processing. Therefore, a portion of the domestic beef industry needs to improve its quality standards and monitoring system (Frost & Sullivan, 2012).

In this context, China needs to assure the origin of the meat and its organoleptic quality. We suggest that a breed allocation test based on DNA markers is a suitable tool to achieve both goals. The objective of this study was to evaluate the performance of a set of 22 STRs as a mean to certify foreign meat for the Chinese market. Specifically, data from commonly raised breeds across the world was used to train the test for breed allocation, whereas meat samples from Chinese commercial slaughterhouses and Argentinean butchers' shops were used to assess its performance.

2. Materials and methods

2.1. Chinese Yellow Cattle samples

Meat samples were collected in four Chinese commercial slaughterhouses (Supplementary Fig. S1) from animals classified as Chinese Yellow Cattle. The four Chinese slaughterhouses were located in the most important cattle producing areas of China: one in the Chinese North region (Jilin Province, named herein Ch2) and three in the Central Agricultural region (Shandong, Shaanxi, and Hubei Provinces, named Ch1, Ch3 and Ch4, respectively). A total of 74 samples were collected from animals with the characteristic phenotype of the local cattle, so that each sampling group represents the cattle in the area of influence of that slaughterhouse. The samples were defatted, and 5 g were lyophilized and stored at room temperature until DNA extraction.

2.2. Foreign breed reference samples

Blood samples (5 ml approximately) were collected with 100 μl of EDTA (6%) as anticoagulant from 256 animals from different foreign breeds. The animals sampled belonged to either one of six *B. taurus* breeds: Angus (ANG), Hereford (HER), Holstein (HOL), Limousin (LIM), Japanese Black (JBL), Japanese Brown (JBR); one of two *B. indicus* breeds: Brahman (BRH) and Nelore (NEL); or one composite breed: Brangus (BRN). All these samples were collected in Argentina, but represent the most commonly raised breeds across the world.

2.3. Unknown breed samples

A second set of 106 meat samples was collected from butchers' shops randomly located in Buenos Aires Province (Argentina). A cube of 1 cm per side was asked from the butcher and stored in a plastic bag at −20 °C until DNA extraction. These samples were from animals of unknown breed and therefore used for assessing the discrimination ability of the allocation test.

2.4. DNA extraction

Total DNA was extracted from blood samples using the Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA) following the manufacturer's instructions. DNA was also extracted from meat samples according to the methods previously reported by Giovambattista et al. (2001).

2.5. Genotyping

In this study, 22 microsatellites were genotyped: BM1818, BM1824, BM2113, BRR, CSRM60, CSSM66, ETH3, ETH10, ETH185, ETH225, HAUT27, HEL1, HEL9, INRA023, INRA063, MM12, RM067, SPS115, TGLA53, TGLA122, TGLA126 and TGLA227. These STRs belong to the current FAO (van de Goor, Koskinen, & van Haeringen, 2011) or ISAG (International Society for Animal Genetics, http://www.isag.us) panels. A self developed kit was used for PCR (detailed protocols available upon request from the corresponding author). Fragments were resolved in an automatic DNA sequencer. Allele sizes were standardized according to the ISAG nomenclature and reference samples were processed in both Chinese and Argentinean labs to validate allele labeling.

2.6. Measures of genetic variability

The average number of alleles, privative alleles, and the Nei's observed (H_o) and unbiased expected heterozygosity (H_e) over all loci were estimated using the ARLEQUIN 3.5 software (Schneider, Roessli, & Excoffier, 2000). The level of genetic differentiation between populations was described through population pairwise F_{ST} index, calculated with the GENEPOP 4.0 software (Rousset, 2007). Principal component analysis (PCA) was conducted using the software smartPCA, included in the package EIGENSOFT (Patterson, Price, & Reich, 2006), and executed using the default parameters ($k = 10$, $m = 5$, $t = 10$, $s = 6$) as recommended by the developers. All these metrics were used to assay the ability of this particular set of STR markers to appropriately group individuals from different breeds.

