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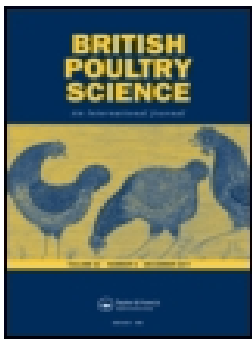
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Genetic variability of two Italian indigenous chicken breeds inferred from microsatellite marker analysis

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Abstract. 1. The objective of this study was to determine the genetic structure and variability of Bionda Piemontese and Bianca di Saluzzo (Piedmont, Northwest Italy) using an international set of microsatellite loci (AVIANDIV-FAO). Differences compared with commercial lines and other Italian breeds were verified to justify the implementation of conservation programmes.

2. Flock contribution to genetic variability was assessed following the approach implemented in the MolKin software. Comparison was performed using the fixation index and the Reynolds genetic distance. The most likely number of different populations was estimated using the clustering procedure implemented in STRUCTURE.

3. The molecular information suggests that management practices could have prevented random mating and produced inbreeding and heterogeneity across flocks. In this respect, Bionda and Bianca show substructuring and are more similar to British breeds than other continental European breeds.

4. Bionda and Bianca fit into the European breeds provided with the highest number of alleles and expected heterozygosity. There is a clear distinction between the Piedmont breeds and the other populations. The Piedmont poultry differ from both commercial lines and other Italian breeds and retain a high level of genetic variability.

5. As for other indigenous breeds, Bionda and Bianca could make an original contribution to the industry in the future. A collective planned approach to restoration is essential, because the flocks are managed with poor regulation. Enhancing connection between breeders with an efficient replacement interchange and mating plan is the right way of controlling inbreeding, preventing substructuring and increasing variability within the flocks.

INTRODUCTION

Biodiversity conservation is a topic of interest and domestic animal diversity is an important component (FAO, 2011). Although scientists are focused on genetic resources of all farm animals, the conservation of poultry has attracted increasing attention for years now (Tadano *et al.*, 2013).

In Italy, 17 breeds have been included in the registry of indigenous poultry (<http://www.aia.it/aia-website/it/home>) and data on genetic variability are available on Ancona, Bianca di Saluzzo, Bionda Piemontese, Ermellinata di Rovigo, Livorno, Modenese, Padovana, Pepoi, Robusta Lionata, Robusta Maculata, Romagnola and Valdarnese (Supplementary Figure 1) (De Marchi *et al.*, 2005; Guidobono Cavalchini *et al.*, 2007; Strillacci *et al.*, 2009; Zanetti *et al.*, 2011a; Ceccobelli *et al.*, 2015).

Supplementary Figures 1-4 to be placed in online version only

In the region of Piedmont (Northwest Italy), two poultry breeds are extant, namely Bionda Piemontese (Bionda) and Bianca di Saluzzo (Bianca); they are well suited to reduced management in grassland-based free-range systems (Ferrante *et al.*, 2005; Schiavone *et al.*, 2009). Bionda is characterised by a buff (in Italian “biondo”) coat with black tail (Supplementary Figure 2), while Bianca is completely white (in Italian “bianco”) (Supplementary Figure 3).

The history of the Piedmont indigenous poultry rests mainly on non-scientific literature and anecdotal information (Di Francesco *et al.*, 2002). In the first half of the 20th century, most indigenous poultry were kept on family farms for both egg and meat production. White poultry were reared in the south-western and central parts of Italy and were considered as a variant of the more widespread buff or golden population; these were ancestors of the present day Bianca. In the years following World War II, use of improved breeds and crossbred lines suitable for industrial farming gave rise to the decline of the local population. Nowadays, there are approximately 16000 Bionda and 4000 Bianca individuals. Relying on geographical distribution and external appearance, farmers distinguish two Bionda ecotypes, namely “Bionda Piemontese Cuneo” (Bionda Cuneo) and “Bionda Piemontese Standard” (Bionda Standard) (Supplementary Figure 1). Bionda and Bianca are reared mainly for meat production under commercial conditions; age at slaughter (d, average \pm standard deviation) is 223 ± 69 for hens, 202 ± 46 for cockerels and 268 ± 8 for capons; slaughter weight (kg, average \pm standard deviation) is 1.8 ± 0.2 for hens, 2.2 ± 0.2 for cockerels and 2.9 ± 0.3 for capons (De Marco *et al.*, 2013). They are suggested for traditional recipes and are included in the Slow Food Presidia (<http://www.fondazione Slow Food.com/>). Although local breeds are a

heritage and reservoir of diversity, actual distinction of individual populations must be proved to justify assignment of conservation funds (Ajmone-Marsan *et al.*, 2010). In addition, analysis of population structure is often impossible due to the lack of genealogical records. In these circumstances, molecular information becomes pivotal to characterise genetic resources. A number of different marker types have been used for this purpose; in practice microsatellite loci and dense SNP panels are available and widespread (Lenstra *et al.*, 2012). Nevertheless, marker types describe different phylogenetic histories, related to different timescales. To assign individuals to populations of relatively recent origin (breeds) and to provide insights into differences among individuals, the microsatellite loci have higher resolution power than other markers, due to high mutation rate and level of polymorphism (DeFaveri *et al.*, 2013; Granevitze *et al.*, 2014). Therefore the microsatellite loci are still popular nuclear DNA markers for the investigation of genetic variability among and within species (Yang *et al.*, 2013). Recently, Italian poultry breeds have been successfully differentiated by a proteomic approach, which could give promising results to explain differences among breed products (Zanetti *et al.*, 2011b).

The objective of this study was to determine the genetic structure and variability of Bionda and Bianca using an international set of microsatellite loci. Differences against commercial lines and other Italian breeds were verified to justify the implementation of conservation programmes.

MATERIALS AND METHODS

Sample collection and genotyping

Blood samples were collected from 89 Bionda Cuneo chickens (15 flocks, 5 to 10 individuals per flock), 124 Bionda Standard (17 flocks, 2 to 13 individuals) and 86 Bianca (6 flocks, 5 to 18 individuals). Commercial farms provided reference samples belonging to 61 broilers (Ross 708) and 180 laying hens of three lines (Eureka, Hy-Line and ISA Brown, 60 individuals each). The samples

were collected into K3 EDTA Venoject tubes (Terumo, Tokyo, Japan). DNA was extracted using the NucleoSpin Blood QuickPure kit (Macherey-Nagel, Dueren, Germany).

