

# Genetic traceability of two local chicken populations, *Bianca di Saluzzo* and *Bionda Piemontese*, versus some current commercial lines

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## Abstract

The aims of this investigation were to analyse the genetic variation of two Piemonte chicken local breeds, *Bionda Piemontese* and *Bianca di Saluzzo*, and to set them against some commercial lines. A panel of 19 microsatellite markers was used. On the overall, the results of different analyses highlight the genetic uniqueness of the two breeds; therefore they should be considered genetic resources worthy of preservation. The panel of microsatellites used in this investigation turns out to be a consistent and reliable tool for traceability. In fact, these markers are able to distinguish the two local populations from the commercial lines and they are able to confirm the existence of two genetically different clusters within the *Bionda Piemontese*, namely the ecotypes *standard* and *Cuneo*. Mating policies implemented to avoid inbreeding and, if necessary, a marker assisted conservation scheme would be sufficient to solve the problem of inbreeding.

## Introduction

The quality, rather than the quantity, of food of animal origin met recently the attention of the consumers, together with the growing perception that regional and traditional products could be healthier and tastier. This novel tendency is based on a reduced confidence towards food of animal origin, as a consequence of the outbreak of diseases like bovine spongiform encephalopathy, followed by avian influenza crisis (Ciampolini *et al.*, 2000; Goffaux *et al.*, 2005). Moreover, Opara and Mazaud (2001) reported a rising incidence of foodborne disease caused by microbial contamination of processed food, leading to additional mistrust in consumers.

Therefore an increasing need to check food processing aimed to prevent frauds and adulterations is arisen. The idea of traceability, intended to be a method able to identify animal or animal products through different steps of the food chain (McKean, 2001), became familiar to consumers, and the development of a reliable traceability system started to play a key role in this area of interest.

The EU always considered food safety a purpose of primary importance, first of all because it is the biggest producer of food and beverage of the world (European Commission, 2000). Following the regulation 178/2002 of the European Parliament, *the traceability of food, feed, food-producing animals, and any other substance intended to be, or expected to be, incorporated into a food or feed shall be established at all stages of production, processing and distribution*.

Then the concept of traceability turned out to be crucial both in safeguarding public and animal health and enhancing the value of typical products; consequently since 2005 it became mandatory for all member countries (Dalvit *et al.*, 2005).

The efficiency of a traceability system is based on three pivotal points, i.e. the identification of the product, the collection of information on the product, and an integrated information management system (Nicoloso *et al.*, 2013). The main biological source of information is DNA, which is inalterable, detectable, and permits the identification of individual, breed, and species, and whose analysis overtakes the limits of the traditional methods like ear tags (Cunningham and Meghen, 2001). In this regard, the use of microsatellite markers is one of the most common strategies, and it proved to be efficient in genetic characterization and traceability of breeds belonging to different species like chicken (Rosenberg *et al.*, 2001; Zanetti *et al.*, 2010; Granewitz *et al.*, 2014), sheep (Bramante *et al.*, 2011; Lasagna *et al.*, 2011), pig (Boitard *et al.*, 2010; Wilkinson *et al.*, 2011; Oh *et al.*, 2014), cattle (Maudet *et al.*, 2002; Moioli *et al.*, 2004; Ciampolini *et al.*, 2006; Orrù *et al.*, 2006; Dalvit *et al.*, 2008; Rodríguez-Ramírez *et al.*, 2011; Rogberg-Muñoz *et al.*, 2014), and fish (Yue *et al.*, 2012).

Chicken meat is an important component of human nutrition. According to Zanon and Sabbioni (2001), the number of Italian local or autochthonous chicken breeds has dealt with a severe reduction in

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size, caused by replacement with cosmopolitan lines and highly productive crosses. The most dramatic effect of the intensive farming was the extinction of many local breeds (Gandini and Villa, 2003). Of the rural breeds present fifty years ago, 61% are currently extinct, 11% survive but are endangered, whereas only 7% have been involved in conservation programs.

In Piemonte region, two autochthonous breeds exist: *Bianca di Saluzzo* and *Bionda Piemontese*, both reared in the Asti and Cuneo provinces for meat production. In past decades, both breeds were subjected to a strong reduction in population size and were frequently replaced by fast-growing lines.

The aims of this investigation were to analyse the genetic variation of these breeds and to set them against some commercial lines. A panel of 23 microsatellite markers was used.

