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MOLECULAR GENETIC DIVERSITY OF SOME RABBIT BREEDS BASED ON MITOCHONDRIAL 16S rRNA SEQUENCES

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Abstract: The present study was performed to assess the genetic variations among six rabbit breeds in Egypt based on mitochondrial 16S rRNA sequences. The length of partial mitochondrial 16S rRNA in the six rabbit breeds ranged from 546 bp to 558 bp. The sequenced regions were submitted to GenBank/NCBI under accession numbers (MW052052 - MW052057). The average content of A+T was 57% in all breeds. Among breeds, the percentages of genetic distance values were ranged from 0.000 to 0.004. The highest *P*-distance (0.004) was found between the New Zealand White breed and all other breeds. The results support the suitability of mitochondrial 16S rRNA for genetic diversity analysis of rabbit breeds and the applicability for future research on genetic relationships and the phylogeny of rabbit breeds.

Key Words: rabbit breeds, DNA, genetic diversity, mitochondrial 16S rRNA.

INTRODUCTION

Many rabbit breeds in Egypt are threatened by extinction due to the low number of living individuals, and some breeds already no longer exist (Khalil and Baselga, 2002). The assessment of genetic variations in a species is important for its conservation and further breeding (Rahimi *et al.*, 2005). Estimates of the genetic variabilities in rabbits could be useful for designing suitable breeding strategies that aim to adapt breeds to meet the changeable markets and consumers' requirements (Groeneveld *et al.*, 2010). Genetic variability was crucial for breeders that aimed to improve the quality and characteristic of the existing breeds in response to environmental changes and disease outbreaks (Galal *et al.*, 2013). Rabbits display special phenotypic diversity and have great commercial societal value as production animals. They also serve as important animal models in biomedical research (Carneiro *et al.*, 2011). Studying the genetic diversity among breeds facilitates proposals for preservation and breeding plans. It provides us with valuable information to help understand domestication and evolution history. Furthermore, it raises studies that aim to make links between phenotype and genotype (Ben Larbi *et al.* 2012; Badr *et al.* 2016).

The phylogeography and genetic diversity of European rabbit have been widely studied through several markers such as mitochondrial DNA (Biju-Duval *et al.*, 1991), as well as proteins (Ferrand, 1995) and microsatellites (Mougel, 1997; Queney, 2000). Mitochondrial DNA (mtDNA) is a commonly-used tool for the identification of animal species because of the high copy number per cell and high mutation rate, which reveal sequence diversity among closely-related species (Dooley *et al.*, 2004; Girish *et al.*, 2004; Pakendorf and Stoneking, 2005). Of the mitochondrial genes, the 16S rRNA gene has been considered a suitable marker for species identification (Rojas *et al.*, 2009). In the same context, Yang *et al.* (2014) mentioned that the rRNA genes code for homologous structures in different species ranging from bacteria to humans because many nucleotides are very similar, whereas these genes exhibit inter- and intraspecific nucleotide variations.

We used 16S rRNA to reveal the molecular relationships of rabbit breeds and assess the molecular relationships of domestic rabbit breeds of *Oryctolagus cuniculus* and other species of the Leporidae family.

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MATERIALS AND METHODS

Study animals

In this study, we tested thirty rabbits from six breeds; Baladi Black (BB), California (CAL), Chinchilla (CHN), Flander (FLA), New Zealand White (NZW), and Rex (REX). All rabbits were healthy. The muscle tissues were separated and preserved at -20°C until used.

DNA extraction

Total genomic DNA was extracted from the preserved muscle tissues using Mini kit (Qiagen, Hidden, Germany) following the manufacturer's instructions and protocol.

PCR amplification and Sequencing

The target region was amplified using polymerase chain reaction (PCR) with primers 16sar and 16sbr (Simon *et al.*, 1991). The PCR reactions were carried out with 10 pmol of each primer, ~100 ng of genomic DNA, and 25 µL PCR master mix in a final reaction volume of 50 µL. PCR reaction was carried out with an initial denaturation for 4 min at 95°C, followed by 35 cycles for 30 s at 95°C, annealing for 30 s at 48°C, and an extension at 72°C for 5 min. The PCR products were run on 1.2% agarose gel stained with ethidium bromide.