A total of 32 microsatellite markers were used. Of these, 29 were recommended by the AVIANDIV project (Hillel *et al.*, 2003; FAO, 2011). To improve structural analysis of the Piedmont indigenous breeds, three loci were added, namely LEI0228, LEI0258 and MCW0080. The first two because they exhibited a high number of alleles (Rosenberg *et al.*, 2001), whereas the third has been used frequently for analysing breed diversity (Zanetti *et al.*, 2011a). Multiplex PCR and amplicon processing were carried out according to Sartore *et al.* (2014) (Supplementary Table 1). Allele-calling was standardised using AVIANDIV samples. Error rate assay per locus was performed by replicating the genotyping on a randomly chosen 10% of individual samples.

Supplementary Tables 1-2 to be placed in online version only

Within-population genetic variability of the Piedmont poultry

Genetic variability was estimated per locus and across all loci for each population by number of observed alleles, allelic richness, observed and expected heterozygosity and coefficient of inbreeding (F_{IS}) using FSTAT v2.9.3.2 (Goudet, 2001). Allelic richness was calculated as a measure of allele number independent from size differences among populations; sample size was fixed as the smallest number of individuals typed for a locus in a population after removing individuals showing missing genotypes (Petit *et al.*, 1998). F_{IS} was calculated as a measure of departures from expected heterozygosity; the corresponding locus x population P -values were based on 196000 randomisations performed by FSTAT. The probability test implemented by GENEPOP v4.2 (Rousset, 2008) was used (default settings) to estimate the extent of linkage disequilibrium (LD) within pairs of loci. Significant F_{IS} (locus x population tests) and LD (pairs of loci) P -values were evaluated after correction for multiple tests using the Bonferroni method ($P = 0.05$ nominal value) (Rice, 1989). Number of private alleles (alleles found in only one population due to lack of gene flow between populations) was computed using GenAIEx v6.501 (Peakall and Smouse, 2012).

Genetic structure of Bionda and Bianca

The model-based clustering procedure implemented in STRUCTURE v2.3.4 was used (Pritchard *et al.*, 2000) to detect the existence of different clusters of flocks within Bionda and Bianca. Ancestry model with admixture, correlated allele frequencies and no prior information were assumed. Number of genetic clusters (K) was tested for all values from 1 to n , where n was the total number of pre-defined flocks. For each value of K , 50 independent runs with 50000 Markov Chain Monte Carlo iterations and a burn-in period of 100000 were carried out. The most plausible number of clusters was determined by the ΔK statistic distribution using Structure Harvester v0.6.94 (Earl and vonHoldt, 2012). Coefficient of similarity over runs was obtained using CLUMPP v1.2.2 (Jakobsson and Rosenberg, 2007). Each individual received a membership or fraction Q of its genome within each of the K inferred clusters and was then associated with the cluster containing its greatest value of Q . If an individual was assigned with $0.5 < Q < 0.8$, some degree of within-individual admixture could be supposed. Graphical representation of assignment was obtained using DISTRUCT v1.1 (Rosenberg, 2004).

The genetic differentiation among the flocks (variance of allele frequencies) was estimated using the F_{ST} index as implemented by FSTAT (Weir and Cockerham, 1984; Goudet, 2001). According to Wright (1978), $F_{ST} = 0$ to 0.05 indicated little differentiation, 0.05 to 0.15 moderate differentiation, 0.15 to 0.25 great differentiation and > 0.25 very great differentiation.

Flock contribution to genetic variability was assessed following the approach implemented in MolKin v3.0 (Gutiérrez *et al.*, 2005) using allelic richness (Petit *et al.*, 1998) and gene diversity, an estimate of expected heterozygosity, as proposed by Nei (1987). Total value (T) of both gene diversity and allelic richness was divided into within-flock (W) and between-flocks (B, divergence between the flocks) component so that $W + B = T$. Each flock was once removed from the data set and each time gene diversity and allelic richness were re-quantified in order to identify the farms whose removing had the most effects on the overall variability conservation.

Relationships with other Italian populations

Bionda and Bianca were also compared with 30 genotype data of Livornese Bianca, 23 of Modenese and 30 of SASSO obtained from a previous investigation (Ceccobelli *et al.*, 2015). The breeds for comparison were specifically selected to maximise the number of shared marker loci.

Analysis of genetic variability and differentiation was performed as above. Locus \times population F_{IS} P -values were based on 240000 randomisations performed by FSTAT (Goudet, 2001).

The Reynolds *et al.* (1983) genetic distance between populations (linear approximation of F_{ST} for short divergence time assuming that sizes did not remain constant and equal in all populations) was computed using Microsat (Minch, 1997) and the robustness of distribution was evaluated by 1000 bootstrap replicates. PHYLIP v3.6 package (Felsenstein, 1989) was used to calculate a consensus statistical support and to construct a Neighbour-Joining cladogram. A Neighbour-Net network was visualised using Splits Tree4 v4.13.1 (Huson and Bryant, 2006).

To detect the most likely number of different populations within the overall data set, the clustering procedure implemented in STRUCTURE v2.3.4 was used (Pritchard *et al.*, 2000) as above. Number of genetic clusters (K) was tested for all values from 1 to n , where n was the number of pre-defined populations.

RESULTS

Quality of the marker loci

MCW0123, LEI0234, MCW0295 and MCW0165 were discarded because they scored high error rates (>10%); the other loci showed no error except three (rate = 1.7%). The proportion of missing data per locus did not exceed 0.6% of genotypes. Finally, the samples were genotyped using 28 loci.

All microsatellites were polymorphic except MCW0081 in Hy-Line. The highest number of alleles (28) was observed at LEI0258, the lowest (2) at MCW0098 (Supplementary Table 1).

Within-population variability of Bionda and Bianca

Descriptive statistics over the full set of 28 loci are presented in the Table 1. Bionda and Bianca exhibited a higher average number of alleles and allelic richness per locus than broilers and layers. The average observed heterozygosity per locus ranged from 0.547 in Bionda Standard to 0.613 in Eureka, while the average expected heterozygosity ranged from 0.540 in ISA-Brown to 0.654 in Bianca. After Bonferroni correction, some locus x population F_{IS} tests showed heterozygosity deficiency in the indigenous breeds and heterozygosity excess in the commercial lines. The overall F_{IS} values confirmed this trend ($P < 0.001$); the removal of some loci that emphasised departures from the expected heterozygosity within more than one population did not change the pattern. There were 53 private alleles in Bionda and Bianca (35 observed in at least two individuals) whereas the overall commercial lines had only 10 (6 observed in at least two individuals). Several private alleles (33) had frequencies ≤ 0.01 ; two alleles in Bionda Cuneo, one in Bionda Standard and 7 in Bianca had frequencies ≤ 0.05 . The highest numbers of private alleles were at LEI0228 (12), LEI0258 (10) and LEI0192 (9). In particular, LEI0258 exhibited 4 alleles in Bionda Cuneo (two with frequency ≤ 0.05), two in Bionda Standard and two in Bianca (one with frequency ≤ 0.05). After Bonferroni correction, some pairs of loci maintained LD mainly in Bionda Cuneo and Bianca; no LD was observed in the commercial lines analysed at the same 28 loci.

