Development and validation of a novel SNP panel for the genetic characterization of Italian chicken breeds by next-generation sequencing discovery and array genotyping

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ABSTRACT The aim of this study was to compare the intra and inter genetic variability and population structure of 7 indigenous chicken breeds of the Veneto region, through a novel panel of 64 SNP, each located in an exonic region and mostly on different chromosomes. A total of 753 blood samples from 7 local chicken breeds (Ermellinata di Rovigo, Millefiori di Lonigo, Polverara, Pepòi, Robusta Lionata, Robusta Maculata, and Padovana) was collected and analyzed. Two strains of Polverara (Nera and Bianca) and Padovana (Dorata and Camosciata) were included in the study. The observed heterozygosity ranged from 0.124 (Pèpoi) to 0.244 (Ermellinata di Rovigo), and the expected heterozygosity varied from 0.132 (Millefiori di Lonigo) to 0.300 (Ermellinata di Rovigo). Global F_{IS} results (0.114) indicated a low-medium inbreeding effect, with values ranging from 0.008 (Millefiori di Lonigo) to 0.223 (Ermellinata di Rovigo). Pairwise F_{ST} values (0.167) for all populations ranged from 0.020 (Polverara Nera and Polverara Bianca) to 0.193 (Robusta Lionata and Polverara Nera), indicating that the studied breeds were genetically highly differentiated. The software STRUCTURE was used to detect the presence of population substructures, and the most probable number of clusters (K) of the 10 chicken populations was at K = 8. The affiliation was successful in all Veneto chicken breeds. The present SNP marker results, compared with previous data obtained using microsatellites, provided a reliable estimate of genetic diversity within and between the studied breeds, and demonstrated the utility of the proposed panel as a rapid, efficient, and cost-effective tool for periodical monitoring of the genetic variability among poultry populations. In addition, the present SNP panel could represent a resource for a systematic approach with relevant impact on breeding program decisions and could turn out to be a reliable tool for genetic traceability of indigenous chicken meat. Adoption of a periodical monitoring system of genetic diversity is a fundamental tool in conservation actions and should increase the value of typical and niche products.

Key words: local chicken breed, genetic diversity, SNP, population structure

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INTRODUCTION

Conservation of animal genetic resources has become a topic of interest worldwide during the last couple of decades (FAO, 2011). Rapid genetic progress in production traits of farm animals has been observed as a result of developments in reproductive technology, application of modern genetic tools in breeding programs, and improved logistics. However, an increase in global use of highly selected and productive breeds has been associated with a loss of genetic diversity in most domestic animals species (Dalvit et al., 2009; Ciampolini et al., 2013), especially in poultry (Hillel et al., 2003; Blackburn 2006; Zanetti et al., 2011a, b). Indigenous breeds, maintained locally in small-sized populations, are often replaced by high productive breeds with a subsequent loss of both genetic diversity within and between breeds and are under pressure (Woelders et al., 2006). As a consequence of this genetic loss, many local chicken breeds reared in Italy until some decades ago have disappeared or are under threat of extinction (Blackburn 2006; Bianchi et al., 2011). Awareness of the value of genetic resources for traditional local farming, combined with many consumers' preference for their eggs and meat, has assumed great importance in the last yr and has stimulated the study of the genetic diversity of indigenous breeds, which can be assessed

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using phenotypic and genotypic information to prioritize for conservation. Information on genetic diversity is a fundamental requirement to establish better management plans for the conservation and recovery of indigenous populations (Fulton and Delany, 2003).

Over the past 10 yr in Italy, several conservation actions in safeguarding local avian breeds from different regions have been undertaken aiming to preserve biodiversity of these unique resources: in Veneto (De Marchi et al., 2006; Zanetti et al., 2010a), in Piemonte (Sartore et al., 2014, 2016), in Toscana (Gualtieri et al., 2006; Strillacci et al., 2009), in Marche, and Emilia Romagna (Bianchi et al., 2011; Ceccobelli et al., 2013) regions. Genetic distance and heterozygosity at DNA level have been effectively and conveniently investigated in the chicken breeds using microsatellite markers as the most common strategy, which provides an efficient support to prioritize breeds for conservation on several species. In recent yr, the availability of the genome assemblies for the relevant livestock species, including chicken (Hillier et al., 2004), the decreasing cost and the rapid progress in next-generation sequencing (**NGS**) and related bioinformatics computing resources, have allowed large-scale discovery of SNPs for animal genetic research and breeding (Ramos et al., 2009; Kumar et al., 2012). In fact, NGS generates massive sequence data that can be used for the efficient identification of de novo and reference-based SNP, currently considered the marker of choice in evaluating genetic diversity and relationships in farm animals due to their large number and genome-wide availability over highly variable microsatellites.