The PCR amplification resulted in a single band in all breeds. All sequences were achieved by Macrogen (South Korea), using the same primer used in amplification. The sequenced regions of 16S rRNA in six rabbit breeds were submitted to the (GenBank/NCBI) to obtain accession numbers for each breed.

Phylogenetic tree construction

Sequence alignment was performed using MUSCLE (Edgar, 2004) with default settings. We used MEGA version 7.0 18 (Kumar *et al.*, 2016) to perform the analyses of the phylogenetic trees by using two phylogenetic methods; Maximum likelihood (ML) and UPGMA method (UM) using 1000 bootstrap iterations (Felsenstein, 1985). Sequence divergences were calculated using Kimura 2-parameter distances (Kimura, 1980) to supply a graphical representation of divergence.

RESULTS

From the PCR amplification, we obtained a single band in each breed. The sequenced regions of 16S rRNA in the six breeds were deposited in the (GenBank/NCBI) under accession numbers (MW052052 - MW052057). Sequences of 16S rRNA after BLAST/N at (NCBI) revealed a relationship with 11 species of Family Leporidae, Order Lagomorpha: *Lepus timidus*, *Lepus arcticus, Lepus californicus*, *Lepus othus*, *Lepus europaeus, Lepus americanus*, *Lepus microtis*, *Lepus brachyurus*, *Oryctolagus cuniculus*, *Sylvilagus floridanus* and *Sylvilagus bachmani,* in addition to three outgroup species; *Ochotona thibetan*a, *Ochotona macrotis* and *Ochotona cansus* (Table 1).

The sequencing length of 16S rRNA in the six rabbit breeds ranged from 546 bp to 558 bp. The results indicated that FLA breed had the longest nucleotide sequences (558 bp), while both breeds BB and CHN had the shortest nucleotide sequences (546 bp). The average nucleotide frequencies of adenine (A) thymine (T), cytosine (C), and guanine (G) were 30.3, 26.7, 22.2, and 20.8%, respectively. In all breeds the A+T contents were higher than C+G contents. The average content of A+T was 57%. The base pair length, nucleotide number percentages, and average content of A+T and C+G are summarised in (Table 2).

Pairwise genetic distances among breeds and other species of Leporidae family additions to the outgroup were estimated by MEGA version 7.0 18 (Kumar *et al.*, 2016) using the K2P method with gamma correction. The *P*-distances among all samples ranged from 0.000 to 0.027%. Overall, the distance value was 0.93%. The genetic distance among breeds ranged from 0.000 to 0.004. The highest *P*-distance (0.004) was found between the NZW and all other breeds (Table 3). Based on the 16SrRNA sequences, the genetic distance values among breeds and

Table 1: The understudying rabbit breeds and their related species with outgroup from the GenBank/ NCBI based on (16S rRNA) gene sequences.

No.	Breeds / Species	Accession number
1	Baladi Black	MW052052
2	California	MW052053
3	Chinchilla	MW052054
4	Flander	MW052055
5	New Zealand White	MW052056
6	Rex	MW052057
7	*Oryctolagus cuniculus*	DQ334838.1
8	*Sylvilagus floridanus*	DQ334836.1
9	*Sylvilagus bachmani*	DQ334837.1
10	*Lepus timidus*	DQ334832.1
11	*Lepus arcticus*	DQ334831.1
12	*Lepus californicus*	DQ334834.1
13	*Lepus othus*	DQ334830.1
14	*Lepus europaeus europaeus*	DQ334835.1
15	*Lepus americanus*	DQ334833.1
16	*Lepus microtis*	KJ193116.1
17	*Lepus brachyurus*	LC500147.1
Out group	*Ochotona thibetana*	MN547475.1
	Ochotonamacrotis	MN547451.1
	Ochotona_cansus	MN547420.1

species of Family Leporidae from GenBank/NCBI ranged from 0.000 to 0.027%. The species most related to the domestic rabbit breeds were *Oryctolagus cuniculus* from GenBank (DQ334838.1) followed by *Sylvilagus bachmani* and *Sylvilagus floridanus* (Table 3).

