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Genetic structure of Tunisian sheep breeds as inferred from genome-wide SNP markers

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Highlights

- Tunisian sheep diversity by genome-wide SNP markers highlighted:
- Relatively high levels of polymorphism in the considered breeds, comparable to the levels previously observed for Mediterranean sheep populations.
- A relative genetic closeness between Barbarine and Queue Fine de l'Ouest despite they are a fat and a thin-tail sheep breed, respectively.
- A marked genetic differentiation of the Noire de Thibar breed. The origin of Noire de Thibar as a composite breed was confirmed.

Abstract

Assessing the status of genetic variability of native sheep breeds could provide important clues for research and policy makers to devise better strategies for the conservation and management of genetic resources. In this study, a genetic investigation of Tunisian sheep breeds using a

genome-wide scan of approximately 50,000 SNPs was performed. To reconstruct genetic structure and relationships among four sheep breeds, 40 samples belonging to fat-tailed Barbarine, Queue Fine de l'Ouest, Noire de Thibar and D'Man breeds were genotyped using Illumina *Ovine* SNP50 BeadChip. Tunisian breeds averaged 96% polymorphic loci with an expected heterozygosity (H_e=0.36). Genetic analysis of relationship between breeds using Bayesian clustering, MDS and Neighbor-Network analysis, and estimation of F_{ST} genetic structure, highlighted the genetic differentiation of Noire de Thibar breed from the other local breeds, reflecting the effect of past events of introgression of European gene pool. The Queue Fine de l'Ouest breed showed a genetic heterogeneity and was close to Barbarine and D'Man breeds, as evidenced by MDS and the lowest level of differentiation with Barbarine breed (F_{ST}=1.8%). The D'Man breed shared a considerable gene flow with the thin-tailed Queue Fine de l'Ouest breed. Possible factors explaining the genetic patterns observed, such as considerable gene flow probably due to anthropogenic activities in the light of population management and conservation programs.

Keywords: Tunisian sheep, SNP data, population structure, diversity.

1. Introduction

The characterization of livestock breeds is considered as one of the strategic priorities for the development of a national plan for the management of animal genetic resources (FAO, 2007). In Tunisia, sheep is the most important livestock species, representing 77% of the total number of reared animals. Sheep farming in Tunisia is an important economic and social activity contributing to 39% of the national red meat production (OEP, 2017).

The current Tunisian sheep populations mainly comprise four meat sheep breeds (Fig.1): Barbarine, Queue Fine de l'Ouest, Noire de Thibar and D'man. The Barbarine represents the

only fat-tail breed and is considered the most important native sheep breed in Tunisia (Atti, 2004). It was probably introduced from the steppes of Central Asia in the Carthaginian period (around 400 years B.C.E.) by the Phoenicians and then reintroduced from the Near-East by the Arabs around 900 years B.P (Masson, 1967). Indeed, local breeds constitute a major genetic resource which is well adapted to diverse agro-ecological conditions. Indeed, the Barbarine and Queue Fine de l'Ouest are locally adapted to harsh conditions in arid and semi-arid regions in Tunisia and heavily concentrated in the central semi-arid regions (Bedhiaf-Romdhani *et al.,* 2008a). However, Noire de Thibar, a composite black coated sheep breed found in the northern sub-humid region because of its higher nutritional requirements and non tolerance of the prevailing harsher condition. The D'Man breed, originated from Morocco, is mainly located in the southern oases of the extreme arid ecosystem. A more comprehensive description of breed-specific phenotypic traits in Tunisian meat sheep breeds is summarized in Table S1. In the last decades, many trends contribute to major changes on sheep genetic diversity. Indeed, the breed genetic integrity is threatened by a clear cross-border gene flow *via* uncontrolled introduction of Lybian Barbarine and Algerian Ouled Djallel from neighboring countries (Rekik et al., 2005). Moreover, an increasing of anarchic crossbreeding between local populations is mainly adopted by small flock owners leads to creation of new population of crosses by systematic crossbreeding between Barbarine and Queue Fine de l'Ouest breeds. This practice is very common because of the demand for non fat-tailed sheep, better response to fattening and easier reproduction management of crossbreeds (Rekik *et al.,* 2005; Bedhiaf-Romdhani *et al.,* 2008b). Genetic diversity of Tunisian sheep breeds was previously investigated through morphologic and morphometric descriptors (Khaldi *et al.,* 2011). Moreover, molecular markers were used to study the genetic structure of Tunisian sheep populations, such as RAPD-PCR (Khaldi *et al.,* 2010, El Hentati *et al.,* 2012), microsatellites markers (Ben Sassi-Zaidy *et al.,* 2014; Kdidi *et al.*, 2015a), and sequence polymorphisms at the Prion protein (*PRNP*) gene (Kdidi *et al.,* 2014)

