Variation of genetic diversity over time in local Italian chicken breeds undergoing in situ conservation

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ABSTRACT The aim of this study was to analyze the variation of different genetic diversity measures in 5 Italian local chicken breeds over a 4-yr period of conservation. The local breeds were Ermellinata di Rovigo, Padovana, Pépoi, Robusta Lionata, and Robusta Maculata. A total of 368 samples were chosen for the analysis among the birds hatched in the years 2002 and 2006. Genetic variation over 30 microsatellite loci was analyzed. All 30 microsatellites were polymorphic, with a total number of alleles equal to 251, a mean (±SD) of 8.367 ± 3.378 across populations and 3.233 ± 1.338 within population. After 4 yr of conservation, a loss of alleles occurred for all the microsatellites, with the exception of 4 loci. The total number of alleles and expected heterozygosity estimates significantly decreased during the 4 yr of conservation, whereas no significant differences were detected for the microsatellites polymorphism information content or for the observed heterozygosity estimates. A decrease of the inbreeding coefficient occurred for all the breeds, with the exception of Padovana and Robusta Lionata. All populations showed evidence of a persistent significant deviation from Hardy-Weinberg equilibrium caused by an excess of homozygotes, except Robusta Maculata, which reached the equilibrium in 2006. For Pépoi, Padovana, Ermellinata di Rovigo, and Robusta Maculata, molecular coancestry increased significantly ($P < 0.001$) after the 4 yr of conservation. No evident genetic structures were detectable within breed, both for 2002 and for 2006 individuals. However, a slight increase in the proportion of membership for each breed had occurred in the year 2006 compared with the data obtained in the year 2002. As consequence, within breed, individuals in 2006 appear more homogeneous, producing clearer, more distinctive and separated groups. Molecular markers analysis helped us monitor the genetic variability of local breeds involved in a conservation scheme, enabling the planning of new strategies for the improvement of in situ conservation schemes.

Key words: biodiversity, conservation, livestock, microsatellite

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INTRODUCTION

Chicken breeds play an important role as a resource for human nutrition. After centuries of rearing and adaptation to different environments, the result has been the development of multiple chicken breeds such as unselected breeds or ecotypes, standardized breeds selected for morphological traits (the case of European and American pure breeds), commercial strains and crosses selected for the particular productive and reproductive traits, and experimental breeds (FAO, 2007). The animal genetic resources include all the species, breeds, and strains that become of enormous economic, scientific, and cultural interest to humans in terms of agricultural production and food. Biodiversity conservation plays an important role in the genetic management of these breeds, aiming to minimize inbreeding and the loss of genetic diversity. There is no risk of extinction for the cosmopolitan domestic breeds, so the main attention and efforts go to the local and less diffused local breeds reared in marginal and restricted areas. In the poultry sector a reduction in the number of the local breeds occurred, caused by replacement with cosmopolitan poultry breeds and highly productive crosses, suggesting an urgent need for conservation of these endangered genetic resources. In Italy, since 2000, some local poultry breeds have been put through a governmental in situ marker assisted conservation scheme (De Marchi et al., 2006b). Among them, 5 local chicken breeds were involved and reared in 3 conservation flocks of the Veneto region (northeast Italy).

The use of molecular tools can potentially improve conservation and management of these populations by aiding in the analysis of population structure, gene flow, parentage, and diseases and by controlling the

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Table 1. Microsatellite markers with corresponding number of singleplex or multiplex PCR reaction (MPX/SPX), chromosomal location, fragment size, annealing temperatures (Ta), and the fluorophore used

