GENETICS AND GENOMICS

Distinguishing industrial meat from that of indigenous chickens with molecular markers

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ABSTRACT The aim of investigation was to evaluate a traceability system to detect industrial chicken meat among indigenous products, considering issues that could affect assignment accuracy. The dataset included 2 Italian indigenous meat breeds, namely Bionda Piemontese (2 ecotypes) and Bianca di Saluzzo, one broiler line, and 3 layer lines. Assignment tests were performed using a standard panel of 28 microsatellite loci. To evaluate effects of inbreeding and substructure on assignment accuracy, a simulated dataset was prepared. Broilers and layers belong to homogeneous populations and never enter the clusters of indigenous breeds. Ambiguity or misallocation are expected between the Bionda ecotypes and between the 2 indigenous breeds, but it is unlikely that niche products provided by Bionda and Bianca will compete with one another. Non-random mating reduces accuracy, but only populations having weak genetic differentiation are involved, namely those that are less interesting to discriminate. The dataset can be used as a reference population to distinguish commercial meat from indigenous meat with great accuracy. Misallocations increase as number of loci decreases, but only within or between the indigenous breeds. A subpanel of the most resolving 14 loci keeps sufficient informative content to provide accuracy and to correctly allocate additional test samples within the reference population. This analytical tool is economically sustainable as a method to detect fraud or mislabeling. Adoption of a monitoring system should increase the value of typical products because the additional burden of molecular analyses would improve commercial grade and perception of quality.

Key words: Traceability, microsatellite, chicken, substructure, inbreeding

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INTRODUCTION

In recent years, consumers have paid attention to the quality of food. As regards the food of animal origin, this tendency has arisen as a result of the outbreak of potential zoonoses and has increased due to incidence of foodborne diseases (Mateus and Russo-Almeida, 2015). An important topic of food assurance is authentication, which avoids fraud or mislabeling, thus preserving quality and enhancing confidence in the market. The authentication attracts additional attention with the perception that typical food, niche or traditional, obtained from indigenous breeds would offer guarantees for safety and flavor. In the context of sustainable use of animals, organic and extensive practices are perceived to be more respectful of animals and the environment than industrial farming. Demand for high quality food opens up a new market based on expensive products, whose authentication becomes the key issue.

Chicken plays an important role in a balanced human diet. Several niche products are relatively new and need to be safeguarded; they contribute to the economy of marginal rural areas and to biodiversity conservation (Fontanesi, 2009). The main tool of the authentication is traceability through all steps of the food chain by means of individual, line, breed, and species identification (Dalvit et al., 2007; Dávila et al., 2009; Bottero and Dalmasso, 2011; Tadano et al., 2011; Tadano et al., 2012). This topic is particularly important if a label or brand is restricted to animals belonging to a single breed reared in a well-defined area. The breed traceability contributes to transparency of the supply chain using verifiable records and guarantees control over the origin of animals. The most sensitive and specific source of information for traceability is DNA, which overtakes the limits of documents and tags (Nicoloso et al., 2013). Different marker loci have been used to implement molecular traceability. Microsatellite loci and dense panels of single nucleotide polymorphisms (**SNP**) are currently available. To allocate individuals to populations of recent origin (breeds), microsatellites exhibit good resolution power due to a high polymorphic content, whereas phylogenetic relationships between populations from ancient evolutionary divergence (strictly related species) are better detected by SNP

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into coding genes (Gärke et al., 2012; Granevitze et al., 2014).

In poultry, the molecular traceability has been specifically pursued using tools such as protein two-dimensional electrophoresis (Zanetti et al., 2011b), amplified fragment length polymorphisms (Soattin et al., 2009), SNP (De Marchi et al., 2003), and microsatellites (Rikimaru and Takahashi, 2007; Tadano et al., 2008; Sartore et al., 2014). The microsatellites have been mainly used for breed conservation purposes so far (Granevitze et al., 2007; Bianchi et al., 2011; Riztyan et al., 2011; Zanetti et al., 2011a; Wilkinson et al., 2012; Tadano et al., 2013; Tadano et al., 2014); a large amount of data are based on the microsatellites recommended by the Food and Agriculture Organization (FAO, 2011) and information from the available datasets may be easily combined also for traceability purposes to arrange reference populations.

In the 20th century, all Italian indigenous poultry breeds have dealt with a decline due to preference for high-performing lines. In the Piedmont region (Northwest Italy), the Bionda Piemontese (Bionda) and the Bianca di Saluzzo (Bianca) have been preserved in spite of replacement by broilers; they are medium-sized meat breeds. Around 16,000 Bionda and 4,000 Bianca are still extant, and they provide an alternative to industrial management due to adaptation to free-range systems (De Marco et al., 2013). On proposal of the Slow Food Organization (2016), Bionda and Bianca have been included in the Italian registry of indigenous poultry with conservation purposes (Associazione Italiana Allevatori, 2014). Their products have been officially recognized as traditional and are sold as carcasses and processed foods (Italian Ministry of Agriculture and Forestry, 2015). When carcasses are marketed, the conformation of an indigenous chicken is very different from that of an industrial broiler, therefore it would seem easy to distinguish the products of an industrial broiler by morphological analysis, but males of layer lines may have rather similar body weight and conformation as Bionda and Bianca chickens and the distinction could be more difficult. On the other hand, when pieces or cuts are brought to market in processed products, authentication may fail to distinguish even layer types from industrial broilers. Therefore, an assignment test is a specific need of breeders even before customers complain about possible mislabeling cases.

