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Assessment of the population structure and genetic diversity of Denizli chicken subpopulations using SSR markers

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ABSTRACT

The aim of this study was to characterise the genetic diversity, genetic relationship and population structure within local Turkish Denizli chicken subpopulations in rural areas using 19 microsatellite markers. To assess the uniqueness and relations of the Denizli subpopulations we used six local Italian chicken breeds' samples, which were genotyped in the same laboratory with the same molecular markers. In total, 105 alleles were found across 19 microsatellite loci with a mean number of 5.53 alleles per locus. Considering all subpopulations and loci, genetic differentiation based on global F_{ST} was 0.030 ($p < .01$). Global F_{IS} values (0.200) indicated that non-random mating occurred in all subpopulations of Denizli fowl and all subpopulations deviated significantly ($p < .01$) from HWE. Over all subpopulations, the mean observed heterozygosity was 0.473, ranging from 0.399 to 0.562. Genetic differentiations between pairs of subpopulations based on the proportion of shared alleles ranged 0.140–0.297. The neighbour-net tree, based on marker estimated kinship distances, separated Denizli subpopulations and Italian breeds into two main clusters. The most likely number of different populations was estimated using the clustering procedure implemented in STRUCTURE. Structure analysis showed a clear separation of the Denizli fowl subpopulations from the Italian populations. A second step sub-clustering allowed discriminating among the six subpopulations of Denizli breed. The results of this study can be used as baseline genetic information to place breeders' flocks in conservation programmes, controlling inbreeding and safeguarding the genetic variability of the populations.

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Introduction

Over the past 50 years, global animal production has increasingly been based on a limited number of high output breeds and this has led to the marginalisation of traditional production systems and the associated local breeds (Food and Agriculture Organisation [FAO] 2007). This trend has caused an increase in the loss of farm animal genetic resources, especially as it refers to avian species and breeds, due to the extraordinary development of the commercial poultry industry (Weigend et al. 2004).

In developing countries, such as Turkey, the effects of genetic erosion are more severe in poultry breeds and species for similar reasons: (i) the contribution of commercial breeds to overall poultry meat and egg consumption; (ii) the destruction of poultry breeds and species in rural gene reservoirs due to the highly pathogenic *Avian influenza* (Food and Agriculture Organisation [FAO] 2004a; Özdemir et al. 2013).

Regardless of the erosion in its reservoir of poultry, Turkey, which has acted as a crossroads for the distribution of domesticated animals from Asia to Europe since the beginnings of agriculture in Mesopotamia or Asia, has preserved its animal biodiversity potential in rural areas. Although it is known that many local chicken breeds live in the rural parts of Turkey, only two of these have officially been registered and conserved against the danger of extinction by the Ministry of Food Agriculture and Livestock of Turkey (MFALT) since 1997.

Denizli fowl, one of the two registered Turkish chicken breeds that are at risk of extinction, is locally reared in Denizli city and its prefectures in the western part of Turkey. Although there are no exact records concerning the origins of this breed, historical ruins from the ancient city of Laodicea show that this breed has lived in Denizli since the second century AD (Turkish Radio and Television Corporation [TRT], 2013). Denizli fowl are reared for their eggs and as a hobby,

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but especially for the long crowing feature of the cocks (approximately 15–25, s); this long crowing behaviour, which is the most important selection criterion for Denizli fowl breeders, has unwittingly assisted in preserving the breed up to the present time. Moreover, there are five known varieties of the breed (Pekmez kefi, Demirkır, Pamukkır, Al and Siyah), named according to the distribution of differently coloured feathers on the hackle, neck and breast of the males.

Currently, Denizli fowl are under conservation in closed flocks through an *ex-situ in vivo* method employed by the MFALT institute. However, the benefits of this kind of conservation are debateable as it may cause increased homozygosity and genetic loss of the breed and it is also potentially a risk for epidemic diseases. In the complex structure of conservation activities, breeders and breed societies are the most important players in the conservation of local poultry breeds (Özdemir et al. 2013). In this respect, bringing breeders' flocks into the conservation scheme for Denizli fowl would be helpful to ensure the genetic variability of the breed and also to safeguard some flocks against epidemic diseases.

It is widely accepted that microsatellite markers are still one of the most powerful molecular tools for assessment of genetic diversity, population structure and differentiation. These highly polymorphic markers have been successfully used in genetic conservation of chickens by enabling tracking individuals in conservation programmes (Tadano et al. 2014). However, very few studies have been carried out with microsatellite variations in Turkish native chicken breeds. With this approach in mind, the aim of this study is to characterise the genetic variability, genetic relationship and population structure of six rural subpopulations of Denizli chicken breeds using 19 microsatellite markers. This study is also the first to investigate the genetic characteristics of Denizli fowl in breeders' stocks in

rural areas and the results of this study can be used as baseline genetic information for bringing breeders' flocks into conservation activities to control inbreeding and safeguard the genetic variability of the populations.