MPX/SPX	Locus	Chromosome	Fragment size (bp)	Ta (°C)	Fluorophore
SPX1	Mcw284	4	239–247	62	D2
MPX1	Mcw216	13	141–151	60	D3
	Mcw248	1	195–243	60	D3
	Lei166	3	251–263	60	D2
MPX2	Mcw20	1	181–199	60	D3
	Mcw37	3	151–159	60	D2
	Mcw295	4	82–118	60	D4
	Mcw111	1	90–118	60	D3
	Mcw103	3	260–272	60	D3
	Lei234	2	215–321	60	D2
	Mcw14	6	164–188	60	D4
	Lei94	4	249–283	60	D4
MPX3	Mcw165	23	108–118	60	D4
	Mcw78	5	130–150	60	D4
MPX4	Mcw222	3	217–229	60	D4
	Adl268	1	101–119	60	D3
	Adl278	8	108–126	60	D4
MPX5	Mcw123	14	112–150	60	D3
	Mcw81	5	111–155	60	D2
MPX6	Mcw330	17	257–289	62	D3
	Mcw69	26	154–178	62	D4
	Mcw34	2	222–246	62	D2
	Lei192	6	255–297	62	D4
MPX7	Mcw98	4	255–259	62	D3
	Mcw67	10	168–180	62	D4
	Mcw206	2	226–250	62	D4
	Mcw80	15	265–281	62	D3
MPX8	Adl112	10	124–132	62	D3
	Mcw183	7	295–339	62	D4
	Mcw16	3	130–152	62	D4

mating system and introduction of unrelated individuals (Frankaham, 2010) and contribute to the valorization of the livestock products of that breeds (Dalvit et al., 2008b). Although nowadays other techniques, such as high-throughput SNP analysis techniques, are being used more (Shen et al., 2005), microsatellite markers analysis is still the standard method of choice to estimate genetic diversity and relationships in livestock populations (Baumung et al., 2004; Muchadeyi et al., 2007; Dalvit et al., 2008a, 2009; Zanetti et al., 2010). In the last few years several studies have used microsatellite markers to study genetic diversity of different chicken breeds or ecotypes species (Wimmers et al., 2000; Muchadeyi et al., 2007; Roushdy et al., 2008), but until now no authors have engaged over-time analysis of genetic variation in conserved breeds or populations. The objective of this study was to analyze the variation of different genetic diversity measures and changes in population structure in 5 Italian local chicken breeds over a 4-yr period of in situ conservation.

MATERIALS AND METHODS

In Situ Conservation Program

Experimental procedures complied with the national and European regulations on animal welfare. Moreover, good handling and manipulation practices were adopted.

The conservation scheme involves 5 chicken breeds and 3 different flocks. The in situ conservation program was begun in 2000, and its management strategies were described by De Marchi et al. (2006a). In brief, each flock contains about 54 individuals for each breed: 34 females and 20 males. Males are divided in 2 groups based on genetic differences. The groups are alternately mated with the females to ensure a high degree of genetic variation to the progeny, which will replace the parents as breeding animals. Each year males are exchanged among the different flocks in a circular scheme.

Bird Sampling

A total of 368 samples were chosen for the analysis among the birds hatched in the years 2002 (a) and 2006 (b). The 5 breeds were Ermellinata di Rovigo [**ER** = 26 (a), 45 (b)], Padovana [**PD** = 26 (a), 49 (b)], Pépoi [**PP** = 40 (a), 45 (b)], Robusta Lionata [**RL** = 24 (a), 43 (b)], and Robusta Maculata [**RM** = 25 (a), 45 (b)]. Blood samples were taken from the wing vein into a sterile collecting vacuum tube (BD Vacutainer, Milano, Italy) containing sodium citrate and citric acid and then stored until use at 4°C.