The Veneto region (in northeastern Italy) is the custodian of a long tradition in the poultry sector. In 2012, the Bionet Program, an Italian regional network for conservation of poultry biodiversity, has been carried out with the main objective of providing genetic information useful for the preservation of local poultry breeds. Thirteen breeds of 5 poultry species distributed in 7 conservation flocks were involved: duck, guinea fowl, chicken, turkey, and goose.

The present study deals with 7 indigenous chicken breeds: Pépoi, Padovana, Polverara, Ermellinata di Rovigo, Robusta Lionata, Robusta Maculata, and Millefiori di Lonigo. These breeds are registered in the list of traditional Italian products recognized by the Ministry of Agricultural and Forestry Policy (MIPAF, 2003) and are distinguished by a strong connection to the territory and their gastronomic traditions. The descriptions and the historical origins of these breeds were previously reported by Cassandro et al. (2004) and De Marchi et al. (2005). All of these breeds are dual-purpose (meat and egg production) and are characterized by a slow growing rate, a high resistance to diseases, and good environmental adaptability (Cassandro et al., 2004; Zanetti et al., 2010a, b; Riovanto et al., 2012; Rizzi et al., 2013; Verdiglione and Cassandro, 2013). The Pépoi, Padovana, and Polverara are small-sized chicken breeds (average maturity weight for male and female of 1.8 kg and 1.4 kg, respectively), whereas the other 4 breeds, Ermellinata di Rovigo, Robusta Maculata, Robusta Lionata, and Millefiori di Lonigo, are medium sized with heavier mature weight (average maturity weight for male and female of 3.6 kg and 2.6 kg, respectively). Previous studies have reported peculiar meat quality characteristics of these local breeds with respect to commercial hybrids (Castellini et al., 1994; Cassandro et al., 2002).

In relation to previous studies based on microsatellite markers (Zanetti et al., 2010a; Özdemir et al., 2016), the aim of the present work is to develop and validate a novel panel of 64 SNP identified by NGS technologies and to provide a reliable, rapid, and cost-effective genetic tool for monitoring genetic aspects of these chicken breeds to be used in conservation programs.

MATERIAL AND METHODS

Bird sampling and DNA collection

A total of 753 individuals representing 7 local chicken breeds was randomly collected from 2013 to 2014 among 5 different conservation flocks located in the plains, hills, and mountains of Veneto region (north-east of Italy). Local populations involved in the present study were: Ermellinata di Rovigo (**ER**), Polverara Bianca (**PB**) and Nera (**PN**), Pèpoi (**PP**), Robusta Lionata (**RL**), Robusta Maculata (**RM**), Padovana Dorata (**PD**), and Camosciata (**PC**), which were previously investigated by Zanetti et al. (2010a) using microsatellite markers, and Millefiori di Lonigo (**ML**). Table 1 reports the number of individuals analyzed for each breed and the conservation flocks in which they were conserved. Moreover, 10 reference samples of Bresse chicken (**BS**) breed, a French purebred broiler line under the AOC (Controlled Designation of Origin) status, also were collected to include in the analysis of another European chicken population.

Blood samples were taken from brachial vein puncture into a sterile collecting vacuum tube (BD Vacutainer, Milano, Italy) containing sodium citrate (3.2%) and then stored until use at 4°C.

Genomic DNA was isolated from blood using BioSprint 96 DNA Blood Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. After isolation, DNA was assayed for concentration and purity by microfluidic gel electrophoresis with the Agilent 2200 TapeStation system (Agilent Technologies, Santa Clara, CA). The average DNA yield was 50 ng μl with an average 260:280 ratio of 1.85. The integrity of high molecular weight DNA also was confirmed by agarose gel analysis stored at −20°C until analyses were performed.

