World Rabbit Sci. 2022, 30: 201-207
doi:10.4995/wrs.2022.17235
WRSA, UPV, 2003

MITOCHONDRIAL D-LOOP SEQUENCES AND HAPLOTYPES DIVERSITY IN EGYPTIAN RABBIT BREEDS

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Abstract: Rabbit breeds in Egypt are local and adapted foreign breeds that have been imported since the middle of the last century. Stressful environmental conditions including climatic changes, exposure to diseases and breeding selection have an influence on how gene flow has shaped the genetic diversity of the breeds. Mitochondrial DNA D-loop is a genetic marker used to trace the geographic distribution of genetic variation for the investigation of expansions, migrations and other gene flow patterns. The study aimed to determine the genetic diversity of the mitochondrial DNA D-loop (mtDNA D-loop) in Black Baladi, Red Baladi, Gabali, APRI line and New Zealand breeds to gather the scientific data required to create a proper conservation and sustainable management plan. Blood samples were taken from animals unrelated to each other. A 332-bp of mtDNA D-loop was successfully amplified and alignment sequences were deposited in the GenBank database. The results detected six haplotypes in the five breeds. Haplotype diversity within individual breeds varied from 0 (Red Baladi) to 0.551±0.114 (Gabali). The nucleotide diversity (π) value was relatively low (0.001-0.006), with greater values in APRI and New Zealand. Pairwise distances between breeds yielded varying values ranging from 0 to 0.254, and the values between the Red Baladi and other breeds were comparatively high, with pairwise distances from 0.172 to 0.254. The phylogenetic analysis involved 74 nucleotide sequences of the Egyptian rabbit and thirty-one sequences retrieved from GenBank of the reference samples of different haplogroups. The results of the phylogenetic analysis correlated to the reference mtDNA GenBank database showed that the five Egyptian rabbit breeds were grouped into haplotypes A, B and K. The results of the genetic diversity using mtDNA shed light on the importance of the local breed's genetic diversity information and revealed unique mtDNA haplotypes, which is an important finding for breeding strategies designed to conserve genetic variants and provide sustainable management.

Key Words: rabbit breeds, mitochondrial DNA, genetic diversity, haplogroup.

INTRODUCTION

Egypt is the fourth country in rabbit production, with 7.6 million head of rabbits (Emam *et al.*, 2020). Rabbit breeds in Egypt consist of local (Native and Gabali rabbits) and adapted foreign breeds that have been imported since the middle of the last century for breeding and crossbreeding to improve the fast productive and reproductive performance (Emam *et al.*, 2020). The exotic foreign breeds (New Zealand White, California, Buscat, Chinchilla, Flemish Giant, Papillon, and Rex) and lines (Spanish V-line and French Hy-plus) became a popular target among smallholders as a result of the widespread increase in the number of rabbit farms (Galal and Khalil, 1994).

Correspondence: S. Ahmed, *selnahta@yahoo.com*. Received *March 2022* - Accepted *July 2022*.
https://doi.org/10.4995/wrs.2022.17235

Cite as: Ahmed S., Ali N.I., Abdelhafez M.A., Darwish H.R., El-Keredy A. 2022. Mitochondrial D-loop sequences and haplotypes diversity in Egyptian rabbit breeds. *World Rabbit Sci., 30: 201-207. https://doi.org/10.4995/wrs.2022.17235*

Egyptian local rabbit breeds, lines and strains number eight in total (FAO, 2013; Emam *et al.*, 2020). The native (Baladi) rabbit breed is an Egyptian domestic rabbit breed found in rural areas of Delta, Middle and Upper Egypt. In 1934, native rabbits breeding black and albino colours were isolated. In 1937, selective breeding took place for the albino strain that has a faster growth rate and larger litter size, currently known as the Giza White breed (Khalil, 1997). To improve Egyptian native rabbits, crossbreeding was carried out for several generations that began with inbreeding, followed by crossbreeding between native rabbits does and Flemish Giant (FG) bucks. The offspring were separated according to fur colour into three groups (red, black and white). The Egyptian Red Baladi (RB), Black Baladi (BB) and White Baladi (WB) breeds continued to evolve according to the colours, and the does of each breed have been mating with bucks of the same colour for several generations. (Galal and Khalil, 1994; Khalil, 1997 and 2002). The Ministry of Agriculture and Land Reclamation in Egypt and FAO reported that the Egyptian White Baladi breed had become extinct (MA and LR, 2003; FAO, 2010). Another report by Emam *et al.* (2016) demonstrated that the native Red and Black Baladi breeds are endangered. In 2005, the Alexandria line was established from the crossing of Spanish V-line rabbits (maternal line) with Black Baladi (paternal line) rabbits (El-Raffa, 2007; El-Sabrout *et al.*, 2017).