Phylogenetic analysis

We used two phylogenetic methods; Maximum likelihood (ML) and UPGMA method (UM) to execute the phylogenetic analysis based on 16S rRNA sequences among the selected domestic rabbit breeds along with 11 related species of Family Leporidae from GenBank/NCBI and three outgroup species. The methods showed six main features: (1) The outgroup species formed a separate cluster; (2) all species from genus *Lepus* grouped together with sister groups within; (3) species from the genus *Sylvilagus* formed a separate cluster; (4) *Oryctolagus cuniculus* from GenBank (DQ334838.1) was related to the included domestic rabbit breeds; (5) NZW breed formed a separate cluster apart from the other rabbit breeds, and *Oryctolagus cuniculus* (DQ334838.1) and (6) NZW breed formed a basal clade for all other rabbit breeds, and *Oryctolagus cuniculus* (DQ334838.1) (Figures 1 and 2).

Table 2: Nucleotide frequencies and their average of 16S rRNA sequence in 6 Rabbit breeds.

		Base pair	Nucleotide Number %				A+T Content	C+G Content
No.	Breeds	length	A	T	C	G	(%)	(%)
1-	Baladi Black	546	30.6	26.6	22.2	20.7	57	43
2-	California	547	30.0	27.1	22.1	20.8	57	43
3-	Chinchilla	546	30.4	26.6	22.2	20.9	57	43
4-	Flander	558	30.1	26.9	22.2	20.8	57	43
5-	New Zealand White	556	30.6	26.4	22.1	20.9	57	43
6-	Rex	553	30.2	26.6	22.4	20.8	57	43
	Average %	551	30.3	26.7	22.2	20.8	57	43