showing the presence of relatively high frequencies of the ARR haplotype associated to scrapie resistance. Analysis of genome-wide SNP markers has been shown to provide an unprecedented resolution in reconstructing genetic relationships, inferring genetic structure, and assessing breed distinctiveness and conservation status in sheep (Kijas *et al.,* 2009; Zhao *et al.,* 2017). The aim of this study was to contribute to satisfy the growing need to exploit local sheep breeds in sustainable manner under the existing production system. This could be possible through assessment of intra-breed genetic variation and population structure using a whole-genome genotyping data via Illumina *Ovine* SNP50 BeadChip.

2. Materials and Methods

2.1. DNA samples

Jugular blood samples (5 ml per sample) were collected from sheep samples belonging to four Tunisian sheep breeds: Barbarine (n=19), Noire de Thibar (n=14), Queue Fine de l'Ouest (n=10) and D'man (n=2) using EDTA as an anticoagulant and stored at -20°C. The sampled animals were raised in 28 flocks from different types of farms (public, cooperative, and private farms) and from a research station belonging to INRAT (Institute National de la Recherche Agronomique de Tunisie). The geographical distribution of sampled animals is shown in the map based on corresponding GPS coordinates (Fig.1). In order to avoid sampling related animals, information about relatedness among animals was checked with farmers when pedigree data was not recorded. Compliance of the sampled animals with breed standards was assessed based on phenotypic appearance (Fig. 2; Table S1-Supplementary material). Genomic DNA was isolated using a standard phenol-chloroform protocol (Sambrook *et al.,* 1989). The obtained average range of DNA concentration was 20-50 ng/µl and samples with A260/280 ratio between 1.7 and 2.0 were selected for further genotyping.

2.2. Genotype data and quality control

The genotyping was performed at the facilities of the Department of Agricultural, Food and Forest Sciences, University of Palermo, Italy. The genomic DNA samples were typed at 54,241 SNPs using the OvineSNP50 BeadChip (Illumina, Inc., San Diego, CA, USA). Genotypes were called on the AB system and using Illumina GenomeStudio® software. Data quality control was performed using PLINK 1.07 software (Purcell *et al.*, 2007). Only SNPs located on autosomes were considered in further analyses. A SNP was removed from data if the following criteria were not met: (i) a SNP call rate greater than 90%, (ii) minor allele frequency (MAF) greater than 0.01. Animals with more than 10% of missing SNPs were also removed from further analyses.

2.3. Genome-wide SNP data analysis

The within-breed genetic diversity was estimated using the following parameters: proportion of polymorphic loci (P_p), distribution of loci in MAF intervals, gene diversity or expected heterozygosity (H_e), the inbreeding coefficient (F_{IS}), and a test for SNP deviation from Hardy-Weinberg equilibrium (HWE). All the above statistics were estimated, using PLINK 1.07, without including D'man breed, due to the reduced sample size. The Arlequin 3.5 software (Excoffier and Lischer, 2010) were used to calculate pairwise differentiation F_{ST} between different breeds. To further understand the genetic relationships between breeds and single individuals, we calculated the average proportion of alleles shared between animals (A_s) via the formula implemented in PLINK 1.07:

IBS2 + 0.5*IBS1/N, where IBS1 and IBS2 are the number of loci that share either one or two alleles identical by state (IBS), respectively, and N is the number of loci tested. The pairwise individual IBS distances were visualized though multi-dimensional scaling (MDS) and Neighbor-Net (Bryant and Moulton, 2004) analysis implemented, respectively, in PLINK1.07 and in the Splits Tree 4.13.1 software (Huson and Bryant, 2006). Population sub-structuring was evaluated through the model-based clustering algorithm implemented in

ADMIXTURE 1.22 (Alexander and Lange, 2011). The most probable number of populations in the dataset (K) was estimated using the default (5-fold) ADMIXTURE's cross-validation procedure, by which estimated prediction errors are computed for each K value. The K value that minimizes the estimated prediction errors was then assumed to be the most suitable.