NGS and SNP selection

The whole genomes of PD and ER breeds were sequenced at the IGA Technology Services (Udine, Italy)

Table 1. Chicken populations analyzed and number of individuals sampled in each conservation flock.

Breed	Code	N	F1	F2	F3	F4	F5
Bresse	BS	10					
Padovana Camosciata	PC	78		38		40	
Padovana Dorata	PD	75		36		39	
Ermellinata di Rovigo	ER	103	33	36	34		
Millefiori di Lonigo	ML	33					33
Pepoi	PP	100	34	33	33		
Robusta Lionata	RL	87	16	37	34		
Robusta Maculata	RM	104	33	36	35		
Polverara Bianca	PB	89		34	34	21	
Polverara Nera	PN	84		37	32	15	
Total		763	116	287	202	115	33

F1: Agricultural Secondary School "Domenico Sartor," Castelfranco Veneto, Treviso, Italy.
F2: Veneto Agricultural Agency "Sasse Rami," Ceregnano, Rovigo, Italy.
F3: Agricultural Secondary School "Antonio Della Lucia," Feltre, Belluno, Italy.
F4: Agricultural Secondary School "Duca degli Abruzzi," Padova, Italy.
F5: Experimental farm, "La Decima," Montecchio Precalcino, Vicenza, Italy.

using Illumina technology. To prepare the libraries for each breed, DNA blood samples belonging to 10 individuals were randomly selected and mixed in a final pool. The libraries were constructed using the TruSeq DNA Sample Prep kit (Illumina) and verified with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Finally, the samples were loaded in estimated equal proportions for their 100 bp paired-end sequencing on a HiSeq2000 platform (Illumina).

The reference genome for the variants' identification and for the recovery of the flanking regions of each polymorphism was obtained through the Ensembl repository, version Galgal4.72. Reads were trimmed for adapter leftover at their ends using cutadapt (v 1.8) with default parameter (Martin, 2011), following quality trimming with an erne-filter (Del Fabbro et al., 2013). Clean reads were aligned to the reference genome with the BWA ALN algorithm (Li and Durbin, 2009). Low mapping quality reads (<10) and duplicates were removed with samtools (Li, 2011). Alignments were further refined around INDELS with the IndelRealigner from Genome Analysis Toolkit software (GATK, v 2.4-9) (McKenna et al., 2010). SNP detection and filtering were obtained using UnifiedGenotyper module, and SNP calls were flagged according to INFO field attributes. Annotation of SNP and INDELS was carried by converting Ensemble gff3 annotation file (Ensembl release version 72) to RefSeq format with custom scripts and processed with the Annovar pipeline (Wang et al., 2010).

SNP genotyping

SNP genotyping was performed using real-time polymerase chain reaction (**PCR**) with high-throughput OpenArray genotyping plates. Each OpenArray plate includes 64 preformatted TaqMan assays (48 samples per panel). Each assay contains two allele-specific probes and a primer pair to detect the specific SNP target. A total of 10 ng of isolated DNA sample was mixed with 2.5 μl of TaqMan OpenArray Genotyping Master Mix in a 384-well plate. The samples were subsequently loaded onto the OpenArray chip using the QuantStudio 12K Flex OpenArray AccuFill System. Each reaction mixture was covered by immersion oil. The PCR conditions were as follows: 10 min at 93°C; 50 cycles of 45 s at 93°C, 13 s at 94°C, and 2.14 min at 53.5°C; and incubation at 25°C for 2 minutes. Data were analyzed with OpenArray SNP Genotyping Analysis software and then imported into TaqMan Genotyper software.

Statistical analysis

The estimated expected (H_E) and observed heterozygosity (H_O), Wright's F-statistics (F_{IS} and F_{ST}), and polymorphism information content (**PIC**) were calculated using the software GenAlEx (Peakall and Smouse, 2012). The inbreeding coefficient (F_{IS}) was calculated according to the correction of Weir and Cockerham (1984). Exact tests for deviation from the Hardy–Weinberg equilibrium (**HWE**; Guo and Thompson, 1992) were executed using Markov Chain Monte Carlo (**MCMC**) simulations (a dememorization number of 10,000, 100 batches, and 5,000 iterations per batch) as implemented in GENEPOP version 3.4 (Raymond and Rousset, 1995).

