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Full Length Research Paper

Genetic diversity of two Tunisian sheep breeds using random amplified polymorphic DNA (RAPD) analysis

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Random amplified polymorphic DNA (RAPD) markers were used to study genetic diversity and population structure in six sheep populations belonging to two native Tunisian breeds (the Barbarine and the Western thin tail). A total of 96 samples were typed using eight RAPD primers. 62 bands were scored, of which 44 bands (70.97%) were polymorphic. For all populations, Nei's gene diversity, Shannon index and percentage of polymorphic loci are respectively in the range of 0.17-0.25, 0.25 to 0.35, 43.55 to 53.23. Between breeds, higher heterozygosity value (0.24) was found within the Western thin tail breed. Using unweighted pair-group method with arithmetic average (UPGMA) dendrogram, the six populations clustered into two groups, each one contained populations of the same breed. Analysis of molecular variance (AMOVA) showed that the variation between breeds is 30.80%, and that the variation between populations of Barbarine breed and populations of Western thin tail breed are 6.06 and 11.83%, respectively.

Key words: Genetic diversity, RAPD, Tunisian sheep, Bioclimatic zones.

INTRODUCTION

Sheep breeding has an essential role in the food safety of Tunisia. This sector provides more than 41% of the country's total red meat production (Rekik et al., 2005). The sheep population is estimated to be about four million female units (Rekik et al., 2005) distributed in four breeds; Barbarine (B), Western thin tail (W), Black of Thibar and the Sicilo Sarde which represent respectively 60.3, 34.6, 2.1 and 0.7% of the Tunisian sheep population (Rekik et al., 2005). On the other hand, Tunisian climatic conditions are characterized by a wide variation. Different bioclimatic zones can be distinguished from humid, in the extreme north-west, with an annual rainfall upper than 800 millimeters per year, to the Saharan area, in the extreme south, where annual rainfall is less than 100 mm per year (Daget, 1977). Given the variability of climatic conditions and amount of available grazing, different breeding practices are adopted in different bioclimatic regions. Moreover, most genetic improvement programs of sheep breeding in Tunisia are based on quantifying some production traits such as growth and reproduction (Djemali et al., 1994; Lassoued and Rekik, 2001; Lassoued et al., 2004). However, estimation of genetic relationship among sheep populations at the molecular level is necessary to speed up genetic improvement and reliable breeding strategy in this species. Several molecular techniques can be used for genetic diversity studies such as restriction fragment length polymorphism (RFLP) (Lu et al., 1996; Gauthier et al., 2002), variable number of tandem repeat (VNTR) (Farid et al. 2000; Arranz et al., 2001; Pariset et al., 2003), DNA sequencing (Parker et al., 1998; Yang et al., 2003; Chapman and Burke, 2007; Wickert et al., 2007) and random amplified polymorphic DNA (RAPD) (Bronzini et al., 2002; Fu et al., 2003; Hovmalm et al., 2004). The latter was used in this study.

Indeed, RAPD markers have advantages such that

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Abbreviations: RAPD, Random amplified polymorphic DNA; **UPGMA,** unweighted pair-group method with arithmetic average; **AMOVA,** analysis of molecular variance.

they are neutral, widely distributed throughout the genome, highly polymorphic and easy to use (Welsh and McClelland 1990; Williams et al. 1990; Wooliams and Toro, 2007). It is based on the study of deoxyribonucleic acid (DNA) polymorphism after amplification of random segments of DNA with arbitrarily primers. RAPD markers are inherited by Mendelian law and are suitable for studying taxonomic identity (Castiglione et al., 1993; Costa et al., 2004), assessing genetic variation and relationships (Gimenes et al., 2000; Vucetich et al., 2001), creating specific probes (Bazzicalupo and Fani, 1995; Comes et al., 1997; Voigt and al., 1998) in both plants and animals. In sheep, RAPD markers have been widely used in genetic diversity studies (Gong et al., 2002; Ali, 2003; Elmaci et al., 2007). The aim of this work was to estimate genetic variability in the two most common native Tunisian sheep breeds representing the major sheep population and to verify the existence of possible divergence between studied populations of different bioclimatic areas using RAPD markers.