3. Results

3.1. Data genotyping

Out of 54,241 SNPs, a total of 42,613 SNPs mapping onto 26 sheep autosomes were retained and passed data quality control. The distribution of retained SNPs across autosomes is available in supporting information (Fig.S1). Five samples with more than 10% missing genotypes were removed and the remaining 40 samples (18 belonging to Barbarine, 9 to Queue Fine de l'Ouest, 11 to Noire de Thibar, and 2 to D'Man). The results after each filtering criteria were summarized in supplemental TableS2. The final genotyping data subset (42,613 SNPs) present a mean call rate of 99.78% (Table S3).

3.2. Genetic diversity within breeds

When considering the whole SNP set (42,613 filtered loci), the percentage of within-breed polymorphic SNPs ranged from 95% to 98%, with the highest value found in Barbarine breed (Table 1). In general, no evidence of inbreeding was observed. The mean estimates (F_{IS}) for each breed showed negative values, ranging from -0.03±0.01 (Barbarine) to -0.05±0.01 (Noire de Thibar). The Barbarine breed show the lowest level of inbreeding, where all individual inbreeding coefficients were negative (data not shown). The estimates of expected heterozygosity showed that the Barbarine breed had a slightly higher level of genetic diversity than Noire de Thibar and Queue Fine de l'Ouest breeds, with H_e=0.37±0.13 vs 0.36±0.14 and 0.35±0.14, respectively (Table 1). All sheep breeds showed high levels of heterozygote excess, ranging from 57.41% to 62.93%. The Noire de Thibar breed had the highest proportion of

markers with excess of heterozygote genotypes (62.93%). For all breeds, few loci (ranging from 53 to 109, Table 1) were significantly deviating from HWE ($p<0.01$). The fat-tailed Barbarine breed show the lowest percentage (81.65%) of loci deviating from HWE due to heterozygote deficiency. Moreover, the highest proportion (100%) of loci deviating due to heterozygote deficiency was observed in the Queue Fine de l'Ouest breed. The minor allele frequency (MAF) distributions for different intervals in Barbarine, Queue Fine de l'Ouest and Noire de Thibar breeds were represented in Figure (3). Five MAF intervals were defined, based on allele frequency values, with rare alleles falling in the class MAF≤ 0.10. The distribution of common alleles (0.10<MAF ≤0.5) was comparable within the three populations, with high proportion of loci falling in intermediate MAF intervals (0.3<MAF ≤0.4). The Noire de Thibar breed displayed the highest proportion of loci with rare alleles.

3.3. Genetic relationship among Tunisian populations

The F_{ST} values computed for each pair of Tunisian breeds ranged from 0.018 to 0.072 (Table 2). The pairwise breed comparisons involving the Queue Fine de l'Ouest breed displayed among the lowest F_{ST} values. The smallest average pairwise F_{ST} value was observed between the fat-tailed Barbarine and the thin-tailed Queue Fine de l'Ouest (F_{ST}= 0.018), reflecting a relative genetic closeness between the two breeds. The second smallest F_{ST} value was observed between Queue Fine de l'Ouest and Noire de Thibar breeds (F_{ST} = 0.035).

The multi-dimensional scaling (MDS) plot analysis of the four Tunisian sheep breeds (Fig. 4) revealed a marked genetic differentiation of the Noire de Thibar breed. Along the second component (C2, on the y-axis), two clusters were highlighted, one including Barbarine, Queue Fine de l'Ouest, and D'Man samples and the second (bottom left side of the MDS plot) including four Queue Fine de l'Ouest individuals which are considered as outliers from the main Queue Fine de l'Ouest group. In order to refine the results observed in the MDS analysis, we reconstructed the relationship between samples using Neighbor-Net graph based on IBS

distance matrix (Fig. 5). The topology highlighted three clusters: The first group included Noire de Thibar animals which are well isolated from the other samples, the second group presented by Barbarine animals and the third cluster regrouped two D'Man and all Queue Fine de l'Ouest individuals (with the exception of one individual, QF73, which clustered close to Barbarine). The four Queue Fine de l'Ouest animals (QF58, QF1, QF8 and QF16) displaying closer relationships among each other (terminally reticulated branches) which are originated from the same flock (Office de l'Elevage et des Pâturages, OEP). Indeed, this clustering was previously supported in MDS analysis (Fig. 4). Indeed, the topology of the Neighbor-Net plot is consistent with the MDS and F_{ST} results, confirming the clear differentiation of Noire de Thibar and the genetic heterogeneity of the Queue Fine de l'Ouest animals, mainly admixed with the Barbarine and the D'Man breeds. When looking at the ADMIXTURE results at K=2 (Fig. 6), the four Tunisian sheep breeds included in this study clustered consistently with the previous results (F_{ST}, MDS, Neighbor-Net plot). Indeed, the Noire de Thibar breed as well as Barbarine, was again confirmed as a separated population, while the remaining breeds (Queue Fine de l'Ouest and D'Man) shared genomic components at various extent.