Table 2. Microsatellite markers with corresponding total number of alleles (TNA), observed heterozygosity (Ho), average number of alleles per population (PNA), the variation of the number of alleles from 2002 to 2006 (ΔNA), and inbreeding coefficient (Fis)

Locus	TNA	Ho	PNA	ΔNA	Fis
Mcw69	11	0.528	2.120	−5	0.515*
Mcw20	9	0.737	3.806	−5	0.481***
Mcw34	10	0.846	6.488	0	0.451***
Lei192	9	0.779	4.515	−3	0.271***
Mcw330	5	0.711	3.459	−1	0.447**
Mcw216	5	0.619	2.622	−1	0.196***
Mcw248	9	0.455	1.834	−3	0.525***
Lei166	5	0.721	3.582	−2	0.454
Adl278	9	0.740	3.840	0	0.498***
Mcw222	6	0.600	2.500	−2	0.446
Adl268	10	0.755	4.089	−3	0.489
Mcw16	11	0.749	3.991	−6	0.622***
Mcw183	14	0.700	3.333	−7	0.199***
Mcw165	7	0.619	2.625	−3	0.628***
Mcw78	12	0.666	2.998	−4	0.541***
Mcw123	8	0.700	3.334	0	0.365**
Mcw98	3	0.206	1.259	−1	0.385***
Mcw81	10	0.685	3.170	−2	0.439***
Mcw67	7	0.638	2.759	−4	0.520***
Adl112	4	0.427	1.746	−1	0.222
Mcw80	5	0.701	3.348	0	0.393***
Mcw37	5	0.410	1.695	0	0.309
Mcw295	12	0.730	3.698	−4	0.496***
Mcw111	14	0.693	3.259	−10	0.450**
Mcw284	4	0.527	2.114	−1	0.298**
Mcw206	7	0.691	3.237	−2	0.508*
Mcw103	4	0.422	1.729	−2	0.281
Lei234	16	0.870	7.668	−1	0.219**
Mcw14	10	0.732	3.725	−5	0.752***
Lei94	10	0.593	2.456	0	0.444*
Mean ± SD	8.367 ± 3.378	0.642 ± 0.144	3.233 ± 1.338		0.428

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Amplification and Genotyping of Microsatellite Markers

The DNA extraction was carried out employing a modified DNA purification kit (Gentra System PUREGENE DNA purification kit (Quiagen S.r.l., Milan, Italy) The DNA samples were amplified by performing multiplex and singleplex PCR reactions in correspondence to 30 microsatellite loci, listed in Table 1, at the following conditions: an initial denaturation step of 30 s at 98°C, 40 cycles of 7 s at 98°C, 15 s at X°C and 20 s at 72°C, and the final extension of 7 min at 72°C, with X°C being the annealing temperature for each multiplex and singleplex. The investigated loci were chosen according to ISAG/FAO Standing Committee Recommendations (FAO Standing Committee, 2004). After the fragments amplification, for each bird 4 different poolings were set and analysis was performed using an automated DNA sequencer (CEQ 8000 Genetic Analysis System, Beckman Coulter, Brea, CA). The following software elaboration of electropherograms was carried out using CEQ 8000 (Beckman Coulter).

Statistical Analysis

The total number of alleles, average number of alleles per population, polymorphic information content (Botstein et al., 1980), and the mean molecular coancestry coefficient (**Fij**) were calculated using MOLKIN version 3.0 (Gutiérrez et al., 2005) by considering both the global population and separately the 2 years 2002 and 2006. The Fij weighted for the polymorphic informative content was considered. The estimated expected (**He**) and observed heterozygosity (**Ho**), mean number of alleles, and Wright's *F*-statistics (**Fis**, **Fst**, and **Fit**) were calculated using the software GENETIX (Belkhir et al., 1996–2002). The He has been corrected for sampling bias as performed by GENETIX. The inbreeding coefficient (Fis) was calculated according to the correction of Weir and Cockerham (1984). Exact tests for deviation from Hardy-Weinberg equilibrium (Guo and Thompson, 1992) were executed using Markov Chain Monte Carlo 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). Within-breed significant differences between the 2 yr of conservation for Ho, He, mean number of alleles, and Fij were calculated using the MULTTEST procedure of the software SAS (SAS Institute Inc., 2008), performing the HOLM correction.

