Analysis of genetic variability within Tunisian Barbarine and Western thin Tail sheep using RAPD-PCR Method

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Abstract: Genetic diversity among two breeds (Barbarine and Western thin Tail) of sheep in Tunisia was detected by Random Amplified Polymorphic DNA (RAPD) markers. The relationship between genetic diversity and larger geographical distance among populations has been attributed to a number of different factors mostly breeding systems. We assessed this relationship among 12 populations belonging to two Tunisian sheep breeds using RAPDs by including eight randomly selected individuals from each population. Eight primers produced 62 clear and reproducible polymorphic bands. In populations, the percentage of polymorphic bands ranged between 32.26 and 51.61%. The range of Nei's gene diversity within-population was wide, varying from 0.1522 to 0.2157. Analysis of molecular variance (AMOVA) showed that the variation between breeds is 29.54%, and that the variation between populations within breeds and within populations are 8.47 and 61.99%, respectively.
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1.Introduction

Sheep are easy to breed and tame and therefore they were among the first species domesticated and used as a source of meat and wool (Ryder, 1983; Clutton-Brock, 1987). As in other species, the domestication of sheep resulted in a variety phenotypic much greater than that observed in the wild (Simm, 1998). Sheep farming plays a vital role in food security in Tunisia. Indeed, this sector provides more than 48% of the annual production of red meat estimated at 120000 tones (Mohamed Brahmi et al., 2010). The Tunisian sheep population count approximately 4000.000 female units distributed in four main breeds: Barbarine (B), Western thin tail (W), Black of thibar and Sicilo sarde which represent respectively 60.3%, 34.6%, 2.1% and 0.7% of the total population (Rekik et al., 2005). Assessment of genetic variability in domestic animals is an important issue to preserve genetic resources and maintain future breeding options in order to satisfy the demands of changeable markets. Animal breeding practices have emphasized productivity and specialization that favored the prevalence of a relatively small number of breeds. As a result, locally adapted native breeds have largely been neglected and/or replaced by more efficient and productive modern breeds. Attempts to hybridize with local breeds have failed because of the difficulties of raising this breed under normal Tunisian grazing conditions. For cons, the two predominant native breeds (the Barbarine and the Western thin tail) are better suited to growing conditions in the country and often raised as pure breeds. The primary unit in animal genetic resources is a breed, strain or geographically defined groups, the members of which share particular morphological characteristics which distinguished them from other groups. Hence, identification and characterization of breeds is a must to identify our genetic resources and also to prioritize breeds for conservation. Assessing genetic variability, as well as relatedness within and among the population, parentage determination, possible bottlenecks, linkage disequilibrium, inbreeding coefficients are also essential for analyzing complete population structure. The complete population structure helps us to plan strategies for conservation and development of a breed. Randomly Amplified Polymorphic DNAs (RAPD) is a polymerase chain reaction (PCR) based technique that has been used for the study of populations (Williams et al., 1990). PCR amplification on a random primer has been used extensively for genetic characterization of bacteria, plants and mammalian species. This technique utilizes short (9-10 bases) primers, designed on a random basis with the sole constraint being high GC content. The principal advantage of the approach is that the levels of detectable polymorphism are generally high. The principal disadvantage of the methodology is that the PCR results are very sensitive to amplification

conditions and consequently can be variable between laboratories and even between assays.

The aim of this work is to evaluate genetic status, genetic diversity and population structure in the two most common Tunisian sheep breeds, to investigate possible relations between genetic structure and geographical distribution of individuals and to examine whether it exist an isolation by distance process making use of RAPDs markers.

2.Material and Methods

Animal material: In this study, a total of 96 blood samples were randomly collected from the breeding tract of Barbarine and Western thin tail sheep which are very vast and wide covering about six districts (Beja, Bizerte, Tunis, Sousse, Sfax, Gabes) of Tunisia (Figure 1). Animals were classified into 12 populations (B1, B2, B3, B4, B5, B6, W1, W2, W3, W4, W5 and W6) according to their breed and origin. Eight animals were randomly selected from each population.

