COMPARATIVE ANALYSIS OF SEQUENCE DIVERSITY DIAGRAM FOR FIVE RABBIT’ BREEDS IN EGYPT USING SINGLE NUCLEOTIDE POLYMORPHISM

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SUMMARY

The genes coded and referred to as 18S rDNA has been selected as a universal marker. Sequence data from 18S rDNA was used to estimate the genetic variants on 128 rabbits belonging to five different breeds in Egypt, New Zealand White (NZW, n=35), California (Ca, n=35), Chinchilla (C, n=19), Flander (F, n=19) and Babion (B, n=20). Blood samples for sequencing were collected and sequence diversity diagram for multiple sequence alignments was performed. In addition, the comparative analysis according to detected SNPs was implemented. The results revealed that a 1115 bp fragment of DNA sequence was constructed using ABI5100 automated sequencer.

Random small DNA sequence mismatches (74 nucleotides; 1-29, 1071-1115) has been detected. The similarity between Ca and NZW breeds was 92%, additionally, higher similarity percentage was observed between C and both of B, and F (97.02, 97.5 respectively). Nine SNPs were detected; five single nucleotide polymorphisms were spread to (SNP: A>T, and T>A) for B and F located at 185, and 508 bp, also C>A and G>C for C located at 268, and 919 bp. In addition, A>T for B located at 267 bp, and four SNPs between Ca and NZW located at 880, 886, 889, and 900 bp were detected. The Maximum Likelihood was used to estimate the Transition/Transversion (ti/tv) Bias resulted in the estimated (ti/tv) bias (B) was 0.67. Substitution pattern and rates were estimated, the nucleotide frequencies were A = 22.12%, T/U = 21.13%, C = 26.72%, and G = 29.99%. In conclusion, the utilized five rabbit breeds possess a good reservoir for diversity which is essential for genetic improvement. Formal conservation plan is needed and must be implemented in Egypt to achieve such proper genetic improvement.

Keywords: Sequence reads; Rabbits; Diversity; Single nucleotide polymorphism

INTRODUCTION

The genetic map of rabbits is still very limited with only one partial projection (Korstanje et al. 2001, 2003). During the last two decades, only markers detectable by conventional biochemical, immunological, and morphological methods were in use for linkage studies in the rabbit (Korstanje 2000). Throughout the process of evolution, populations of different species preserved some common features while dropped others. This progression, referred to as natural selection, promotes survival of the strong genes which offer organisms more chances of surviving under lively habitats, reproducing vigorously and encountering longer lives. This sort of genetic change produces variations in genes over an extended duration of time, although genetic diversity happens within the momentary, neutralizing the characteristics between parents and offspring.

During the time of inheritance, nucleotides, but not necessarily entire genes, are rearranged and recombined to frame new mixes that are not quite the same as the parents. Also, different occasions can make new variety in the DNA sequences. Transformations or mutations are changes to at least one nucleotide in the DNA sequence. Mutations in somatic cells can cause issues, for example, growth, in a living being yet are not heritable. Transformations in regenerative cells (gametes) are acquired by the descendants. These changes in the DNA sequence can be gainful, inconvenient, or neutral. Furthermore, artificial and flavored genetic variation afterwards helps species survive with support of lowering threat of initiating defects, disorder and harmful genes. Unfortunately, the procedure of domestication tends to diminish the hereditary assorted variety of selected species, due to some degree to the hereditary bottleneck forced when just couple of animals are chosen. In addition, genetic diversity is essential since it keeps up the strength of a population, by including alleles that might be significant in resisting diseases, climate changes and other burdens.

In a small isolated population, individual might be compelled to breed with close relatives. To ensure that the breeding program practically remains on basics to screen and keep up genetic diversity, hereditary decent variety takes into account determination of prevalent animals for breeding. In the event that there is no genetic diversity, if all animals would be hereditarily the same, selection won't bring any changes or improvements in the coming generations. Recently, molecular markers have proven to be superior to the conventional methods for determining gene flow, moreover, data on genetic variation can provide crucial input to plan effective strategies for conservation and rehabilitation of natural populations. The essential
tools for advance of sequencing, eg. dense-linkage maps, genomic libraries, etc. were adapted by agricultural scientists in Europe and US (Aerts et al., 2003; Lee et al., 2003, Kersten et al., 2011). Nowadays, a first-generation microsatellite-based integrated genetic and cytogenetic map for the European rabbit (Oryctolagus cuniculus) was constructed by Chantry-Darmon et al. (2006). Although, microsatellite markers are used most widely in the analysis of genetic diversity of livestock, and population structure of rabbits (Zenger et al., 2003; Tian-Wen et al., 2010, El-Aksher et al. 2016), the sequence reads has been used in this study. Formerly, few efforts were existed related to Egyptian indigenous rabbits’ germplasm (Rabie 2012; Emam et al. 2017; Abdel-Kafy et al., 2018; Badr et al. 2019). Accordingly, this study was planned to estimate the specific molecular variants between five breeds of rabbits in Egypt within the genome causing this genetic variation by using sequence reads, and single nucleotide polymorphism analysis.