2.7. Allocation test

Three allocation tests were performed based on the software Structure 2.3.4 (Pritchard, Stephens & Donnelly, 2000) to meet different goals. The software employs a Hidden Markov Chain algorithm to cluster input data in one of *K* predefined clusters with some probability. In our implementation, the reference samples helped to cluster animals of known breed, thus training the algorithm to allocate samples from animals of unknown breed. All the allocation tests were executed with the admixture model, correlated allele frequencies, and a burn-in of 100,000 iterations followed by 1,000,000 Markov Monte Carlo iterations. The number of clusters, *K*, was defined in each case according to the number of populations involved. The three allocation tests were:

Test 1 (population differentiation):
This test was executed to evaluate the degree of differentiation between the foreign breeds and the Chinese populations. The test was performed including all nine foreign breeds (6 *B. taurus*, 2 *B. indicus* and 1 composite) and the four Chinese populations. The *K* parameter values were set sequentially from 2 to 9.

Test 2 (CYC structure and relationship with foreign breeds):
The second analysis was executed to evaluate the existence of admixture and/or common ancestry among the Chinese cattle, three of the *B. taurus* breeds (Holstein, Limousin and Japanese Brown), and one of the *B. indicus* breeds (Nelore). In this case, as the breed of each individual was known, this information was used to set an *a priori* cluster for each sample (LOCPRIOR model). The *K* parameter values used were set sequentially from 2 to 6.

Test 3 (allocation test for unknown breed samples):
Finally, this test was carried out to evaluate the performance of the algorithm to allocate unknown breed samples into clusters. The analysis included all foreign breeds, the four Chinese populations, and the samples collected in Buenos Aires Province, Argentina. The number of clusters/breeds was set to $K = 8$. An animal was allocated to a particular breed when the probability of membership to any individual cluster was greater than 0.8. Alternatively, if the proportion was lower than 0.8, the sum of the four European

breeds (European component) was computed, and whenever the value was greater than 0.8 the sample was classified as "European crossbreed". In this latter case, when the contribution of any of the Europeans breeds was greater than 0.4, this specific breed was also made explicit. Finally, if the sample was neither allocated to a specific breed nor classified as a European crossbreed, the sum of the proportions of all foreign *B. taurus* breeds was calculated. If the value was above a threshold of 0.85 the sample was classified as "*Bos taurus* not Chinese".

3. Results and discussion

3.1. Estimates of genetic variability within populations

The average number of alleles, the number of privative alleles, and the expected (H_e) and observed heterozygosity (H_o) for each of the foreign breeds and Chinese populations studied are presented in Table 1. Values ranged between 3.09 (JBR) and 8.27 (CH1) for the average number of alleles, from 0 (JBR and JBL) to 6 (CH1) for the number of privative alleles, between 0.510 (JBR) and 0.806 (CH1) for H_e, and from 0.555 (JBR) to 0.740 (CH4) for H_o. As expected, the nonselected Chinese populations were the most diverse, followed by the composite Brangus breed. Conversely, Japanese breeds were the less diverse, probably due to the historically limited number of animals (and genetics) exported from Japan, creating thus a bottleneck effect. In the case of JBR, the reduced amount of genotyped animals should also be considered.

3.2. Estimates of genetic variability between populations

The F_{ST} index and the exact G-test for population differentiation were calculated to measure the degree of genetic differentiation among all the cattle breeds and populations studied. The exact G-test is a likelihood ratio test that evaluates if the distribution of diploid allele frequencies between populations is significantly different. The result for this test indicated that the differences between any pair of populations were highly significant (exact p value for all loci ≤ 0.0001). In turn, the pairwise F_{ST} statistic measures the fraction of the genetic variability that is explained by differences between the populations relative to the total genetic variability: the higher the value, the greater the distance between the populations. The lowest value of F_{ST} (0.007) was found between the two Chinese populations located in the northern and eastern territories (Ch1 and Ch2), whereas the highest value (0.334) was found between the Japanese Brown and the Brahman breeds. In general, greater pairwise F_{ST} values (F_{ST} greater than 0.2) were found between pure *B. indicus* breeds and pure *B. taurus* breeds, evidencing the divergence between the two main domestication centers. Japanese breeds and European breeds have also shown large F_{ST} values. Genetic differences among European breeds resulted in F_{ST} values ranging from 0.082 and 0.107, which are in agreement with previous reports (Kantanen et al., 2000; Machugh, Loftus, Cuninngham, & Bradley, 1998). Chinese breeds presented the lowest pairwise F_{ST} values (F_{ST} between 0.007 and 0.062) and hence the genetic variability among populations was explained almost entirely by the differences between individuals (see Supplementary Table ST1). The low level of differentiation observed between CYC populations could be a consequence of either the lack of any selection program, the exchange of genetic material across regions, or both. A similar pattern was obtained with the PCA (see Supplementary Fig. SF2): the first component accounted for *B. taurus*–*B. indicus* genetic content, while the second accounted for the differences between European and Asian *B. taurus*. Chinese populations where placed in a central position, evidencing its ancient *B. indicus*–*B. taurus* admixture and the recent introgression of European breeds, predominantly Holstein and Limousin. Accordingly, there was an overlap observed between the distribution of those breeds and some of the Chinese individuals.