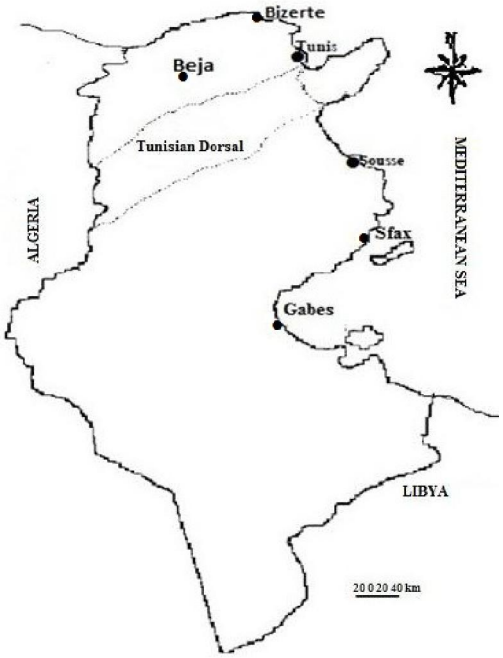

Figure 1. Map indicating localities of sheep genotypes sampled.

DNA extraction: Whole blood was collected from each animal in 7 ml Vacutainer tubes containing the anticoagulant, ethylenediaminetetra-acetic acid (EDTA). The blood samples were kept cold at -20°C until the isolation of total DNA. DNA was extracted from the whole blood with the 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 distilled 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 step 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.

PCR amplification: 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 MgCl2, 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 separated 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→PA20; Operon technologies). Only the primers producing polymorphic and repeatable bands were selected for the individual typing of all animals.

Statistical analysis: Analysis of the results of RAPD polymorphism was based on the phenotypic interpretation of revealed electrophoretic profiles. First, amplified fragments by all primers and in all individuals were identified Then, a contingency table with n rows (individuals) and p columns (amplified band) was established by assigning the value (1) when the band of a given level (molecular weight in bp) is present and (0) when it is absent and served as a data matrix for the calculation of various statistical parameters. Since RAPD markers are dominants, it was assumed that it was impossible to distinguish homozygote and heterozygote genotypes at individual loci. 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), the coefficient of gene differentiation (Gst) (Nei, 1973) 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. Mantel test was conducted using xlstat version 2013.2.03.

3.Results and Discussion

Primer Screening: RAPD-PCR method was used in the present study and the genetic variation among two sheep breeds was analyzed using eight primers. The eight primers have produced a total of 62 clear, reproducible polymorphic bands, ranging in size from 150 to 2500 bp. All the eight primers showed polymorphism in producing bands. Contrary to the present study, low number of fragments was amplified (57 amplified bands using 5 random primers with a mean of 11.4 bands per primer) as reported by Mahfouz et al. (2008). On average, 7.75 polymorphic bands were produced by each primer, with a maximum of 11 bands for primer OPA 10 and a minimum of 6 bands for primers OPA06 and OPA12. The number of DNA fragments amplified with each primer found in the present study is higher than found in many studies (Saifi et al., 2004; Mahfouz, 2008; Tariq et al., 2012).

Heterozygosity analysis: Genetic variability can be measured as the amount of heterozygosity. The percentage of polymorphic RAPD bands among populations ranged from 32.26 to 51.61% in W5 and W2, respectively (Table 1). The results of the present study are lower than the finding of Tariq et al. (2012) who revealed that percentage of polymorphism was 60.8% for indigenous sheep breeds of Balochistan in Pakistan and Elmaci et al. (2007) who revealed that percentage of polymorphic loci ranged from 80.49 to 73.17% for Turkish breeds. As shown in table 1, the heterozygosity values (H) calculated according to Nei (1978) generally varied from 0.1522 for W5 population with a Shannon's index of 0.2143 to 0.2157 for W4 population with a Shannon's index of 0.3102. The high heterozygosity value (0.2157) indicates low level of inbreeding as a result of wider geographical distribution of the breed. The average genetic diversity or heterozygosity values found indicate that the broader genetic diversity between and within populations was highest within B2, B3 and W5 and the lowest value was recorded within W4 individuals (Table 1). Our results are higher than the heterozygosity defined by Stephen et al. (2001), in a study they made with 4 RAPD primers to identify the genetic relations between 5 Tanzanian sheep ecotypes, who reported a highest average heterozygosity value of 0.203 and a lowest value of 0.137. In the other hand, they are lower than finding of Paiva et al. (2005) who stated that heterozygosity values ranged between 0.3229 and 0.4050.