MATERIALS AND METHODS

Samples collection and DNA extraction

Samples were collected from 96 animals of the two breeds; B and W in three areas belonging to different bioclimatic zones defined according to Emberger's classification (Emberger 1966, cited by Daget, 1977): Sub-humid (SH) (Beja, Bizerte), semi-arid with mild winter (SA) (Tunis, Sousse) and arid with mild winter (A) (Sfax, Gabes), where annual rainfall is respectively in the range of 600 to 800, 400 to 600 and 100 to 200 mm. Blood samples from both sexes of pure B and W breeds were collected from the jugular vein in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes and kept at -20°C until the isolation of total DNA. One sample was collected per herd to avoid inbreeding. Animals were classified into six populations (B1, B2, B3, W1, W2 and W3) according to their breed and bioclimatic zone origin. 16 sheep were typed by population. DNA extraction was carried out using a genomic purification kit (blood DNA preparation kit, Jena Bioscience). In order to improve the quality and quantity of extracted DNA, some modifications to the standard protocol were conducted. The red blood cell lyses step was followed by additional washes by adding 900 µl of bidistilled sterilized water to the sample, vortexing vigorously for 5 min, centrifuging at 13000 rotations per minute for 5 min and removing the supernatant. This stage was repeated until obtaining a clean pellet rid of any trace of hemoglobin. The DNA hydration time was prolonged to three days and conducted in dark at room temperature.

According to the kit manual, a 300 µl sample of whole blood yields 10 to 20 µg of DNA. DNA quality and quantity were controlled using analysis on agarose gels and spectrophotometry.

RAPD-PCR conditions

PCR amplifications were performed in 50 µl reaction mixtures containing 30 ng of genomic DNA, 0.8 µM of the arbitrary primer, 100 µM of dNTP (dNTP Mix, Jena Bioscience), 3 mM of $MgCl_2$, 1.25 unit of Taq DNA polymerase (ULTRATOOLS DNA Polymerase, Biotools) and 5 µl of 10X Taq DNA polymerase buffer. In order to detect any DNA contamination, control reactions were set up without genomic DNA. Amplifications were performed using a thermal cycler (Eppendorf, Mastercycler gradient) programmed for 40 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min and extension at 72°C for 2 min. An initial denaturation step of 2 min at 94°C and a final extension step of 2 min at 72°C were included in the first and last cycles, respectively. The amplification products were size-fractionated in a 1.2% agarose gel containing ethidium bromide in Tris-borate EDTA buffer and visualized under UV transillumination. Specific DNA pools of each population were amplified using twenty RAPD primers (OPA01→OPA20; Operon technologies). Only the primers producing polymorphic and repeatable bands were selected for the individual typing of all animals.

Statistical analyses

Data were recorded as binary matrix by assigning the value (1) when the band of a given level is present and (0) when it is absent. To estimate the genetic diversity within and among populations and breeds, Nei's gene diversity (H) (Nei, 1973) under Hardy-Weinberg equilibrium (Clark and Lanigan, 1993), Shannon index (I) (Lewontin, 1972), the percentage of polymorphic loci (P) and unbiased Nei's distance (Nei, 1978) corrected for small samples were all calculated. In addition, an unweighted pair-group method with arithmetic average (UPGMA) dendrogram containing the six studied populations was constructed on the basis of the matrix of genetic distance using Popgene (Population Genetic Analysis) version 1.32 (Yeh and Boyle, 1997) software. In order to evaluate the amount of population genetic structure, an analysis of molecular variation (AMOVA) was conducted using Arlequin program ver. 3.0 (Excoffier et al., 2005), significance of genetic structure indices was evaluated after 1000 random permutations.

RESULTS AND DISCUSSION

As a preliminary result, eight primers (OPA02, OPA06, OPA07, OPA10, OPA12, OPA15, OPA16 and OPA18) out of 20 (40%) generated reproducible and polymorphic bands and were selected for DNA amplifications from 96 individuals (Table 1). An example of RAPDs banding pattern has been shown in Figures 1 and 2.

Ali (2003), studying four Egyptian sheep breeds, indicates that five of 19 random primers (26.31%) generated polymorphic bands. Likewise, Gaali and Satti (2009) found a percentage of polymorphic primers of 46.6% in two Sudanese goat breeds. The number of RAPD fragments detected by each primer ranged from six to 11 with a size range varying from 150 to 2500 base pairs length. These band sizes confirm results of studies carried out by Cushwa et al. (1996) and Kumar et al. (2003). The eight selected primers generated 62 bands (loci), 44 of them (70.97%) were polymorphic. This level of polymorphic loci is comparable to results found by Yu et al., (2004) and Devrim et al., (2007) in Anatolian sheep and Yunnan cattle breeds, respectively. Results show that H, I and P values were highest in the Western thin tail breed than in Barbarine breed (Table 2). Within population, values for H, I, and P were highest in Western thin tail of SA (W2) and lowest in Western thin Tail of A (W3). Barbarine of A (B3) had also the lowest value of P

Table 1. Sequences of the random primers selected for the individual typing of the studied animals.