## Materials and methods

### Sample collection

A total of 540 blood samples belonging to the two Piemonte breeds and to four different commercial lines were collected: 213 from *Bionda Piemontese* (BP), 86 from *Bianca di Saluzzo* (BS), 61 from broiler chickens (BR), and 60 from each of three egg-laying commercial lines (CL), *i.e.* Hy-line, Isa Brown, and Eureka. Within the BP, the farmers usually distinguish two ecotypes based on different sampling areas and morphological traits, *i.e.* *Bionda Piemontese standard* (BPS) and *Bionda Piemontese Cuneo* (BPC); therefore we divided the BP into BPS (124 individuals) and BPC (89). The animals were chosen in different farms, namely 15 for BPC, 18 for BPS, and 6 for BS, in order to obtain a representative sample of each breed. DNA was extracted from blood specimens using the NucleoSpin QuickPure extraction kit (Macherey-Nagel, Dueren, Germany).

### Genotyping

A set of 23 microsatellite loci was used, ADL0268, ADL0278, LEI0094, MCW0216, MCW0248, MCW0034, MCW0069, MCW0081, MCW0222, MCW0295, LEI0166, LEI0234, MCW0016, MCW0037, MCW0111, MCW0020, MCW0104, MCW0123, MCW0165, ADL0112, LEI0192, MCW0014, and MCW0183. These were part of the loci recommended by the Food and Agriculture Organisation (<http://www.fao.org/docrep/meeting/022/am652e.pdf> Rome, 18-22 July 2011) and by the AVIANDIV project (2011) ([http://aviandiv.tzv.fal.de/primer\\_table.html](http://aviandiv.tzv.fal.de/primer_table.html)) and they are suitable for multiplex polymerase chain reaction (PCR) protocols. The markers were subjected to a multiplex PCR amplification in 10 mL reactions using the following final concentrations: 1X buffer Qiagen (Hilden, Germany), 0.4 mM dNTPs, and 0.05 mM HotStartTaq Qiagen. The following thermo-cycling conditions were used: an initial denaturation step of 15 min at 95°C, 31 cycles of 30 s at 95°C, 1 min at the annealing temperature specific to of each multiplex PCR, 1 min at 72°C, and a final extension of 7 min at 72°C. Analyses of fragments were performed using the automated DNA Genetic Analyzer ABI PRISM 310 (Applied Biosystems, Foster City, CA, USA) and the computer software GeneMapper 4.0 (Applied Biosystems). Allele calling was adjusted to Aviandiv project nomenclature including nine DNA reference samples. An error assay was performed by replicating the genotyping on a randomly chosen 10% of individual samples. The average error rate per locus was computed (Pompanon *et al.*, 2005).

## Descriptive statistics for genetic diversity

The main statistics were obtained using the software Fstat version 2.9.3.2 (Goudet, 1995) and GenAIEx version 3.2 (Peakall and Smouse, 2006, 2012). Observed number of alleles (A), allelic richness (R), number of private alleles (Ap), and observed (Ho) and unbiased expected (He) heterozygosity were calculated by locus, overall loci, and by breed/line. Fis statistics per locus and breed/line and the significance of their non-zero values were performed (alleles were randomized among individuals within populations).

## Genetic differentiation and cluster analysis

The Wright's F-statistics were calculated. Allele frequency differences across and between breeds/lines were tested with Fst statistics across all loci using the analysis of molecular variance. The deviation from the null hypothesis was tested with 999 permutations (GenAIEx 3.2). Average molecular coefficient of kinship (coancestry),  $f_{ij}$ , between  $i$  and  $j$  individuals within breed/line was also computed by bootstrapping (1000), equalising for sampling size (77), and weighted by the polymorphism information content of the marker loci (Molkin version 3.0, Gutiérrez *et al.*, 2005).

The relationship between breeds/lines and individuals were represented using the FCA (factorial correspondence analysis) performed by the Génétix software (Lewis and Zaykin, 2001). A frequency-based population assignment test (Paetkau *et al.*, 1995) was carried out and the leave-one-out procedure was used (GenAIEx 3.2).

The Bayesian methodology implemented by the Structure version 2.0 software was employed to determine the level of structure and substructure in the dataset (Pritchard *et al.*, 2000). The assignment of individuals to breeds/lines assumed an ancestry model with admixture and correlated allele frequencies without prior information. Ten independent runs with 1,000,000 MCMC (Markov chain Monte Carlo) iterations and a burn-in period of 300,000 were carried out for  $3 \leq K \leq 8$  number of clusters to estimate the most likely number of clusters (K) present in the dataset. The number of clusters was established by calculating  $\Delta K$  by the Structure Harvester software (Evanno *et al.*, 2005; Earl and vonHoldt, 2012).