Sartore et al. (2014) and Sartore et al. (2016) showed the possibility of distinguishing these 2 breeds from commercial lines and other Italian indigenous populations using microsatellites from the FAO (2011) panel. Nevertheless, when products are monitored with a trace-back approach to detect fraud or mislabeling, allocation accuracy is an important issue. The accuracy could be reduced by inbreeding, substructure, and coancestry; nevertheless, these issues have been poorly investigated (Tadano et al., 2007a; Tadano et al., 2008; Bianchi et al., 2011; Wilkinson et al., 2012; Sartore et al., 2016). Confidence values should also be considered to know probability distribution of correct allocations in addition to proportion of correctly classified individuals (Maudet et al., 2002; Mateus and Russo-Almeida, 2015). In poultry, this approach has been used by Soattin et al. (2009). Cost of each marker is another limiting factor because the tool, when compared with the market value of a meat chicken (a Bianca carcass costs EUR 9 to 12 per kilogram), must be economically sustainable as a routine test. On the other hand, the marker loci provide different contributions to the distribution of correct allocations.

In the present study, effects of non-random mating and robustness of allocation were considered at the same time to define the properties of a reference population. The objectives were to arrange a test to detect industrial chicken meat among indigenous products and to produce an accurate traceability system applicable to the products of the 2 indigenous Italian Piedmont chicken breeds. To reduce the cost of analyses without decreasing accuracy, the efficacy of a subpanel of loci was also tested.

MATERIALS AND METHODS

Sampling, Genotyping, and Data Analysis for Genetic Differentiation

Sample collection, genotyping process, and descriptive statistics for variability have been presented earlier in Sartore et al. (2014) and Sartore et al. (2016). Briefly, DNA was extracted from 540 blood samples of Bionda (213 chickens), Bianca (86), Ross 708 broilers (61), and 60 each of 3 brown laying lines, namely Hy-Line, ISA Brown, and Eureka. The Bionda samples were divided into the Bionda Cuneo (89 chickens) and Bionda Standard (124) ecotypes relying on geographical distribution. All the populations provide chickens that are sold in the Piedmont market; layers were added because males are usually used for meat production. In the present investigation, 39 additional samples were collected from local stocks, namely from 19 Bionda Standard and 20 Bianca chickens, and they were used as anonymous samples to validate the allocation. DNA was extracted using the NucleoSpin Tissue extraction kit (Macherey-Nagel, Düren, Germany; Ref. 740952.50).

The number of marker loci was increased compared to Sartore et al. (2014); to be able to compose a subpanel of high resolving loci, the 28 autosomal microsatellites used by Sartore et al. (2016) were chosen because of the high polymorphic content (number of alleles and heterozygosity). The $\boldsymbol{F}_{\mathrm{ST}}$ fixation index, namely the difference of allele frequencies among and between the source populations, was calculated and the differentiation was classified as detailed in Supplementary data (Data Analysis for Genetic Differentiation).

Cluster Analysis and Allocation Test

Chicken genotypes were clustered using the model-based procedure implemented by the Structure v2.3.4

software (Pritchard et al., 2000) and the performance of assigning test was explored. Using an ancestry model with admixture, correlated allele frequencies, and no prior information about source populations (unsupervised mode), the number of genetic clusters, K, was tested for all values from 1 to n (number of source populations). For each K-value, 50 independent runs with a burn-in period of 10,000 followed by 10,000 Markov chain Monte Carlo iterations were made. The most likely solution was obtained by the ΔK statistic and, for the best K-value, a graphical display of assignments was obtained (detailed explanation is given in Supplementary data, Cluster Analysis and Allocation Test). Following the step-wise algorithm of Rosenberg et al. (2001), each individual received a membership or fraction Q of its genome within each of the K inferred clusters and then it was allocated to the cluster containing its greatest Q-value. Association of clusters with source populations was performed according to the allocation of highest percentage of individuals of a population. Any individual classified in the cluster of its source population was considered as correctly allocated, whereas chickens classified in a cluster associated with a population other than its own were considered as misallocated**.** The performance of the method was evaluated in terms of sensitivity, calculated as the proportion of correctly allocated individuals, and specificity as the number of correctly allocated individuals divided by the number of all individuals classified in that cluster, including misallocations. The percentage of assigned individuals according to different Q-values was also obtained (Soattin et al., 2009).

To detect possible effects of inbreeding, substructure, and coancestry, a dataset of 7 linkage-equilibrium randomly-mating populations was obtained using the Hybridlab software v1.0 (Nielsen et al., 2006). Simulated populations were obtained by drawing alleles at random from the frequencies of the actual dataset and equal numbers of genotypes were generated. The cluster analysis was then repeated under the conditions previously described.

In addition, using the actual genotypes, 60 crossbred chickens were simulated with the Hybridlab software v1.0 (Nielsen et al., 2006) (20 Bionda Cuneo × broilers, 20 Bionda Standard × broilers, and 20 Bianca × broilers). The cluster analysis was then performed using the overall actual dataset as a reference population in agreement with the best K-values obtained by the ΔK statistic under the outlined running conditions. Each crossbred genotype received a Q-value within each of the K clusters and it was allocated as previously described.