Kinship distances among breeds were measured according to Caballero and Toro (2002) using MolKin 3.0 (Gutiérrez et al., 2005). Genetic distances among breeds (D_A-distances) were estimated following Nei et al. (1983) and were plotted as a neighbor network using SplitsTree4 (Huson and Bryant, 2006). The genetic structure and the degree of admixture of the 10 populations were investigated using the Bayesian clustering algorithm of STRUCTURE v.2.3.3 (Pritchard et al., 2000) by performing 50 independent runs for each number of clusters (K) to estimate the most likely number of genetic groups present in the dataset, where $2 \leq K \leq 12$. For each value of K, a burn-in period of 250,000 iterations followed by 500,000 MCMC repeats of each run and the admixture model with the

option of correlated allele frequencies were used along with an additional option of location prioritization. Results were then analyzed with Structure Harvester (Earl and VonHoldt, 2012) to detect the number of genetic clusters (i.e., K) that best fit the data, according to the "Evanno" method (Evanno et al., 2005). The alignment of 50 repetitions for each cluster was performed by CLUMPP v.1.1 (Jakobsson and Rosenberg, 2007), and results were visualized using DISTRUCT (Rosenberg, 2004).

RESULTS AND DISCUSSION

Genetic variability at SNP loci

The numbers of reads generated by the NGS sequencing were 209,571,836 and 262,803,820 in total, covering 97% of the reference genome at an average of 15- and 19-fold coverage for PD and ER, respectively (data reported in Supporting Information Table S2). Out of a total of 32,998 and 30,991 exonic variants identified for PD and ER, respectively, we decided to include only coding regions at regular distances along each chosen autosomal chromosome for the selection of the final SNP list. Information about the variability of the investigated loci is shown in Table 2. For the 64 SNP markers selected, PIC values ranged from 0.004, for a C/G SNP (SNP54), to 0.375, for a T/A SNP (SNP30), with an average of 0.212 ± 0.014 (Table 2). Similar low PIC values were detected in Abdalhag et al. (2015). Due to the bi-allelic nature of SNP, their PIC ranges from 0 to 0.5, whereas for microsatellite markers, which are multi-allelic, PIC varies from 0 to 1.0. Because of their lower PIC, more SNP are required than microsatellites to obtain the same power of exclusion. In this context and in our study, SNP markers with a mean PIC value 0.212 were informative. The observed heterozygosity and F_{IS} over all loci varied from 0.004 (SNP54) and −0.015 (SNP08) to 0.325 (SNP51) and 0.771 (SNP04), with a mean value of 0.152 ± 0.011 and 0.133 ± 0.028, respectively. As expected, due to differences between the marker types, results for SNP were lower than those for microsatellite studies of Zanetti et al. (2010a) on local Italian chicken breeds, Maretto et al. (2013) on local Polish chicken breeds, and Özdemir et al. (2016) on Turkish and Italian local chicken breeds. In parallel to PIC results, single-loci SNP analyses presented a loss of information due to the bi-allelic nature of the markers, as compared to the multi-allelic microsatellites having larger numbers of alleles per locus, and hence higher frequency of heterozygotes. The results demonstrated that the SNP panel was successfully used for genetic assignment in the present study. The OpenArray assays were sensitive and reproducible with input genomic DNA extracted from chicken blood. An average call rate of 92% was reached, within expected and acceptable levels. The cost of SNP analysis strongly depends on the number of loci and individuals tested (Nickerson, 2012). With the proposed set of SNP markers, it has been estimated

Table 2. Information of SNP selected for the analysis, heterozygosity (H), polymorphism information content (PIC), and inbreeding coefficients (F_{IS}).