## Results

Four microsatellites out of the 23 analysed were discarded because they scored a high error rate ( $>0.02$ ), so the genotyping data from 19 loci were finally used. The rate of missing genotypes was only 0.06%.

All loci showed to be polymorphic and an overall number of 162 alleles was detected. The number of alleles per locus ranged from 26 (LEI0192) to 3 (MCW0248 and MCW0037).

The genetic variation within breed/line was quantified using the descriptive statistics of Table 1. The average number of alleles was very similar to the average allelic richness; therefore differences in sample size across breeds/lines did not affect the genetic analyses.

Observed heterozygosity ranged from 0.64 in CL to 0.59 in BP, expected heterozygosity ranged from 0.67 in BPC to 0.55 in BR. The combined Fis values were significantly different from zero. Seven loci out of 19 in BPS, 8 in BPC, and 6 in BS contributed to heterozygosity deficiency. Removal of MCW0014, which showed the most difference ( $P < 0.0001$ ), did not reduce the value of significance. BR and CL showed heterozygosity excess. The average coefficient  $f_{ij}$  within breed/line was higher ( $>0.4$ ) in BR and CL than in the two local breeds (Table 2).

Variation among the breeds/lines, as estimated by the global Fst index, was highly significant ( $Fst = 0.109$ , std. err. 0.017,  $P < 0.001$ ). All

pairwise  $F_{ST}$  values contributed with significant differences included the two ecotypes of BP ( $F_{ST}=0.035$ ,  $P<0.001$ ) (Table 3). The largest differences were obtained between the two local breeds and the other lines ( $F_{ST}>0.100$ ). Most private alleles were observed in the two local breeds, i.e. 13 in BP (4 in BPS and 9 in BPC) and 15 in BS (Table 4). Two alleles out of 4 showed a frequency above 0.01 in BPS, 6 out of 9 in BPC, and 10 out of 15 in BS. The FCA plot of individual genotypes is presented in Figures 1 and 2. The distribution based on axis 1 (45% of global genetic diversity) supported a clear difference between EL and the meat-producing chickens. Axis 2 (24% of diversity) separated BP from BS and BR. The third factorial component (axis 3, 21% of diversity) provided a further difference between BS and BR.

The pair wise assignment tests, as performed with the GenAIEx software, corroborated these results: BP and BS differed from BR and EL (*data not shown*). The assignment test gave 97% of correct individual assignments to the five clusters (Table 5). Four BPS individuals clustered together with BPC and 8 BPC with BPS. Two BP were assigned to EL and one BS to BR.

The Bayesian analysis performed by the Structure software showed that estimated posterior probability ( $\ln \Pr(\text{GK})$ ) reached plateau value at  $K=7$  (24,672) but the modal value of the distribution of DK statistics was located at  $K=5$  (-24,959) (Figure 3). The clustering pattern was as follow: BP split the two ecotypes BPS and BPC, BS, BR, and overall EL (Figure 4). Average membership of all breeds/lines except BPC was >0.900 in one of the five clusters (*data not shown*). In the present investigation, we were not able to distinguish the three lines of EL each other. Most incorrect individual assignments of BPS and BPC corresponded to the results obtained with the GenAIEx software, but no BP and BS were assigned to EL or BR.

## Discussion

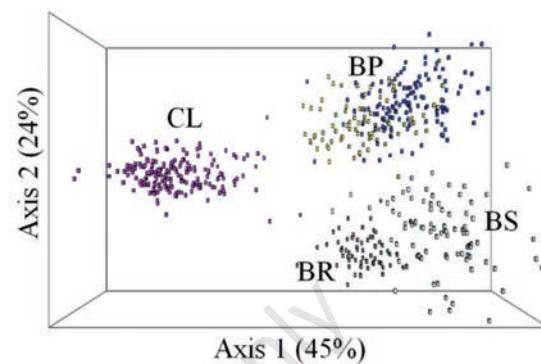
The panel of selected microsatellite markers revealed to be a good tool for diversity analyses of our local breeds. In a survey carried out on Spanish chicken breeds, Dávila *et al.* (2009) detected less than 4 alleles per locus. Hillel *et al.* (2003) assessed the genetic variation within and among 52 populations from a wide range of chicken types (local and commercial) and found an average of 3.52 alleles per locus. In Italian breeds, Bianchi *et al.* (2011) detected 3.73 alleles per locus in the Livorno, 3.50 in the Ancona, and 4.03 in Sasso.