Materials and methods

A total of 155 both sexes individuals were randomly selected from six different farms that locate in and around Denizli city in the Aegean region of Turkey (Table 1). The sampled subpopulations were drawn from different breeder flocks in the centre of Denizli (DN_D, $n=25$), Honaz (DN_H, $n=25$), Tavas (DN_T, $n=25$), Acipayam (DN_A, $n=25$) and Serinhisar (DN_S, $n=25$) and the Adnan Menderes University Denizli Fowl flock in Aydın (DN_U, $n=30$) (Table 1). The DN_U and DN_D subpopulations originated from the conservation flock of the Denizli Cock Rearing and Conservation Farm Station, which obtained its conservation animals from breeders' stocks in the past. All local breeders are members of the Denizli Fowl Breeders' Association under the Turkish Ornamental and Garden Animals Federation. Breeders' farms in this study were selected based on their Denizli fowl stocks, namely individuals representing the best morphological traits of pure Denizli fowl.

Blood samples were preserved on FTA cards (Whatman, No.WB120205). Total genomic DNA was isolated from a portion of each blood-soaked FTA card using the NaOH method described by Zhou et al. (2006), which involves only one step of incubation in 20, mM of NaOH solution, followed by a single wash with TE^{-1} buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). In line with Zhou et al.'s (2006) NaOH method, quadruplicate 1.2 mm diameter disks were punched from each blood spot and each disk was transferred into a 0.2 mL PCR tube. To extract DNA using the NaOH method, an aliquot of 200 μ L of 20 mM NaOH solution was added to the tube containing the blood

Table 1. List of sampled chicken breeds.

| Breeds | Acronym | <i>n</i> | Sampling location | |
|-----------------------|---------|----------|---------------------|---------------------------|
| Denizli | DN_D | 25 | Denizli | 37°46'41.8"N 29°06'41.5"E |
| Denizli | DN_H | 25 | Denizli, Honaz | 37°45'17.9"N 29°16'20.0"E |
| Denizli | DN_T | 25 | Denizli, Tavas | 37°34'30.1"N 29°04'13.9"E |
| Denizli | DN_A | 25 | Denizli, Acipayam | 37°25'56.8"N 29°20'46.3"E |
| Denizli | DN_S | 25 | Denizli, Serinhisar | 37°34'18.1"N 29°16'34.0"E |
| Denizli | DN_U | 30 | Aydın, Kocarlı | 37°45'30.5"N 27°45'18.9"E |
| Reference set | | | | |
| Padovana | PD | 30 | Padua | |
| Ermellinata di Rovigo | ER | 21 | Rovigo | |
| Pepoi | PP | 30 | Rovigo | |
| Robusta Lionata | RL | 30 | Rovigo | |
| Robusta Maculata | RM | 30 | Treviso | |
| Polverara | PV | 30 | Padua | |

disk and then incubated for 30 min at 50 °C. The solution was then discarded and the disk was washed in 200 µl TE⁻¹ buffer for 2 min. After the removal of the TE⁻¹, the disk was air-dried and stored at room temperature until polymerase chain reaction (PCR) analysis. Genomic DNA concentrations were determined by fluorescence assay and the integrity of the DNA was visually inspected on 1% agarose gels.

Amplification and genotyping of microsatellite markers

The DNA samples were amplified by performing multiplex and singleplex PCR reactions using 19 microsatellite loci, listed in Table 2, under the following conditions: initial denaturation step of 5 min at 95 °C, 45 cycles of 30 s at 95 °C, annealing for 1.3 min at X °C (58–64, °C) and 45 s at 72 °C and a final extension of 30 min at 60 °C. The loci investigated were chosen according to ISAG/FAO Standing Committee Recommendations (Food and Agriculture Organisation [FAO]. Standing Committee 2004b). Compatible amplicons were pooled before separation on an automated capillary electrophoresis system (CEQ 8000 Genetic Analysis System, Beckman Coulter, Indianapolis, IN). Allele sizing was performed using Genetic Analysis Software version 9.0 (Beckman Coulter, Brea, CA). Data on Italian chicken breeds were provided from previous studies as reference populations (Zanetti et al. 2011) (Table 1). Genotyping of the reference Italian samples and Turkish samples was performed in the same

laboratory and standard alleles were used to adjust the allele scoring between researchers.