Table 1 near here

Genetic structure of Bionda and Bianca

At the first step of cluster analysis of flocks of the Piedmont indigenous breeds (Supplementary Figure 4A), the overall 32 pre-defined Bionda flocks were split into the two ecotypes and exhibited moderate differences ($F_{ST} = 0.087$). Some chickens were allocated within the wrong cluster (9/213) or within the expected cluster but with $0.5 < Q < 0.8$ (26/213). At the second step, Bionda Cuneo and Bionda Standard were analysed separately (Supplementary Figure 4B). The 15 Cuneo and 17 Standard flocks were split into three and two subclusters, respectively. Several flocks (19/32)

exhibited ambiguous membership for any subcluster (mis-allocation or assignment with $0.5 < Q < 0.8$). Within both ecotypes moderate differentiation among flocks was present (Cuneo: $F_{ST} = 0.093$; Standard: $F_{ST} = 0.054$).

The Bianca flocks were less different than the Bionda flocks ($F_{ST} = 0.049$) except one, which is known to be closed to external replacements (Supplementary Figure 4C).

LD and F_{IS} were then re-estimated within these subclusters (Supplementary Figure 4, B and C) and the results were compared with the values of the respective overall populations described in the Table 1. Within the Bionda subclusters LD disappeared; within Bianca, after excluding the chickens of the most different flock, LD decreased. All subclusters showed deficiency of heterozygosity (F_{IS} with $P < 0.001$).

Removal of any flock of Bionda and Bianca resulted in a decrease of gene diversity within the flocks (W) and an increase of divergence between the flocks (B). The number of flocks critical for total gene diversity conservation (T) was 10/15 in Bionda Cuneo and 7/17 in Bionda Standard. Regarding Bianca, the least contribution to increase the gene diversity within the flocks and the similarity among the flocks was provided by the most different farm, which clustered apart and had poor variability.

After removal, the flocks had different contributions to allelic richness components. Nevertheless, the flocks that increased total richness (T) were 11/15 of Bionda Cuneo and 4/17 of Bionda Standard. All Bianca flocks but one increased richness within the flocks (W) and decreased the divergence between the flocks (B); all flocks increased total richness, including the most different.

Relationships with other Italian populations

In a previous investigation (Ceccobelli *et al.*, 2015), Livornese, Modenese and SASSO were analysed using 24 out of 28 microsatellite loci used in the present work. The comparison among Livornese, Modenese, SASSO, commercial lines and the Piedmont breeds was then performed

using the 24 shared loci (Table 2). The Piedmont chickens had an average advantage of 2-2.8 alleles (number of alleles) and of 1-1.7 alleles (allelic richness) per locus over Livornese and Modenese. On the average, the highest observed heterozygosity was exhibited by SASSO (0.647) and Eureka (0.592), while the highest expected heterozygosity was observed in Bianca (0.632) and Bionda Cuneo (0.618). After Bonferroni correction, the highest number of loci showing heterozygosity excess was observed in the layers. The overall F_{IS} values showed no departure from the expectation in Modenese and deficiency of heterozygosity in the other breeds ($P < 0.001$); excess of heterozygosity was obtained in the commercial lines and SASSO ($P < 0.001$).

Table 2 near here

Bianca exhibited the highest number of private alleles (10 observed in at least two individuals). There were 6 private alleles in Bionda Cuneo (4 observed in at least two individuals) and 15 in Livornese, Modenese and SASSO (13 observed in at least two individuals). Most private alleles showed frequencies ≤ 0.01 , especially in Bianca; 4 private alleles in Livornese and Modenese, three in Bianca and two in SASSO had a frequency ≤ 0.05 .

The overall F_{ST} (0.152) showed great differentiation. All pairwise F_{ST} values showed differences between populations (Supplementary Table 2, above the diagonal). Little to moderate genetic divergence was observed between the two Piedmont breeds that also showed the least average differences towards the other populations. Very great differentiation ($F_{ST} > 0.25$) was detected between Modenese and layers.

The Reynolds distance (Supplementary Table 2, below the diagonal) ranged from the lowest value of Bionda Cuneo vs. Bionda Standard to the highest of Modenese vs. SASSO. In the Neighbour-Net network (Figure 1), the two Bionda ecotypes gathered in the same branch near Bianca that clustered with SASSO; Livornese and Modenese divided away from the other populations with long branches; the commercial lines were split into broilers and layers.

Figure 1 near here

The STRUCTURE clustering solutions are depicted in Figure 2. The overall data set was divided into two distinct clusters (Figure 2A); the layers were clearly differentiated from the meat chickens ($Q > 0.98$) and no individual was miss-allocated. In the dataset without layers, the

distribution of K statistic showed two peaks ($K = 3$ and $K = 7$) but the maximum value indicated that a pattern of 7 clusters was the most likely (Figure 2B). SASSO separated from the single Livornese-Modenese cluster, within which it kept some membership ($Q = 0.25$). Bianca and broilers formed different clusters ($Q = 0.91$ and 0.96 , respectively). Bionda was split into three clusters, namely one associated with Bionda Cuneo, another with Bionda Standard and a third having membership within both ecotypes; several Bionda chickens (85/213) were allocated with $Q < 0.8$, but no miss-allocations or high membership values within the clusters containing the other populations were obtained.

Figure 2 near here

DISCUSSION

The European Union established measures to support breeds in danger of being lost to farming (EUR-Lex, 2006). The threshold under which a chicken breed is considered in danger is 25000 breeding females; the sizes of Bionda and Bianca are below this value (De Marco *et al.*, 2013). The AVIANDIV-FAO microsatellite tool meets the need to establish a standard approach to characterise animal genetic resources and the number of loci ensures high differentiation power (Hillel *et al.*, 2003; FAO, 2011; Gärke *et al.*, 2012). The widespread use of these markers provides the largest amount of data to perform comparisons among populations of different origin. Therefore models for linking information are largely based on this tool and new data on indigenous poultry may be combined with available data sets of other breeds and commercial lines (Granevitze *et al.*, 2007; Zanetti *et al.*, 2010; Wilkinson *et al.*, 2012; Abebe *et al.*, 2015).