Table 1
Number of alleles (average and privative), expected (H_e) and observed heterozygosity (H_o) for all breeds and populations.

Population [a]	Breed origin	N	Number of alleles		H_e	H_o
			Average	Privative		
ANG	UK	47	6.55	1	0.694	0.634
HER	UK	20	5.64	3	0.701	0.645
HOL	Netherlands	21	6.45	2	0.725	0.606
LIM	France	24	6.50	1	0.739	0.695
JBR	Japan	9	3.09	0	0.510	0.555
JBL	Japan	35	4.68	0	0.629	0.630
CH1	China	20	8.27	6	0.806	0.725
CH2	China	20	7.14	1	0.772	0.712
CH3	China	20	7.45	1	0.796	0.694
CH4	China	14	7.14	2	0.794	0.740
BRN	Argentina/USA	24	7.50	1	0.764	0.693
BRH	USA	33	5.95	2	0.669	0.611
NEL	Brazil	43	5.68	4	0.630	0.573

[a] Angus (ANG), Hereford (HER), Holstein (HOL), Limousin (LIM), Japanese Brown (JBR), Japanese Black (JBL), Brangus (BRN), Brahman (BRH), Nelore (NEL), and Chinese populations (CH1, CH2, CH3, and CH4).

3.3. Allocation tests

The three allocation tests performed in this study collectively allowed to properly identify foreign meat for the Chinese market. Results from each test are detailed next:

Test 1 (population differentiation):
This first analysis was performed to determine the degree of differentiation of the Chinese cattle against the foreign reference breeds based on the set of markers used in this study. Fig. 1 shows the bar plots for different values of the parameter K, ranging from 2 to 9. When K was set to 2, the first structure level, the procedure detected the two main bovine subspecies, *B. taurus* and *B. indicus*; in this case, the samples from Brangus and the Chinese populations clearly reflected their mixed status. When further K was set sequentially from 3 to 8 the whole *B. taurus* breed structure was revealed. In particular, Japanese Black and Angus were the first breeds to be identified separately ($K = 3$ and $K = 4$, respectively). Next, when $K = 5$, the Chinese cluster arose, even though a degree of admixture was present for some samples. The Ch4 population showed a Zebu introgression of 20% approximately, consistent with its southern geographical localization, closer to the *B. indicus* domestication center. Instead, populations CH1 and CH2 showed a degree of European-origin gene introgression, in agreement with the available historical data (Qiu, Ju, & Chang, 1993). The main introgression source was the Limousin cluster, but some Holstein have also been detected. Next, with K ranging from 6 to 8, Hereford, Holstein, Limousin and Japanese Brown clusters were sequentially separated. All foreign breeds were clustered separately and thus could be properly differentiated when $K = 8$. Finally, when $K = 9$, populations CH1 and CH2 showed a different main component with respect to populations CH3 and CH4, which could suggest a second genetic structure within the *B. taurus* component in CYC. In summary, and considering the genetic structure just described, there is enough genetic diversity in this set of markers to properly differentiate almost all the foreign breeds commercialized in the Chinese market. Furthermore, *B. taurus* breeds Angus, Hereford, Holstein, Japanese Brown and Japanese Black, and *B. indicus* breeds Nelore and Brahman, could be clearly discriminated from CYC. In turn, the composite breed Brangus could also be identified by its Angus and Brahman components. However, a note of caution is in order here, as a few Chinese Yellow Cattle samples were wrongly allocated into Limousin or Holstein clusters. This result was probably due to the degree of introgression