Table 1. Nei's gene diversity (H), Shannon index (I) and percentage of polymorphic loci (P) at the population for the twelve populations studied**.**

	B1	B2	B3	B4	B5	B6	W1	W2	W3	W4	W5	W6
N	8	8	8	8	8	8	8	8	8	8	8	8
P	43.55	33.87	33.87	35.48	43.55	40.32	43.55	51.61	50	50	32.26	43.55
H	0.17	0.15	0.15	0.16	0.18	0.18	0.20	0.19	0.20	0.21	0.15	0.17
I	0.26	0.21	0.21	0.22	0.27	0.25	0.28	0.29	0.29	0.31	0.21	0.25

As can be seen in Table 1, the polymorphism ratio obtained in the study ranged from 32.25 to 51.61% with an average polymorphism of 41.80%. Aytekin and Boztepe (2010) identified the polymorphism ratio, with RAPD method, to be between 75% and 100% with a mean 90.46% in Turkish Güney Karaman Sheep-Breed, using RAPD technique. Yuan Fang et al. (2006) investigated the genetic polymorphism and relationship of 7 indigenous sheep breeds of China and 3 imported sheep breeds using RAPD analysis. They found that the percentage of polymorphic markers was 66.24%, which meant that the RAPD was very efficient at detecting polymorphism, and sensitive in the study on the genetic variation among the sheep breeds. Diversity indices provide more information about community composition than simply species richness; they also take the relative abundances of different species into account. The Shannon diversity index was computed to provide a relative estimation of degree of variability; its values ranged between 0.21 and 0.31. The values of the Shannon diversity index were consistent with the values of heterozygosity.

Analysis of Molecular Variance: The partitioning of total genetic variation in B and W breeds using AMOVA indicated 29.54% genetic diversity among breeds, 8.47% among populations within breeds and 61.99% within populations. Differentiation between populations in each breed was significant 11.22% ($P<0.001$) and 12.73% ($P<0.001$), respectively for

populations of Barbarine and Western thin Tail breed. In the case of W breed, results showed that 87.27% of total variation (P<0.001) was due to within population differences, and 12.73% was due to inter-population differences (Tables 2, 3 and 4). This result shows a low migration rate or exchange of animals between herds of the same breed. On the other hand, these results are important for management of these breeds, as the Barbarine populations and Western thin Tail behave as if they were independent management units. Our findings are in agreement with those of Paiva et al. (2005) who reported a similar difference between populations of the same breed (9.27%, P<0.01) and the variation between breeds (9.54%, P<0.01) using RAPD method to investigate the genetic structure of the Brazilian hair sheep breeds.

F-Statistics: The fixation indices (F_{CT}, F_{SC} and F_{ST}) values are shown in Tables 2, 3 and 4. F_{CT}, F_{SC} and F_{ST} values over all the population are found to be 0.29539, 0.12025 and 0.38012, respectively, which were extremely significant (P<0.005; P<0.001). The values of the three fixation indices indicated that inbreeding was lower among the populations. The low F_{CT}, F_{SC} values indicated low level of inbreeding within and among the populations and also point towards low genetic differentiation between the populations. Li et al. (2002) estimated F statistics within and among 12 Chinese goat populations. The mean F_{IS}, F_{IT} and F_{ST} values were found to be 0.030, 0.132 and 0.105, respectively which indicated low level of inbreeding within the population. Similarly Grigaliunaite et al. (2003) used F_{IS} to determine level of inbreeding in Baltic sheep breeds, where its value was 0.178. The values obtained were lower and significantly different from zero. Araujo et al. (2006) studied genetic diversity between herds of Alpine and Saanen dairy goats where mean differentiation among populations (F_{ST}) was 0.0717.

Table 2. Analysis of molecular variance (AMOVA) in B and W breeds

Source of variation	Df	SS	VC	PV
Among breeds	1	159.458	3.04349 Va	29.54
Among populations within breeds	10	133.708	0.87299 Vb	8.47
Within populations	84	536.500	6.38690 Vc	61.99
Total	95	829.667	10.30339	
Fixation indices F_{CT}: 0. 29539 P(Va and FCT) <0.005 F_{SC}: 0. 12025 P(Vb and FSC) < 0.001 F_{ST}: 0. 38012 P(Vc and FST) < 0.001				

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

Table 3. Analysis of molecular variance (AMOVA) in B breed

Source of variation	Df	SS	VC	PV
Among populations	5	60.625	0.76190 Va	11.22
Within populations	42	253.250	6.02976 Vb	88.78
Total	47	313.875	6.79167	
Fixation index F_{ST}: 0. 11218 P(Va and FST) < 0.001				

Table 4. Analysis of molecular variance (AMOVA) in W breed

Source of variation	Df	SS	VC	PV
Among populations	5	73.083	0.98408 Va	12.73
Within populations	42	283.250	6.74405 Vb	87.27
Total	47	356.333	7.72812	
Fixation index F_{ST}: 0. 12734 P(Va and FST) < 0.001				

Genetic relationship between populations: The relationship among the populations can be examined using the genetic distances/identities. The genetic distances and genetic identity among the twelve populations are shown in Table 5. Genetic identities between populations, computed from combined data of all eight primers, ranged from 0.7829 between W1 and B2 to 0.9962, between B1 and B5. Similar genetic distances were found between W1 and B4 and between W1 and B5. The results of genetic identities of the present study are higher than the findings of Hoda et al. (2009), who reported that the gene identity ranged from 0.75 to 0.78. Genetic

distance ranged from 0.0038 between B1 and B5 to 0.2447, between B2 and W1.