Table 3: Pairwise distances based on (16S rRNA) gene among six rabbit breeds and related species in addition to the outgroup.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	MW052052.1_Baladi_Black		0.000	0.000	0.000	0.004	0.000	0.000	0.014	0.015	0.016	0.017	0.016	0.016	0.016	0.018	0.016	0.016	0.024	0.025	0.025
2	MW052053.1_California	0.000		0.000	0.000	0.004	0.000	0.000	0.014	0.015	0.016	0.017	0.016	0.016	0.016	0.018	0.016	0.016	0.024	0.025	0.025
3	MW052054.1_Chinchilla	0.000	0.000		0.000	0.004	0.000	0.000	0.014	0.015	0.016	0.017	0.016	0.016	0.016	0.018	0.016	0.016	0.024	0.025	0.025
4	MW052055.1_Flander	0.000	0.000	0.000		0.004	0.000	0.000	0.014	0.015	0.016	0.017	0.016	0.016	0.016	0.018	0.016	0.016	0.024	0.025	0.025
5	MW052056.1_New_Zealand	0.007	0.007	0.007	0.007		0.004	0.004	0.015	0.016	0.017	0.018	0.017	0.017	0.017	0.019	0.017	0.017	0.026	0.026	0.026
6	MW052057.1_Rex	0.000	0.000	0.000	0.000	0.007		0.000	0.014	0.015	0.016	0.017	0.016	0.016	0.016	0.018	0.016	0.016	0.024	0.025	0.025
7	DQ334838.1_*Oryctolagus_cuniculus*	0.000	0.000	0.000	0.000	0.007	0.000		0.014	0.015	0.016	0.017	0.016	0.016	0.016	0.018	0.016	0.016	0.024	0.025	0.025
8	DQ334837.1_*Sylvilagus_bachmani*	0.074	0.074	0.074	0.074	0.082	0.074	0.074		0.008	0.018	0.018	0.017	0.017	0.017	0.018	0.017	0.017	0.022	0.024	0.023
9	DQ334836.1_*Sylvilagus_floridanus*	0.074	0.074	0.074	0.074	0.083	0.074	0.074	0.029		0.016	0.016	0.016	0.015	0.015	0.016	0.016	0.016	0.024	0.025	0.024
10	DQ334834.1_*Lepus_californicus_melanotis*	0.086	0.086	0.086	0.086	0.094	0.086	0.086	0.109	0.091		0.007	0.007	0.010	0.008	0.007	0.007	0.007	0.026	0.026	0.024
11	DQ334833.1_*Lepus_americanus*	0.097	0.097	0.097	0.097	0.106	0.097	0.097	0.105	0.094	0.024		0.009	0.011	0.010	0.010	0.009	0.009	0.026	0.027	0.024
12	DQ334831.1_*Lepus_arcticus*	0.089	0.089	0.089	0.089	0.097	0.089	0.089	0.103	0.091	0.024	0.031		0.009	0.009	0.009	0.000	0.000	0.025	0.027	0.024
13	LC500147.1_*Lepus_brachyurus*	0.089	0.089	0.089	0.089	0.097	0.089	0.089	0.099	0.085	0.036	0.047	0.034		0.009	0.011	0.009	0.009	0.026	0.026	0.026
14	DQ334835.1_*Lepus_europaeus_europaeus*	0.092	0.092	0.092	0.092	0.100	0.092	0.092	0.102	0.088	0.026	0.039	0.032	0.034		0.010	0.009	0.009	0.026	0.026	0.024
15	KJ193116.1_*Lepus_microtis*	0.104	0.104	0.104	0.104	0.112	0.104	0.104	0.109	0.097	0.021	0.036	0.031	0.044	0.039		0.009	0.009	0.028	0.027	0.025
16	DQ334830.1_*Lepus_othus*	0.089	0.089	0.089	0.089	0.097	0.089	0.089	0.103	0.091	0.024	0.031	0.000	0.034	0.032	0.031		0.000	0.025	0.027	0.024
17	DQ334832.1_*Lepus_timidus*	0.089	0.089	0.089	0.089	0.097	0.089	0.089	0.103	0.091	0.024	0.031	0.000	0.034	0.032	0.031	0.000		0.025	0.027	0.024
18	MN547420.1_*Ochotona_cansus*	0.164	0.164	0.164	0.164	0.174	0.164	0.164	0.153	0.160	0.182	0.182	0.179	0.179	0.185	0.194	0.179	0.179		0.013	0.009
19	MN547451.1_*Ochotona_macrotis*	0.171	0.171	0.171	0.171	0.182	0.171	0.171	0.170	0.170	0.179	0.186	0.194	0.182	0.178	0.194	0.194	0.194	0.063		0.013
20	MN547475.1_*Ochotona_thibetana*	0.171	0.171	0.171	0.171	0.182	0.171	0.171	0.167	0.170	0.164	0.171	0.171	0.179	0.167	0.179	0.171	0.171	0.034	0.060	