Cluster Analysis and Allocation Test Using a Subpanel of Loci

Clustering success as a function of a subpanel of microsatellites was determined using the Bels software (Bromaghin, 2008). The performance measure was the proportion of correctly allocated chickens; 200 chickens per population were simulated (1,000 replications). At the end of the backward locus elimination procedure (detailed explanation is given in Supplementary data, Cluster Analysis and Allocation Test Using a Subpanel of Loci), the loci were ranked from the most to the least able to resolve individual populations. To validate the choice of the number of loci, pair-wise comparisons of each indigenous breed with each commercial line were carried out using subpanels of loci from 24 to 4; the less useful loci were gradually discarded from the list sorted by the software (4 at a time) and the cluster analysis was performed as described above. Proportions of correctly allocated individuals with $Q \geq 0.90$, 0.95, and 0.99 were computed.

A subpanel containing the most resolving loci was finally arranged and data analysis for descriptive statistics was performed (Supplementary data, Cluster Analysis and Allocation Test Using a Subpanel of Loci). For multiple comparisons, statistical significance levels were Bonferroni-corrected. The allelic richness (number of alleles independently from sample size variation) and the observed and expected heterozygosity per locus were computed. The $\boldsymbol{F}_{\mathbf{IS}}$ inbreeding coefficient was computed to estimate departures from the expected heterozygosity (locus × populations P-values were obtained after 98,000 randomizations). Number of private alleles (alleles found in individual populations due to lack of gene flow) was obtained by direct counting. The F_{ST} index was also computed as previously; a Mantel test for correspondence was performed to compare the 2 F_{ST} matrices, namely subpanel vs. full panel.

To validate the allocation, the additional 39 samples of Bionda Standard and Bianca were used as anonymous samples and were analyzed with the subpanel of loci. The cluster analysis was carried out as outlined in clustering the crossbred genotypes.

RESULTS

Data Analysis for Genetic Differentiation

There was moderate differentiation among populations, that is $F_{ST} = 0.13$, with the contribution of all 28 loci, therefore 87% of the differences of allele frequencies was explained by variability within the source populations. The pair-wise comparison (Table 1) showed little to moderate distance between the 2 Bionda ecotypes and between Bionda and Bianca, whereas there was moderate to great differentiation between indigenous breeds and commercial lines.

Cluster Analysis and Allocation Test

At the first step of the cluster analysis, the best number of clusters was $K = 2$, namely meat chickens separated from layers (Supplementary Figure S1A) and

Table 1. Matrix of the F_{ST} genetic distance between the poultry populations using the full panel of 28 loci (above the diagonal) and the subpanel of 14 loci (below the diagonal).

	BPC	BPST	BS	BR	EK	HL	ISA
BPC		0.04	0.07	0.11	0.15	0.15	0.15
BPST	0.04		0.07	0.13	0.18	0.19	0.18
BS	0.08	0.07		0.11	0.15	0.16	0.15
BR	0.12	0.15	0.14		0.18	0.18	0.19
EK	0.12	0.15	0.14	0.17		0.07	0.03
HL	0.12	0.15	0.15	0.16	0.07		0.06
ISA	0.13	0.16	0.15	0.18	0.04	0.07	

BPC = Bionda Piemontese, ecotype Cuneo; BPST = Bionda Piemontese, ecotype Standard; BS = Bianca di Saluzzo; BR = Ross 708 broiler; EK = Eureka layer; HL = Hy-Line layer; ISA = ISA Brown layer.