4.Discussion

4.1. level of SNP polymorphism and ascertainment bias

In this study, a set of 50K Illumina SNPs was found to be highly informative in an initial cohort of Tunisian sheep breeds. Similarly, this tool has been largely adopted in several genetic diversity studies on sheep breeds in North Africa (Gaouar *et al.,* 2017), Europe (Deniskova *et al.,* 2018; Michailidou et al., 2018; Mastrangelo *et al.,* 2017; Beynon *et al.,* 2015; Ciani *et al.,* 2013), Asia (Zhao *et al.,* 2017) and south America (Grasso *et al.,* 2014) showing generally good performances. The survey of Kijas et al. (2012), reported by a collection of 74 sheep breeds from each continent, highlighted the presence of an ascertainment bias phenomenon when

contrasting allele frequency-dependent diversity estimates among groups of breeds from different continents. This ascertainment is also supported by the relatively similar number of SNPs that passed quality control in our study (42,613) compared to the study performed by Gaoaur *et al.,* (2017) in 46 individuals from 8 Algerian sheep breeds (45,755), where the slightly higher value may be due to the larger number of sheep breeds (eight *vs* four breeds), thus likely allowing them to retain a larger number of polymorphic loci. An additional concern may derive from the use of small samples sizes to infer population genetic parameters. While this is legitimate for within-breed diversity estimates, it has been empirically demonstrated via a sub-sampling procedure applied to a set of three different population datasets, that, at least for the Neighbor-Net and the ADMIXTURE analyses, the patterns observed using 6 randomly extracted animals per breed closely mirrors those inferred from 20-24 animals per breed (the most usual population sample size in genome-wide SNP genotyping projects) (Gaouar *et al.,* 2017). Moreover, as for genetic differentiation measured by F_{ST} estimators, it has been shown via computer simulations that the population sample size can be significantly reduced (as small as n= 4-6) when using a large number of bi-allelic genetic markers (k>1,000) without significant bias or accuracy drop in F_{ST} estimation (Willing *et al.,* 2012).

4.2. Genetic diversity within Tunisian sheep breeds

Throughout its history, North Africa passed by several waves of migrations (Phoenicians, Romans and Arabs, etc) accompanied by hybridization of imported and local breeds (Jemaa *et al.,* 2019). Thus, it would be tempting to speculate that this observation may reflect a general homogeneity among Mediterranean sheep populations, likely a legacy of the continued historical intermingling of civilizations at the "mare nostrum" crossroad. This hypothesis seems to be supported by the higr level of SNP polymorphism (P_p) and genetic diversity (H_e), and

lower inbreeding (F_{IS}) observed in Tunisian sheep breeds compared to Asian and central African sheep populations.

At the national level, comparable values of gene diversity (H_e) were observed in the three considered breeds, with a slightly higher level of genetic diversity (measured both as H_e and as P_p) for Barbarine compared to Noire de Thibar and Queue Fine de l'Ouest. Despite we cannot rule out a sample-size bias. These results explain the genetic variability of the Barbarine breed at larger population size showing different "ecotypes" adapted to various environmental conditions (Bedhiaf *et al.*, 2006). Our results were also consistent with microsatellites data that highlighted Barbarine as the breed displaying the highest level of genetic diversity when contrasted to Queue Fine de l'Ouest, Noire de Thibar, D'man and Sicilo sarde (Ben-Sassi Zaidy *et al.*, 2014). The Noire de Thibar breed displayed the highest proportion of loci with rare alleles and the highest proportion of loci, out of the total SNP set, showing heterozygote excess. These results are consistent to previous genetic diversity studies of Tunisian sheep populations using nuclear microsatellites (Kdidi *et al.*, 2015a) and Y chromosome markers (Kdidi *et al.*, 2015b). In addition, the analysis of genetic relationships among Tunisian breeds (see section below) showing that the current genetic make-up may be the result of past introgression of differentiated gene pools from Europe into local sheep genetic stocks which are reported by historical knowledge (Kallel, 1968; Djemali and Alhadrami, 1997). For all breeds, few loci were significantly deviating from HWE showing that the sampled animals are not exposed to random fluctuation of genetic variation, thus no evidence of genetic drift or/and inbreeding are shown . This could be partly explained by the sampling design where unrelated animals were sampled from different geographic areas and flocks. In addition, for the Barbarine breed, due to the mating difficulties related to the presence of a large fat-tail, the operation is usually assisted by shepherds who also avoid the mating between relatives (Ben Sassi-Ziady *et al.*, 2016; Lassoued *et al.*, 2017).