The markers of the present investigation are suitable to evaluate genetic relationships among populations and to assess whether a supported plan should be implemented. Although different markers were used, the number of alleles and the expected heterozygosity of the present analysis are in agreement with the investigation of Guidobono Cavalchini *et al.* (2007) on the same Piedmont breeds. Moreover, our results show that genetic variability has not changed in the short term.

To justify the implementation and development of a conservation programme, the Piedmont indigenous poultry variability may be compared to other breeds. Hillel *et al.* (2003), Granevitze *et al.* (2007), Berthouly *et al.* (2008), Zanetti *et al.* (2010) and Ceccobelli *et al.* (2015) studied local breeds from different countries of Europe, Asia and Africa using the AVIANDIV-FAO tool and found average number of alleles per locus ranging from 2 to 6.7 and average expected heterozygosity per locus ranging from 0.17 to 0.67. Bionda and Bianca fit into the European breeds provided with the highest number of alleles and expected heterozygosity; furthermore, they have more genetic variability than the British breeds (Wilkinson *et al.*, 2012), the Swedish breeds (Abebe *et al.*, 2015) and the commercial lines. The perspective that microsatellite variability reflects whole genome diversity is a matter of debate (DeFaveri *et al.*, 2013); nevertheless the breeds exhibiting high genetic variability at neutral loci (like Bionda and Bianca) are also assumed to have a large amount of diversity in coding genes (FAO, 2011).

Farmers of Bionda and Bianca use different supplying procedures, namely within-flock replacement and purchasing from few suppliers (Dr M. De Marco, unpublished data). The molecular information suggests that these practices could have prevented random mating; the heterozygosity deficiency could be due to inbreeding and heterogeneity across flocks. The differences among flocks could also give rise to LD (Nei and Li, 1973). On the other hand, the foundation of commercial stocks is based on different parent lines from highly selected nuclei (Hillel *et al.*, 2003); when the lines are crossed, this breeding practice causes high observed heterozygosity within homogeneous stocks of individuals that share many identical-by-descent alleles.

The population structure and the splitting into collection sites of the Piedmont chickens have been analysed to provide some management advice. Continental European breeds have been observed to be generally homogeneous populations (Zanetti *et al.*, 2010; Bianchi *et al.*, 2011); in this respect, Bionda and Bianca show substructuring and are more similar to British breeds described by Wilkinson *et al.* (2012). The flocks with high gene diversity and allelic richness

contribute to the variability within flocks and to the similarity between flocks and may provide useful replacements to planned exchanges of breeding animals. Interestingly, even the Bianca flock with poor allelic richness could provide a favourable contribution to total variability; the most likely explanation is that drift and/or directional selection for different objectives have combined a less frequent allele arrangement than within the other flocks.

Little differences across the Bionda chickens are due to discontinuity between the two ecotypes. A very recent common origin is evident, as the cluster analysis confirms through mutual miss-allocations or correct allocations but with low membership ($0.5 < Q < 0.8$). The assignment of the Bionda chickens to each ecotype is made by farmers and is only based on geographical distribution, but the present study show that the within-ecotype variability is higher than the between-ecotype variability. Existence of dichotomy is worth analysing in detail with further investigations. Flocks that provide favourable contributions to variability are more frequent in the Cuneo ecotype than in the Standard ecotype. If these ecotypes show very attractive peculiarities, they could be preserved and exploited; otherwise the segregation will decrease variability.

The comparison among Livornese, Modenese, SASSO and the Piedmont breeds shows that Bionda and Bianca exhibit the least genetic differences from the other populations probably because breeds having high heterozygosity also show low genetic distinction (Berthouly *et al.*, 2008). Despite this, there is a clear distinction between the Piedmont breeds and the other populations.

The proximity of populations in the network and some membership sharing may be the consequence of common origins from stocks that were involved, to some extent, in the evolution of populations; for example, SASSO towards Bianca, or SASSO towards Livornese and Modenese. Nevertheless, SASSO is a modern strain of meat chickens that received gene flow from other breeds whereas broilers come from a wide range of founder stocks; this may emphasise genetic similarities of the synthetic lines with other populations (Granevitze *et al.*, 2007).

On the other hand, many breeds received the contribution of migration and admixture in the near or remote past; afterwards, migrant and native genes found their optimal proportions for a

particular environment and production system (Ceccobelli *et al.*, 2015). If the performances meet the demand of the market (traditional and niche products), the merit of this gene heritage must be conserved.

Bionda and Bianca are always separated from each other by all genetic analyses, but they are very close in the Neighbour-Net trunk and have a smaller distance than that between Livornese and Modenese, which share a well-known past crossbreeding (Ceccobelli *et al.*, 2015). The hypothesis that Bianca comes from a white variety of ancient Piedmont poultry seems plausible, therefore discontinuity between the two breeds seem to be due to preferences of the farmers.

The distinction and high genetic variability, which is a useful evolutionary potential, warrant the sense and feasibility of a conservation programme of the Piedmont indigenous breeds.

A fair amount of private alleles shows a frequency ≤ 0.05 in Bianca, Livornese and Modenese. The presence of private alleles with intermediate frequencies is not exceptional in poultry (Hillel *et al.*, 2003; Zanetti *et al.*, 2010). If the gene flow is restricted, frequencies of rare private alleles could increase into different populations due to drift (Granevitze *et al.*, 2007), but also other explanations could be assessed.

Bionda and Bianca have most private alleles at the hypervariable LEI0258 locus when compared to the commercial lines. In different breeds, this marker has been used to evaluate genetic variability of the MHC region on chromosome 16 and its genotypes have been correlated with serology (Chazara *et al.*, 2013). Alleles have been associated with intensity of infection with *Ascaridia galli*, immune response to *Salmonella enterica serovar Enteritidis* and mortality due to *Pasteurella multocida* (Schou *et al.*, 2010). Association studies also revealed influences of MHC haplotypes on production traits (Nikbakht and Esmailnejad, 2015). Although the same allele is not necessarily associated with the same serological variant in different breeds and LEI0258 has not been included in the AVIANDIV project, its richness of alleles suggests the existence of some unique or interesting haplotypes in the Piedmont poultry that would be worth verifying by re-

sequencing to provide a dense SNP map of the region. The richness of private alleles justifies the purpose of conserving Bionda and Bianca.