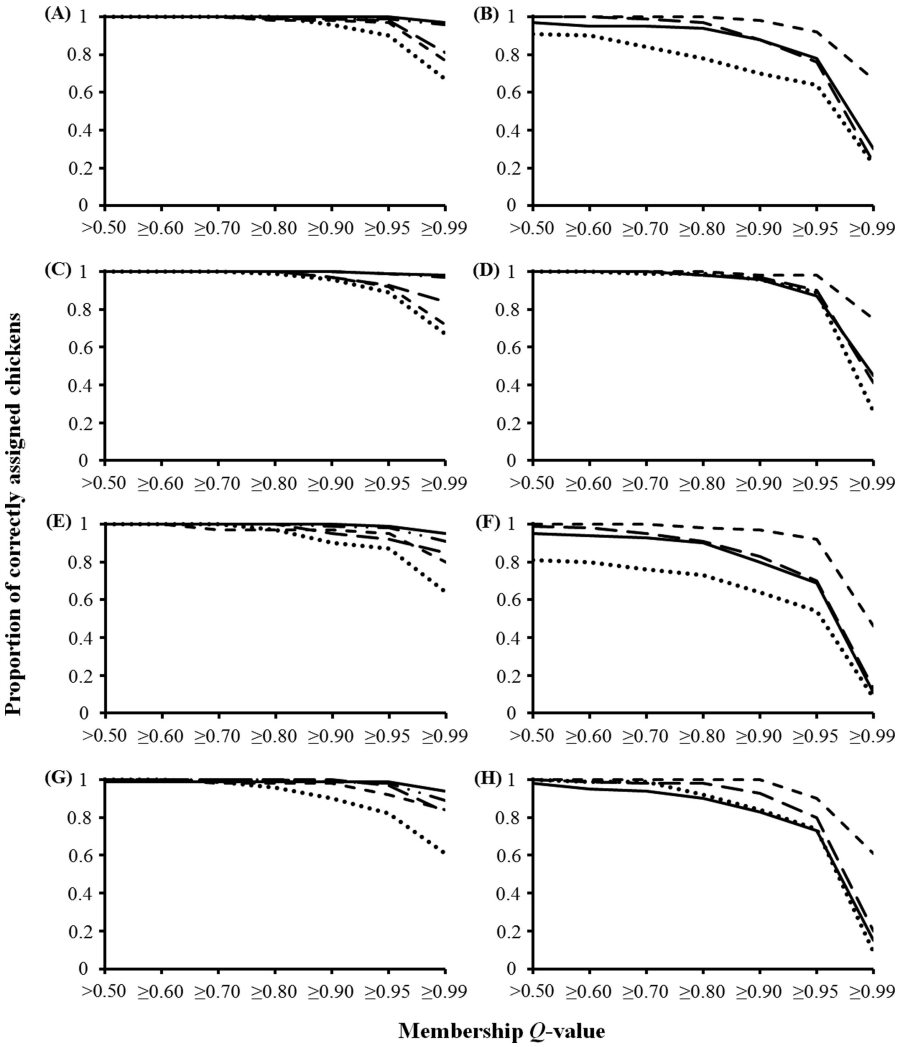

Figure 1. Proportions of correctly allocated chickens distributed according to different membership Q-values. (A) Overall actual dataset at $K = 2$ (28 loci). (B) Actual meat chickens at $K = 4$ (28 loci). (C) Overall simulated dataset at $K = 2$ (28 loci). (D) Simulated meat chickens at $K = 4$ (28 loci). (E) Overall actual dataset at $K = 2$ (14 loci). (F) Actual meat chickens at $K = 4$ (14 loci). (G) Overall simulated dataset at $K = 2$ (14 loci). (H) Simulated meat chickens at $K = 4$ (14 loci). Bionda Cuneo (•••); Bionda Standard (———); Bianca (—— ——); Broiler (— —); overall layers (— • • —).

all individuals were correctly allocated. Two inflection points were observed on the curves of proportion of correctly allocated chickens distributed according to different Q-values, namely at 0.80 to 0.90 and 0.95 (Figure 1A). Most chickens exhibited $Q \geq 0.95$ and most Bionda Standard and layers showed $Q \geq 0.99$. All chickens had $Q \geq 0.80$ except one Bianca and one broiler. In the second step (meat chickens only), the clustering solution exhibited a modal value at $K = 4$, and each cluster was associated with a distinct population (Supplementary Figure S1B). All chickens were correctly classified except 7 Bionda out of 213; these misallocations concerned only the assignment to ecotype within the Bionda breed. In Figure 1B, 2 main inflection points were observed at Q-values 0.80 and 0.95. All broilers and 97% of Bianca had $Q \geq 0.80$. As regards the 2

Table 2. Descriptive statistics of genetic variability for the poultry populations using the subpanel of 14 loci.

Population	AR^1	N_{PA}	$H_O{}^1$	$H_E{}^1$	$F_{IS}{}^2$
Bionda Cuneo	7.71 ± 1.26	12 (9)	0.62 ± 0.04	0.71 ± 0.03	$+0.14^{***}$
Bionda Standard	6.71 ± 0.92	9 (3)	0.64 ± 0.03	0.71 ± 0.02	$+0.11^{***}$
Bianca	7.93 ± 1.18	17 (11)	0.62 ± 0.05	0.71 ± 0.03	$+0.13^{***}$
Broiler	5.49 ± 0.77	7 (5)	0.58 ± 0.05	0.57 ± 0.05	−0.01
Eureka	5.14 ± 0.60	0	0.71 ± 0.06	0.61 ± 0.04	-0.14^{***}
Hy-Line	4.57 ± 0.50	2 (1)	0.74 ± 0.06	0.63 ± 0.04	-0.17^{***}
ISA Brown	4.71 ± 0.55	0	0.69 ± 0.04	0.61 ± 0.04	-0.11^{***}

AR = allelic richness; N_{PA} = number of private alleles and number of private alleles with frequency ≥ 0.01 in parenthesis; H_O = observed heterozygosity; H_E = expected heterozygosity; F_{IS} = inbreeding coefficient.
[1]The numbers indicate: average across loci ± standard error (13 df).
[2]Positive and negative values indicated heterozygosity deficiency and excess, respectively (after Bonferroni correction).
$^{***}P \leq 0.001$.

Bionda ecotypes, 96% of Bionda Standard but only 82% of Bionda Cuneo had $Q > 0.80$ in the proper cluster. If we considered the 2 ecotypes as a whole, 96% of Bionda chickens had $Q \geq 0.80$ within a unique Bionda cluster. The membership of Bionda and Bianca within broilers was always $Q < 0.20$ and most often < 0.10, whereas the membership of broilers within Bionda and Bianca was always $Q < 0.15$.

As regards the simulated dataset, at the first step it exhibited the same clustering and allocation pattern as the actual one, namely $K = 2$ clusters and all correct allocations across meat chickens and layers (Supplementary Figure S1C and Figure 1C). All chickens had $Q \geq 0.80$ except a Bionda Cuneo. In the second step, the simulated meat chickens separated into 4 population-specific clusters (Supplementary Figure S1D). All individuals were correctly classified. In Figure 1D, 2 inflection points were observed at Q-values 0.90 and 0.95. All chickens had $Q \geq 0.80$ except one Bionda Cuneo, 2 Bionda Standard, and 2 Bianca (that is, 5 out of 360 meat chickens). The membership of the indigenous chickens within broilers was $Q \leq 0.09$ except for one Bionda Cuneo and one Bianca, whereas the membership of broilers within indigenous clusters was always $Q < 0.10$. In general, there were small differences between the simulated and actual datasets. The proportion of meat chickens exhibiting high Q-values within the proper clusters was larger in the broilers than in the indigenous poultry.