STRUCTURE version 2.2 (Pritchard et al., 2000) was implemented to detect the most likely number of populations (**K**) fitting the data. This software is widely used for the detection of hidden structures within

populations, the assignment of individuals to reference populations, and the identification of migrants and admixed individuals. For the analysis, a burn-in period of 25,000 iterations and 250,000 repetitions were set, with K ranging from 2 to 9 and 30 independent runs of each K. The analysis, performed for both 2002 and 2006 individuals, involved an admixture model with correlated allelic frequencies. The best number of clusters fitting the data was established by using the ΔK statistics suggested by Evanno et al. (2005). Clustering results from 30 independent runs of each K were averaged, based on the LargeKGreedy algorithm and the G pairwise matrix similarity statistics, using CLUMPP (Jakobsson and Rosenberg, 2007). Clustering results were visualized using DISTRUCT (Rosenberg, 2004). The optimal number of assumed genetic clusters (K) was determined by the ΔK statistic (Evanno et al., 2005).

RESULTS AND DISCUSSION

Microsatellites Genetic Variation

To study the genetic diversity of a population, the use of molecular markers with a high grade of polymorphism and that are not in linkage disequilibrium is important (Goldstein and Pollok, 1997). Here a set of 30 microsatellite markers was chosen. They are located in different chromosomes or separated by large distances, showing no linkage disequilibrium. Information about chromosomal location, fragment size, number of singleplex or multiplex PCR reactions, annealing temperatures, and the list of fluorophores bound to forward primers is shown in Table 1.

The variability of the investigated loci is shown in Table 2. All the selected microsatellites markers were polymorphic, with a total number of alleles equal to 251, showing mean values (±SD) of 8.367 ± 3.378 across populations and 3.233 ± 1.338 within population. These values are evidence of high differentiation between breeds, but moderate within-breed diversity. The most polymorphic markers were Lei234, with 16 alleles across populations and an average 7.668 alleles per population, and Mcw34, with 10 alleles and an average of 6.488. On the other hand, MCW98 was the least polymorphic, with 3 alleles across populations and an average of 1.259 per population. The Ho over all the loci showed a mean value of 0.642 ± 0.144, ranging from 0.206 to 0.870 in Mcw98 and Lei234, respectively. The Fis coefficient showed a global deviation from Hardy-Weinberg equilibrium for 24 microsatellites upon 30 ($P < 0.05$). There are 2 main reasons for this departure from Hardy-Weinberg equilibrium: first, subdivisions of the population into different breeds and second, an excess of homozygotes within breeds. Globally, with a total value of Fit = 0.439, Fis = 0.054 and Fst = 0.407, the division in breeds has the major effect on Hardy-Weinberg equilibrium departures. The results underline a high degree of breed differentiation that is in accordance with the values reported by Tadano et al. (2008)