The APRI line breed is an Egyptian line that was developed by the animal production research institute (APRI) through mating Red Baladi (paternal line) to Spanish V-line rabbits (maternal line), obtaining the F1, F2 and then F3; at this generation, selection for litter weight at weaning was started (Youssef *et al.*, 2008; Abou Khadiga *et al.*, 2010).

The Gabali rabbit is a medium-sized breed that has two strains, one from the north-western desert coast of Egypt (Mariout) and the other in Sinai. Gabali Mariout and Sinai were successfully domesticated from a feral habitat to caged production through cooperation projects between the Ministry of Agriculture, the Desert Research Centre and the Faculty of Agriculture of Moshtohor University from 1992 to 1994 (MA and LR 2003; Galal, 2007).

Diversity of animal genome, including mitochondrial genome (mtDNA), has been described as the basic data required to create a proper conservation and sustainable management plan (Caird *et al.* 2019). MtDNA polymorphism analyses are useful in the studies of genetic variation and species evolution. Moreover, mtDNA is used as a molecular marker for the characterisation of genetic resources to study the genetic diversity and phylogenetic relationships between different breeds (Slaska *et al.*, 2014).

The mitochondrial D-loop region is the most variable part of mtDNA, due to a higher substitution rate than in the rest of the mitochondrial genome (Pierpaoli *et al.*, 1999; Ahmed *et al.*, 2017; Owuor *et al.*, 2019). This study aimed to determine the genetic diversity of the mitochondrial DNA D-loop (mtDNA- D-loop) in Black Baladi, Red Baladi, Gabali, APRI line and New Zealand breeds to obtain the scientific data required to create a proper conservation and sustainable management plan.

MATERIALS AND METHODS

Samples

A total of 74 blood samples were collected from the Black Baladi (BB,15), Red Baladi (RB, 12), Gabali (GB, 17), New Zealand (NB, 15), and APRI line (AP, 15) breeds. The samples were taken from animals that were unrelated to each other, to avoid sampling animals from the same doe.

Genetic analysis

Genomic DNA was extracted according to John *et al.*, (1991). Polymerase chain reaction (PCR) amplifications were conducted using primers, F: CCACCATCAGCACCCAAAGCT; R: ATTTAAGAGGAACGTGTGGG according to Nguyen *et al.*, (2003). The reactions ran in Thermal Cycler Bio-Rad (T100, USA) and were cycled for 5min at 95°C, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 54°C for 35 s, extension at 72°C for 1 min and final extension at 72°C for 7 min. The PCR products were loaded onto 2% agarose gels (with 100-bp DNA ladder) for electrophoresis separation to gauge the success of PCR reactions. Successfully obtained amplicons were purified by GeneJET gel extraction Kit (Thermo Lithuania). All purified PCR products were sent to the Macrogen Company (South Korea) for forward direction sequencing.

Statistical analysis

Sequences were aligned, trimmed and inspected using GENEIOUS 6.0 software (New Zealand; Kearse *et al*., 2012) and exported in FASTA format. Thirty-one sequences representative of different haplogroups were downloaded from GenBank (Table 1) and added to our dataset as comparison of global rabbit diversity. The haplotype diversity (π) was calculated in DNA SP6 (Rozas *et al*., 2017); and nucleotide diversity (π), in ARLEQUIN (Excoffier *et al*., 2010). The hierarchical distribution of total genetic diversity was determined using an analysis of molecular variance (AMOVA), as implemented in ARLEQUIN. Pairwise differences between breeds were determined using ARLEQUIN (Barcelona, Spain). The DNA SP6 was used to generate a haplotype file, and to identify haplotypes occurring in more than one individual, to ensure that each unique haplotype occurs only once during phylogenetic analysis. The consolidated dataset was then used for further analysis. MEGA X (Tamura *et al*., 2018) was used to determine the model of nucleotide substitution that best suited our dataset and to construct a Maximum Likelihood tree to identify the relationships between the breeds sampled and global breeds, with 1000 bootstrap replications.