Statistical analysis

The total number of alleles, mean number of alleles, effective number of alleles, allelic richness, expected (H_E) and observed heterozygosity (H_O) were calculated using the GenAEx version 6.5 software (Peakall and Smouse 2012; Oxford University Press Oxford, UK) and PICcalc (Nagy et al. 2012) was used to calculate the polymorphic information content (PIC). Nei's gene diversity and Wright's fixation indices (F_{IT} , F_{IS} and F_{ST}) within and across populations were calculated using MSA version 4.05 (Dieringer and Schlötterer 2003; University of Vienna, Vienna, Austria). Exact tests for deviation from the Hardy–Weinberg equilibrium were applied using the Markov chain Monte Carlo (MCMC) simulation (5000 iterations, 100 batches and a dememorisation number of 10,000) implemented in GENEPOP version 4.2 (Rousset 2008; Laboratoire de Genetique et Environment, Montpellier, France). Genetic distances between subpopulation pairs based on the proportion of shared alleles (D_{PS}) (Bowcock et al. 1994) and Nei (D_A) (Nei 1972) were calculated using MSA 4.05 (Dieringer and Schlötterer 2003) to investigate the genetic distances within breed among subpopulations. Employing these distances, relationships among Denizli fowl subpopulations were visualised in a phylogenetic network using SPLITSTREE version 4.8 (Huson and Bryant 2006; Universität Tübingen, Tübingen, Germany).

Table 2. Microsatellite loci with corresponding chromosomal location (CL), fragment size (FS), total number of alleles (TNA), mean number of effective alleles over subpopulations (MNE), polymorphism index content (PIC), Wright's inbreeding coefficient (F_{IS}) and the overall inbreeding coefficient (F_{IT}) for all loci across subpopulations.

| Locus | CL | FS (bp) | TNA | MNE | PIC | F_{IS} | F_{IT} |
|---------|----|---------|------|------|-------|----------|----------|
| ADL0268 | 1 | 101–119 | 5 | 2.95 | 0.654 | –0.087 | –0.001 |
| MCW0216 | 13 | 141–151 | 4 | 2.08 | 0.448 | 0.602 | 0.612 |
| MCW0248 | 1 | 195–243 | 5 | 2.30 | 0.516 | 0.664 | 0.673 |
| ADL0278 | 8 | 110–126 | 8 | 2.93 | 0.628 | 0.256 | 0.261 |
| MCW0081 | 5 | 111–139 | 5 | 2.89 | 0.612 | 0.372 | 0.379 |
| MCW0069 | 26 | 154–178 | 4 | 2.05 | 0.459 | –0.040 | –0.020 |
| MCW0222 | 3 | 217–229 | 5 | 2.92 | 0.614 | 0.556 | 0.557 |
| MCW0034 | 2 | 222–246 | 7 | 3.05 | 0.678 | 0.268 | 0.302 |
| LEI0166 | 3 | 251–263 | 6 | 2.99 | 0.672 | 0.229 | 0.287 |
| LEI0094 | 4 | 251–285 | 6 | 2.01 | 0.456 | 0.156 | 0.163 |
| MCW0111 | 1 | 90–118 | 6 | 2.42 | 0.564 | 0.072 | 0.138 |
| ADL0112 | 4 | 122–132 | 6 | 2.99 | 0.652 | 0.228 | 0.247 |
| MCW0103 | 4 | 260–272 | 3 | 1.81 | 0.359 | –0.090 | –0.077 |
| MCW0295 | 12 | 82–118 | 5 | 2.22 | 0.498 | –0.331 | –0.314 |
| MCW0037 | 5 | 151–159 | 3 | 2.06 | 0.462 | 0.012 | 0.021 |
| MCW0016 | 11 | 130–152 | 3 | 2.05 | 0.435 | –0.599 | –0.521 |
| MCW0014 | 10 | 164–188 | 2 | 1.16 | 0.119 | –0.082 | –0.067 |
| MCW0183 | 14 | 295–339 | 8 | 2.58 | 0.593 | 0.572 | 0.575 |
| LEI0234 | 16 | 189–321 | 14 | 5.44 | 0.831 | 0.415 | 0.431 |
| | | Mean | 5.53 | 2.57 | 0.539 | 0.167 | 0.192 |
| | | SD | 2.63 | 0.86 | 0.153 | 0.332 | 0.319 |

Between and within kinship coefficients for Denizli subpopulations and reference populations were estimated using MSA 4.05 (Dieringer and Schlötterer 2003). To construct a neighbour-net tree based on marker estimated kinship (MEK), the matrix was converted to a kinship distance matrix as in Yu et al. (2006). Relationships between Denizli fowl subpopulations and reference populations were visualised in a phylogenetic network using SPLITSTREE 4.8 (Huson and Bryant 2006).