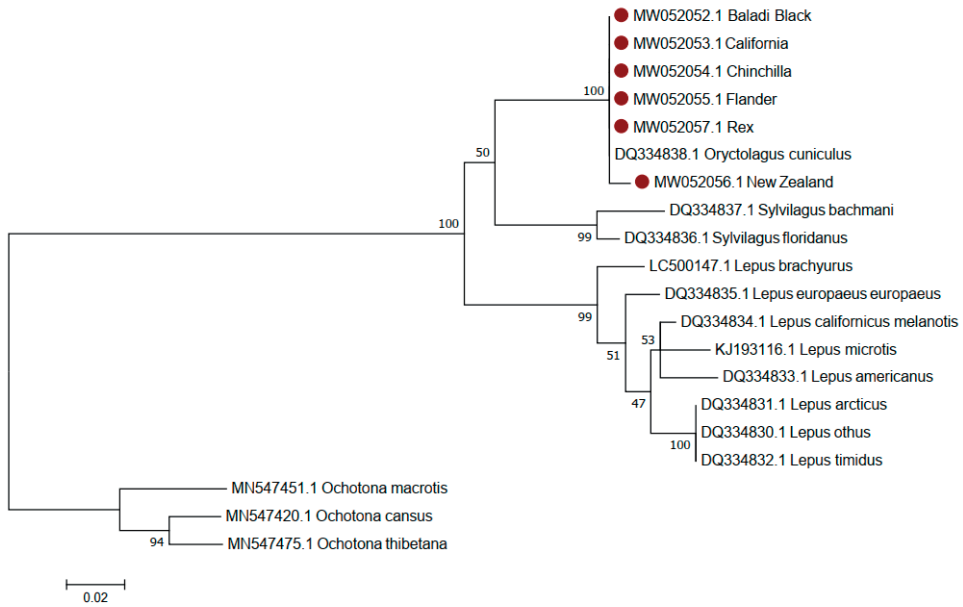

Figure 1: Phylogenetic tree using the Maximum Likelihood method among understudying Rabbit breeds and its related species with outgroups from the GenBank/ NCBI based on (16S rRNA) sequences.

DISCUSSION

Investigation of genetic variation among rabbit breeds and the linkage between their genetic information and performance may facilitate the conservation of genetic resources of rabbits (Wilkinson *et al.*, 2011) and would be useful in rabbit breeding programmes (El-Sabrout and Aggag, 2015). Numerous DNA-based markers are used as tools for estimating genetic diversity among and within animal breeds and populations (Mohamed and Abdelfattah, 2018).

Several traits make mtDNA a good marker to study the origin and diversity of high rates of mutation, maternal inheritance and large quantities in the cell. The high rates of mutation enable accountability for the diversity that occurred in a species through time, and maternal inheritance allows tracing of all the animals to their ancestor(s) (Gupta *et al.*, 2015; Owuor *et al.*, 2019).

16S rRNA is a ribosomal RNA encoded by the mitochondrial genome, necessary for the translation of messenger RNA into mitochondrial proteins. The length of the 16S rRNA gene is almost 1570 bp (Chen *et al.*, 2011). Regardless of its small size, it is indispensable for the translation of mRNA into mitochondrial proteins, and is used as a genetic marker to identify many animals; invertebrates, mammals, poultry, and using universal primers (Mitani *et al.*, 2009; Sarri *et al.*, 2014; Yang *et al.*, 2014).

Several studies have been carried out on rabbit breeds using different genetic markers. El-Bayomi *et al.* (2013) used Random Amplified Polymorphic DNA Markers to investigate the genetic diversity and phylogenetic relationship among three rabbit breeds; NZW, Cal and FLA. Likewise, Badr *et al.* (2016) used RAPD-DNA to assess the genetic variability among four rabbit breeds: NZW, Gabali (G), Baladi Red (BR) and BB. They stated that the NZW breed clustered alone, whereas the local breeds BB, BR and G were grouped together in one cluster. Mohamed and Abdelfattah (2018) used RAPD and SRAP markers to assess the genetic diversity and similarity among six rabbit breeds: G, Bouscat, Chinchilla, Flemish, Cal and NZW. Badr *et al.* (2019) applied 12 microsatellite loci to investigate the genetic diversity