4.3. Genetic relationships among Tunisian sheep breeds

Among the four considered breeds, Noire de Thibar showed a clear differentiation. This evidence was consistently observed with all the adopted approaches (F_{ST}, MDS, Neighbor-Net, ADMIXTURE) thus confirming its origin as a composite breed originated via introduction of exotic genetic material (Chalh *et al.,* 2007). The remaining breeds (Barbarine, Queue Fine de l'Ouest and D'Man) were not clearly differentiated. This results are consistent with the genetic diversity study performed by Kdidi *et al.* (2015a) showing that an extensive gene flow between Tunisian sheep breeds prevented their substantial genetic differentiation explained by uncontrolled reproduction management and absence of breed development programs. However D'Man, which originated from Morocco, displayed relatively higher fixation indices, suggesting that weak phylogeographic gradients may exist in sheep populations in North Africa (Belabdi *et al.,* 2019). In our study, MDS and Neighbor-Net analyses highlighted the stratification of Queue Fine de l'Ouest into two distinct groups. All the four divergent Queue Fine de l'Ouest individuals were breeding males sampled at the public farm station belonging to the Office de l'Elevage et des Pâturages (OEP). This peculiar clustering pattern seems to support the following considerations (i) since these animals are belonging to the same strictly controlled flock, they clustered in the MDS plot as an isolated group (ii) the above mentioned four animals may be considered as representatives of purebred (uncrossed) Queue Fine de l'Ouest rams. Theses rams were used in state farms for implementation of breeding schemes where pedigree and recording performances are rigorously controlled (Rekik *et al.,* 2005). While, the remaining Queue Fine de l'Ouest individuals, that clustered, in the MDS plot and in the Neighbor-network, close to Barbarine and D'Man, have been sampled from private farms. This insight is based on the known information about the tendency to adopt crossbreeding practices, by small private farms, in order to reduce the fat tail of the Barbarine breed according to the preferences of farmers or consumers (Bedhiaf-Romdhani *et al.,* 2008b) or increasing the prolificacy of the

Queue Fine de l'Ouest breed high prolific D'man breed (Rekik *et al.*, 2005; Bedhiaf-Romdhani *et al.*, 2013). In particular, in our study, the homogenization process looked more pronounced between Barbarine and Queue Fine de l'Ouest, as suggested by the lowest F_{ST} value. Therefore, the phenotype of recent crossbred animals (Barbarine x Queue Fine de l'Ouest) is very similar to one of parental breeds but relatively different in term of genetic distance. Based on this observation, some samples were admixed animals were considered as a pure bred animal because it does not show any phenotypic distinction when compared to purebred sheep breed. A similar homogenization scenario was observed in Algerian sheep populations, with Rembi and Taâdmit having seemingly lost most of their genetic originality because of intensive cross-breeding with Ouled-Djellal (Gaouar *et al.*, 2015).

5. Conclusions

The present study of Tunisian sheep diversity by genome-wide SNP markers highlighted relatively high levels of polymorphism in the considered breeds. No evidence of inbreeding was detected, although validation on a larger number of animals will be required prior to using data generated here in genetic management and conservation decisions. Analysis of genetic relationships allowed to confirm the origin of Noire de Thibar as a composite breed, and to give evidence of a general closeness among the other three Tunisian breeds. Our findings represent a starting point for the characterization of Tunisian sheep breeds suggesting the need to set up accurate conservation measures aiming to safeguard and monitor their genetic variability and provide appropriate conservation strategies.

CONFLICT OF INTEREST

The authors have declared that no competing interests exist.