The simulated crossbred genotypes were then allocated using the actual dataset as a reference population; only 2 out of 60 (3%) exhibited $Q > 0.80$ within one of the indigenous parental clusters.

Cluster Analysis and Allocation Test Using a Subpanel of Loci

When the backward elimination procedure was performed from the original set of 28 microsatellites, the proportion of correctly allocated chickens fell using less than 8 loci (Supplementary Figure S2). In the list of loci sorted by decreasing ability to resolve individual populations, the first 6 microsatellites were also the most polymorphic (data not shown): number of alleles and expected heterozygosity ranged from 7 to 28 and from 0.71 to 0.81, respectively. The pair-wise comparison of breeds and commercial lines showed that the proportion of correctly allocated chickens distributed according to different Q-values fell using less than 8 loci (Supplementary Figure S3), whereas some misallocations appeared using less than 12 loci. The proportion of Bianca chickens exhibiting $Q \geq 0.99$ within the correct cluster started to decrease below 12 loci.

The first 14 microsatellites were then carefully selected and assembled in 2 PCR reactions (Supplementary Table S1). At the selected loci, the indigenous breeds showed higher allelic richness than the commercial lines (Table 2); Hy-Line exhibited the highest observed heterozygosity, but Bionda and Bianca had the highest expected heterozygosity. A total of 47 private alleles were detected and 38 were observed in the indigenous breeds (24 in at least two chickens). Several private alleles showed frequency ≥ 0.01, in particular 23 in the indigenous breeds; furthermore, 6 private alleles in Bianca, 2 in Bionda Cuneo and broilers and 1 in Bionda Standard and Hy-Line had frequency > 0.05. The overall and locus × population F_{IS} values outlined heterozygosity deficiency in the indigenous breeds and excess in the layers after Bonferroni correction. Overall genetic difference was $F_{ST} = 0.12$ ranging from 0.04 to 0.18 in the pair-wise comparisons (Table 1); differentiation pattern between populations was very similar to that based on the full panel (matrix correspondence was $r = +0.9$, $P = 0.01$).

Using the subpanel of 14 loci in the cluster analysis, the actual and simulated datasets exhibited similar proportions of correctly allocated chickens distributed according to the Q-values; on the curves (Figure 1E to H), the presence of 2 inflection points was confirmed (at Q-values 0.70 to 0.80 and 0.95). Both overall datasets (actual and simulated) divided into 2 clusters, namely meat chickens and layers (Figure 2A and C). All allocations were correct except a simulated Bionda Standard, which was classified in the layers (correctly allocated using 28 loci). Only 3 Bionda Cuneo exhibited $Q < 0.80$; using 28 loci, they had $Q > 0.80$.

The meat chickens split into 4 clusters. In the actual dataset (Figure 2B), mutual 18 out of 213 misallocations were obtained between the Bionda ecotypes and

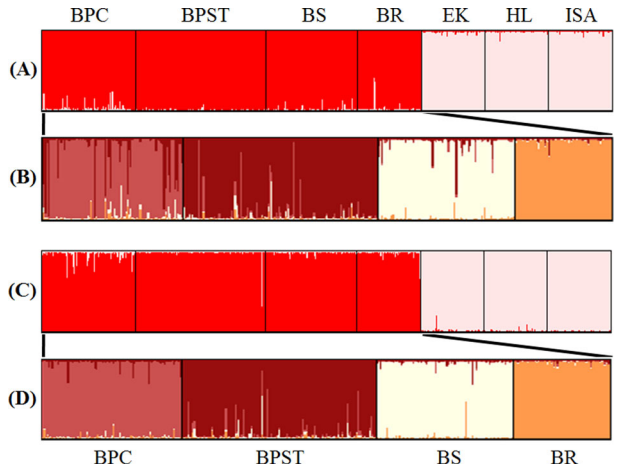

Figure 2. Cluster analysis and allocation tests of the poultry populations using the subpanel of 14 loci. Overall actual (A) and simulated (C) dataset clustered at $K = 2$. Actual (B) and simulated (D) meat chickens clustered at $K = 4$. BPC = Bionda Piemontese, ecotype Cuneo; BPST = Bionda Piemontese, ecotype Standard; BS = Bianca di Saluzzo; BR = Ross 708 broiler; EK = Eureka layer; HL = Hy-Line layer; ISA = ISA Brown layer.

a Bianca was classified as a Bionda Standard (correctly classified using 28 loci). One broiler, 7 Bianca, 9 Bionda Cuneo, and 7 Bionda Standard had $Q < 0.80$ (that is, 24 out of 360 meat chickens) (Figure 1F). In the simulated meat dataset (Figure 2D), all correct allocations were obtained except 3 Bionda Standard (classified in Bionda Cuneo); 22 indigenous genotypes exhibited $Q < 0.80$, that is 93% of the meat simulated chickens had $Q > 0.80$ within the proper cluster (Figure 1H). Once again, the membership of the indigenous chickens within broilers was $Q < 0.20$ and most often < 0.10.