RESULTS

A successful 332-bp mtDNA D-loop was amplified from all the breeds sampled. A total of 74 alignment sequences were deposited in the GenBank database (accession numbers OK491137-OK491210). Parsimony informative sites

Table 1: Sequence data list from Gene bank used in this study.

Breed	Haplotype	Accession Number
Domestic French Lop	B	AJ293839.1
Wild rabbit (Australia)	D	U62927.1
European Wild Rabbit (Spain)	B	KT029920.1
Spanish common rabbit	B	KT029974.1
Zika (Germany great line)	A	AF534100.1
Domestic French Lop	A	AJ293840.1
Black Baladi (Egypt)	A	KT030001
Gabali (Egypt)	A	KT030031.1
Black Baladi (Egypt)	A	KT029985.1
Japanese White (China)	A	AF534104.1
Yufeng Brown (China)	A	AF534105.1
Qixing (China)	A	AF534080.1
Wild rabbit (Australia)	B	U62925.1
Spanish breed	B	Z83367.1
Yufeng Brown (China)	A	AF534099.1
Zhenhai thick-hair Angora (China)	A	AF534103.1
Spanish breed	B	Z83366.1
Black Baladi (Egypt)	K	KT029980.1
Red Baladi (Egypt)	K	KT030044.1
Gabali (Egypt)	K	KT030011.1
Zika (Germany great line)	A	AF534107.1
Zika (Germany great line)	A	AF534106.1
Spanish breed	B	AJ293834.1
French breed	B	AJ535787.1
Domestic Australia rabbit	A	AF003190.1
Spanish breed	B	AJ535812.1
Spanish breed	B	Z83354.1
Spanish breed	B	AJ535802.1
French breed	B	AJ535811.1
French breed	A	Z83341.1
French breed	A	Z83344.1

revealed 16 site positions: 14, 62, 74, 96, 118, 121, 125, 130, 139, 166, 223, 235, 286, 287, 293, 313. The results detected six haplotypes in the five breeds. The RB breed showed only one haplotype, while there were two haplotypes in NZ and BB, and three haplotypes in AP and GB. Hap_2 was observed with the highest frequency among the Egyptian rabbits, in all breeds under study compared to other haplotypes (Table 2).

The average value of the haplotype diversity between breeds (Hd) was 0.417, with a standard deviation of 0.069. Haplotype diversity within individual breeds varied from 0 (RB) to 0.551±0.114 (GB). The nucleotide diversity (π) value was a relatively low (0.001-0.006), with greater values in AP and NZ (Table 3).

The phylogenetic analysis involved 105 nucleotide sequences of the Egyptian rabbit (74) and sequences retrieved from GenBank of the reference samples (31) of different haplogroups (Table 1). Maximum Likelihood method was used for the phylogenetic analysis based on the Hasegawa-Kishino-Yano model (HKY+G+I), as determined by running a model test in MEGA X. The phylogenetic tree was obtained by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The circular phylogram tree shows the relationships between all rabbits included in this study and the GenBank reference samples for domestic and wild rabbits (Figure 1). All reference samples for haplogroups A, B, D and K were grouped according to previously identified haplogroups. The phylogenetic analysis correlated to the reference mtDNA GenBank database showed that the five Egyptian rabbit breeds were grouped into haplotypes A, B and K.

Pairwise distances between breeds yielded varying values ranging from 0 to 0.254 (Table 4); the values between the RB and other breeds were comparatively high, with pairwise distances of 0.172 to 0.254. The results demonstrated that the same value of pairwise distances (0.045) were observed in GB with AP and NB, while the lowest pairwise distance (0.04) was between NB and AP. Pairwise F_{ST} values were determined as an estimator of the mean genetic distance between rabbit breeds. In pairwise comparisons of F_{ST}, the GB pairwise distance values varied from 0 to 0.176 and with support of significant differentiation at $P<0.05$ with BB. For RB, pairwise distance values were 0 to 0.254 and with a significant difference from NB. No significant difference was detected among all other comparisons (Table 4).