The genetic structure and the degree of admixture of Denizli chicken subpopulations and reference populations were investigated using the Bayesian clustering algorithm in STRUCTURE version 2.3.3 (Pritchard et al. 2000; Stanford University, Stanford, CA) by performing 100 independent runs for each K value ranging from 2 to 14. We used a burn-in period of 50,000 iterations followed by 250,000 MCMC repeats of each run and the admixture model with the option of correlated allele frequencies. To investigate the population substructures of Denizli breed further, subpopulations were reanalysed in STRUCTURE (Pritchard et al. 2000) using the same settings but different clustering range between $1 \leq K \leq 8$ with an additional option of location prioritisation (LOC PRIOR). The LOC-PRIOR model, which uses sampling locations as prior information to assist in the clustering of data sets, is better than other models for detecting the genetic structure even with low levels of genetic divergence or a limited number of loci (Hubisz et al. 2009). The results were analysed using Structure Harvester (Earl and vonholdt 2012) to identify the optimal number of clusters fitting the data, established using the ΔK statistics suggested by Evanno et al. (2005). The alignment of 100 repetitions for each cluster was performed in CLUMPP version 1.1 (Jakobsson and Rosenberg 2007) and the results were visualised using DISTRUCT (Rosenberg 2004).

Results

Variability of microsatellites

In total, 105 alleles were observed for Denizli breed subpopulations, with an average of 5.53 (standard deviation [SD] = 2.63). As is apparent from Table 2, the locus with the highest number of alleles is LEI0234 (14 alleles). The effective number of alleles range from 1.16 to 5.44, with a mean of 2.57 (SD = 0.86), whereas the PIC per marker ranges from 0.119 (MCW0014) to 0.831 (LEI0234) (Table 2).

The inbreeding coefficient within subpopulations (F_{IS}) across loci ranges from -0.599 (MCW0016) to 0.664 (MCW0248), with a mean of 0.167 ($p < .01$), while the overall inbreeding coefficient (F_{IT}) is in the range of -0.522 (MCW0016) to 0.673 (MCW0248) over all loci across subpopulations (Table 2).

Genetic diversity of Denizli subpopulations

The genetic variability in each subpopulation of Denizli fowl was studied in terms of the mean number of alleles (N_a), allelic richness (N_{AR}), Wright's fixation index (F_{IS}), observed (H_O) and expected (H_E) heterozygosity and deviations from the Hardy – Weinberg equilibrium ($dHWE$) (Table 3). As is apparent from the table, the DN_A subpopulation shows the least number of alleles, whereas the DN_T subpopulation has the largest N_a value. In terms of the allelic richness results, DN_U shows the lowest variability while DN_T has the highest allelic richness. The expected heterozygosity was higher than the mean observed heterozygosity in all Denizli fowl subpopulations. Over all subpopulations, the mean observed heterozygosity is 0.473, ranging from 0.399 in DN_D to 0.562 in DN_T. The F_{IS} values are positive for all subpopulations, ranging from 0.011 (DN_T) to 0.295 (DN_D).

Table 3. Genetic variability of each subpopulations of Denizli fowl in terms of sample size (N), mean number of alleles (N_a), Allelic richness per population (N_{AR}), observed (H_O) and expected (H_E) heterozygosity, Wright's inbreeding coefficient (F_{IS}) and deviations from Hardy–Weinberg equilibrium (HWE) for each Denizli fowl subpopulation.

| Subpopulation ^a | N | N_a | N_{AR} | $H_O \pm SD$ | $H_E \pm SD$ | F_{IS} | $dHWE$ |
|----------------------------|-----|-------|----------|-------------------|-------------------|----------|--------|
| DN_D | 25 | 4.21 | 4.09 | 0.399 ± 0.242 | 0.590 ± 0.167 | 0.295 | *** |
| DN_H | 25 | 4.21 | 4.03 | 0.423 ± 0.152 | 0.583 ± 0.147 | 0.235 | *** |
| DN_T | 25 | 4.26 | 4.21 | 0.562 ± 0.250 | 0.593 ± 0.151 | 0.011 | * |
| DN_A | 25 | 4.11 | 4.05 | 0.537 ± 0.204 | 0.584 ± 0.151 | 0.035 | * |
| DN_S | 25 | 4.16 | 4.04 | 0.498 ± 0.222 | 0.576 ± 0.148 | 0.096 | ** |
| DN_U | 30 | 4.16 | 3.91 | 0.417 ± 0.252 | 0.550 ± 0.173 | 0.214 | ** |

^aDN_D: Subpopulation from centre of Denizli city; DN_H: Subpopulation from Honaz prefecture; DN_T: Subpopulation from Tavas prefecture; DN_A: Subpopulation from Acipayam prefecture; DN_S: Subpopulation from Serinhisar prefecture and DN_U: Subpopulation from Adnan Menderes University flock in Aydin.

* $p < .05$; ** $p < .01$; *** $p < .001$.