Primer	Sequence 5' to 3'
OPA-02	TGCCGAGCTG
OPA-06	GGTCCCTGAC
OPA-07	GAAACGGGTG
OPA-10	GTGATCGCAG
OPA-12	TCGGCGATAG
OPA-15	TTCCGAACCC
OPA-16	AGCCAGCGAA
OPA-18	AGGTGACCGT

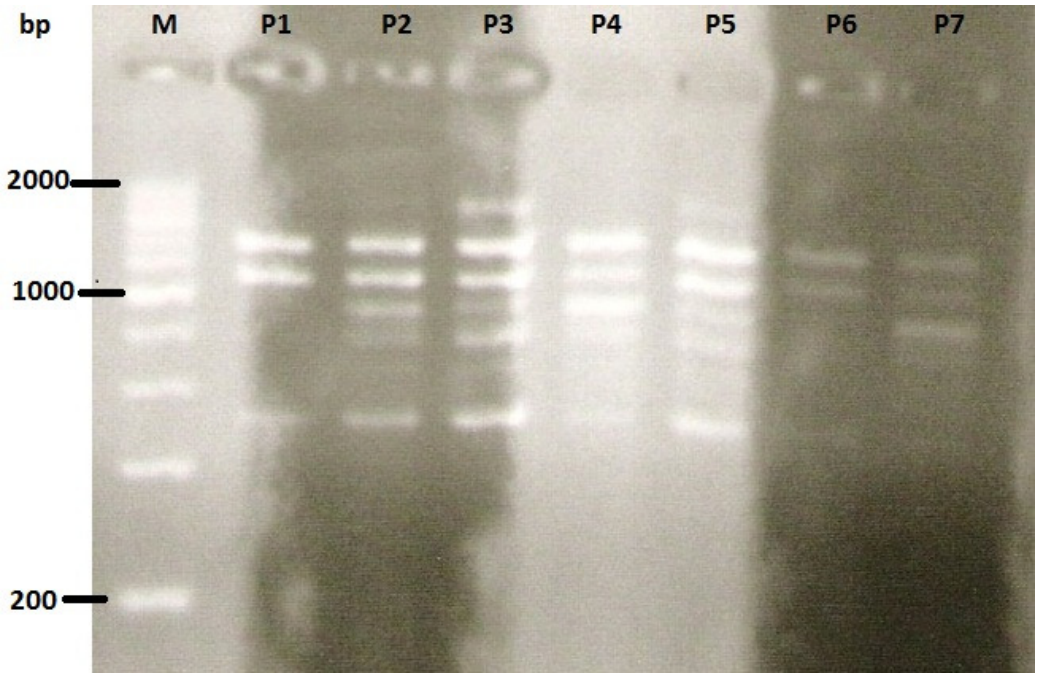

Figure 1. RAPD profiles of 7 Tunisian sheep individuals generated with OPA07 primer. M, 200 bp DNA ladder (200 bp to 2 Kb); P1, DNA sampled from B1 population; P2 and P4, DNA sampled from B2 population; P3, DNA sampled from B3 population; P5, DNA sampled from W1 population; P6, DNA sampled from W2 population; P7, DNA sampled from W3 population. RAPD, Random amplified polymorphic DNA.

(43.55%). Genetic diversity in the overall population was 0.2788.

Nei's unbiased genetic distance between the two studied breeds was 0.146, considering populations, it was ranged from 0.010 between Barbarine of SH (B1) and Barbarine of SA (B2) to 0.190 between B1 and Western thin tail of SH (W1) (Table 3). Genetic identity and genetic distance indices are used to measure respectively, the similarity and dissimilarity between pairs of populations. Shorter genetic distances indicate a close genetic relationship, whereas small genetic identities indicate a more distant genetic relationship. Elmaci et al. (2007) found value of total genetic diversity of 0.226 and a genetic distance average of 0.050 in three Turkish sheep breeds using RAPD markers.