Table 5. Nei's original measures of genetic identity and genetic distance between the studied sheep populations.

Population	B1	B2	B3	B4	B5	B6	W1	W2	W3	W4	W5	W6
B1	****	0.9545	0.9720	0.9665	0.9962	0.9519	0.8244	0.8480	0.8424	0.7950	0.8804	0.8488
B2	0.0466	****	0.9915	0.9421	0.9606	0.9294	0.7829	0.8061	0.7924	0.7989	0.8213	0.7947
B3	0.0284	0.0086	****	0.9605	0.9689	0.9395	0.7986	0.8224	0.8131	0.7927	0.8576	0.8326
B4	0.0340	0.0597	0.0403	****	0.9417	0.8820	0.8186	0.8611	0.8326	0.8343	0.8834	0.8634
B5	0.0038	0.0402	0.0316	0.0601	****	0.9561	0.8182	0.8400	0.8337	0.7859	0.8582	0.8293
B6	0.0493	0.0732	0.0624	0.1255	0.0449	****	0.8299	0.8237	0.8301	0.8151	0.8244	0.8238
W1	0.1931	0.2447	0.2249	0.2002	0.2006	0.1865	****	0.9912	0.9871	0.8951	0.9451	0.9496
W2	0.1649	0.2155	0.1955	0.1495	0.1743	0.1939	0.0088	****	0.9683	0.8998	0.9432	0.9445
W3	0.1715	0.2327	0.2069	0.1832	0.1818	0.1862	0.0130	0.0322	****	0.9157	0.9577	0.9428
W4	0.2295	0.2245	0.2323	0.1811	0.2410	0.2044	0.1108	0.1056	0.0880	****	0.8837	0.9160
W5	0.1274	0.1969	0.1536	0.1240	0.1529	0.1931	0.0565	0.0585	0.0432	0.1236	****	0.9902
W6	0.1639	0.2298	0.1832	0.1468	0.1872	0.1938	0.0517	0.0571	0.0589	0.0877	0.0098	****

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

A UPGMA dendrogram, grouping all populations was constructed using Nei genetic distances to better visualize the structure of all populations (Figure 2). It shows two differentiated branches, the first one included populations of the B breed and the second included populations of the W breed. Phylogenetic relationships among the two genetic groups are shown in Figure 2. The dendrogram can be divided into two main clusters. The first cluster contained three sub-clusters, the first one with 4 Barbarines populations (B1 and B5; B2 and B3). The second and third sub-clusters contained one Barbarine population, B4 and B6, respectively. While, the second cluster contained Western thin Tail breed (W). As seen in Figure 2, the second cluster was divided into three main sub-groups, one with W4 individuals, one with W5 and W6 and the other with all the other individuals. The individuals that made up the other group apart from W5 and W6 again were sub-grouped with W1, W2 and W3 individuals.

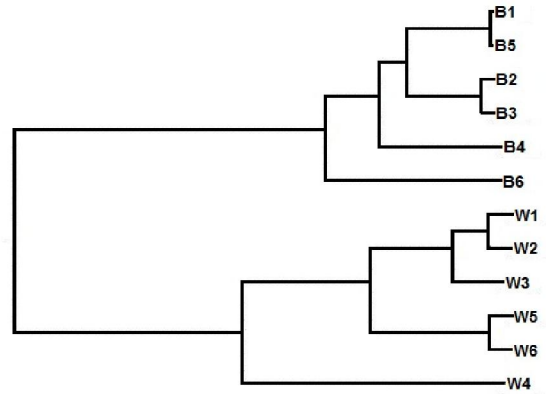

Figure 2. UPGMA dendrogram of twelve Tunisian sheep populations based on Nei's (1978) genetic distance. UPGMA, unweighted pair-group method with arithmetic average.

The relationship between genetic and geographical distances was examined to assess isolation-by-distance (Slatkin 1993). The result show that no isolation-by-distance relationship was detected with both genetic distance measures (r= 0.248, P= 0.393) for Barbarine breed and (r= 0.121; P=0.658) for Western thin tail breed (Figures 3 and 4). Our finding are not in agreement with those of Worley et al. (2004) who detected a significant correlation (r= 0.747, P= 0.0001) between genetic and geographic distances in North American thinhorn sheep.