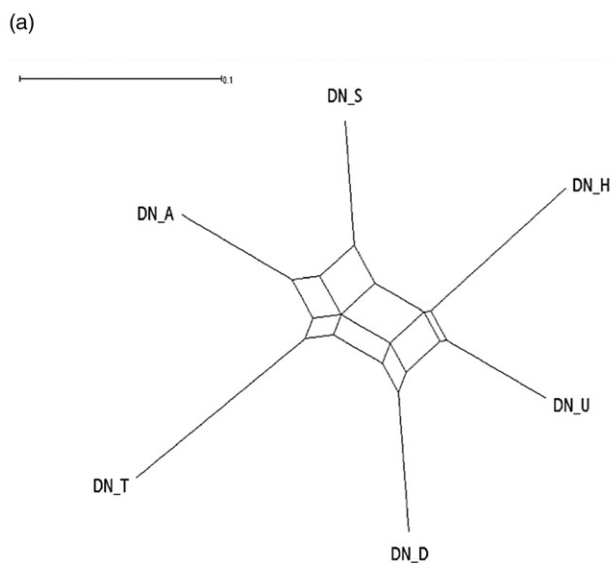
All subpopulations deviate significantly ($p < .01$) from the Hardy–Weinberg equilibrium (Table 3).

The genetic distances between subpopulation pairs calculated using Nei's genetic distance (D_A) and the proportion of shared alleles (D_{PS}) are given in Table 4. Nei's genetic distance shows a range from 0.035 (between DN_D and DN_U) to 0.110 (between DN_T and DN_U), while the genetic distances estimated by the logarithmic proportion of shared alleles (D_{PS}) range from 0.140 (between DN_D and DN_U) to 0.297 (between DN_T and DN_H) (Table 4). The neighbour-net tree constructed on D_{PS} is shown in Figure 1(a). Although the genetic differences among subpopulations based on Nei's distances are not high, the neighbour-net tree shows a clear separation among

Table 4. Nei's genetic distance (D_A) (upper diagonal) and proportion of shared alleles (D_{PS}) (lower diagonal) between Denizli fowl subpopulation pairs.

| Subpopulation ^a | DN_D | DN_H | DN_T | DN_A | DN_S | DN_U |
|----------------------------|-------|-------|-------|-------|-------|-------|
| DN_D | – | 0.067 | 0.089 | 0.061 | 0.068 | 0.035 |
| DN_H | 0.182 | – | 0.108 | 0.084 | 0.065 | 0.050 |
| DN_T | 0.257 | 0.297 | – | 0.079 | 0.095 | 0.110 |
| DN_A | 0.195 | 0.225 | 0.235 | – | 0.054 | 0.076 |
| DN_S | 0.190 | 0.184 | 0.258 | 0.143 | – | 0.051 |
| DN_U | 0.140 | 0.149 | 0.296 | 0.210 | 0.157 | – |

^aDN_D: Subpopulation from centre of Denizli city; DN_H: Subpopulation from Honaz prefecture; DN_T: Subpopulation from Tavas prefecture; DN_A: Subpopulation from Acipayam prefecture; DN_S: Subpopulation from Serinhisar prefecture and DN_U: Subpopulation from Adnan Menderes University flock in Aydın.



subpopulations. The genetic distances between subpopulations with DN_T are significant ($p < .01$), therefore DN_T is placed on the longest branch Figure 1(a).

The results of STRUCTURE analysis for Denizli breed subpopulations and reference populations are presented in two steps in Figure 2(a,b). In the first step of analysis, the Denizli subpopulations were analysed with Italian chicken populations and a clear distinction can be observed between Denizli breeds and Italian breeds at $K=2$ or higher (Figure 2(a)). The most probable clustering for the first step is found at $K=6$ with the highest ΔK value (3.22). Although Denizli breed subpopulations show a homogeneous population structure and form one common cluster against the reference populations, the highest ΔK value (140.94) is found at $K=2$ employing a second step of analysis in line with Evanno et al. (2005) within subpopulations (Figure 2(b)).

Relationship between Denizli chicken breed and reference chicken populations

The relationships between six subpopulations of the Denizli chicken breed and six local Italian chicken breeds are presented in Figure 1(b) as a network tree derived from MEK distance matrices of 12 populations. The Denizli breed subpopulations are predominantly clustered away from the Italian chicken breeds.