Allocation of Anonymous Samples

The 39 Bionda Standard and Bianca chickens assessed as anonymous samples were then genotyped at the subpanel of 14 loci, and the actual dataset was used as a reference population in the cluster analysis. At $K = 2$ (meat vs. egg), all the anonymous chickens allocated within the meat cluster with $Q \geq 0.90$. At $K = 4$ (meat chickens only), all these anonymous samples correctly allocated and 36 out of 39 exhibited $Q \geq 0.80$ within the proper cluster, whereas the other 3 samples exhibited up to 0.30 within the other indigenous clusters. The membership of anonymous chickens within broilers was always $Q < 0.10$.

DISCUSSION

The 28 microsatellites used in our investigation are recommended by FAO (2011) and most available datasets are based on this panel (Granevitze et al., 2007; Wilkinson et al., 2012). The loci are highly polymorphic, reproducibility is satisfactory, and most chickens have complete multilocus genotypes (Sartore et al., 2016). The broilers and layers included in our investigation show relatively high coancestry and heterozygosity excess (Sartore et al., 2016). Bionda and Bianca exhibit a very different pattern because they have heterozygosity deficiency and low coancestry, even lower than that reported for other Italian breeds (Zanetti et al., 2010; Sartore et al., 2014). Within Bionda and Bianca, heterozygosity deficiency is likely to result from inbreeding. In addition, the flocks are grouped into subclusters that retain heterozygosity deficiency, whereas linkage disequilibrium may arise as a result of differences in allele frequencies among subclusters (Sartore et al., 2016).

The clustering method implemented by the Structure software is efficient to group individuals into putative source populations based on genetic differences and to detect immigrants (or cases of mislabeling) (Cornuet et al., 1999; Pritchard et al., 2000). Inbreeding, substructure, and coancestry could generate misleading signals and influence the allocation accuracy. Although Tadano et al. (2008) showed that the procedure implemented by the Structure software performs well despite some deviations from the expected properties, we thought it was appropriate to arrange a dataset of simulated genotypes with the same size of the actual one and no discrepancies from the expectations (random mating).

Correct allocation may be affected also by variability of loci, number of animals, and differentiation across populations (Dalvit et al., 2007). Cornuet et al. (1999) suggest that a full correct assignment can be achieved using 10 microsatellites on populations with 50 individuals, 0.60 of heterozygosity, and $F_{ST} \approx 0.10$. Tadano et al. (2007a) obtained 97% of correct allocations using 40 microsatellites on 12 populations with 0.65 of expected heterozygosity and $F_{ST} \approx 0.30$. Our set of samples and marker loci basically meets these conditions, therefore we continued the investigation with the cluster analysis.

The layers separate from the meat chickens, irrespective of coancestry or lack of random mating, and most exhibit proportion of genome at the highest memberships. The meat chickens split into 4 clusters with moderate differentiation. The membership value, Q, of the correctly allocated individuals is not a trivial problem (Maudet et al., 2002; Mateus and Russo-Almeida, 2015). According to Wilkinson et al. (2012), $Q < 0.80$ could be considered as a clue to within-individual admixture; nevertheless, the proportion of ambiguous membership is also important. All chickens are correctly allocated, no broilers and few indigenous individuals (3 to 4%) exhibit $Q < 0.80$. Inbreeding and substructure within the actual dataset weakly reduce the proportion of chickens exhibiting high membership. Any breed keeping high natural variation could be made up of different flocks providing uneven contribution to replacements (Rosenberg et al., 2001; Sartore et al., 2016); this may be the explanation of the small number of indigenous individuals with ambiguous membership (such individuals are absent in the more homogeneous broilers and layers): the few indigenous-declared

chickens that exhibit $Q < 0.80$ within the expected cluster could be confused with the other indigenous chickens, but not with the commercial lines ($Q < 0.20$ within both broilers and layers). Bionda and Bianca are similar to each other and different from the main commercial lines that are sold in the Piedmont market for chicken meat supplying.

Broadly speaking, our results are in accordance with investigations on other Italian populations. Using amplified fragment length polymorphisms, no chickens belonging to 6 Veneto breeds and one commercial line are assigned at $Q > 0.99$, whereas the proportion increases up to 83% at $Q > 0.95$ (Soattin et al., 2009). The same breeds exhibit little to moderate differentiation using 20 microsatellites (Zanetti et al., 2010). Almost all Ancona, Livorno, and SASSO chickens are correctly allocated at $Q > 0.90$ using 30 microsatellites with great differentiation (Bianchi et al., 2011). The most important issue is that broilers and layers come from homogeneous populations and never enter the clusters of our indigenous breeds. They exhibit high membership within the proper clusters, no ambiguity within individuals, and satisfactory proportions of membership up to the highest values. Our results are also in accordance with several investigations that show genetic distance between commercial lines and indigenous chickens and, in particular, between broilers and some local breeds used for meat consumption (Hillel et al., 2003; Tadano et al., 2007b; Berthouly et al., 2008; Berthouly et al., 2009; Bodzsar et al., 2009; Granevitze et al., 2009; Mtileni et al., 2011; Leroy et al., 2012; Shimogiri et al., 2012; Lyimo et al., 2014; Ceccobelli et al., 2015).

