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POPULATION STRUCTURE IN TUNISIAN INDIGENOUS RABBIT ASCERTAINED USING MOLECULAR INFORMATION

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Abstract: Understanding the genetic structure of domestic species provides a window into the process of domestication. This study attempts to offer an insight into the prevailing genetic status of Tunisian indigenous rabbit breeds using molecular markers. Thirty-six microsatellite loci were used to provide a comprehensive insight into the genetic status and relationship among 12 Tunisian indigenous rabbit populations. A total of 264 rabbits from villages of the Tozeur and Kebili regions were studied. Standard statistics parameters of genetic variability within and between populations were calculated. The observed heterozygosity, unbiased expected heterozygosity and the effective number of alleles were used to assess the genetic variation of each indigenous breed. Results show a high genetic diversity and observed heterozygosity ranged between 0.3 and 0.5, which implies that there is an abundant genetic variation stored in Tunisian indigenous rabbit breeds. Significant population differentiation was observed (F_{ST}=0.11), which means that most of the genetic variation resides within breeds. The percentage of individuals correctly classified to their population was 85%. Breeds with more than one breeder origin were divided into subgroups, due to differences in gene frequencies between breeders, which in some cases creates a genetic differentiation even higher than that observed between distinct breeds. The current study is the first detailed analysis of the genetic diversity of Tunisian indigenous rabbit populations. The data generated here provides valuable information about the genetic structure of the 12 rabbit populations and this can be used to designate priorities for their conservation.

Key Words: Tunisian rabbit, microsatellite, genetic differentiation, genetic diversity.

INTRODUCTION

In the history of domestication, the rabbit presents two specific characteristics: it is a very recent practice (less than 2000 yr) and it was the only species that was domesticated in Western Europe. Both wild and domestic European rabbit belong to the single species *Oryctolagus cuniculus*. The history of wild populations is well documented through both archaeological and genetic studies (Monnerot *et al.,* 1994; Ferrand, 2008). A consequence of ancestral breeding and recent selection practices on more than 60 breeds were described, where size, fur type and colour vary greatly (Rogel-Gaillard *et al.,* 2009). Genetic studies have focused on the European geographical expansion of this species (Monnerot *et al.*, 1994; Branco *et al.,* 2000 and Queney *et al.,* 2001). A very strong phylogeographical pattern of two highly divergent mtDNA lineages in Iberian wild rabbits was observed by Monnerot *et al.* (1994) and Branco *et al.* (2000), while Queney *et al.* (2001) defined the main routes of rabbit migration and observed structuring of genetic diversity in French wild rabbit populations. Geographical isolation of the populations could lead to substructuring through drift, mutation and different natural selection forces (Muchadeyi *et al.*, 2007). Social and reproductive

organisation and viral epizootics can have an impact on genetic diversity (Mounolou *et al.,* 2003). Genetic markers are also powerful tools to assess genetic variation within and between domestic stocks in a conservation programme for genetic resources (Bolet *et al.,* 2000; 2002; Berthouly *et al.,* 2008).

In Tunisia, indigenous rabbits are raised by smallholder farmers with few resources (Ben Larbi *et al.*, 2008). Village rabbit kept under smallholder-low input systems are considered important genetic resources that should be protected against production threats. Characterisation of these genetic resources will serve as an essential prerequisite for the identification and effective management and utilisation of Tunisian indigenous rabbits, which will facilitate their conservation.

In the present study, we used 36 microsatellite markers, to elucidate the degree and pattern of genetic variability in 12 indigenous rabbit populations in Tunisia, and to explain their genetic relationship.

MATERIALS AND METHODS

Sample selection

A random sample of local rabbit populations was conducted from 12 villages of Tozeur and Kebili regions (Figure 1), with the support of the Office of Livestock and Pastures regional agencies. Samples from the same village were considered as a population. A total of 264 rabbits were tested using molecular markers. Details about the breed origin and the number of animals per breed are given in Table 1.