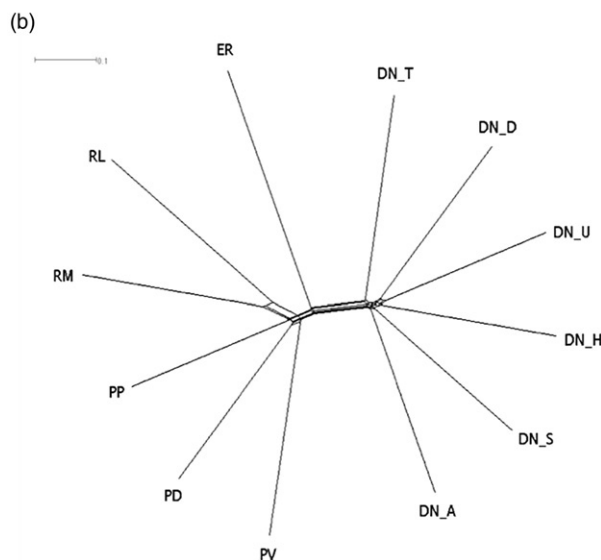


Figure 1. (a) Neighbour-net network of D_{PS} distances among Denizli fowl subpopulations (DN_D: Subpopulation from centre of Denizli city; DN_H: Subpopulation from Honaz prefecture; DN_T: Subpopulation from Tavas prefecture; DN_A: Subpopulation from Acipayam prefecture; DN_S: Subpopulation from Serinhisar prefecture and DN_U = Subpopulation from Adnan Menderes University flock in Aydın). (b) Network tree of the 12 populations based on MEK distances (DN_D: Subpopulation from centre of Denizli city; DN_H: Subpopulation from Honaz prefecture; DN_T: Subpopulation from Tavas prefecture; DN_A: Subpopulation from Acipayam prefecture; DN_S: Subpopulation from Serinhisar prefecture; DN_U: Subpopulation from Adnan Menderes University flock in Aydın; PD: Padovana; ER: Ermellinata di Rovigo; PP: Pèpoi; RL: Robusta Lionata; RM: Robusta Maculata; PV: Polverara).