The present investigation was carried out with the same panel of markers used by Hillel *et al.* (2003) and Bianchi *et al.* (2011) and reveals a similar or greater level of variation in the two Piemonte breeds, i.e. 5.89 alleles per locus in BPS, 6.37 in BPC, and 6.11 in BS,

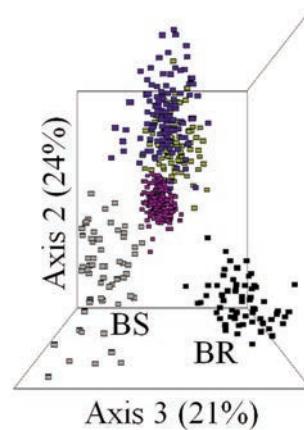
whereas less than 5 are present in broilers and layers.

In BP and BS the numbers of private alleles are not very high (13 and 15, respectively, vs 17 in Ancona and 26 in Livorno), nevertheless most of them show a frequency above 0.01, so they may play a role in clustering success.

Dalvit *et al.* (2009) found a high and significant deficiency of heterozygosity in two Veneto local breeds, *Ermellinata di Rovigo* and



**Figure 1.** Distribution of individuals based on the factorial correspondence analysis, axes 1 and 2. BP, *Bionda Piemontese*; BS, *Bianca di Saluzzo*; BR, broiler chickens; CL, egg-laying commercial lines.



**Figure 2.** Distribution of individuals based on the factorial correspondence analysis, axes 2 and 3. BS, *Bianca di Saluzzo*; BR, broiler chickens.

**Table 1.** Genetic diversity estimates over 19 marker loci for each breed/line included in this investigation.

	N	A	R	Ap	Ho	He	Fis	P
BPS	112	5.89	5.47	4	0.59	0.66	+0.104	***
		0.68			0.04	0.03		
BPC	121	6.37	6.17	9	0.59	0.67	+0.113	***
		0.81			0.04	0.03		
BS	116	6.11	5.86	15	0.60	0.66	+0.089	***
		0.67			0.04	0.03		
BR	85	4.47	4.47	4	0.61	0.55	-0.112	***
		0.44			0.04	0.03		
CL	88	4.63	4.37	3	0.64	0.59	-0.087	***
		0.47			0.07	0.04		

N, number of alleles; A, average observed number of alleles per locus; R, average allelic richness per locus; Ap, overall number of private alleles; Ho, observed heterozygosity; He unbiased expected heterozygosity; BPS, *Bionda Piemontese* standard; BPC, *Bionda Piemontese* Cuneo; BS, *Bianca di Saluzzo*; BR, broiler chickens; CL, egg-laying commercial lines. \*\*\*P<0.001 (significance of non-zero values of Fis).

*Robusta Maculata*. Over a 3-year period, the expected heterozygosity decreased from 0.54 to 0.42 and from 0.40 to 0.28, respectively.

The average expected heterozygosity in the two Piemonte breeds is rather high and similar to the value of the *Sasso* breed (0.66 vs 0.60), a population which received a gene flow from other breeds (Bianchi *et al.*, 2011). The average coefficients  $f_{ij}$  values within BP and BS were lower than those reported by Zanetti *et al.* (2011) for the other local Italian chicken breeds. Interestingly, compared to BP and BS, BR and EL show both heterozygosity excess and more co-ancestry. Foundation of commercial lines is based on different grandparent stocks from highly selected nuclei (Hillel *et al.*, 2003; Tadano *et al.*, 2007): if a limited number of stocks is used, these breeding practices may cause high observed heterozygosity in chickens sharing most alleles identical by descent. Actually, we have poor information about the phylogenetic relationships of BP and BS, nevertheless the presence of private alleles and the differences emphasized by the cluster analysis shows that no recent introgression of allochthonous germplasm took place, in particular from commercial lines.