Microsatellite genotyping

Blood samples were collected from the marginal ear vein into 5 mL vacutainer tubes containing EDTA as anticoagulant and stored at 4°C until molecular analyses were performed. To determine levels and patterns of genetic variation, a set of 36 microsatellites was used, distributed throughout the rabbit genome. The selection of the microsatellites panel was obtained from previous studies (Mougel, 1997; Chantry-Darmon *et al.*, 2005). Genomic DNA samples were extracted from white blood cells. Blood samples obtained from Tunisian rabbits were transported to the animal
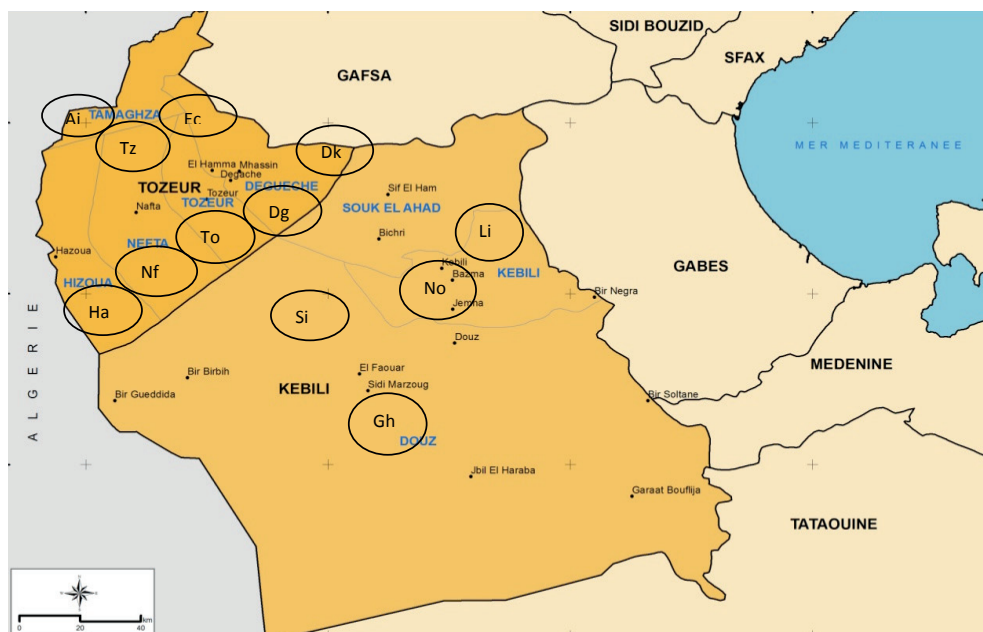

Figure 1: Geographical sampling locations.

Table 1: Rabbit samples used in this study.

Region	Localities	Code	Sample Size (n)
Tozeur	Aïn Karma	Ai	26
(n=182)	Dkoumes	Dk	25
	Dguech	Dg	24
	Echbika	Ec	11
	Hazoua	Ha	13
	Nefta	Nf	39
	Tozeur	To	19
	Tamaghza	Tz	25
Kebili	Ghlissia	Gh	13
(n=82)	Limaguess	Li	41
	Nouail	No	17
	Siidaine	Si	11

genetic analysis laboratory LABOGENA in Jouy-en-Josas, France. DNA extraction was performed in automatically with a QiASymphony DNA kit (Qiagen). The PCR amplifications were carried out by the GIE LABOGENA. After amplification using fluorescent primers, PCR products were migrated on capillary sequencer (3730xl DNA Applied Biosystems Analyzer).

Genetic data analysis

Within population genetic diversity

To estimate genetic variation within populations, the total number of alleles, number of observed alleles per locus (Ao), expected heterozygosity (He) estimated by Nei (1987) and observed heterozygosity (Ho) were calculated with GENETIX 4.05 software (Belkhir *et al.,* 2004). Reduction in heterozygosity (F_{IS}) due to inbreeding per population was determined using the GENEPOP 4.1 program (Raymond and Rousset 1995; Rousset, 2008). Significance of non-zero F_{IS} values per population was established by permutation (1000 permutations per population).

Population subdivision and relationships among breeds.