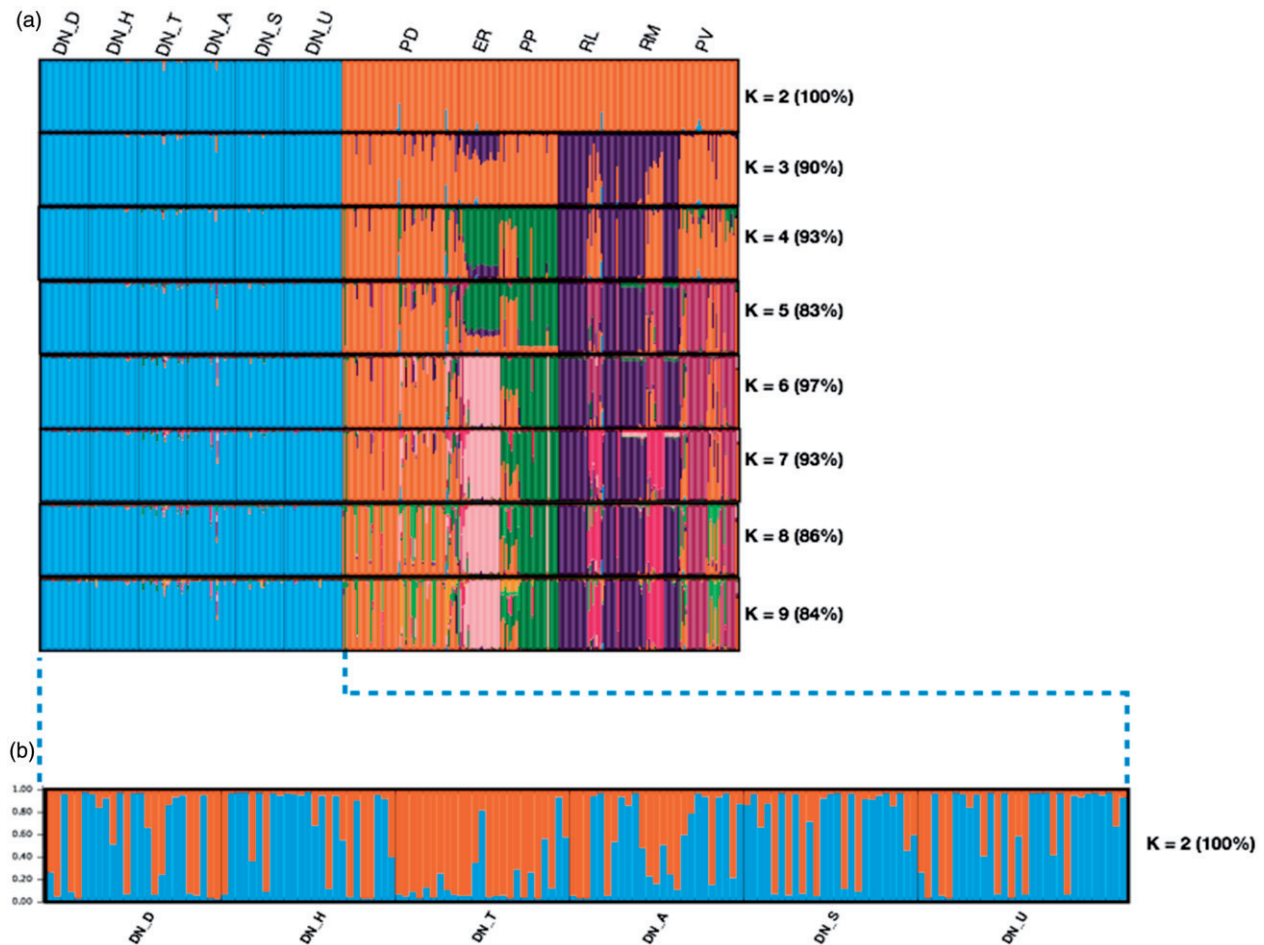


Figure 2. (a) Graphical representation of clustering assignment of Denzli breed and Italian chicken breeds. DN: Denizli; PD: Padovana; ER: Ermellinata di Rovigo; PP: Pèpoj; RL: Robusta Lionata; RM: Robusta Maculata; PV: Polverara. Numbers in the parenthesis indicate the average similarity index between individuals assigned into the same cluster. (b) The second step analysis of Denzli breeds subpopulations without Italian populations. DN_D: Subpopulation from centre of Denizli city; DN_H: Subpopulation from Honaz prefecture, DN_T: Subpopulation from Tavas prefecture; DN_A: Subpopulation from Acipayam prefecture; DN_S: Subpopulation from Serinhisar prefecture and DN_U: Subpopulation from Adnan Menderes University flock in Aydın. Numbers in the parenthesis indicate the average similarity index between individuals assigned into the same cluster.

There is evidence in the neighbour-net tree of a closer relationship between DN_D and DN_U, between PD and PV and between RL and RM (Figure 1(b)).

Discussion

We used 19 microsatellite loci recommended by the FAO (Food and Agriculture Organisation [FAO]. Standing Committee 2004b) for chicken genetic diversity analysis. According to the PIC, most of the microsatellite markers used in this study were informative in estimating the genetic diversity and population structure of Denizli fowl. Barker (1994) suggested that the microsatellites investigated should display a minimum of four alleles per locus for proficient judgement of genetic differentiation. In this study, four microsatellites of the 19 show lower values, whereas the mean

number of alleles (5.53) per locus is higher than 4 (Table 2). These results are similar to those reported in different chicken populations by Dávila et al. (2009), and Abebe et al. (2015). However, Hillel et al. (2003) and Suh et al. (2014) reported higher values for mean allele numbers per locus. The number of alleles observed across the loci is greater than the effective number of alleles, which is as expected and also as reported by Liu et al. (2008).

The mean allele number of Denizli fowl over all subpopulations is 4.19 (Table 3), lower than the figure reported by Kaya and Yıldız (2008) for Denizli fowl subpopulations (6.1). Comparing the results of the two studies, the reduction in the number of alleles of Denizli fowl could be viewed as remarkable, but the markers used in these studies in terms of the numbers and characteristics are completely different from each

other. Therefore, it is not possible to interpret our results as showing a genetic loss in Denizli fowl. Allelic richness (N_{AR}) is a measure of genetic diversity indicative of a population's long-term potential for adaptability and persistence (Greenbaum et al. 2014). The results highlight that all of the studied subpopulations have higher levels of N_{AR} than those reported by Maretto et al. (2013) in local Italian and Polish chicken breeds, by Tadano et al. (2012) in closely related Japanese native Nagoya chicken breeds.

The observed heterozygosity (H_O) values indicate greater diversity in the subpopulations derived from the breeders' farms than in the DN_D and DN_U obtained from conservation flock. Mtileni et al. (2011) reported similar results in a study comparing the genetic variability of village chicken populations to conservation flocks in South Africa. In our study, the lowest H_O is observed in DN_D, with a value of 0.399; in contrast, Kaya and Yıldız (2008) reported an observed heterozygosity value for the same population of 0.512. Although the significant differences between the two studies could be due to the number and characteristics of the microsatellite markers used, it may be necessary investigating the loss of heterozygosity to protect the genetic variability of this breed in conservation flock. Our mean observed heterozygosity value (0.473) over all subpopulations is in the range of the heterozygosity values reported by Lyimo et al. (2014) for European chicken breeds (0.360–0.510).

Wright's F -statistics (F_{IS} , F_{IT} , F_{ST}) provide important insights into the evolutionary processes that influence the structure of genetic variation within and among populations and they are among the most widely used descriptive statistics in population and evolutionary genetics (Holsinger and Weir 2009). In fixation indices, F_{ST} is directly related to the variance in allele frequency among populations and conversely to the degree of resemblance among individuals within populations (Holsinger and Weir 2009). In our study, the global F_{ST} over all loci across Denizli fowl subpopulations is 0.030, indicating a low but significant ($p < .01$) degree of genetic differentiation among subpopulations. Similar to our findings, low F_{ST} values were reported by Bouzat et al. (1998) in Greater Prairie chickens (0.044), by Berima et al. (2013) in native Sudanese chicken breeds (0.026), by Lyimo et al. (2013) in five Tanzanian chicken ecotypes (0.048) and by Touko et al. (2015) in Cameroon chickens (0.040).