In fact, *Sasso* showed an excess of observed heterozygosity, just what happens to BR and CL in our investigation, which are obtained by specific crossings. On the opposite, significant deficiency of observed heterozygosity is present in BP and BS, as in other local breeds, which is usually a signature of inbreeding or sub-structuring in closed populations. This does not exclude that different populations could have curred to foundation of BP and BS. All breeds received the genetic contribution of migration and admixture in the near or remote past. Afterwards, breeders concentrated on selection and genes *migrant* and *native* found their optimal proportions for a particular environment and production system (Nicholas, 2010).

This gene pool must be conserved. Mating policies and, if necessary, a marker assisted conservation scheme would be sufficient to limit the inbreeding. The existence of genetic differentiation between the two BP ecotypes is worth analysing in detail with further investigation. If such ecotypes have some attractive peculiarities, they would be preserved and exploited, otherwise their reproductive isolation just increases genetic subdivision, which will lead to additional homozygosity excess (Bianchi *et al.*, 2011).

**Table 2. Coefficient  $f_{ij}$  within breed/line.**

	$f_{ij}$	Standard error
BPS	0.317	0.005
BPC	0.327	0.005
BS	0.333	0.006
BR	0.435	0.008
CL	0.415	0.006

BPS, *Bionda Piemontese* standard; BPC, *Bionda Piemontese Cuneo*; BS, *Bianca di Saluzzo*; BR, broiler chickens; CL, egg-laying commercial lines.

**Table 3. Fst (above the diagonal) and its significance (below the diagonal) as a measure of genetic differentiation between pairs of the breeds/lines.**

	BPS	BPC	BS	BR	CL
BPS	-	0.035	0.067	0.128	0.136
BPC	***	-	0.072	0.110	0.103
BS	***	***	-	0.124	0.131
BR	***	***	***	-	0.151

BPS, *Bionda Piemontese* standard; BPC, *Bionda Piemontese Cuneo*; BS, *Bianca di Saluzzo*; BR, broiler chickens; CL, egg-laying commercial lines. \*\*\*P<0.001 (significance of non-zero values).

The analysis of genetic diversity within and among breeds/lines was improved by the FCA and assignment test. The first three axes explained a high proportion of the total variation; therefore the separation of the two Piemonte breeds from each other and from the commercial lines is very reliable.

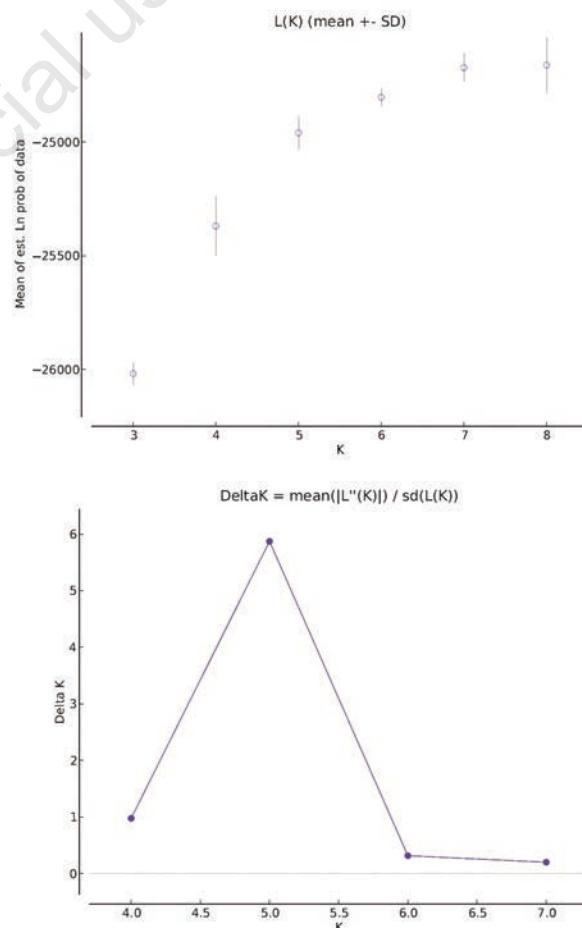
These results are confirmed by the Bayesian approach using the DK of Evanno *et al.* (2005), whose modal value is at the true K for most situations.

In the assignment tests, only few individuals moved away from the alleged cluster, two BP towards EL and one BS towards BR, but only with the Paetkau *et al.* (1995) test. The most important result is that no BR or EL were incorrectly assigned to BP or BS.