SNP Code	H	PIC	F_{IS}
SNP01	0.165	0.279	−0.054
SNP02	0.166	0.265	0.101
SNP03	0.162	0.167	−0.086
SNP04	0.067	0.374	0.771
SNP05	0.185	0.218	0.071
SNP06	0.096	0.165	0.155
SNP07	0.249	0.321	0.200
SNP08	0.310	0.305	−0.015
SNP09	0.097	0.145	−0.146
SNP10	0.167	0.268	0.000
SNP11	0.052	0.067	0.000
SNP12	0.189	0.320	0.293
SNP13	0.260	0.374	0.138
SNP14	0.073	0.187	0.547
SNP15	0.164	0.138	−0.102
SNP16	0.059	0.058	−0.306
SNP17	0.131	0.161	0.027
SNP18	0.025	0.040	0.180
SNP19	0.071	0.034	−0.118
SNP20	0.197	0.373	0.077
SNP21	0.178	0.240	0.149
SNP22	0.062	0.094	0.268
SNP23	0.105	0.198	0.096
SNP24	0.278	0.358	0.155
SNP25	0.306	0.334	0.179
SNP26	0.128	0.164	0.107
SNP27	0.188	0.238	0.234
SNP28	0.217	0.293	0.088
SNP29	0.045	0.095	0.096
SNP30	0.195	0.375	0.171
SNP31	0.065	0.298	0.753
SNP32	0.175	0.184	0.108
SNP33	0.104	0.170	0.165
SNP34	0.324	0.355	−0.022
SNP35	0.019	0.031	0.556
SNP36	0.055	0.087	0.042
SNP37	0.037	0.045	0.067
SNP38	0.271	0.351	0.126
SNP39	0.123	0.180	0.109
SNP40	0.085	0.137	0.172
SNP41	0.242	0.366	0.020
SNP42	0.301	0.311	−0.041
SNP43	0.109	0.170	0.016
SNP44	0.090	0.126	0.232
SNP45	0.102	0.169	0.178
SNP46	0.181	0.186	−0.267
SNP47	0.094	0.191	0.426
SNP48	0.265	0.373	0.218
SNP49	0.044	0.040	−0.072
SNP50	0.099	0.154	0.054
SNP51	0.325	0.373	0.063
SNP52	0.254	0.266	0.062
SNP53	0.193	0.236	−0.090
SNP54	0.004	0.004	−0.018
SNP55	0.249	0.346	0.175
SNP56	0.231	0.266	−0.106
SNP57	0.010	0.016	0.399
SNP58	0.163	0.268	0.131
SNP59	0.183	0.248	0.008
SNP60	0.037	0.068	0.118
SNP61	0.075	0.121	0.200
SNP62	0.129	0.156	−0.068
SNP63	0.168	0.279	0.292
SNP64	0.315	0.374	0.061
Mean	0.152	0.212	0.133
±SE	0.011	0.014	0.028

Table 3. Observed (H_O) and expected (H_E) heterozygosity, inbreeding coefficient (F_{IS}), and number of loci deviated from Hardy–Weinberg equilibrium (HWE) across chicken breeds.

Breed[1]	H_O	H_E	F_{IS}	HWE
BS	0.135	0.145	0.039	3
PC	0.183	0.209	0.112	12
PD	0.174	0.201	0.117	13
ER	0.244	0.300	0.223	29
ML	0.135	0.132	0.008	8
PP	0.124	0.155	0.171	11
RL	0.135	0.141	0.062	11
RM	0.138	0.154	0.070	10
PB	0.124	0.154	0.153	12
PN	0.124	0.159	0.180	17

[1]Abbreviations are as follows: Bresse chicken (BS). Padovana Camosciata (PC). Padovana Dorata (PD). Ermellinata di Rovigo (ER). Millefiori di Lonigo (ML). Pèpoi (PP). Robusta Lionata (RL). Robusta Maculata (RM). Polverara Bianca (PB). Polverara Nera (PN).

that the cost per data point is about \$0.06 (\$42.5 per individual) for the analysis of a few dozen samples, but the effective cost could decrease to \$0.03 (\$22.4 per individual) considering more than one thousand of processed samples (Soglia et al., 2017). Therefore, the proposed genotyping method is highly competitive and cost effective with respect to other available genotyping approaches.