In synthesis, the present Piedmont poultry differ from both commercial lines and other Italian breeds and retain a high level of genetic variability and some interesting properties. As other indigenous breeds, Bionda and Bianca could make an original contribution to the industry in the future.

A collective planned approach to restoration is essential, because the flocks are managed with poor regulation. Enhancing connection between breeders with an efficient replacement interchange and mating plan is the right way of controlling inbreeding, preventing substructuring and increasing variability within the flocks. Planning of new strategies may also include cryopreservation and artificial insemination (Silversides *et al.*, 2012).

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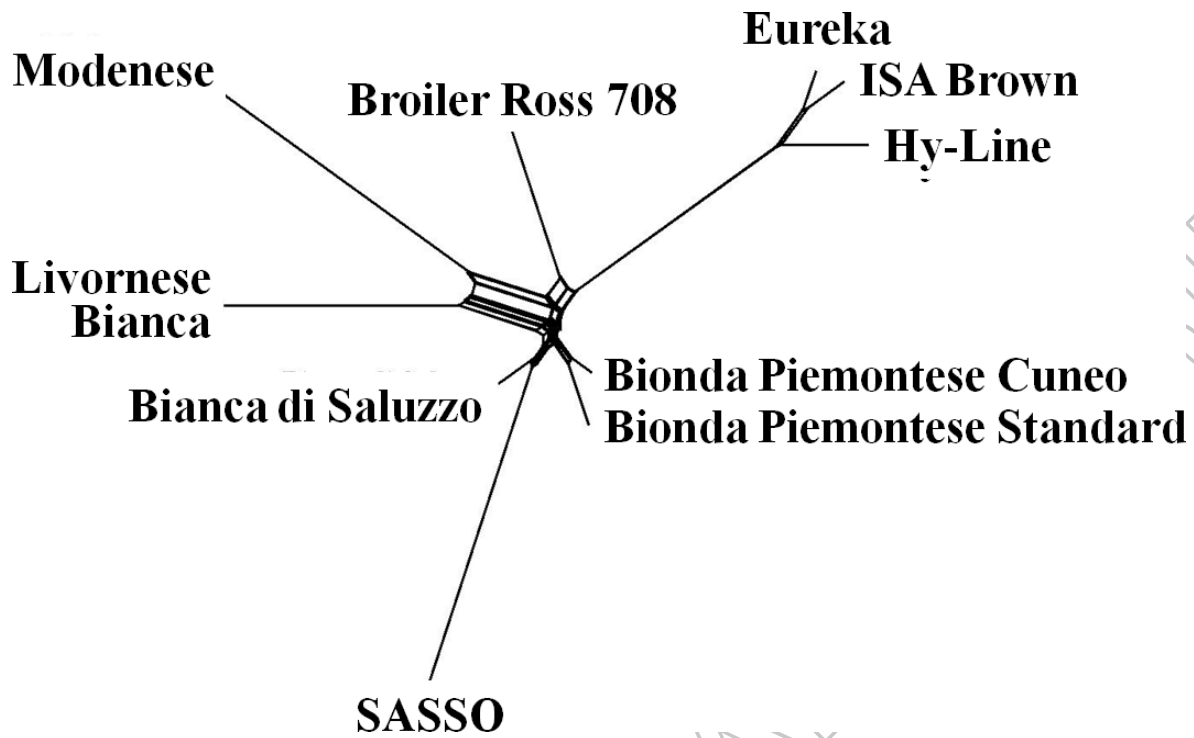
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FIGURE CAPTIONS

Figure 1. Neighbour-Net network based on the Reynolds distance for the breeds and the commercial lines over 24 loci shared with Ceccobelli et al. (2015).



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Figure 2. Cluster analysis of the breeds and the commercial lines over 24 loci shared with Ceccobelli et al. (2015). (A) The first step over the overall data set. (B) The second step over the data set without layers at $K = 7$. LB: Livornese Bianca, MD: Modenese, SA: SASSO, BPC: Bionda Piemontese Cuneo, BPST: Bionda Piemontese Standard, BS: Bianca di Saluzzo, BR: Broiler, EK: Eureka, HL: Hy-Line and ISA: ISA Brown.