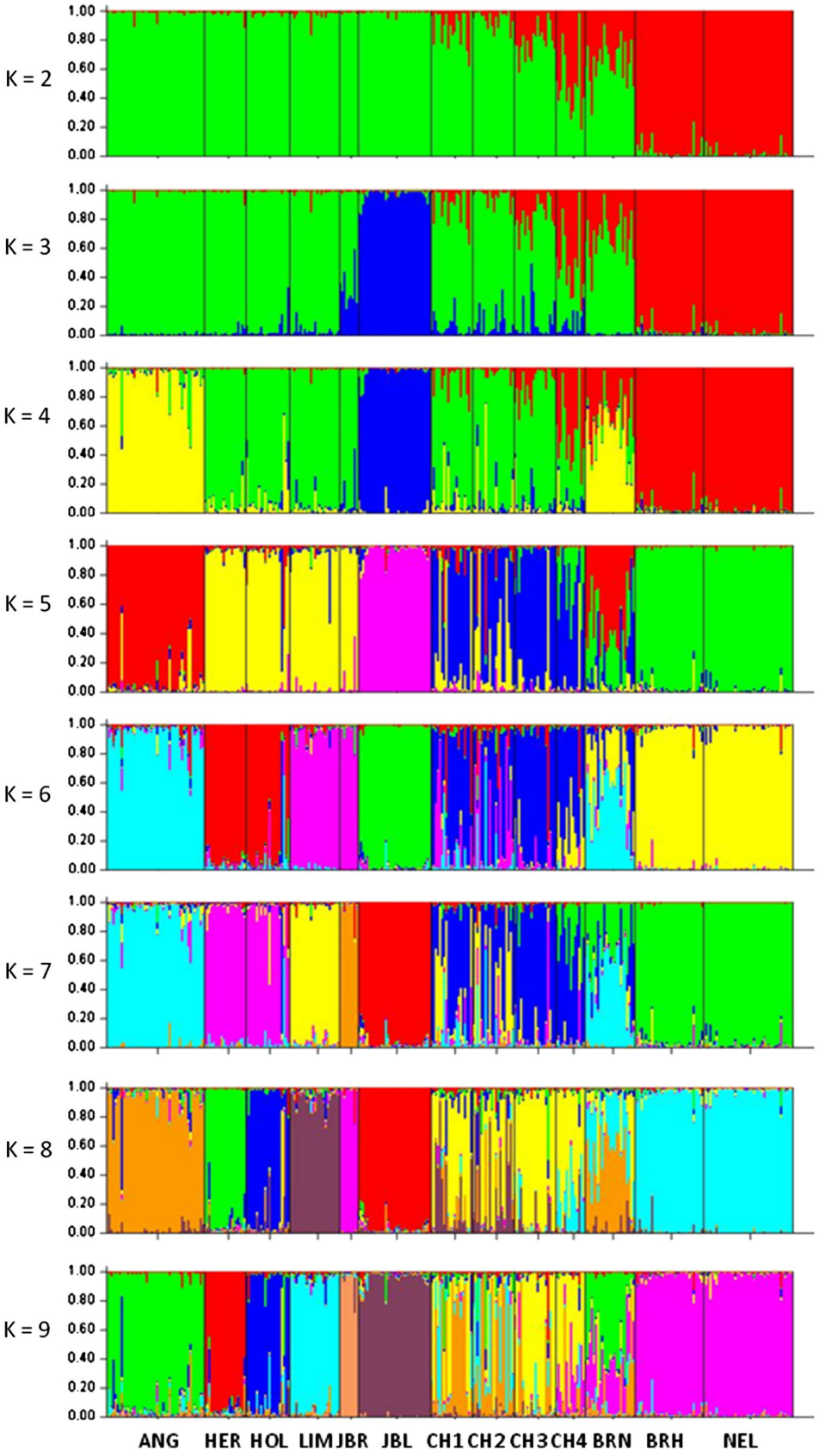

Fig. 1. Structure plot for clustering results considering all studied breeds and populations. The parameter *K* set the number of clusters to be simulated. Angus (ANG), Hereford (HER), Holstein (HOL), Limousin (LIM), Japanese Brown (JBR), Japanese Black (JBL), Brangus (BRN), Brahman (BRH), Nelore (NEL),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

of these breeds into the modern Chinese cattle. Nevertheless, as the current possibility of fraud in China is the use of Chinese meat labeled as "imported beef" (mainly from Argentina, Australia, New Zealand or Uruguay), those wrong allocations would not affect significantly the application of this method.