The population differentiation pattern was described by a factorial correspondence analysis (FCA) of the individual multilocus genotypes. Pairwise distances between individuals were estimated from the proportion of shared alleles according to Bowcock *et al.* (1994).

Genetic differentiation among and within populations was estimated with the F-Statistics defined by Wright (1951); they were calculated according to Weir and Cockerham (1984) using Genetix 4.0 and FSTAT 2.8: intrapopulation structure was investigated using the F_{IS} parameter (observed individual heterozygosity compared to the theoretical one within breed; 0 means that the samples are from a panmictic population at Hardy-Weinberg equilibrium). Genetic differentiation between populations was estimated from the F_{ST} parameter (breeds heterozygosity compared to the overall heterozygosity; 0 means no differentiation between breeds). Moreover, values of pairwise genetic differentiation (F_{ST}) were computed for all pairs from the 12 populations. The Reynolds genetic distance (D_R) was calculated for each pair of populations based on allele frequencies (Reynolds *et al.*, 1983) using the GENETIX software, version 4.03 (Belkhir *et al.,* 2004).

An unrooted neighbour-joining (NJ) tree (Saitou and Nei, 1987) based on the genetic distance matrix of Reynolds was constructed with the NEIGHBOR program in the PHYLIP package version 3.69 (Felsenstein, 2005). In addition, reliability of the phylogenetic tree was tested with a bootstrap analysis (Felsenstein, 2005): NJ trees were constructed for each replicate (over 1000 bootstrap replicates) using the CONSENSE program to create a majority-rule consensus tree.

Table 2: Within-population summary statistics.

| Population	n	He	Ho	Ao	F_{IS}
Aïn Karma	26	0.45	0.4	3.16	0.13[a]
Dkoumes	25	0.54	0.56	4.47	0.14[a]
Dguech	24	0.58	0.46	4.25	0.22[b]
Echbika	11	0.42	0.37	3	0.15[a]
Ghlissia	13	0.39	0.3	2.86	0.26[b]
Hazoua	20	0.52	0.53	3.58	0.01[NS]
Limaguess	41	0.54	0.47	4.11	0.14[a]
Nefta	39	0.58	0.44	4.55	0.26[b]
Nouail	17	0.56	0.42	3.77	0.27[b]
Siidaine	11	0.46	0.35	3.33	0.29[b]
Tamaghza	25	0.57	0.51	4.08	0.12[a]
Tozeur	18	0.57	0.47	4.19	0.20[b]

Mean number of observed alleles (Ao), observed heterozygosity (Ho), expected heterozygosity (He) and intra-population structure (F_{IS}). [NS]non significant. Significant deviation from 0 at : [a]$P<0.01$ and [b]$P<0.001$.

Breed assignments.

To assign individuals to populations, we used a Bayesian model implemented in the STRUCTURE software version 2.2 (Pritchard *et al.*, 2000), which infers genetic clusters from a collection of individual multilocus genotypes and estimates the proportion of each individual genome pertaining to each cluster. A Markov chain Monte Carlo algorithm was used to group similar genotypes into clusters. The analysis was performed using the admixture model in STRUCTURE and assuming that allele frequencies were correlated among populations.