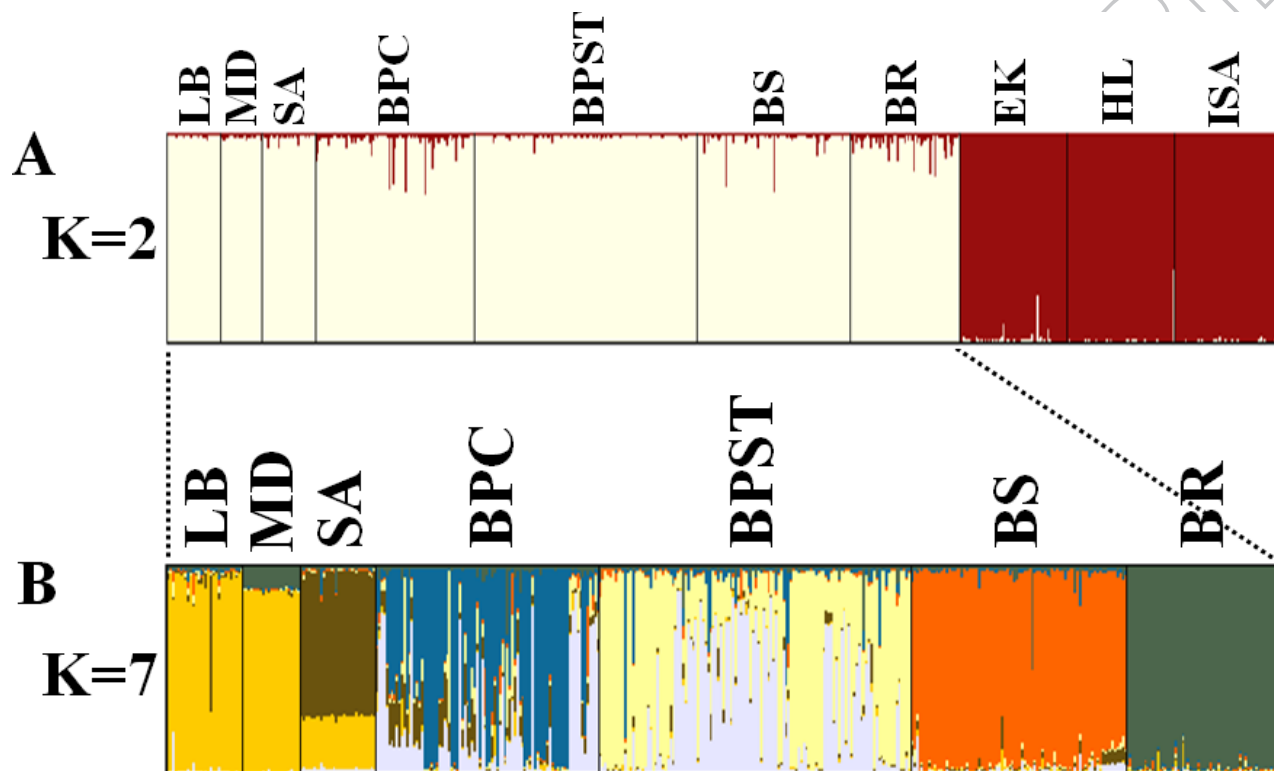


Table 1. Descriptive statistics of the Piedmont indigenous breeds and the commercial lines over 28 loci(average \pm standard error)

| | <i>A</i> | <i>AR</i> | <i>PA</i> | <i>H_O</i> | <i>H_E</i> | <i>F_{IS}</i> | <i>LD</i> |
|-------------------|------------------|------------------|-----------|----------------------|----------------------|-----------------------|-----------|
| BPC ¹ | 6.50 \pm 0.819 | 6.19 \pm 0.755 | 19 (13) | 0.552 \pm 0.0373 | 0.635 \pm 0.0323 | +0.136 | 7 (1) |
| BPST ² | 5.89 \pm 0.753 | 5.32 \pm 0.609 | 11 (4) | 0.547 \pm 0.0353 | 0.626 \pm 0.0264 | +0.129 | 1 |
| BS ³ | 6.29 \pm 0.740 | 6.02 \pm 0.720 | 23 (16) | 0.579 \pm 0.0333 | 0.654 \pm 0.0246 | +0.120 | 5 (1) |
| BR ⁴ | 4.50 \pm 0.447 | 4.48 \pm 0.452 | 7 (5) | 0.589 \pm 0.0379 | 0.554 \pm 0.0307 | -0.055 | 0 |
| EK ⁵ | 4.21 \pm 0.376 | 4.20 \pm 0.380 | 0 | 0.613 \pm 0.0479 | 0.547 \pm 0.0323 | -0.113 | 0 |
| HL ⁶ | 3.82 \pm 0.334 | 3.81 \pm 0.338 | 3 (1) | 0.597 \pm 0.0653 | 0.541 \pm 0.0387 | -0.095 | 0 |
| ISA ⁷ | 3.93 \pm 0.341 | 3.92 \pm 0.343 | 0 | 0.579 \pm 0.0519 | 0.540 \pm 0.0324 | -0.065 | 0 |

¹Bionda Piemontese Cuneo. ²Bionda Piemontese Standard. ³Bianca di Saluzzo. ⁴Broiler. ⁵Eureka. ⁶Hy-Line. ⁷ISA Brown.

A = average number of alleles per locus, *AR* = average allelic richness per locus, *PA* = number of private alleles and number of private alleles with frequency ≥ 0.01 (in parentheses), *H_O* = average observed heterozygosity per locus, *H_E* = average expected heterozygosity per locus, *F_{IS}* = inbreeding coefficient, *LD* = number of pairs of loci in LD after sequential Bonferroni correction and number of syntenic loci in LD (in parentheses).

Table 2. Descriptive statistics of the breeds and the commercial lines over the 24 loci shared with Ceccobelli *et al.* (2015) (average \pm standard error)

| Breed | A | AR | PA | H_O | H_E | F_{IS} |
|-------------------|------------------|------------------|---------|--------------------|--------------------|----------|
| LB ¹ | 3.04 \pm 0.204 | 2.83 \pm 0.180 | 5 (5) | 0.407 \pm 0.0446 | 0.444 \pm 0.0393 | +0.104 |
| MD ² | 2.58 \pm 0.199 | 2.45 \pm 0.173 | 5 (5) | 0.372 \pm 0.0524 | 0.359 \pm 0.0426 | -0.013 |
| SA ³ | 3.78 \pm 0.301 | 3.49 \pm 0.235 | 5 (5) | 0.647 \pm 0.0517 | 0.552 \pm 0.0378 | -0.156 |
| BPC ⁴ | 5.33 \pm 0.530 | 4.14 \pm 0.315 | 6 (4) | 0.550 \pm 0.0377 | 0.618 \pm 0.0339 | +0.116 |
| BPST ⁵ | 5.00 \pm 0.571 | 3.88 \pm 0.307 | 3 (1) | 0.559 \pm 0.0343 | 0.613 \pm 0.0268 | +0.091 |
| BS ⁶ | 5.29 \pm 0.476 | 4.12 \pm 0.282 | 15 (10) | 0.580 \pm 0.0346 | 0.632 \pm 0.0240 | +0.088 |
| BR ⁷ | 3.83 \pm 0.293 | 3.34 \pm 0.182 | 2 (0) | 0.581 \pm 0.0412 | 0.521 \pm 0.0303 | -0.107 |
| EK ⁸ | 3.75 \pm 0.320 | 3.37 \pm 0.229 | 0 | 0.592 \pm 0.0528 | 0.523 \pm 0.0349 | -0.123 |
| HL ⁹ | 3.50 \pm 0.289 | 3.22 \pm 0.258 | 1 (0) | 0.584 \pm 0.0714 | 0.529 \pm 0.0422 | -0.096 |
| ISA ¹⁰ | 3.50 \pm 0.295 | 3.18 \pm 0.222 | 0 | 0.558 \pm 0.0563 | 0.519 \pm 0.0337 | -0.065 |

¹Livornese Bianca. ²Modenese. ³SASSO. ⁴Bionda Piemontese Cuneo. ⁵Bionda Piemontese Standard. ⁶Bianca di Saluzzo. ⁷Broiler. ⁸Eureka. ⁹Hy-Line. ¹⁰ISA Brown.

A = average number of alleles per locus, AR = average allelic richness per locus, PA = number of private alleles and number of private alleles with frequency ≥ 0.01 (in parentheses), H_O = average observed heterozygosity per locus, H_E = average expected heterozygosity per locus, F_{IS} = inbreeding coefficient.