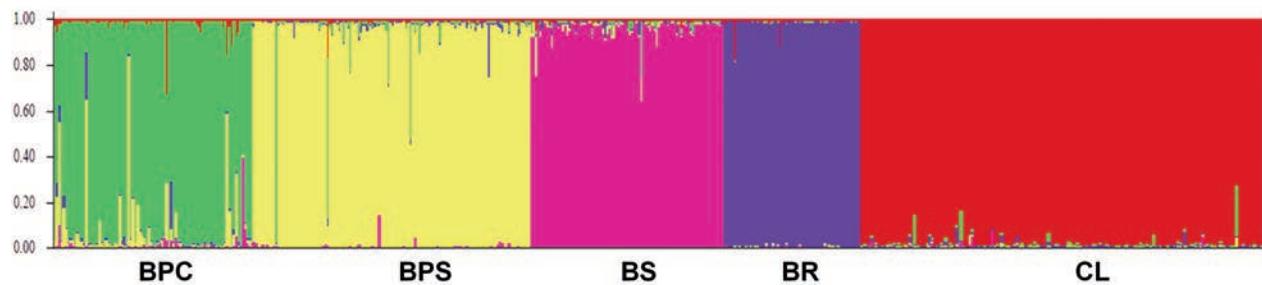
The clustering algorithm implemented by the Structure software has a great potential to correctly assign individuals of unknown origin (Rosenberg *et al.*, 2001).

The panel of markers used in our investigation is a useful tool for traceability. This result is in agreement with the conclusions of Granevitze *et al.* (2014), who stated that microsatellite loci may perform even better than other marker types in the assignment tests applied to wild and domestic chicken populations.

In practice, the assignment tests show to be suitable tools to certify breed origin of meat and to detect adulterations of labelled products in chicken (Nakamura *et al.*, 2006; Rikimaru and Takahashi, 2007), and also in pig (Oh *et al.*, 2014), cattle (Ciampolini *et al.*, 2006; Orrù *et al.*,



**Figure 3. Graphic visualisation of estimated posterior probability of  $\ln \Pr(G/K) = L(K)$  and delta K distribution.**



**Figure 4.** Bayesian cluster analysis at K=5. BPC, *Bionda Piemontese Cuneo*; BPS, *Bionda Piemontese standard*; BS, *Bianca di Saluzzo*; BR, broiler chickens; CL, egg-laying commercial lines.

**Table 4. Summary of private alleles in the two local breeds.**

	Locus	Allele (bp)	Frequency
BPS	MCW0104	204	0.028
	MCW0014	164	0.012
	ADL0112	122	0.008
	MCW0034	226	0.004
BPC	LEI0192	245	0.028
	MCW0014	170	0.028
	LEI0192	249	0.022
	MCW0111	112	0.017
	LEI0192	283	0.017
	MCW0183	326	0.011
	LEI0094	241	0.006
	LEI0094	259	0.006
	LEI0192	257	0.006
BS	MCW0034	212	0.064
	ADL0278	120	0.052
	MCW0081	122	0.052
	LEI0192	413	0.052
	MCW0016	206	0.041
	LEI0166	362	0.035
	LEI0094	275	0.029
	LEI0192	405	0.023
	MCW0014	182	0.017
	MCW0104	198	0.012
	ADL0278	112	0.006
	ADL0278	119	0.006
	MCW0216	149	0.006
	MCW0034	218	0.006
	LEI0166	358	0.006

BPS, *Bionda Piemontese standard*; BPC, *Bionda Piemontese Cuneo*; BS, *Bianca di Saluzzo*.

**Table 5. Assignment test to correct (Self) or incorrect (Others) breed/line (Paetkau *et al.*, 1995).**

	Self	Others
BPS	119	5
BPC	80	9
BS	85	1
BR	61	0
CL	180	0
Total	525	15
Proportion	0.97	0.03

BPS, *Bionda Piemontese standard*; BPC, *Bionda Piemontese Cuneo*; BS, *Bianca di Saluzzo*; BR, broiler chickens; CL, egg-laying commercial lines.

2006; Negrini *et al.*, 2008; Rogberg-Muñoz *et al.*, 2014), and fish (Yue *et al.*, 2012).

## Conclusions

On the overall, the results of different analyses highlight the genetic uniqueness of the two Piemonte breeds; therefore they should be considered genetic resources worthy of preservation. Secondly, the panel of 19 microsatellites used in this investigation turns out to be a consistent and reliable tool for traceability. In fact, these markers are able to distinguish the two local populations, BP and BS, from commercial lines and, moreover, they are able to confirm the existence of two genetically different clusters within the BP, namely BPS and BPC.

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