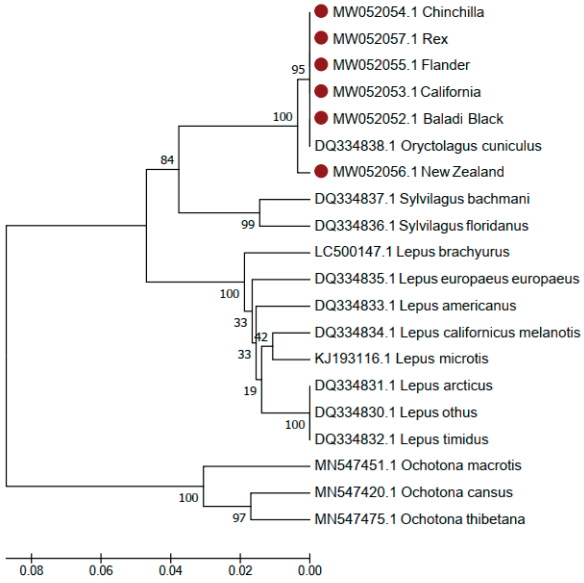

Figure 2: Phylogenetic tree using the UPGMA method among understudying rabbit breeds and its related species with outgroups from the GenBank/ NCBI based on (16S rRNA) sequences.

among three Egyptian rabbit breeds (BR, BB and G) in addition to NZW. Owuor *et al.* (2019) used an mtDNA genetic marker to assess the genetic diversity and the origins of domesticated rabbits in Kenya. Here, we illustrated the phylogenetic relationships of rabbit breeds using mitochondrial 16S rRNA sequences.

The results of the partial sequence of 16S rRNA showed that A+T contents of all understudying rabbit breeds tend to be higher than C+G contents (Table 2). The 16S rRNA sequences comprised 569 nucleotides sites of which 547 sites (96%) are conserved (Figure 3). This was in agreement with van der Kuyl *et al.* (1995) and Saikia *et al.* (2016) who reported that mitochondrial genes are highly conserved in different animal species, which enabled the design of universal primers for PCR amplification of mitochondrial genes.

According to Klomtong *et al.* (2016), the universal primers facilitate the PCR amplification of 16S rRNA in several animal species. The primers used in this study developed by Simon *et al.* (1991) were successfully amplifying a single band in all rabbit breeds, thus revealing their usefulness as universal primers for 16S rRNA. The average size of the amplified band was 551 bp. which was within the expected range according to Simon *et al.* (1991) who stated that the 16Sar and 16Sbr primers amplify a 500-650 base fragment.

The results showed that the NZW breed separated from the others, which was concordant with El Sayed (2010), who reported that NZW and Hyplus were genetically separated breeds. El-Sabrout and Aggag (2015) reported that genetic similarity reflects the range of homogeneity and inbreeding within each genotype studied. Results for 16S rRNA showed that the NZW breed had the highest polymorphism and was distinctly different from the other breeds, while FLA and Rex breeds were the most related breeds.

BB breed was genetically diverged from the NZW breed, which was in agreement with Grimal *et al.* (2012), who found that four Egyptian breeds were structurally separated from the Spanish NZW line. Moreover, this result was consistent with Badr *et al.* (2019), who reported that G, BR and BB breeds were more distinct from the NZW breed, using microsatellite markers.

Galal *et al.* (2013) stated that the genetic variation in domestic breeders allows the evolution of new features for environmental changes or adaptation to diseases. El-Sabrout and Aggag (2015) used ISSR to genetically determine