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Supplementary Table 1. *Microsatellite loci, chromosome location (Chr), PCR information and number of alleles (A) of 28 loci across the Piedmont indigenous breeds*

| Locus | Chr | Multiplex | Annealing (°C) | Size range (bp) | A |
|---------|-----|-----------|----------------|-----------------|----|
| ADL0268 | 1 | 1 | 60 | 104-116 | 5 |
| MCW0248 | 1 | 1 | 60 | 215-223 | 3 |
| LEI0094 | 4 | 1 | 60 | 241-283 | 15 |
| ADL0278 | 8 | 1 | 60 | 112-125 | 11 |
| MCW0216 | 13 | 1 | 60 | 141-149 | 6 |
| MCW0034 | 2 | 2 | 60 | 212-244 | 14 |
| MCW0222 | 3 | 2 | 60 | 220-228 | 5 |
| MCW0081 | 5 | 2 | 60 | 114-136 | 6 |
| MCW0069 | 26 | 2 | 60 | 158-174 | 7 |
| MCW0111 | 1 | 3 | 60 | 98-112 | 5 |
| LEI0166 | 3 | 3 | 60 | 356-366 | 6 |
| MCW0016 | 3 | 3 | 60 | 162-206 | 9 |
| MCW0037 | 3 | 3 | 60 | 154-158 | 3 |
| LEI0192 | 6 | 4 | 58 | 245-425 | 22 |
| MCW0014 | 6 | 4 | 58 | 162-182 | 6 |
| MCW0183 | 7 | 4 | 58 | 296-326 | 9 |
| ADL0112 | 10 | 4 | 58 | 122-132 | 5 |
| MCW0020 | 1 | 5 | 62 | 179-185 | 4 |
| MCW0104 | 13 | 5 | 62 | 190-226 | 13 |
| MCW0098 | 4 | 6 | 60 | 263-265 | 2 |
| MCW0078 | 5 | 6 | 60 | 135-143 | 5 |
| MCW0067 | 10 | 6 | 60 | 176-186 | 6 |
| MCW0330 | 17 | 6 | 60 | 260-290 | 4 |
| MCW0206 | 2 | 7 | 58 | 223-239 | 7 |
| MCW0103 | 3 | 7 | 58 | 262-270 | 3 |

| | | | | | |
|---------|----|---|----|---------|----|
| LEI0228 | 2 | 8 | 62 | 163-489 | 27 |
| MCW0080 | 15 | 8 | 62 | 265-277 | 4 |
| LEI0258 | 16 | 8 | 62 | 195-445 | 28 |

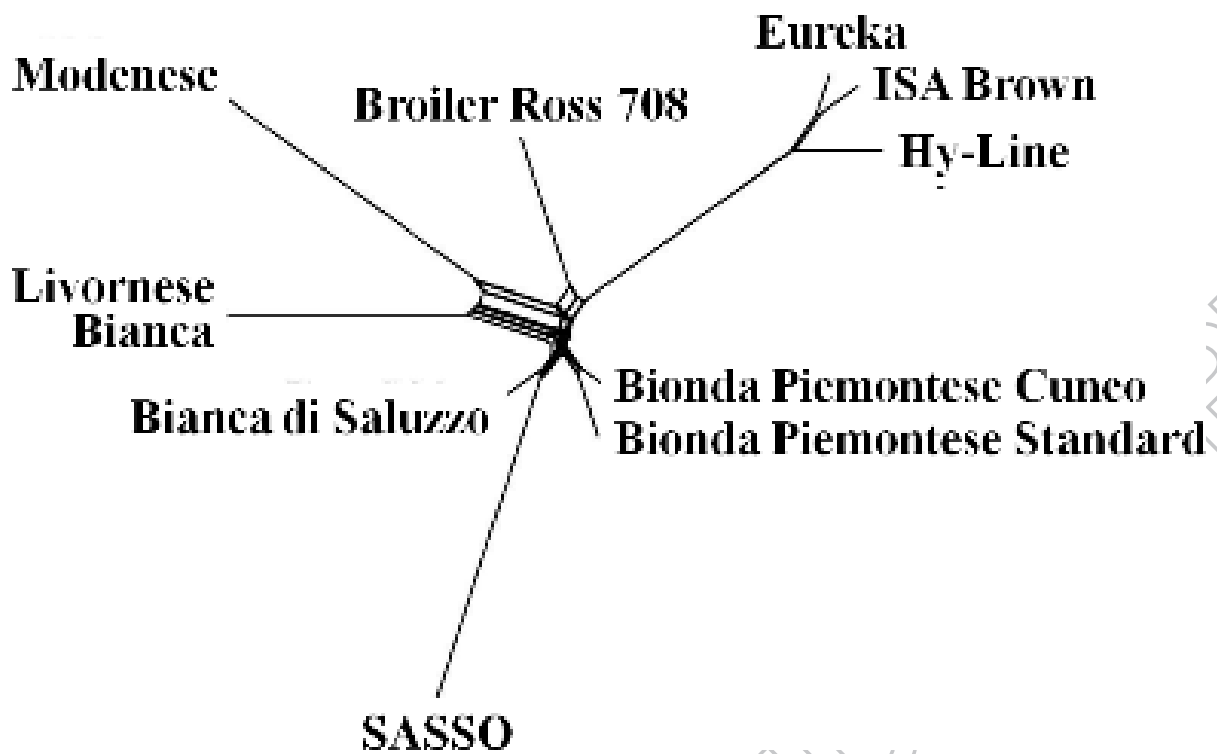
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Supplementary Table 2. Matrix of F_{ST} (above the diagonal) and Reynolds genetic distance (below the diagonal) between the breeds and the commercial lines over the 24 loci shared with Ceccobelli et al. (2015)