Breed variability and differentiation

Descriptive statistics over the full set of 64 loci are shown in Table 3. Results show the maintenance of H_O and H_E for the local chicken populations with values ranging from 0.124 (PP, PB, and PN) to 0.244 (ER), and from 0.132 (ML) to 0.300 (ER), respectively. For the Bresse chicken line, values of H_O and H_E were 0.135 and 0.145, respectively. The ER breed exhibited the highest heterozygosity among the studied populations, followed by the 2 strains of Padovana (PC and PD). Compared to other Italian and European chicken breeds (Granevitze et al., 2007; Bianchi et al., 2011; Maretto et al., 2013; Sartore et al., 2014; Strillacci et al., 2016) and Japanese (Tadano et al., 2007) and Indonesian local populations (Riztyan et al., 2011), heterozygosity estimates detected in the present study were rather low. As reported by Zanetti et al. (2010a), the low level of heterozygosity observed for the Veneto local breeds may be explained by a founder effect occurring in the conservation scheme responsible for a loss of genetic variability. Global F_{IS} results (0.114) indicated a low-medium inbreeding effect. Moreover, positive values for these statistics entailed that individuals of a population were more related than expected under a model of random mating, and a certain level of inbreeding has occurred. Positive values of F_{IS} were observed in all chicken breeds, ranging from 0.008 (ML) to 0.223 (ER). The highest F_{IS} values were detected for ER, PC, PD, PP, PB, and PN populations, which also implies that these populations had the highest deviations from HWE (Tadano et al., 2007). The number of deviated loci from HWE ranged from 3 (BS) to 29 (ER). Since 2000, local chicken breeds of the Veneto region have been reared under an in situ marker-assisted conservation program in a free-range system: it is possible that breeding strategies adopted and non-random mating to maintain the morphological standards of breeds may have caused an increase of F_{IS} values and deviations from HWE. Results are comparable to those reported by Tadano et al. (2007), Zanetti et al. (2010a), Maretto et al. (2013), and Özdemir et al. (2016).

Genetic distances

Average F_{ST} value over all populations was 0.167, indicating that almost 17% of the observed variability was attributable to among-breed variation, and the remaining 83% to differences within breeds, meaning a significant degree of breed differentiation, even if lower than values reported by Zanetti et al. (2010a) using microsatellite markers. Pairwise F_{ST} genetic distances among chicken breeds ranged from 0.020 (PB-PN) to 0.193 (RL-PN; Table 4). The highest differentiation has been observed between the group formed with Padovana (PD, PC) and Polverara (PB, PN) breeds and the group including ER, RM, and RL breeds. Kinship distances between local chicken populations varied from 0.103 (PN-PB) to 0.296 (ER-PC; Table 4). Estimates detected showed very similar results obtained by F_{ST} values, confirming the marked distinction between the observed groups, in agreement with the origin of these breeds (De Marchi et al., 2006).

The neighbor-joining dendrogram plotted in Figure 1 reveals 2 separate clusters, reflecting previous results with microsatellite markers on the characterization of local chicken breeds in the Veneto region (Zanetti et al., 2010a; Özdemir et al., 2016). The first cluster included Polverara (PN and PB) and Padovana (PD and PC) breeds, while the second group was composed of RM, RL, and ER breeds. These results are consistent with the literature on these local breeds (De Marchi et al., 2005, 2006; Zanetti et al., 2010a). The close relationship between the tufted ancient breeds, PD and PC as phenotypically very similar, was expected and consistent with the high genetic similarity reported by the aforementioned authors and consistent with the common origin of the breeds (De Marchi et al., 2005). The strict genetic relationship among RM, RL, and ER estimated in the present study corroborates previous findings of Zanetti et al. (2010a). These 3 breeds are characterized by similar breed traits and historic origin, as reported by De Marchi et al. (2005). Finally, PP, ML, and BS breeds were clearly distinct from the other chicken populations (Figure 1).

Population structure

The genetic structure of breeds was obtained using a Bayesian approach to estimate the most genetically

Table 4. Pairwise F_{ST} (below the diagonal) and kinship (above the diagonal) genetic distances between breeds.

	BS	PC	PD	ER	ML	PP	RL	RM	PB	PN
BS		0.165	0.151	0.240	0.139	0.142	0.138	0.141	0.153	0.153
PC	0.098		0.146	0.296	0.202	0.183	0.201	0.211	0.163	0.159
PD	0.100	0.042		0.280	0.189	0.179	0.191	0.197	0.161	0.156
ER	0.136	0.177	0.164		0.255	0.264	0.241	0.237	0.286	0.291
ML	0.116	0.156	0.156	0.152		0.158	0.159	0.181	0.202	0.197
PP	0.107	0.128	0.137	0.152	0.127		0.159	0.153	0.165	0.165
RL	0.117	0.163	0.163	0.139	0.111	0.134		0.129	0.207	0.207
RM	0.122	0.176	0.162	0.123	0.172	0.129	0.106		0.206	0.200
PB	0.118	0.085	0.100	0.178	0.177	0.122	0.192	0.186		0.103
PN	0.121	0.081	0.094	0.181	0.172	0.126	0.193	0.187	0.020	