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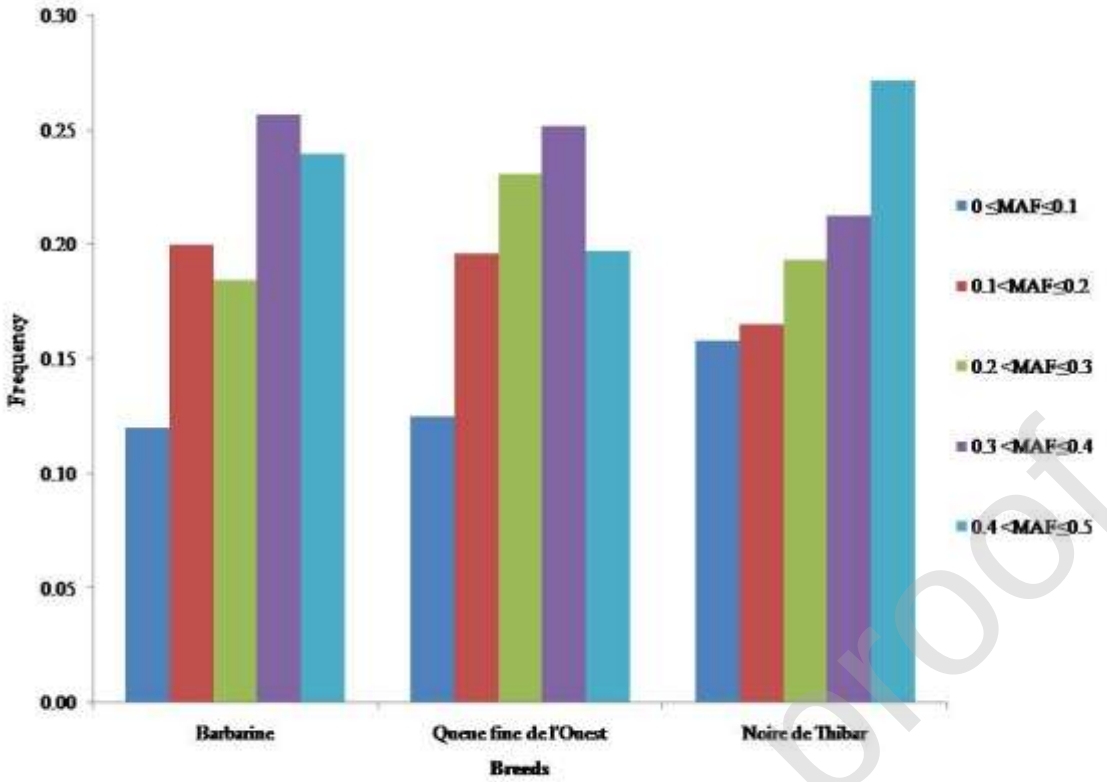
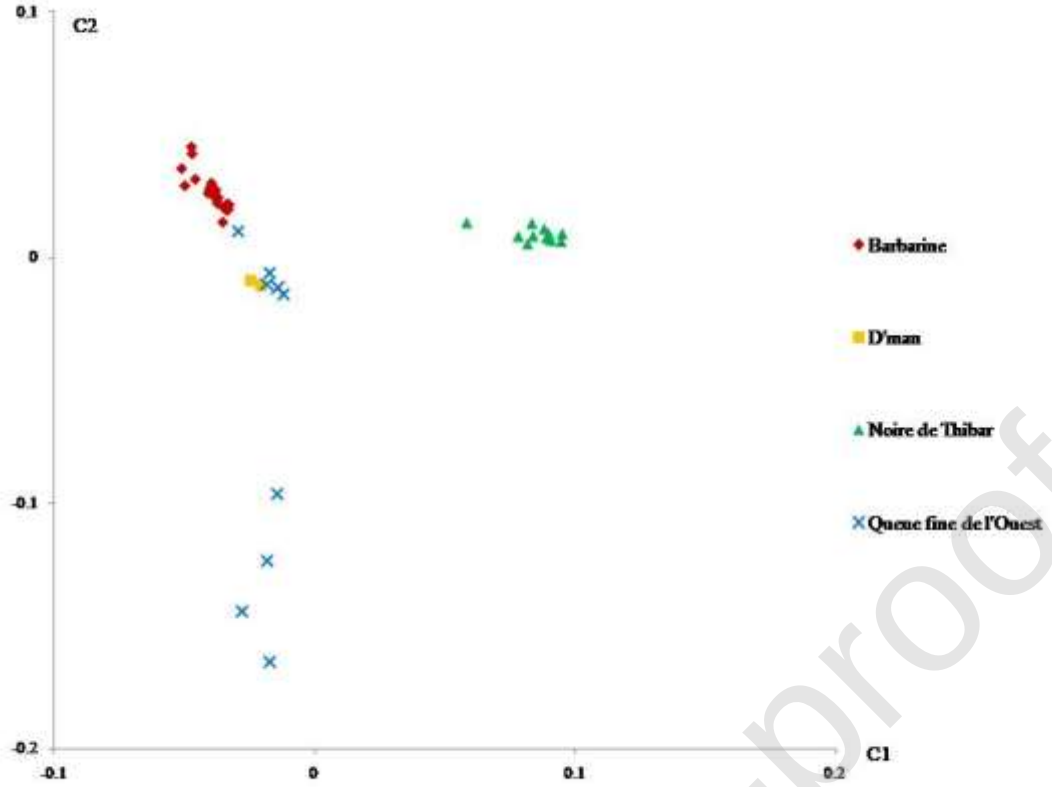
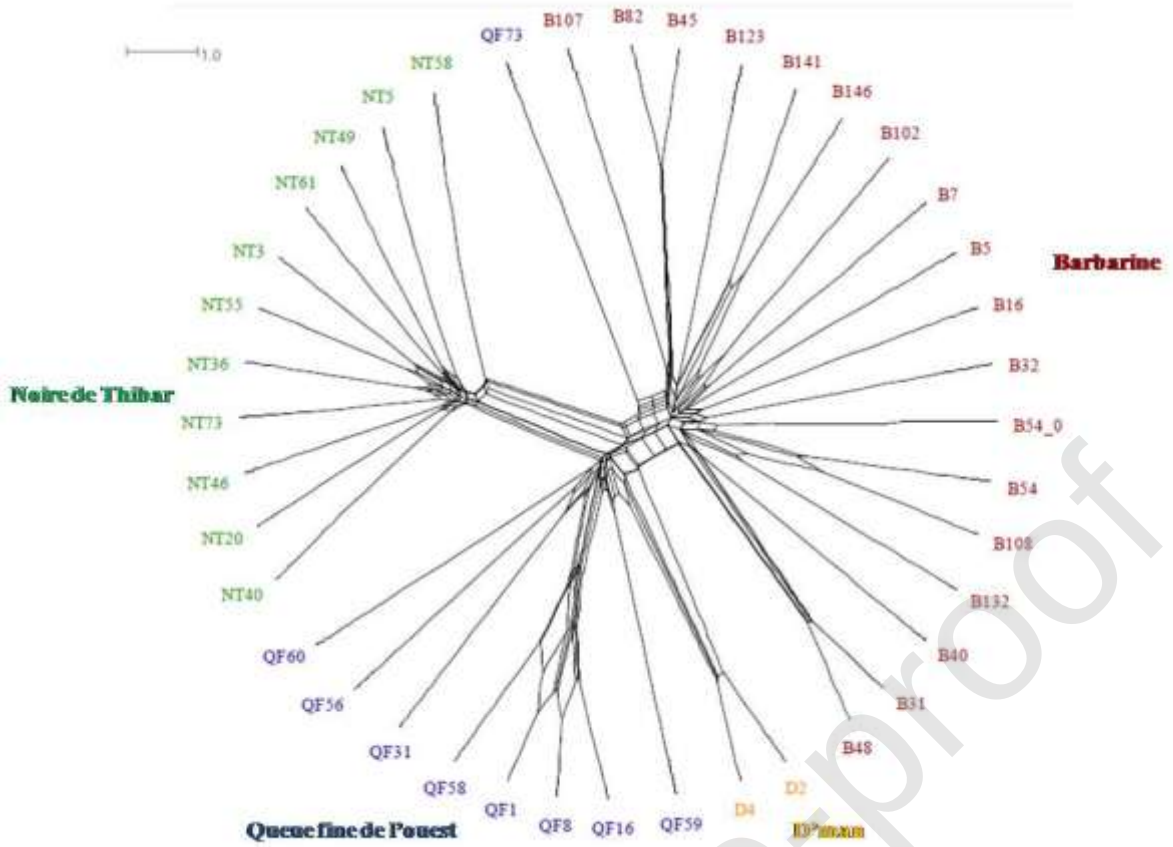
Fig. 1. Map of geographical distribution of sampled animals based on GPS coordinates.
Fig. 2. Pictures of four Tunisian meat sheep breeds. **A)** Barbarine **B)** Queue fine de l'Ouest **C)** Noire de Thibar **D)** D'man.
Fig. 3. Within-breed class distributions of Minor Allele Frequency (MAF) values for the genome-wide SNP loci. Five MAF intervals were arbitrarily defined. Among the three breeds, Noire de Thibar displays the highest proportion of loci with rare alleles (MAF≤ 0.1).
Fig. 4. Multi-dimensional scaling (MDS) plot of the pairwise individual IBS distances for the four Tunisian sheep breeds. The first (C1) and second (C2) components are presented in the x- and the y- axis, respectively. A marked genetic differentiation of Noire de Thibar with respect to the other breeds is evident. Red, Barbarine; blue, Queue fine de l'Ouest; green, Noire de Thibar; yellow, D'man.
Fig. 5. Neighbor-net plot of the pairwise individual IBS distances for the four Tunisian sheep breeds. The plot highlights the complex (reticulated) relationship between the Tunisian sheep breeds. A marked genetic differentiation of Noire de Thibar with respect to the other breeds is evident. Red, Barbarine; blue, Queue fine de l'Ouest; green, Noire de Thibar; yellow, D'man.
Fig. 6. Plot of coefficients of individual membership to the two arbitrarily assumed clusters (K), estimated for the Tunisian breeds using the unsupervised model-based clustering algorithm implemented in ADMIXTURE 1.22. Individuals are represented by single vertical columns, while the length of the colored segment represents the estimated level of admixture. Barbarine and Noire de Thibar/D'man animals are clearly differentiated, as they fall in the red and the green cluster, respectively. On the contrary, animals of the Queue Fine de l'Ouest breed displayed, at different extent, both components, suggesting the existence of genetic relationship with both Barbarine and Noire de Thibar/D'man.