A UPGMA dendrogram, grouping all populations was constructed using Nei genetic distances to better visualize the structure of all populations (Figure 3). It shows two differentiated branches, the first one included populations of the B breed and the second included populations of the W breed. In both breeds, it was observed closer proximity of populations belonging to the SH and the SA bioclimatic areas, while population from A bioclimatic zone had the greatest genetic distance from the two other sheep populations. These findings revealed a population structure within each breed and indicated

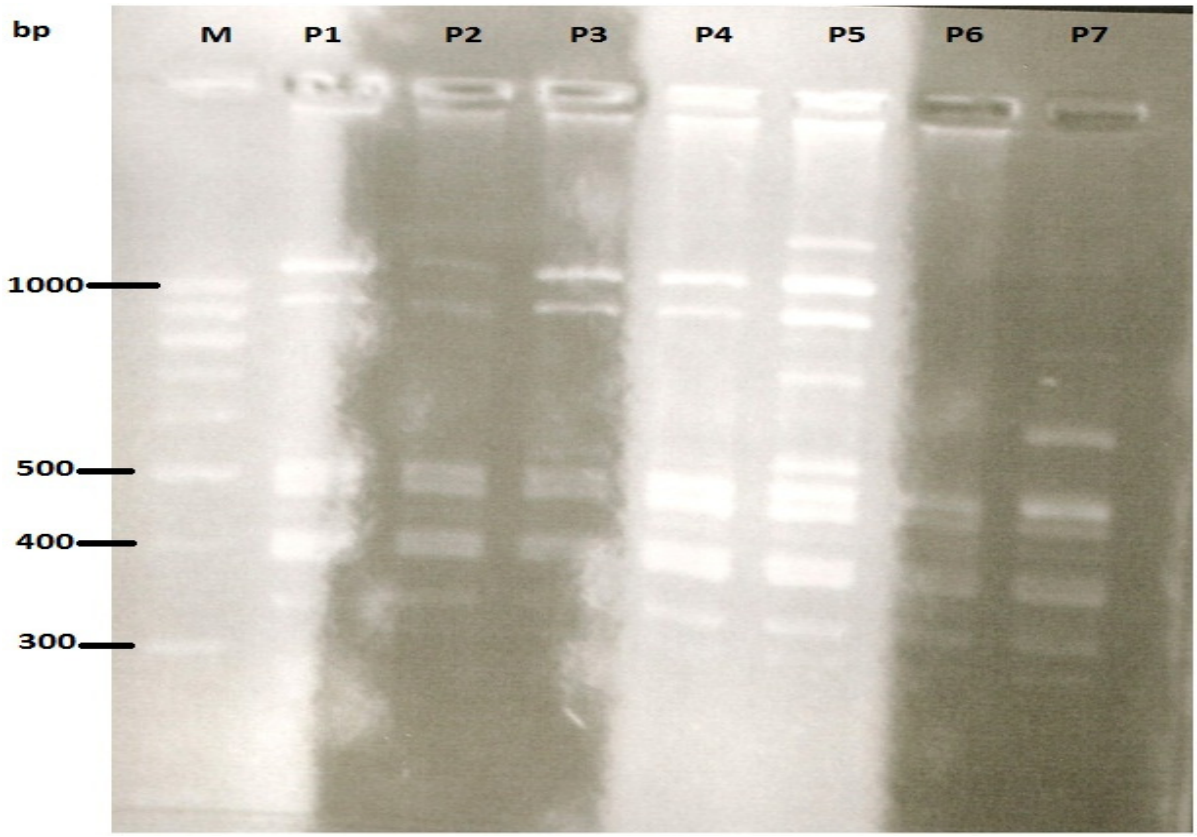

Figure 2. RAPD profiles of 7 Tunisian sheep individuals generated with OPA10 primer. M, 100 bp DNA ladder (100 bp to 1 Kb); P1, DNA sampled from B1 population; P2, DNA sampled from B2 population; P3 and P4, DNA sampled from B3 population; P5, DNA sampled from W1 population; P6, DNA sampled from W2 population; P7, DNA sampled from W3 population. RAPD, Random amplified polymorphic DNA.

Table 2. Nei's gene diversity (H), Shannon index (I) and percentage of polymorphic loci (P) at the population (Hpop, Ipop, Ppop) and breed (Hbreed, Ibreed, Pbreed) level for the 96 individuals typed.

Population (1)	N (2)	Hpop	Ipop	Ppop	Hbreed	Ibreed	Pbreed
B1	16	0.19	0.27	45.16	0.21	0.31	53.23
B2	16	0.18	0.26	41.94			
B3	16	0.21	0.29	43.55			
W1	16	0.21	0.30	51.61	0.24	0.34	59.68
W2	16	0.25	0.35	53.23			
W3	16	0.17	0.25	43.55			

(1) B1, Barbarine of SH; B2, Barbarine of SA; B3, Barbarine of A; W1, Western thin tail of SH; W2, Western thin tail of SA; W3, Western thin tail of A. (2) Number of analysed individuals.

that genetic differentiation was consistent with their bioclimatic location.