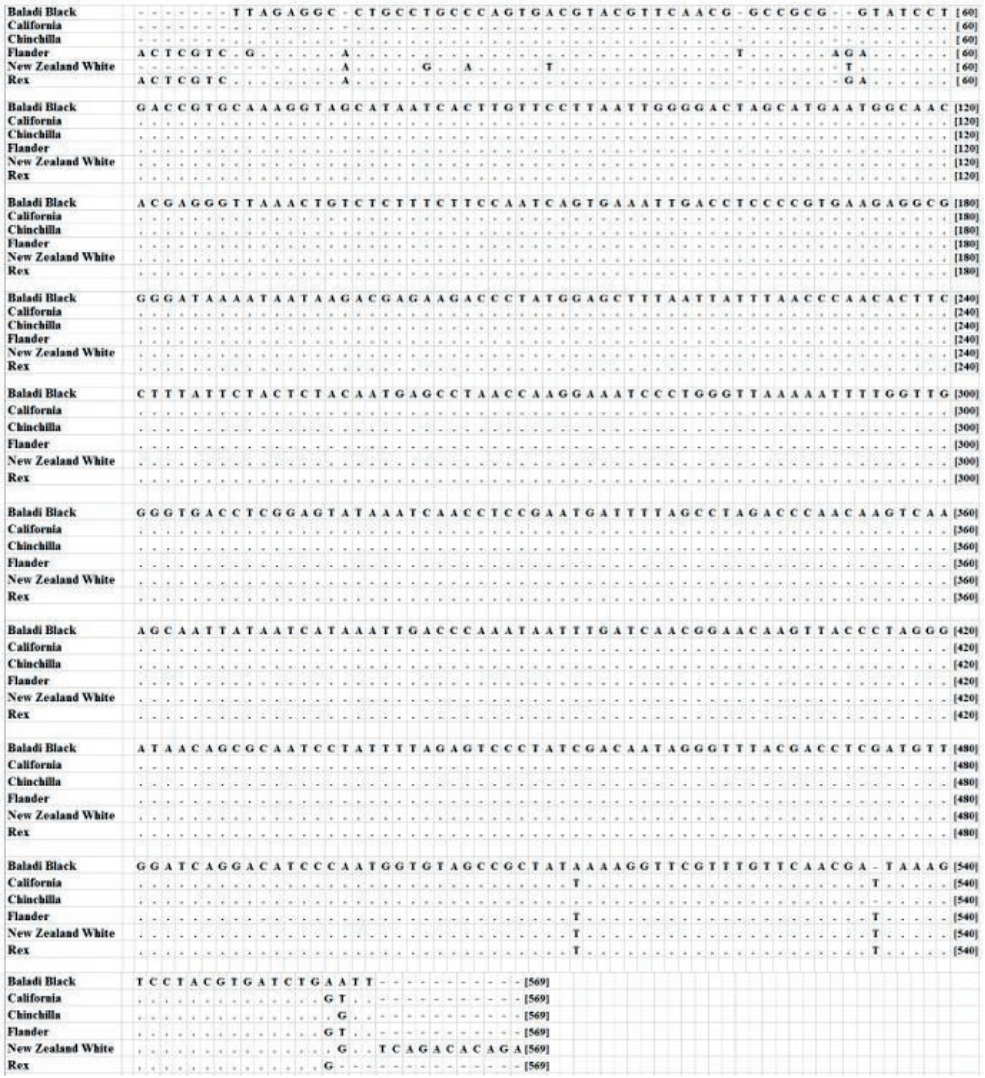

Figure 3: Alignment of partial sequences of 16S rRNA gene among rabbit breeds. Dots indicate identical nucleotides and A, T, C and G indicate the difference nucleotides.

the genetic similarity and diversity in four rabbit breeds; Alexandria, Cal, V-line and NZW. They reported that the Alexandria breed was distinctly different from the Cal and NZW breeds. They expected that because the Alexandria and California breeds were genetically far apart, the crossbreeding between them would be suitable for developing new characteristics to improve the productive performance of rabbits, as well as developing new features to become more adapted to environmental changes. In this research, the phylogenetic analysis of domestic rabbit breeds based on 16S rRNA sequences showed that the NZW breed formed a separate cluster apart from the other rabbit breeds. From these results, it can be expected that the crossbreeding between the NZW breed and each other breed will be more successful in terms of improving the productive capacity of rabbits and providing many quality characteristics so that they become more adaptable to environmental changes.

The results of this study confirmed the applicability and efficiency of 16S rRNA for assessing genetic diversity for rabbit breeds. This information could be used as an initial guide to design further investigations for the development of genetic improvement and conservation programmes for Egyptian rabbit genetic resources.

CONCLUSION

The results of 16S rRNA sequences illustrated that the NZW breed was distinctly different from the other breeds. The results of our work confirmed the qualifications of the 16S rRNA sequences for estimating genetic diversity among rabbit breeds. Furthermore, this study could be useful in further research on the evolution of genetic improvement and conservation of rabbit genetic resources and to help understand the variability in some productive traits in rabbits.

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