Abbreviations are as follows: Bresse chicken (BS). Padovana Camosciata (PC). Padovana Dorata (PD). Ermellinata di Rovigo (ER). Millefiori di Lonigo (ML). Pèpoi (PP). Robusta Lionata (RL). Robusta Maculata (RM). Polverara Bianca (PB). Polverara Nera (PN).

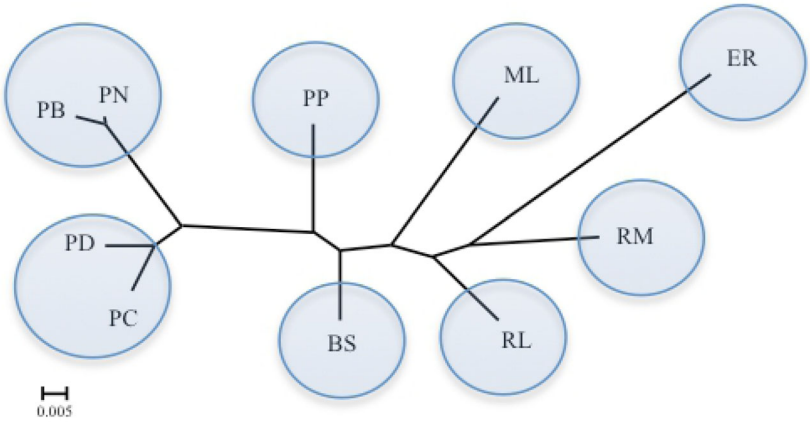

Figure 1. Neighbor-joining dendrogram constructed from Nei's DA-distances (Nei et al., 1983) among analyzed populations. Abbreviations are as follows: Bresse chicken (BS). Padovana Camosciata (PC). Padovana Dorata (PD). Ermellinata di Rovigo (ER). Millefiori di Lonigo (ML). Pèpoi (PP). Robusta Lionata (RL). Robusta Maculata (RM). Polverara Bianca (PB). Polverara Nera (PN).

distinct clusters in the population. The results of STRUCTURE clustering from K = 2 to K = 8 are displayed in Figure 2. At the lowest K-value (K = 2) the tufted ancient breeds, Padovana (PC and PD) and Polverara (PB and PN), split from others and remained together in the same cluster until K = 4. These results agreed with the clustering observed in the neighbor-joining analysis in Figure 1 and with the pairwise F_{ST} values in Table 4. At K = 3, only the ER breed appeared as a discrete population, while the other breeds were grouped in 2 clusters [(PC, PD, and PB, PN) and (ML, PP, RL, RM)]. At K = 4, Padovana (PC and PD) and Polverara (PB and PN) breeds split from each other and clustered independently, whereas other breeds clustered together. At K = 5, RL and RM populations still clustered together, while other breeds formed their own clusters, except for ML, which did not show a unique form until K = 8. According to Evanno et al. (2005), the highest ΔK value was found at K = 8, thus identifying the most probable number of clusters in the population. At this level, all breeds were assigned to a distinct cluster, and the affiliation was successful. Although the BS breed separated clearly from local breeds in the neighbor-joining analysis, it did not form a distinct cluster and acted like an admixed population in STRUCTURE analysis, which might be due to the limited sample size. Subpopulations of Padovana (PC and PD) and Polverara breeds (PB and PV) did not form distinct clusters. In line with F_{ST} values and neighbor-joining dendrogram, STRUCTURE analysis showed the genetic similarity between Padovana and Polverara chicken breeds, which also have similar phenotypic characteristics, such as head tuffs and v-shaped combs. Similar genetic relationships between Padovana and Polverara also were reported in previous studies (De Marchi et al., 2005; Zanetti et al., 2010a), and they were confirmed by genetic distances, population structures, morphology, and known historic origin.