We performed analyses with 100000 steps, after a burn-in period of 500000 steps with an increasing number of clusters (K values), testing all values from 2 to 14, with 3 independent runs each.
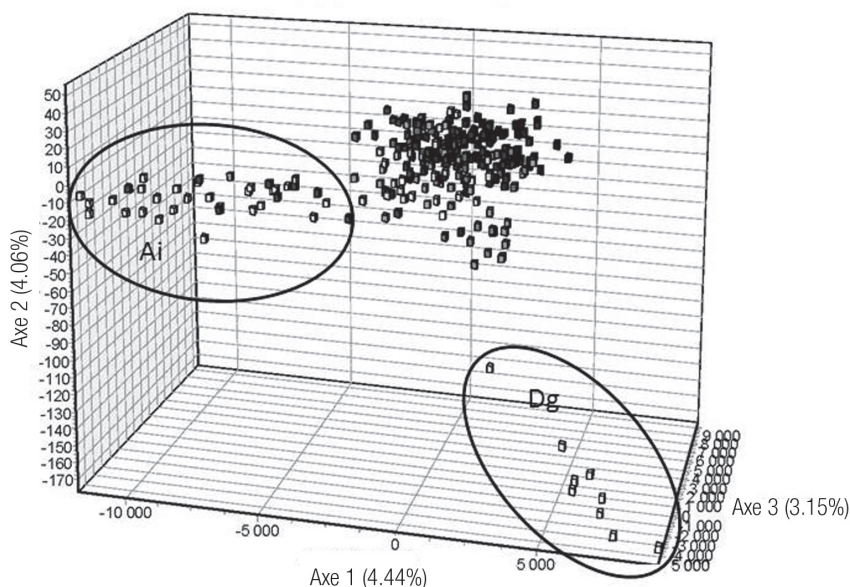
Figure 2: Factorial correspondence analysis based on the allele frequencies from 36 microsatellites loci genotyped in 12 rabbit populations. Ai and Dg populations are defined in table 1. The number between parenthesis represents the percentage of variability explained by each axis.

Table 3: Pairwise Reynolds's genetic distance (D_R) between pairs of rabbit populations (above the diagonal) and genetic differentiation (F_{ST}) between pairs of rabbit populations (below diagonal) as observed in this study.

|	Ai	Dk	Dg	Ec	Gh	Ha	Li	Ne	No	Si	Tz	To
Ai	-	0.17	0.17	0.20	0.23	0.19	0.18	0.16	0.16	0.20	0.10	0.13
Dk	0.20	-	0.09	0.142	0.18	0.10	0.10	0.06	0.09	0.13	0.09	0.07
Dg	0.18	0.08	-	0.15	0.18	0.13	0.11	0.08	0.09	0.11	0.10	0.07
Ec	0.27	0.14	0.15	-	0.19	0.18	0.16	0.13	0.13	0.19	0.16	0.13
Gh	0.28	0.19	0.18	0.23	-	0.23	0.17	0.17	0.16	0.22	0.20	0.17
Ha	0.20	0.08	0.11	0.19	0.23	-	0.12	0.05	0.12	0.13	0.14	0.10
Li	0.20	0.10	0.10	0.16	0.19	0.10	-	0.07	0.09	0.12	0.10	0.09
Nf	0.17	0.05	0.06	0.13	0.16	0.03	0.07	-	0.08	0.11	0.08	0.06
No	0.16	0.08	0.07	0.13	0.16	0.10	0.06	0.05	-	0.11	0.10	0.09
Si	0.25	0.14	0.09	0.24	0.26	0.14	0.13	0.11	0.10	-	0.14	0.13
Tz	0.11	0.08	0.08	0.17	0.21	0.11	0.09	0.06	0.06	0.12	-	0.07
To	0.13	0.06	0.05	0.14	0.17	0.08	0.08	0.04	0.06	0.11	0.05	-

Abbreviations as in Table 1.

RESULTS

Genetic diversity and differentiation

All typed microsatellite loci were polymorphic. The number of alleles per locus expected and observed heterozygosity and F_{IS} per populations are shown in Table 2. A total of 119 alleles were observed for the 36 loci surveyed across the 12 populations. The number of alleles per locus per population ranged between 2 (INRA0105, INRA0143 and INRA0274) and 18 (INRA0172) with an average of 3.30 (Table 2).The Nefta (Nf) population showed the highest mean effective allele number (Ao=4.55), while Ghlissia (Gh) was the population with the lowest locus variability (Ao=2.86). We documented moderate levels of heterozygosity within each study area. Average expected heterozygosity (He) across all the populations varied between 0.39 (Gh) and 0.58 (Dguech [Dg] and Nf). Overall, the highest genetic diversity measured by observed heterozygosity (Ho) was reported in Dkoumes (Dk) population (0.56), while the lowest genetic diversity was shown in Gh population (0.3) (Table 2). All values were statistically different from zero, with the exception of the Hazoua (Ha) population. This deficit of heterozygotes ranged from 13% (P<0.01) for Aïn Karma (Ai) population, to 29% (P<0.001) in the Siidaine (Si) population.