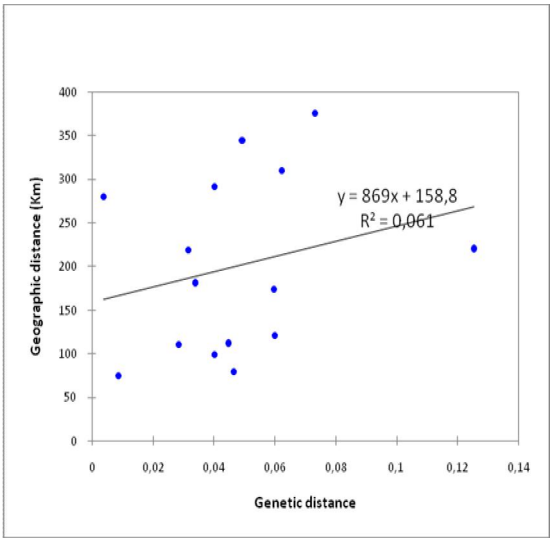

Figure 3. Results of Mantel tests among pairwise estimates of Nei's unbiased genetic distance in B populations and pairwise geographic distance (km); r(AB): 0.248 (P= 0.393).

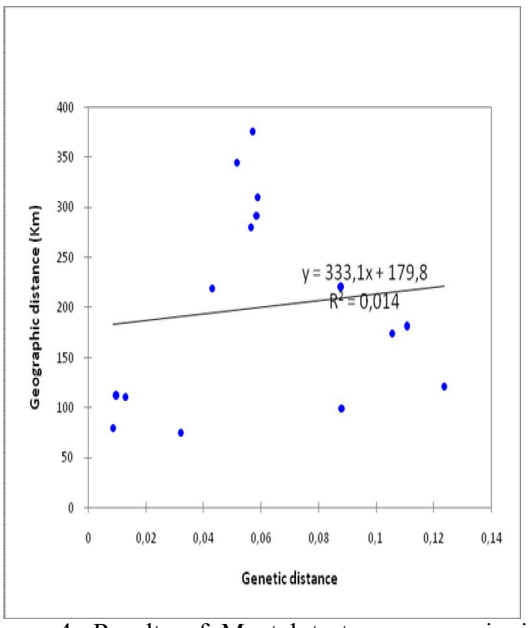

Figure 4. Results of Mantel tests among pairwise estimates of Nei's unbiased genetic distance in W populations and pairwise geographic distance (km r(AB): 0.121; p-value: 0.658; alpha: 0.05; The p-value was calculated from the distribution of r (AB) estimated from 10000 permutations.

Among the 12 populations, the mean coefficient of gene differentiation (Gst) was 0.3615. Gene flow (Nm) was calculated to be 0.9150 between all populations, 2.0887 between populations of the B breed and 1.9379, between populations of the W breed (Table 6). The coefficient of population differentiation (Gst) was 0.3534 which is in agreement with the finding of Askari et al. (2011) who found a mean coefficient of gene differentiation (Gst) of 0.36, for Iranian sheep. The results of Gst in this study reveal that gene variation among the breeds is still low. This found is agreeing with the results of Agaviezor et al. (2012) in Nigerian sheep breeds. This differentiation formed the basis for describing how genetic variation is partitioned within Tunisian sheep breeds.

In conclusion**,** genetic variation is a basic requirement for animal breeding, whereas a high genetic variation is needed for genetic improvement of domestic animals. Knowledge of genetic distance among animals and breeds, and genetic diversity/structure within breeds could be useful for conservation of genetic resources. As a result, it can be said that these 12 populations, which are representative of Barbarine and Western thin Tail sheep, has a heterogenous nature and that this genetic variation has the potential use for purposes of improvement. In this study, the polymorphism ratio ranged from 32.25 to 51.61% with an average polymorphism of 41.80%. The highest genetic diversity (61.99%) was reported within populations. The genetic distance in sheep breeds ranged from 0.7829 to 0.9962. Coefficient of gene differentiation (Gst) was 0.3615 and gene flow (Nm) was 0.9150 between all populations.

Table 6. Genetic differentiation coefficient (Gst) and gene flow value (Nm)

	Gst	Nm
Between all populations	0.3534	0.9150
Between populations of the B breed	0.1932	2.0887
Between populations of the W breed	0.2051	1.9379

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