CONCLUSION

In the present study, intra- and inter-breed genetic variability of 7 indigenous poultry populations under conservation programs was estimated using a novel panel of 64 SNP identified by NGS sequencing for comprehensive characterization and valorization of the chicken breeds. SNP marker information provided a reliable estimate of genetic diversity within and between the studied breeds, and it is a rapid, efficient, and cost-effective genetic tool for biodiversity studies of chicken populations. The continuous monitoring of genetic variability over time is needed to avoid the increase of inbreeding and the loss of genetic variation.

Combining molecular data with other information, such as adaptive features, productive and reproductive performance, extinction probability, and

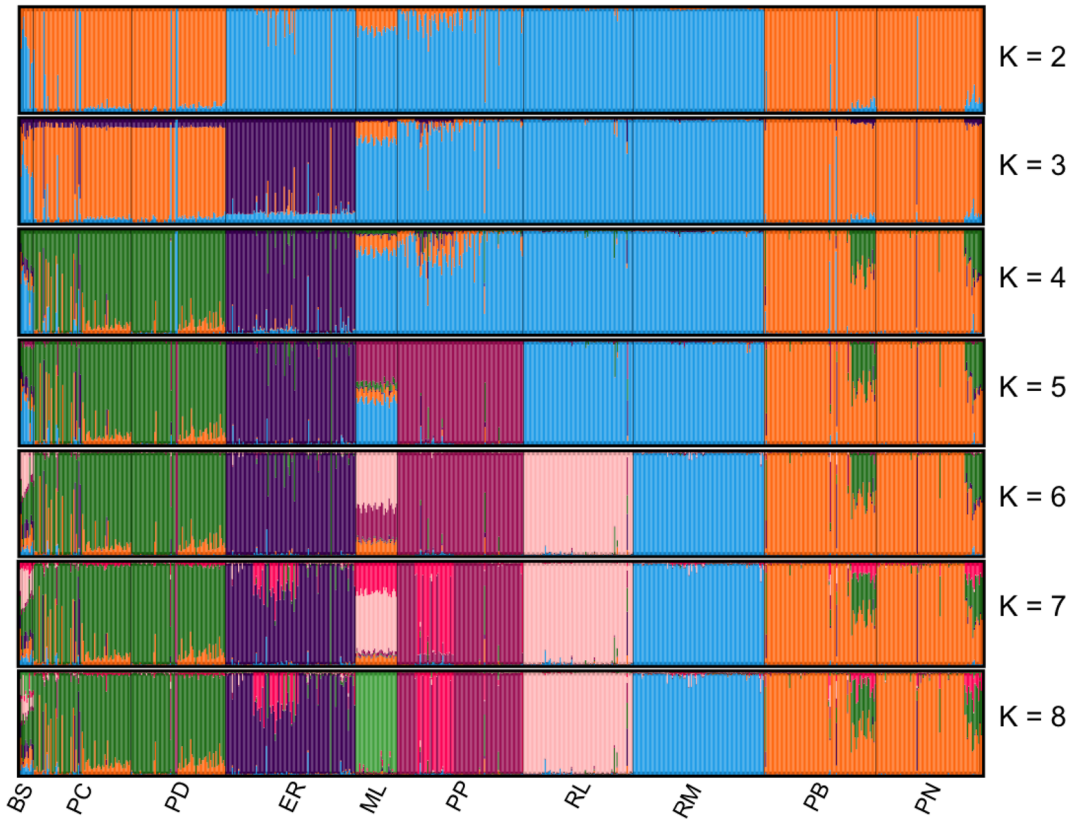

Figure 2. Clustering assignment of the 10 chicken breeds: Bresse chicken (BS). Padovana Camosciata (PC). Padovana Dorata (PD). Ermellinata di Rovigo (ER). Millefiori di Lonigo (ML). Pèpoi (PP). Robusta Lionata (RL). Robusta Maculata (RM). Polverara Bianca (PB). Polverara Nera (PN).

cultural-historical values, would help to improve conservation programs. Moreover, the approach proposed in the present work may be useful to select parents of the future generation within each breed, limiting gene losses and allowing the conservation of the local breeds as an important reservoir of genetic variability. Finally, the SNP panel of the present study could turn out to be a reliable and useful tool for genetic traceability of meat from native chicken breeds and thus certify their origin.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

Table S1. Information on SNP selected, position, mutation and flanking regions.

Table S2. Information on reads generated by the NGS sequencing.

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