A Factorial Correspondence Analysis (FCA) was performed including all animals and loci to summarise individual relationships. The two first dimensions of the FCA analysis showed a clear separation between 3 groups representing Ai, Dg and all other populations (Figure 2). A total of approximately 12% of the variance accounted for the first three dimensions of the FCA (Figure 2). Axis 1 (approximately 4.4% of total variance) separated Dg population from the other populations, while Axis 2 (4%) further separated Ai population from the rest.
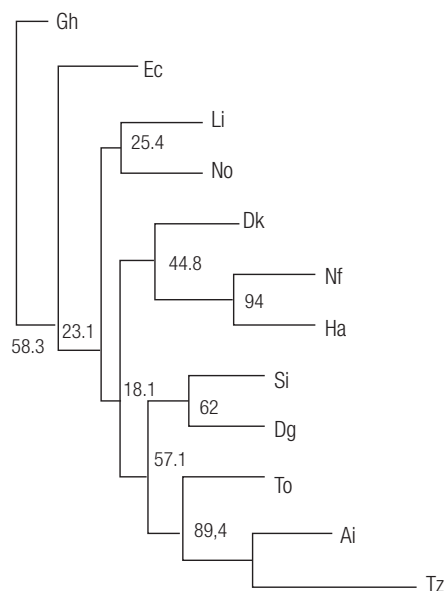
Figure 3: Neighbour-joining tree for the 12 Tunisian rabbit populations. Figures at nodes represent the bootstrap values over 1000 samples. Abbreviations as in Table 1.
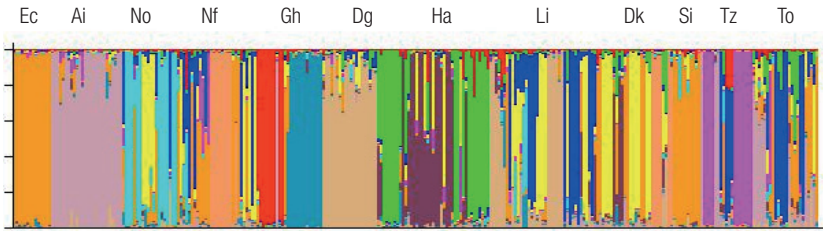
Figure 4: Estimated population structure for 12 indigenous Tunisian rabbit in this work at K=12. The colours represent the percentage of each cluster that is present in each rabbit population. Bayesian clustering of the 264 Tunisian rabbits, for K=12 clusters. Animals are sorted by initial population. Abbreviations as in Table 1.

Population subdivision and relationships among breeds

The F_{ST} values between pairs of breeds, using all data, indicate a generally high level of genetic differentiation, ranging from 0.03 (between Nf and Ha) to 0.28 (between Ai and Gh) (Table 3). The overall genetic differentiation among populations (F_{ST}) value using all data was low (1.1%).This implies that 98.9% of the total genetic variation was explained by individual variability.

Allele frequencies were also used to estimate Reynolds genetic distances for each pair of populations. The NJ tree was obtained using the Reynolds genetic distances (Figure 3).

Bootstrap values indicate that the clustering of Ai and Tamaghza (Tz) (89.4) is stable, as well as that of Nf and Ha (94). All other values were lower than 70. Bootstrap values of 70 or higher are likely to indicate reliable groupings (Baldauf, 2003).

Breed assignment

We investigated the breed assignment of population structure within domestic indigenous rabbits by means of Bayesian clustering. No clear plateau of the likelihood was evidenced for varying K, and convergence became more and more difficult for larger K. Therefore, we chose to present the results for K=12 (Figure 4). The percentage of correct assignment ranged between 85 to 100% depending on the population of origin.