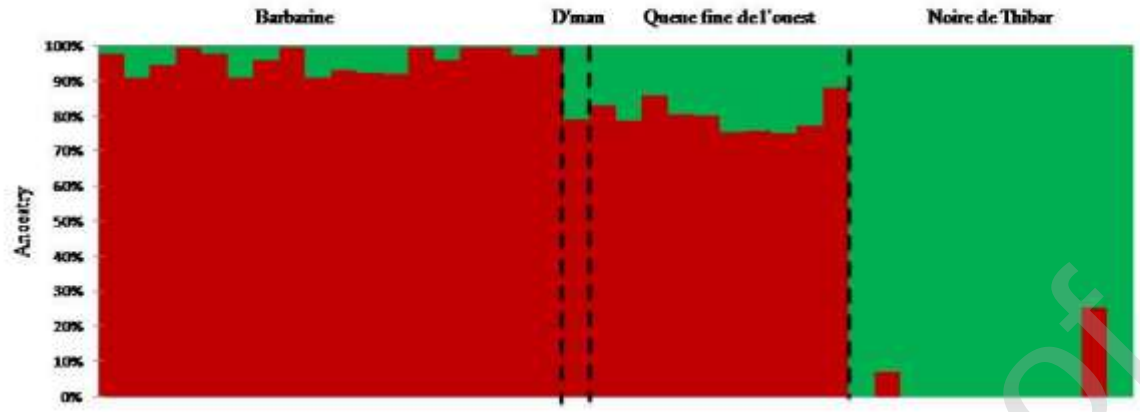


Table 1. Genetic diversity parameters at the breed level

		Total SNPs (42,613 SNP)					SNPs not in HWE (*p<0.01*)		
Breed	n	F_{IS}	P_P	$H_e \pm SD$	Het ex (%)	Het def (%)	SNP (%)	Het ex (%)	Het def (%)
BAR	18	-0.03±0.01	0.98	0.37±0.13	25983 (60.97)	16630 (39.03)	109 (0.26)	20 (18.35)	89 (81.65)
QFO	9	-0.04±0.05	0.95	0.35±0.14	24466 (57.41)	18147 (42.59)	53 (0.12)	0 (0)	53 (100)
NT	11	-0.05±0.01	0.97	0.36±0.14	26817 (62.93)	15796 (37.07)	82 (0.19)	6(7.32)	76 (92.68)

BAR: Barbarine; NT: Noire de Thibar; QFO: Queue fine de l'Ouest; n: number of samples; F_{IS} : inbreeding coefficient; Pp: proportion of polymorphic loci, H_e: expected heterozygosity; number (and proportion) of loci showing excess (Het ex) and deficiency (Het def) of heterozygosity in the total SNP set and in the set of SNPs not in HWE; SD: standard deviation; SNP: number (and proportion) of markers showing significant deviation from Hardy Weinberg Equilibrium (P<0.01).

Table 2. Pairwise genetic differentiation (F_{ST}) between Tunisian sheep breeds.

	Barbarine	**Queue fine de l'Ouest**	**Noire de Thibar**
Queue fine de l'Ouest	0.018		

Noire de Thibar	0.035	0.035	
D'man	0.056	0.058	0.072