| Breed | LB | MD | SA | BPC | BPST | BS | BR | EK | HL | ISA |
|------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| LB ¹ | – | 0.210 | 0.225 | 0.135 | 0.159 | 0.155 | 0.206 | 0.269 | 0.267 | 0.272 |
| MD ² | 0.246 | – | 0.320 | 0.189 | 0.211 | 0.209 | 0.207 | 0.298 | 0.286 | 0.297 |
| SA ³ | 0.264 | 0.392 | – | 0.155 | 0.159 | 0.152 | 0.223 | 0.256 | 0.258 | 0.253 |
| BPC ⁴ | 0.151 | 0.217 | 0.174 | – | 0.032 | 0.070 | 0.111 | 0.156 | 0.157 | 0.159 |
| BPST ⁵ | 0.180 | 0.244 | 0.177 | 0.036 | – | 0.066 | 0.128 | 0.183 | 0.188 | 0.189 |
| BS ⁶ | 0.170 | 0.248 | 0.152 | 0.081 | 0.077 | – | 0.117 | 0.151 | 0.160 | 0.156 |
| BR ⁷ | 0.240 | 0.236 | 0.256 | 0.121 | 0.140 | 0.124 | – | 0.188 | 0.184 | 0.195 |
| EK ⁸ | 0.315 | 0.350 | 0.303 | 0.169 | 0.202 | 0.166 | 0.204 | – | 0.067 | 0.030 |
| HL ⁹ | 0.319 | 0.337 | 0.312 | 0.172 | 0.210 | 0.180 | 0.200 | 0.075 | – | 0.058 |
| ISA ¹⁰ | 0.321 | 0.353 | 0.296 | 0.175 | 0.211 | 0.172 | 0.218 | 0.034 | 0.066 | – |
| F_{ST} ¹¹ | 0.211 | 0.248 | 0.222 | 0.129 | 0.146 | 0.137 | 0.173 | 0.178 | 0.181 | 0.179 |
| Reynolds ¹¹ | 0.0166 | 0.0161 | 0.0181 | 0.0157 | 0.0190 | 0.0144 | 0.0134 | 0.0282 | 0.0257 | 0.0286 |
| | 0.245 | 0.291 | 0.258 | 0.144 | 0.164 | 0.152 | 0.193 | 0.202 | 0.208 | 0.205 |
| | 0.0209 | 0.0206 | 0.0247 | 0.0175 | 0.0214 | 0.0166 | 0.0163 | 0.0336 | 0.0314 | 0.0332 |

¹Livornese Bianca. ²Modenese. ³SASSO. ⁴Bionda Piemontese Cuneo. ⁵Bionda Piemontese Standard. ⁶Bianca di Saluzzo. ⁷Broiler. ⁸Eureka. ⁹Hy-Line. ¹⁰ISA Brown. ¹¹Average and standard error.



Supplementary Figure 1. *Sample collection sites. Not included in the present investigation: 1 = Ancona, 2 = Ermellinata di Rovigo, 3 = Padovana, 4 = Pepoi, 5 = Robusta Lionata, 6 = Robusta maculate, 7 = Romagnola, 8 = Valdarnese.*

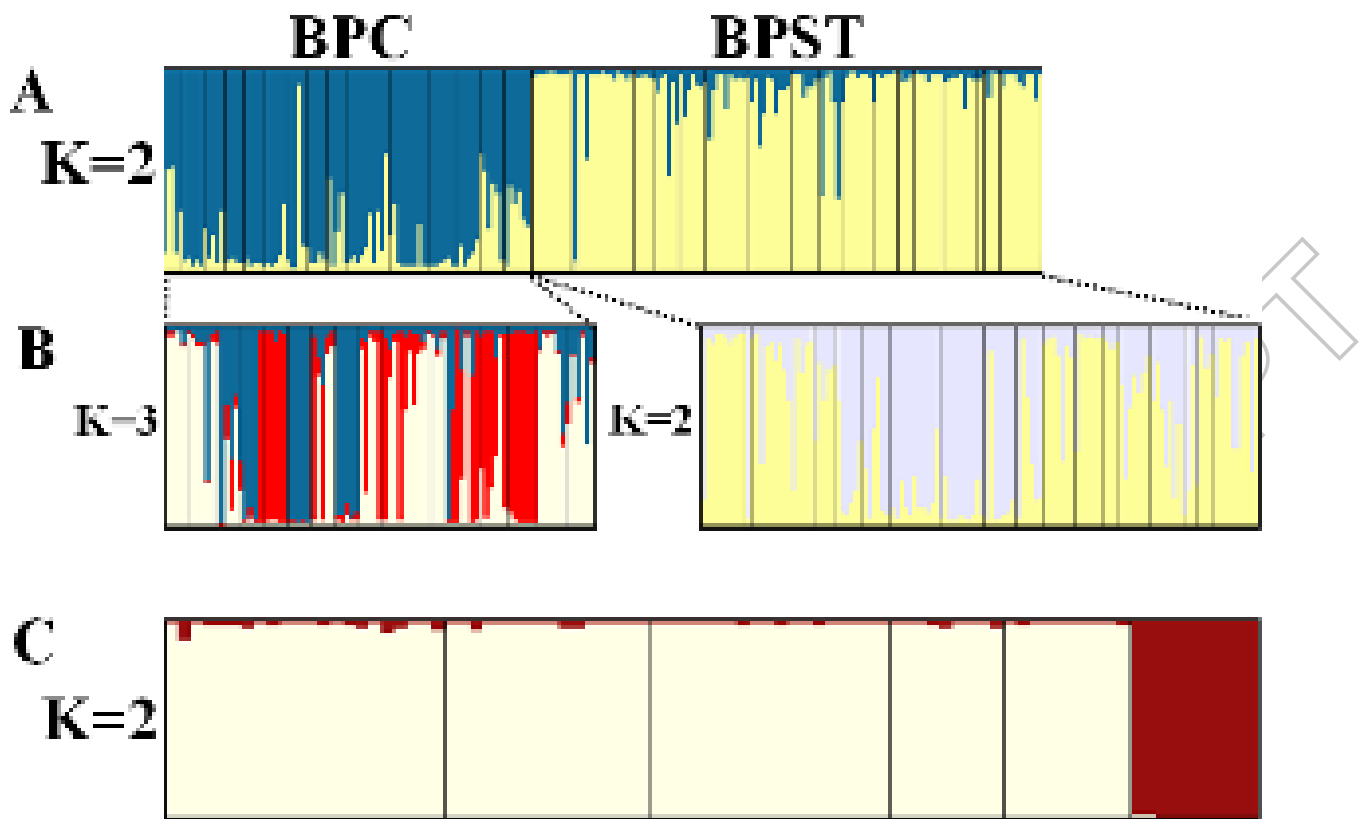


Supplementary Figure 3. *Bianca di Saluzzo chickens.*

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Supplementary Figure 2. *Bionda Piemontese chickens.*



Supplementary Figure 4. Cluster analysis of the pre-defined flocks of the Piedmont indigenous breeds over 28 loci. (A) The 32 flocks of Bionda Piemontese. (B) The 15 flocks of Bionda Piemontese Cuneo (BPC) and the 17 flocks of Bionda Piemontese Standard (BPST). (C) The 6 flocks of Bianca di Saluzzo.