DISCUSSION

The mitochondrial DNA D-loop is a genetic marker used to trace the geographic distribution of genetic variation, for the investigation of expansions, migrations and other gene flow patterns. It is reported to have a high rate of polymorphism (Pierpaoli *et al.*, 1999; Ahmed *et al.*, 2017; Owuor *et al.*, 2019).

The haplotype diversity in the five breeds under study revealed six haplotypes with common haplotype 2 in the five breeds (Table 2). The results of haplotype diversity and nucleotide diversity were identified as low (Table 3), in agreement with the results reported in Spanish, Italian, Kenyan rabbits and three Egyptian breeds (Pierpaoli *et al.*, 1999; Long *et al.*, 2003; Owuor *et al.*, 2019; Emam *et al.*, 2020). Their studies indicated that the low genetic diversity due to domestication and high selection pressure during commercial animal production leads to an inherent decrease in breed variability. The low haplotype diversity detected in Egyptian rabbit breeds could be because of the low genetic diversity in Egyptian rabbits, as the rabbit breeding was approximately dependent on the inbreeding programme of genetically close individuals, as reported in the breeding history, where closely related individuals were involved in breeding BB, RB and AP breeds that originated from the native breed and one maternal origin in rural areas of Delta,

Table 2: Haplotype frequencies between different Egyptian rabbit breeds.

Haplotype	AP	BB	GB	NZ	RB
Hap_1	------	-------	0.176±0.095	-----	-----
Hap_2	0.666±0.125	0.666±0.125	0.647±0.119	0.666±0.125	1.00±0.00
Hap_3	------	------	0.1760±0.095	-----	------
Hap_4	------	------	------	0.200±0.106	------
Hap_5	0.200±0.106	0.333±0.125	------	0.133±0.090	------
Hap_6	0.133±0.090	------	------	------	------

APRI line (AP), Black Baladi (BB), Gabali (GB), New Zealand (NB), and Red Baladi (RB) breeds.