AMOVA analyses showed that both the breeds were significantly different. Indeed, interbreed differences accounted for 30.80% of total variation (P<0.001) (Table 4). This value is higher than that found by Paiva et al. (2005), in which value of 14.92% (P<0.01) is detected in Brazilian sheep breeds using RAPD markers.

Differentiation between populations in each breed was significant 6.06% (P<0.01) and 11.83% (P<0.001), respectively for populations of Barbarine and Western thin Tail breed (Tables 5 and 6). This result shows a low migrants' exchange rate among populations of the same breed and could be the consequence of different bioclimatic zones isolation. Paiva et al. (2005), studying 10 sheep populations belonging to five Brazilian breeds

Table 3. Genetic distance between Tunisian sheep populations.

Population	B1	B2	B3	W1	W2	W3
B1	-	0.989	0.982	0.826	0.835	0.848
B2	0.010	-	0.953	0.835	0.845	0.870
B3	0.018	0.047	-	0.839	0.844	0. 845
W1	0.190	0.179	0.175	-	0.961	0. 950
W2	0.180	0.168	0.169	0.039	-	0.948
W3	0.164	0.138	0.167	0.051	0.053	-

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

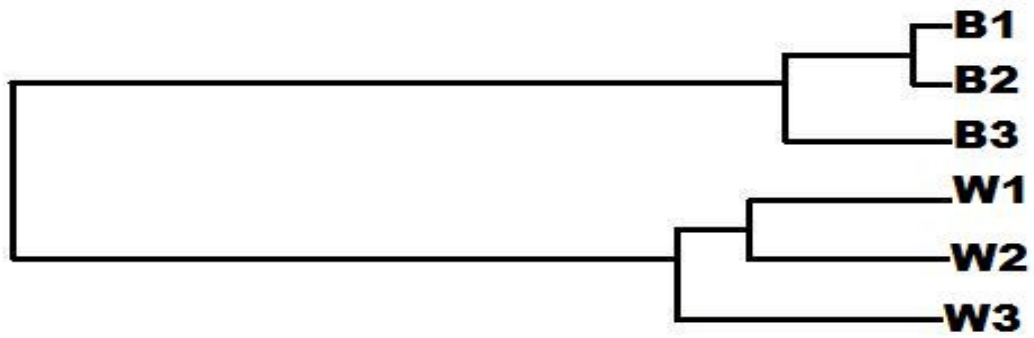

Figure 3. UPGMA dendrogram of six Tunisian sheep populations based on Nei's (1978) genetic distance. UPGMA, unweighted pair-group method with arithmetic average.

Table 4. Analysis of molecular variance for 96 individuals sampled from two Tunisian sheep breeds.

Source of variation	Df	SS	VC	Total (%)	P-value
Between breeds	1	159.45	3.17	30.80	<0.001
Within breeds	94	670.20	7.12	69.20	<0.001

Df: degrees of freedom; SS: sum of squares; VC: variance components.

Table 5. Analysis of molecular variance for 48 individuals sampled from three populations of Tunisian Barbarine breed.

Source of variation	Df	SS	VC	Total (%)	P-value
Between populations	2	26	0.41	6.06	<0.01
Within populations	45	287.87	6.39	93.94	<0.01

Df: degrees of freedom; SS: sum of squares; VC: variance components.

Table 6. Analysis of molecular variance for 48 individuals sampled from three populations of Tunisian Western thin tail breed.

Source of variation	Df	SS	VC	Total (%)	P-value
Between populations	2	43.70	0.93	11.83	<0.001
Within populations	45	312.62	6.94	88.17	<0.001

Df, Degrees of freedom; SS, sum of squares; VC, variance components.

found that differences among populations within breeds contribute for 9.27% (P<0.01) of total variation.

Conclusion

This study helped to assess genetic diversity in the two most common sheep breeds in Tunisia and showed that genetic diversity exists in the analyzed populations. A significant genetic structure of populations within breeds and a high interbreed variation were demonstrated. The results of this work, combined with those found by Khaldi et al. (2010) could be exploited to improve sheep breeding programs in Tunisia. The use of RAPD markers should be generalized to the characterization of other livestock in Tunisia given their efficiency and simplicity in detecting polymorphism between breeds. RAPD technique can be also easily used to identify the species or breed of an animal or its products. This is important when some typical products must be produced from a specific breed or species. In this context, several studies were conducted in different animal species to identify RAPD specific markers in species and breeds (Gwakisa et al., 1994; Kemp and Teale, 1994; Rothuizen and Van Wolferen, 1994).

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