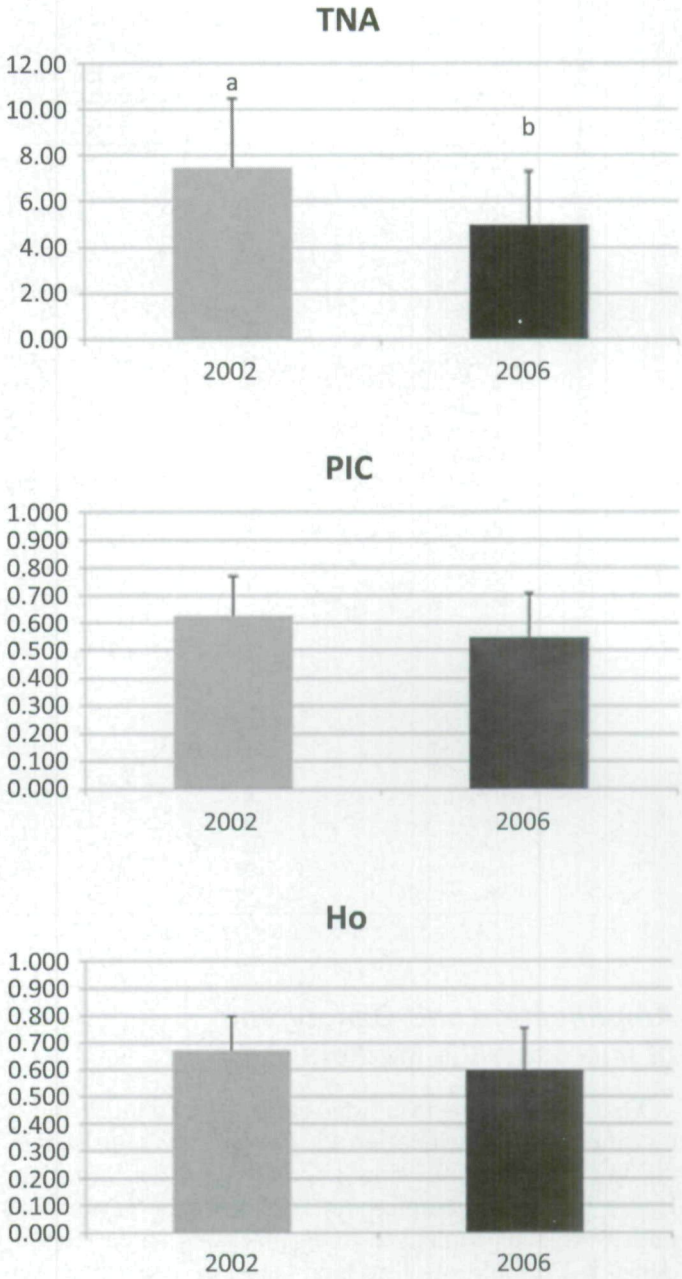

Figure 1. Averages with SD of total number of alleles (TNA), observed heterozygosity (Ho), and the polymorphic information content (PIC) for all breeds reared in 2002 and 2006. Significant differences among years ($P < 0.05$) are marked with letters a and b.

for native Japanese poultry breeds but in contrast with lower genetic differentiation values found in 8 Finnish chicken breeds by Vanhala et al. (1998). After 4 yr of conservation, a loss of alleles occurred for all the microsatellites with the exception of 6 loci (Mcw34, Adl278, Mcw123, Mcw80, Mcw37, Lei94), with decreases ranging from 1 to 10 alleles (Table 2). Comparing the total number of alleles values at the 2 moments of conservation (Figure 1), a significant decrease occurred ($P = 0.002$), with a mean value ranging from 7.467 ± 3.003 in the year 2002 to 5.000 ± 2.289 in the year 2006. The polymorphic information content showed a mean value of 0.625 ± 0.142 in the year 2002 and 0.548 ± 0.159 in 2006, but no significant differences were detected.