Test 2 (CYC structure and relationship with foreign breeds):
The second analysis included all the Chinese populations but only those foreign breeds presumably related with the Chinese cattle. There were two reasons for launching this test: i) some cluster contributions could be underestimated or mistakenly asserted when inaccurate reference populations are included in the analysis (Nicoloso, Crepaldi, Mazza, Ajmone-Marsan, & Negrini, 2013); ii) the test may refine the Chinese cattle population structure detected in the previous analysis, thus allowing to identify cattle from different Chinese regions. The decision to include a foreign breed was based on the following considerations. First, Limousin and Holstein were included, as the historical data (Longworth et al., 2001) suggests a possible introgression of those breeds in the Chinese cattle. Next, the Japanese Brown breed was included as it supposedly descended from Korean cattle which, in turn, is probably related to Chinese cattle, especially to the north-east population (CH2). Finally, the Nelore breed was included to represent *B. indicus* cattle, which is known to have influenced Chinese cattle in proportions that decrease from south to north. In this latter regard, Zhang et al. (2007) demonstrated a geographical cline in *B. indicus* introgression within Chinese cattle. The results of this analysis are presented in Supplementary Fig. SF3. Almost all genetic assignment methods are based on the linkage disequilibrium (LD) generated between loci due to the population genetic structure (*i.e.* genetic drift), and this is generated in few generations after two populations are separated. Additionally, the Chinese concept of breed differs from the Occidental concept; in China the genetic material could be shared between populations (or breeds). Overall, they suggest that even though Chinese populations are geographically separated, their genetic structure is relatively weak. In fact, as previously discussed, it only consists in a south-to-north cline in the amount of *B. indicus* introgression, and a possible *B. taurus* component that should be confirmed with a greater number of samples widely distributed throughout the country.

Test 3 (allocation test for unknown breed samples):
The results of this third test are displayed in Table 2. Almost 60% of the samples were allocated to the Angus breed, whereas 92% were allocated to either Angus, Hereford, or their crossbreed. This is in agreement with the Buenos Aires Province breed cattle stock statistics (IPCVA, 2013). Notice that none of the 106 Argentinean samples was attributed a Chinese component. This latter result suggests that meat from this Argentinean Province could be perfectly discriminated from Chinese cattle meat.

Table 2
Breed allocation of meat samples obtained from randomly located butchers in Buenos Aires Province (Argentina).

Breed assigned[a]	Number	Percentage
ANG	60	57%
HER	16	15%
HOL	1	1%
EUR ANG crossbreed	10	9%
EUR HER crossbreed	10	9%
EUR HOL crossbreed	2	2%
EUR LIM crossbreed	1	1%
EUR ANG × HOL crossbreed	1	1%
EUR crossbreed	2	2%
Bos taurus not Chinese	3	3%
Total	106	100%

[a] Angus (ANG), Hereford (HER), Holstein (HOL), Limousin (LIM), and European *Bos taurus* (EUR).

Labeling has become an important issue in food trading. Consequently, mislabeling (intentional or not) has to be controlled to defend the consumers (Dalvit et al., 2007). In addition, given the recognition of some valuable attributes to local food (*i.e.* European Union Regulation EEU 2081/92), it is important to develop methods to control the origin and processing of that valuable food (García et al., 2006; Negrini et al., 2008). China has become one of the biggest importers of meat in the world and foreign meat is usually considered of superior quality. In turn, CYC is only produced within China and it only has a domestic commercialization. At present, the possible fraud with meat commercialization is to label CYC meat as foreign. The breed allocation strategy used in this study has shown to be a useful tool for monitoring this practice. Moreover, if in the future the local CYC meat becomes protected and/or valuable, the information included in this study will help to develop a more informative test to certify CYC.

In summary, the allocation test assayed has correctly discriminated the foreign breeds from the Chinese cattle, although some CYC individuals have been wrongly allocated into either Limousin, Holstein, or their crossbreeds, probably due to the influence of these breeds into modern Chinese cattle. The native Chinese cattle exhibited a genetic cline south–north of zebu introgression and, possibly, a second genetic structure within *B. taurus*. Finally, an independent test executed with samples of unknown breeds from randomly chosen Argentinean butchers did not allocate any animal into Chinese cattle as expected. In conclusion, the breed allocation test we have described in this study can be confidently used both to differentiate foreign meat and to check for adulterations of breed-labeled meat (or meat products) for the Chinese market.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meatsci.2014.07.028.

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