The estimates of within-subpopulation inbreeding coefficients (F_{IS}) were considerably higher in DN_D, DN_U and DN_H than in other subpopulations (Table 3). Moreover all subpopulations deviated significantly ($p < .01$) from the Hardy–Weinberg equilibrium.

The breeding strategies and non-random mating applied to maintain the morphological standards of breeds may have caused an increase in F_{IS} values and also deviations from the Hardy–Weinberg equilibrium especially in low effective size populations. Furthermore, high F_{IS} values could also be caused by some levels of selection to improve the breed-specific characteristics, such as feather patterns, body weight or long crowing duration. The high F_{IS} results in the study are comparable to those reported by Kaya and Yıldız (2008) in Denizli fowl and in different chicken breeds by Tadano et al. (2007), Zanetti et al. (2011), Maretto et al. (2013) and Touko et al. (2015).

Genetic distances between subpopulations calculated using Nei's genetic distance (D_A) and the proportion of shared alleles (D_{PS}) are given in Table 4. The lowest genetic differentiation according to D_A is found between DN_D and DN_U (0.035). This result is consistent with the breeding history of DN_D and DN_U, which were created from individuals in conservation flock in 2006. Although the highest genetic differentiation is shown between DN_T and DN_U subpopulations, genetic differentiation amongst subpopulations is low in general according to the D_A matrices. Our findings are compatible with those reported by Kaya and Yıldız (2008) for D_A (0.07) between the DN_D and conservation flocks of Denizli fowl and by Bodzsar et al. (2009) in Hungarian native chicken breeds, but are lower than those reported by Tadano et al. (2012) in closely related Nagoya chicken lines. In our study, the sampled populations were the subpopulations of Denizli fowl and genetic similarity amongst these subpopulations was expected. Therefore, the proportion of shared alleles (D_{PS}) was also used to calculate genetic distances between all pairwise subpopulations. This genetic distance measure assumes all alleles are related (Bowcock et al. 1994) and is appropriate to estimate the genetic distance for closely related populations. According to the D_{PS} matrices, DN_T is the most differentiated compared with the other subpopulations, while DN_D and DN_U show the highest allelic similarity (Table 4). The neighbour-net tree, constructed on D_{PS} estimates, highlights the presence of clear genetic separation between subpopulations in different groups and reveals the genetic similarity between DN_H and DN_D–DN_U (Figure 1(a)). DN_T is the most differentiated and is placed at the end of the longest branch.

STRUCTURE-based clustering shows a clear distinction between the Denizli chicken breeds and Italian local chicken breeds (Figure 2(a)). The Denizli subpopulations, that have a common genetic background, did not show substructures when they analysed with

Italian chicken breeds. Although the highest mean similarity score was estimated at $K=2$, the most probable clustering for the first step was found at $K=6$. To investigate the population substructures of Denizli breeds further, a second step STRUCTURE analysis was done with an additional option of location prioritisation. Similar sub-structuring approach was also used by Ceccobelli et al. (2015) in Italian chicken breeds. In the second phase of analysis, the most probable clustering for second step was found at $K=2$ and sub-structuring according to geographic location could not be observed, but all subpopulations showed a similar proportion of admixture (Figure 2(b)). The most differentiated subpopulation was DN_T, which showed less admixed structure than the other subpopulations. Admixture results might be due to some gene flow across the subpopulations of Denizli fowl in the past. This result also displays the signs of possible interbreeding by Denizli fowl breeders, who trade their chickens and cocks amongst themselves through fairs and auctions. Our results are compatible with those reported by Chen et al. (2008) in genetically closely related Chinese indigenous chicken breeds, by Bodzsar et al. (2009) in Hungarian chicken breeds and by Mtileni et al. (2011) in village chicken populations in South Africa.

The phylogenetic network based on MEK distances in 12 populations (Figure 1(b)) shows a clear distinction between the Denizli breed subpopulations and the Italian chicken breeds. Low kinship estimates in the Denizli chicken subpopulations could also be associated with the small flocks managed with continuous but limited gene flow between the subpopulations. The network analysis separated the DN_T, DN_A and DN_S Denizli subpopulations according to their more diverse genetic structure. Despite the high allelic similarity among subpopulations, the neighbour-net clustering split the subpopulations into two main clusters (DN_D-DN_U-DN_H and DN_T-DN_A-DN_S) (Figure 1(a)). While this result seems in agreement with the structure analysis that evidenced the most probable clustering of $K=2$ (Figure 2(b)), DN_T was apart from all subpopulations.

Conclusions

Based on our results, some subpopulations show higher heterozygosity and lower degrees of inbreeding than subpopulations obtained from conservation flock. In this context, bringing the selected breeders' subpopulations into the conservation scheme would help control the increasing homozygosity and reduce

inbreeding, thus safeguarding the survival of the breed and reducing the costs of the conservation process.

Disclosure statement

No potential conflict of interest was reported by the authors.

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