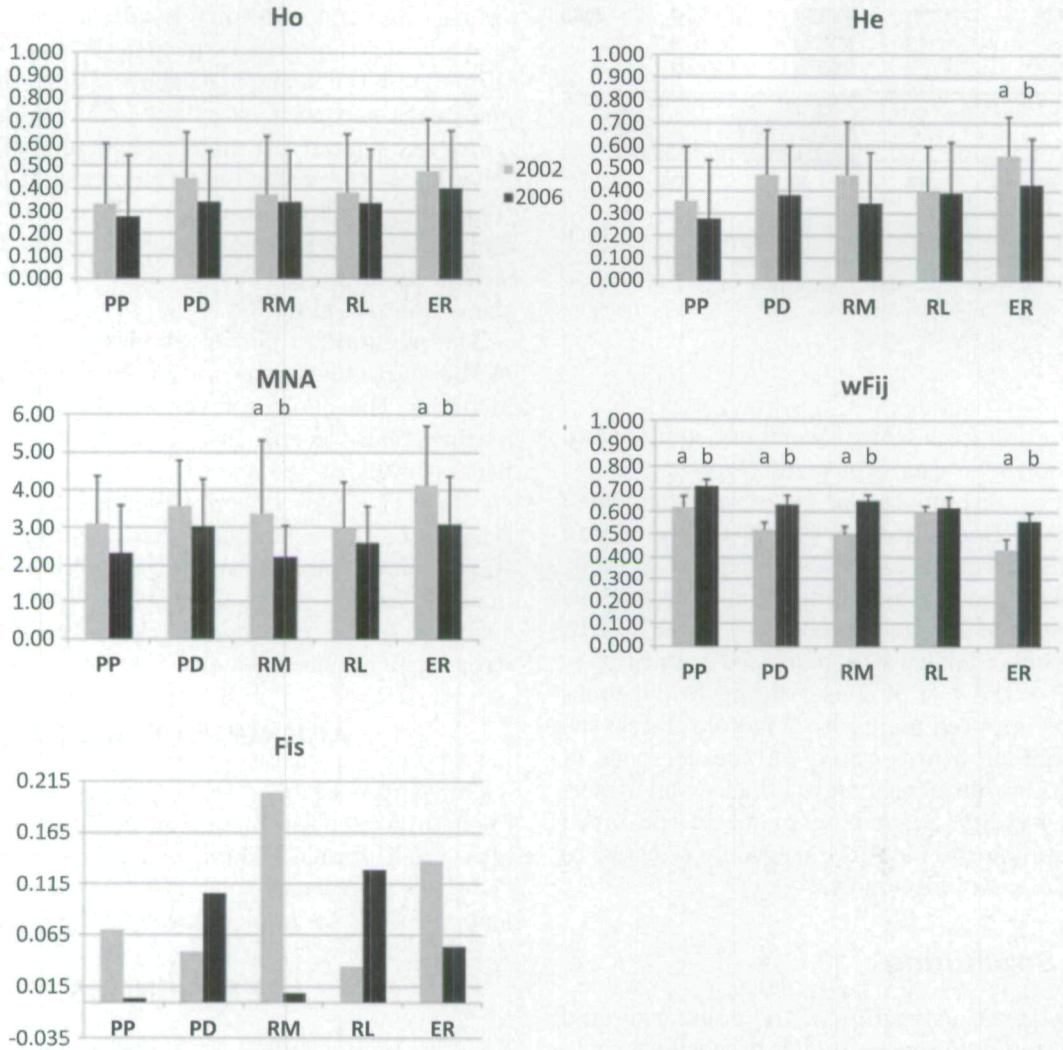

Figure 2. Averages with SD of observed (Ho) and expected (He) heterozygosity, mean number of alleles (MNA), weighted molecular coancestry coefficient (wFij), and inbreeding coefficient (FIS) for each breed sampled in 2002 and 2006. Abreviation PP = Pépoi, PD = Padovana, RM = Robusta Maculata, RL = Robusta Lionata, and ER = Ermellinata di Rovigo. Within a breed, significant differences among years ($P < 0.05$) are marked with letters a and b.

A statistically not significant decrease for Ho, with a mean value of 0.669 ± 0.130 for 2002 and 0.600 ± 0.156 for 2006, occurred (Table 2). These results are consistent with those reported by Tadano et al. (2007) in a study about genetic relationships among native chicken breeds and those reported by Hillel et al. (2003) in a study of the biodiversity of 52 chicken populations.

Within-Breed Variation Over Time

All studied breeds showed not significant decreases in He during the conservation period, with the exception of ER ($P = 0.022$; Figure 2). Also, not significant decreases in Ho were detected in all breeds. Furthermore, among all breeds, only ER and RM showed a significant decrease in the mean number of alleles ($P = 0.021$ and $P = 0.015$ for ER and RM, respectively;). Because no studies were found that aimed to compare changes in genetic variation for livestock species along in situ conservation plans, the discussion here focuses on the breeds characteristics. The Ho values were low compared with those of other studies about Hungarian and Vietnamese indigenous chicken breeds (Cuc et al., 2006; Bodzsar et al., 2009) but similar to those reported by other authors about European pure chicken breeds (Hillel et al., 2003; Granevitze et al., 2007; Dávila et al., 2009). In particular, the PD breed showed values of Ho consistent with those previously reported by those authors.