DISCUSSION

In our study, a set of microsatellite markers, well spread over the genome, were used to analyse the genetic diversity of 12 Tunisian indigenous rabbit breeds in Tozeur and Kebili provinces and the relationship among them. The number of alleles observed in these 12 Tunisian indigenous populations was higher than that observed in European rabbit breeds (from 3 to 4.2) using microsatellite DNA markers (Bolet *et al.*, 2000). Tunisian rabbit populations exhibit the same genetic diversity compared to Egyptian and Spanish breeds (Ho ranging from 0.36 to 0.48) (Grimal *et al.*, 2012). These results are in general similar to those of previous studies (Bolet *et al* 2000; Queney *et al.*, 2001; Carneiro *et al.*, 2011) on patrimonial breeds. This finding supports the utility of microsatellites in genetic diversity evaluation of indigenous Tunisian rabbits. Moreover, this study revealed the fact that all microsatellite markers used were high polymorphic. Significant positive F_{IS} values were observed for all populations investigated, suggesting a lack of heterozygotes. The deficit of heterozygotes with respect to the Hardy-Weinberg hypothesis may be attributed to inbreeding, due to the lack of gene diversity in these breeds, and/or Wahlund effect (population substructure), due to the pooling of samples (within breed) from different breeding flocks.

The average genetic differentiation between all breeds (F_{ST}) was 0.11 (*P*<0.05), revealing moderate discrimination between the 12 indigenous rabbit populations investigated by 36 markers. This level of differentiation, although low, is within the range reported in the literature for F_{ST} values in rabbit (Bolet *et al.*, 2002; Grimal *et al.*, 2012). Higher F_{ST} values were previously reported in studies carried out on European populations (Carneiro *et al.*, 2011). A lack of specific selection strategies, founder effects, genetic drift and geographical isolation of the study area may have

contributed to the moderate level of differentiation among the Tunisian rabbit populations investigated. The largest branch length was seen for the Ai population from Ain Karma village, which is isolated, with difficult access; this explains its difference from the other populations shown in Figure 2: this isolated population does not show great variability. Dg is a population that had migrated from Tozeur area to Kebili. Tz is a population of Tamaghza village with high heterozygosity, and is near to Ai. The Nf population of Nefta village shows a high heterozygosity. Ha is an intermediate population between the populations of the governorate of Tozeur and Kebili. Dk is an isolated population with large variability.

The Bayesian clustering indicated a weak subdivision between indigenous rabbit populations. The data support the clustering of breeds by geographical proximity. Although the Bayesian analysis revealed the highest probability of forming 12 clusters, it needs to be interpreted with caution, as some of the clusters were not very well defined. In this study, the variation between breeds and regions has decreased because of the gradient of geographical proximity between them. The genetic differentiation between the indigenous Tunisian rabbits investigated is therefore mainly not discernible among regions.

These molecular data show that indigenous rabbits represent a high variability, which is consistent with the lack of selection programmes in the past and also the absence of bottleneck effect or high genetic drift. However, we cannot identify a structuring of indigenous rabbits sampled according to the province's geographic origin. The use of allelic frequencies to study genetic differentiation among local populations living and adapting to the natural environment involves several forces (mutation, genetic drift, migration and selection). Geographic isolation is an important factor of differentiation between populations, but no clear geographic pattern was observed in our analysis. In this case, it seems that the migration is related to the socio-cultural role of indigenous rabbit, which may explain the lack of clearly identified structure between local Tunisians, despite the significant F_{ST} values observed between them. This analysis allows us, then, to consider animals from different provinces studied as a single population.

CONCLUSION

This study is the first to use microsatellite DNA markers to understand the genetic diversity of local rabbit populations in Tunisia. Very little information is currently available to compare different rabbit populations from Tunisia. Knowledge of genetic diversity is paramount to the conservation of these populations, but also for the identification of loci involved in economically important traits. This work discussed the genetic variation within the population and among the populations which can contribute to the scientific theory of protecting and using rabbit resource. Microsatellites also reveal a high degree of geographical structuring, although incompatibility of datasets again limits the scope of most studies to the regional scale. The knowledge thus generated would enable prioritisation and monitoring of the indigenous Tunisian rabbit biodiversity for its efficient management, improvement and conservation.

This study therefore provides the basic information for the design of genetic improvement and conservation programmes for the Tunisian indigenous rabbits investigated, which have to date faced general neglect and apathy.

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