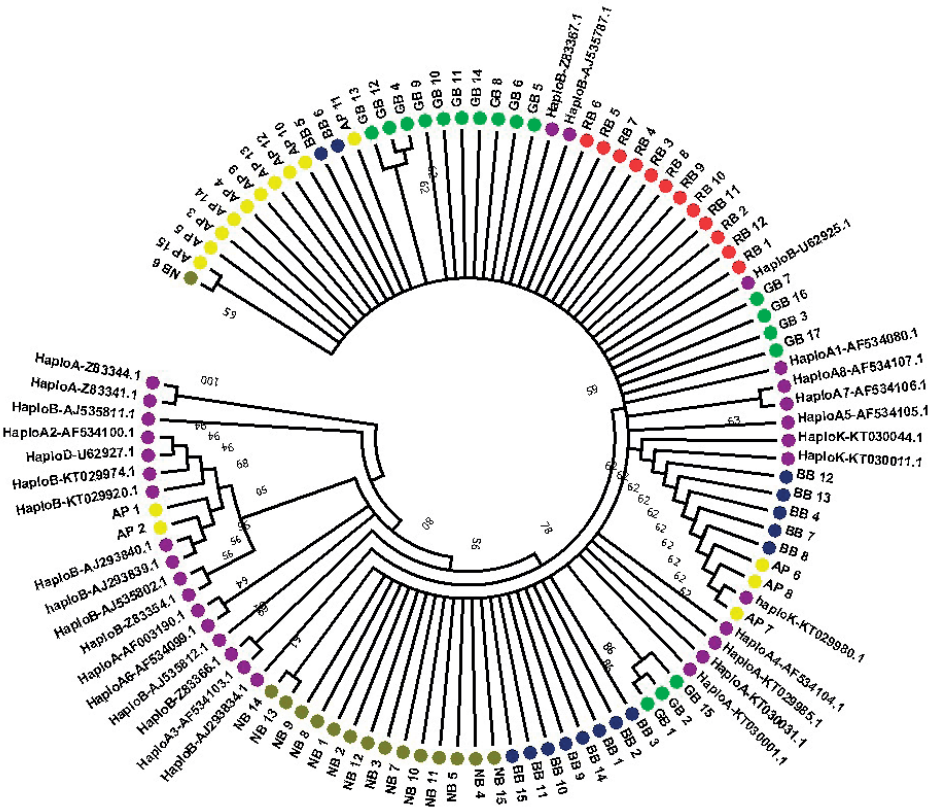

Figure 1: The maximum likelihood circular phylogram showing Egyptian rabbit breeds, and 31 references of the global several lineages of rabbits. APRI line (AP), Black Baladi (BB), Gabali (GB), New Zealand (NB), and Red Baladi (RB) breeds.

Middle and Upper Egypt (Galal and Khalil, 1994; Khalil, 1997, 2002; El-Raffa, 2007; Youssef *et al*., 2008; Abou Khadiga *et al*., 2010; El-Sabrout *et al*., 2017), while the GB is a domesticated native from a feral habitat (Mariout) (MA and LR, 2003; Galal, 2007).

Pairwise distances between breeds showed a significant genetic distance between GB and BB breeds (Table 4), which is most likely related to the different geographical origins of GB and BB. The GB is a domesticated breed from desert areas, compared to the BB from the agroclimatic regions. Moreover, almost all BB grouped to haplotype K and A, whereas almost all GB grouped to haplotype B. The results showed that the genetic distance between RB and NB breeds was significant. This is justified by the RB being from haplotype B, while almost all NB grouped to haplotype A.

Table 3: Number of haplotypes following alignment and trimming, number of different haplotypes, haplotype diversity and nucleotide diversity (means and standard deviation) in five Egyptian rabbit breeds.

Breeds	Number of haplotypes	Haplotype diversity	Nucleotide diversity
AP	3	0.533±0.126	0.006± 0.003
BB	2	0.476±0.092	0.001± 0.0002
GB	3	0.551± 0.114	0.003± 0.001
NZ	2	0.247± 0.131	0.0004± 0.0002
RB	1	0	0

APRI line (AP), Black Baladi (BB), Gabali (GB), New Zealand (NB), and Red Baladi (RB) breeds.

Table 4: Computing conventional F-Statistics from haplotype frequencies expressed as the average number of pairwise differences (F_{ST}) between breeds (below the diagonal), with significance values above the diagonal.

	GB	RB	BB	AP	NB
GB	0	0.99	0.045*	0.189	0.081
RB	0.176	0	0.054	0.063	0.036*
BB	0.095	0.254	0	0783	0.063
AP	0.045	0.172	0.032	0	0.261
NB	0.045	0.172	0.091	0.04	0

Values marked with * denote breed pairs where the hypothesis of a lack of significant differentiation is rejected at $P<0.05$. APRI line (AP), Black Baladi (BB), Gabali (GB), New Zealand (NB), and Red Baladi (RB) breeds.

Other genetic distances between breeds revealed non-significant differences that could be explained by the sharing of haplotypes between breeds.

The phylogenetic analysis correlated to the reference mtDNA GenBank database showed that the five Egyptian rabbit breeds were grouped into haplotypes A, B and K (Figure 1). The majority of Egyptian rabbit breeds sampled grouped with haplogroup A and B, but nine animals grouped with haplogroup K (from BB and AP). Haplotype A was reported in Asian and European domestic rabbit breeds (California, New Zealand White, Rex, Angora, Dwarf, Belgium) and wild rabbits from Australia and Spain (from published sequences and those of this study), while haplotype B presented in wild rabbits (Spain), Spanish common rabbit and French breeds (GenBank database). Emam *et al.* (2020) indicated that the haplotype A was diverged from one site with haplotype K, and haplotype B was differed from haplotypes J and I and French Lop (European domestic breed). The results suggested that the Egyptian rabbit breeds have different haplogroup diversity according to the geographic distribution and admixture of breeding evolution.

CONCLUSION

The results shed light on the importance of the local breed's genetic diversity information and reveal unique mtDNA haplotypes, which is important for breeding strategies designed to conserve genetic variants and provide sustainable management of the local breed's genetic resources. As a next step, genetic characterisation of rabbit breeds using nuclear DNA markers with the association of quantitative traits is required for sustainable management.

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