During the conservation period, 3 breeds out of 5 (PP, RM, and ER) showed a decrease of Fis. The increment of this parameter in RL is probably due to the light selection performed during conservation, to maintain morphological standards and to discard birds presenting physical defects. Recently new strategies have been adopted to reestablish and contain the levels of inbreeding in these 2 breeds, by introducing new unrelated birds for the PD and by backcrossing with the ancestor breeds (e.g., in the case of RL, which was originated by crossing Orpington and White American).

Except for RM, which reached the equilibrium in 2006 (data not shown), all breeds showed a persistent

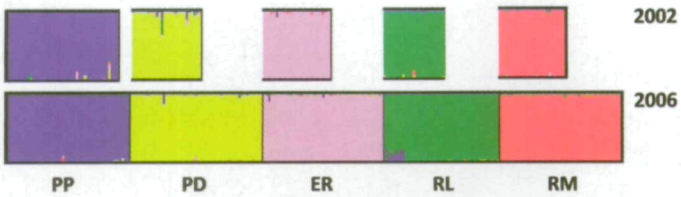

Figure 3. Graphical representation of the estimated membership fractions of individuals of the breeds analyzed in each of the K=5 inferred clusters, for the years 2002 and 2006.Abbreviation PP = Pépoi, PD = Padovana, RM = Robusta Maculata, RL = Robusta Lionata, and ER = Ermellinata di Rovigo.

significant deviation from Hardy-Weinberg equilibrium. This was caused by an excess of homozygotes.

The Fij values within population were higher than those reported by Bodzsar et al. (2009) on Hungarian indigenous chicken breeds and those reported by Marletta et al. (2006) about other domestic species, such as western Mediterranean horse breeds. For PP, PD, ER, and RM, molecular coancestry increased significantly ($P < 0.001$) after the 4 yr of conservation. In our opinion, this can be imputed mainly to 2 factors. First, the cocks have different fitness based on the presence of hierarchies within the conservation flocks and different individual fertility. Second, to maintain the breed phenotypical standards, birds are routinely selected to exclude not adequate individuals.

Population Structuring

To study the genetic structure of the population and to detect over time differences among breeds and within-breed hidden structures, a Bayesian approach was used and the analysis inferred the number of clusters (K) present in the population. The optimal number of assumed genetic clusters ΔK statistics was 5, both for 2002 and for 2006. The graphical representation of the estimated membership fractions of individuals is reported in Figure 3. No evident genetic structures were detectable within breed, both for 2002 and for 2006 individuals. However, a slight increase in the proportion of membership for each breed had occurred in the year 2006 compared with the data obtained in the year 2002. So, within breed, individuals in 2006 appear more homogeneous, producing clearer, more distinctive and separated groups. Similar population structures were observed for the Hungarian chicken breeds (Bodzsar et al., 2009).

Conclusions

Molecular markers show great usefulness for monitoring the genetic variability of breeds involved in a conservation scheme. Information about the family origin, that keeps record of paternity, has already been used to maximize within-breed genetic variation since the beginning of the conservation plan, but it is not possible in the daily managerial practice to gather information about the exact paternity of all birds. To gain direct control over the changes in genetic diversity occurring throughout the years, we believe firmly that molecular analysis is necessary every few generations.

Results showed the maintenance of expected and observed heterozygosity but an increase of the molecular coancestry within breed. Two breeds of chicken, PD and ER, showed a reduction in the mean number of alleles, underlining the critical state of these breeds in the conservation flocks.

The planning of new strategies for the improvement of this particular conservation scheme is now possible, including the choice of the animals, a more efficient mating plan, taking into consideration the different fitness levels of the males within the flocks, and the creation of a new selection index based on the maintenance of the existing genetic variation. In addition, to guarantee higher levels of variability for these Italian local breeds, other approaches should be considered such as sperm cryo-conservation techniques coupled with artificial insemination.

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