The Bionda ecotypes show little differentiation and mutual misallocations. Very recent common origin or high frequency of gene exchange between stocks reared in the same region can explain both misallocations and low proportions of chickens showing the highest Q-values (Rosenberg et al., 2001). A margin of ambiguity is then expected between the Bionda ecotypes and even between the 2 indigenous breeds (moderate differentiation), but it seems unnecessary to trace back products to the ecotype level within small local populations, and it is unlikely that the niche products provided by Bionda and Bianca will compete on the same market. In synthesis, the actual dataset can be used as a reference population with great confidence because the source populations originate genetically distinct clusters without a priori knowledge of sample location; non-random mating and substructure may reduce sensitivity and specificity, but only populations having weak genetic differentiation are involved, namely those that are less interesting to differentiate when products must be traced. It should be possible to distinguish meat of commercial livestock from meat obtained from the overall cluster of indigenous breeds without ambiguity.

As far as we know, the crossing between our indigenous meat breeds and commercial lines seems to be very unlikely, nevertheless we simulated a crossbred population of meat chickens and evaluated the allocation pattern. Some crossbred genotypes exhibit a membership $0.80 < Q < 0.90$ within one of the indigenous parental cluster. As a consequence, adopting a threshold of $Q \geq 0.80$ for the authentication all broilers and layers and 97% of crossbreds are excluded from our indigenous clusters with no ambiguity. Regarding the crossbreds, a residual error of 3% may be expected. A proportion of about 5% of indigenous chickens would remain undetermined because they exhibit $Q < 0.80$ within the proper cluster mainly due to similarity between Bionda and Bianca more than to relationship with the commercial lines.

To reduce the cost of analyses with the least loss of accuracy, different approaches may be taken to arrange subpanels of loci (Tadano et al., 2008). In our investigation, the Bels software (Bromaghin, 2008) provided the most resolving combination of 14 microsatellites, some exhibiting the highest polymorphic content (Chazara et al., 2013; Han et al., 2013). Our choice is in accordance with Rosenberg et al. (2001), who stated that expected heterozygosity and number of alleles are better than F_{ST} to choose markers for cluster analysis and individual allocation, and that 12 to 15 highly variable microsatellites should be genotyped to achieve around 90% allocation accuracy. In addition, 8 of the 15 best loci of Rosenberg et al. (2001) are included in the list of our 14 microsatellites.

A fair amount of private alleles with intermediate frequencies is present. In comparison with the full panel of loci (Sartore et al., 2016), the 14 loci retain 63 to 81% of private alleles, in particular 69 to 75% of private alleles showing frequency ≥ 0.01 and all the alleles with frequency ≥ 0.05 except 1 in Bianca. Although private alleles alone cannot support the traceability, they may play a role as Rosenberg et al. (2001) have pointed out with particular reference to *LEI0228* and *LEI0192* loci, both present in our panel.

Broilers and layers are easy to separate into distinct clusters with high membership, and all chickens may be correctly allocated using a small microsatellite number. Misallocations increase as the number of loci decreases, but they only occur within or between the indigenous poultry. When populations retain little differentiation, the ambiguity is relatively insensitive to the number of loci because most variability depends on differences among individuals. If meat from a commercial chicken was supplied labeled as an indigenous chicken, the mislabeling would be found out. The full microsatellite panel will be applied if, using the subpanel at a first step, any chicken is allocated against the expectation (Figure 3).

The allocation of the anonymous samples gives good results even using the subpanel. Few samples (2 out of 39) are classified below the highest membership and they have some membership within the other indigenous clusters.

The major problem for the feasibility of a molecular system is the cost (Dalvit et al., 2007). The analysis of the best 14 loci (2 multiplex PCR with amplicon

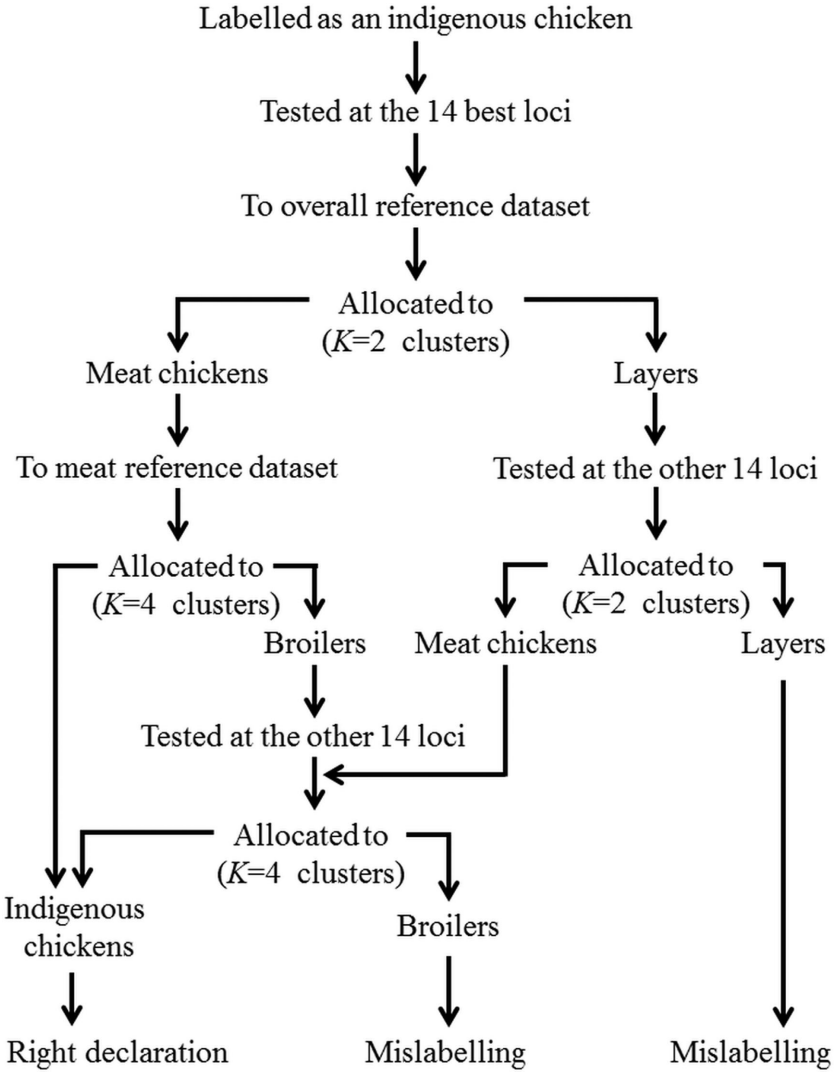

Figure 3. Procedure by which the allocation of a chicken should be evaluated.

processing) costs EUR 20 per chicken. The tool is unsustainable as a routine test, whereas it is sustainable as a method to detect possible fraud or mislabeling. This test should be sufficient in most cases, because no commercial chickens exhibit ambiguous membership within indigenous clusters. For example, when 50 out of 1,000 chickens are randomly tested, the farmer association could bear the cost of EUR 1,000 that would amount to an additional EUR 1 per chicken, if shared among the overall batch. Any chicken allocated against the declaration could be analyzed with the full set, so other two optimized multiplex PCR (the 14 remaining loci) would produce an additional increase by EUR 0.02. Cases like this should become uncommon as the traceability system is developed, because deception should be discouraged.

Granevitze et al. (2014) have found that the microsatellites assign chickens more correctly than any other marker type and 29 loci are better than 152 SNP; nevertheless, the use of higher numbers of SNP would improve resolution. The cost of SNP analysis strongly depends on number of loci and individuals tested (Nickerson, 2012). With 75 SNP having the resolving power of 14 microsatellites, the analysis of a few dozen chickens costs EUR 0.5 per marker, that is, EUR 38 per individual. If all 1,000 chickens were tested with the same panel of SNP, the cost could decrease to about EUR 20 per individual. Therefore, a panel of SNP with the same informative content as a panel of microsatellites is cost efficient only if a high number of individuals are concurrently tested; otherwise, the development of a panel would not be inexpensive for random testing.

Even if our investigation is not innovative from a technical point of view, as far as we know, this is the first contribution that evaluates the accuracy of a genetic traceability system applied to indigenous poultry considering, at the same time, the effects of non-random mating and number of marker loci as confounding issues and the distribution of membership values to optimize accuracy. The results should be of broad relevance and provide a comparative model because substructure and inbreeding are not uncommon within indigenous

European breeds. The genetic distinction of the Piedmont indigenous poultry enables the clustering method to identify the origin of chickens. Non-random mating does not compromise the sensitivity and specificity of allocation. The dataset can be used as a reference population and the molecular tool of 28 loci is useful to verify the origin of a single chicken, carcass, or retail cut. The test with the best 14 loci keeps sufficient resolving power to provide accuracy and cost saving. Irrespective of number of loci, weakly ambiguity may exist between Bionda and Bianca, but there is no economic justification for the competition between 2 indigenous breeds.

In conclusion, the method proposed is effective in detecting industrial meat products and distinguishing them from indigenous products. In addition, it may be used for the breed traceability within the limits of the Piedmont Bionda and Bianca. The adoption of a monitoring system on regular basis should increase the value of typical products because the additional burden of molecular analyses would improve the commercial grade and the quality perception. Moreover, data from this investigation could contribute to conservation of indigenous breeds.

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SUPPLEMENTARY DATA

Supplementary data are available at *PSCIEN* online.

Figure S1. Cluster analysis of the poultry populations using 28 loci. (A) Overall actual dataset clustered at $K = 2$. (B) Actual meat chickens clustered at $K = 4$. (C) Overall simulated dataset clustered at $K = 2$. (D) Simulated meat chickens clustered at $K = 4$. BPC = Bionda Piemontese, ecotype Cuneo; BPST = Bionda Piemontese, ecotype Standard; BS = Bianca di Saluzzo; BR = Ross 708 broiler; EK = Eureka layer; HL = Hy-Line layer; ISA = ISA Brown layer.

Figure S2. Supplementary Figure S2. Performance (proportion of chickens that were correctly allocated to the user-defined populations) as a function of the number of loci. Average (———) and minimum (•••) proportion were provided by the Bels software (Bromaghin, 2008).

Figure S3. Proportions of correctly allocated chickens distributed according to different membership Q-values as a function of the number of loci. (A) Bionda vs. broilers. (B) Bionda vs. layers. (C) Bianca vs. broilers. (D) Bianca vs. layers. $Q \geq 0.90$ (———); $Q \geq 0.95$ (— —); $Q \geq 0.99$ (•••).

Table S1. The 14 most resolving loci combined into 2 multiplex PCR: properties across Bionda and Bianca poultry breeds.

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