MATERIALS AND METHODS

Collecting blood samples and DNA extraction:
The present study was conducted at the laboratory of biotechnology, Department of Animal Production & Fish Resources, Faculty of Agriculture, and the Biotechnology Research Institute, Suez Canal University to identify the genetic variant between five rabbit breeds to detect genetic variations between New Zealand White (NZW, n=35), California (Cal, n=35), Flander (F, n=19), Chinchilla (Ch, n=19), and Babion (B, n=20). Blood samples from the five breeds were collected according to the institutional ethical norms of the Faculty of Agriculture, Suez Canal University, Egypt. About 1ml of blood from the marginal ear vein was individually collected into coated tubes with K3-EDTA (FL medical, Italy) and stored immediately at -20°C until DNA extraction. Quick-gDNA MiniPrep (Zymo Research, USA) was used to extract the genomic DNA to provide high purity DNA yield. Furthermore, the most frequently used method to determine DNA quality is the ratio of absorbance at A260 nm /A280 nm. Ratios around 1.8 indicate good-quality DNA whereas lower values indicate protein contamination since proteins have a peak in absorption of 280 nm resulting from the aromatic amino acids. As a unique assessment of the DNA sample, the NanoDrop® ND-1000 UV-Vis spectrophotometer has been used enabling highly accurate analyses of the extremely small samples with notable reproducibility. Cuvettes and capillaries were needed to accelerate measuring sample cycling time of sample retention system. The extracted DNA was pooled for each breed and two replicates prepared and used for gene sequencing.

Gene sequencing:
The gene coded and symbolized as 18S rDNA has been used as the universal marker (5'-TTAAG CCATGCATGTCTAAG-3', 5'-GACTACGACGGT ATCTAATC-3'). The data from this gene is extensively used in molecular analysis approach due to its slow evolutionary rate which enables reconstruction of the genetic evolutionary history of organisms. Therefore, 18S marker was used for PCR amplification, each 25µl reaction contained 12 µl 2X MasterMix (Qiagen), 40ng of gDNA, 20 pmole of each primer and miliQ water. The PCR was performed at Mastercycler gradient (eppendorf) starting at 94°C for 2 min as an initial template DNA denaturation, followed by 35 amplification cycles (94°C for 30s, then 30s at 52°C, 3 min at 72°C), then 72°C for 10 min as final extension. Furthermore, the gel advanced ver.2 software was used for the capture of gel photo, and the relative band concentration was tested by EgGel-analyzer software. Afterwards, PCR product was purified using QIA quick PCR Purification Kit (QUIGEN). The PCR product was visualized using 1.5% agarose gel documentation system, consequently analyzed by Gel Docu and performed in total volume of 20 µL using BigDye Terminator (v3.1) Cycle Sequencing Kit, each reaction contained DNA template. Template quantity was calculated according to the PCR product size, 8 µL Terminator ready reaction mix, 3.2 pmol primer, and miliQ water. The thermal profile for sequencing cycle was 60°C for one minute, followed by additional purification with CENTRI-SEP columns (Princeton Separations), Therefore, 3500 genetic analyzer (Applied Biosystems) was used for DNA sequencing starting at 96°C, followed by 25 Cycles (96°C for 10s, 50°C for 5s, and 60°C for 4 min).

Data Analysis and manipulation:

Evolutionary relationships and SNP analysis
The obtained sequences were analyzed, and the SNPs were detected according to the results of multiple sequence alignments using Mega X software (Kumar et al. 2018), consequently, phylogenetic trees were charted using the Neighbor-Joining method (Saitou and Nei, 1987) and the genetic distances were determined using the Composite Maximum Likelihood (CML) according to Tamura et al., (2004). This analysis involved 5 nucleotide sequences, and the 1st+2nd+3rd codons +Noncoding were included, wherever, all ambiguous positions were removed for each sequence pair using “pairwise deletion option”.

Maximum Likelihood Estimate (ML) of Transition/Transversion Bias
The initial tree generated by applying NJ was used for the ML search. In addition, BIONJ algorithms to the matrix of pairwise distances was estimated using the maximum composite likelihood approach for nucleotide sequences. To estimate Transition/Transversion (ti/tv) bias, parameters κ, κ1, and κ2 under Maximum Composite Likelihood model, thus substitution pattern and rates, were estimated under the Tamura and Nei (1993) model.
RESULTS AND DISCUSSION

In the present study, the DNA retrieved from the five rabbit breeds has been sequenced to evaluate the prospected variability among them and examine the genetic effects based on sequence reads. The results revealed that a 1115 bp fragment of DNA sequence was constructed using ABI5100 automated sequencer. Random small DNA sequence mismatches (74 nucleotides; 1-29, 1071-1115) have been detected, and may impulsively be developed in the divergent breeds. In order to facilitate the use of this information, multiple sequence alignments have been created using Mega X platform to enhance the discovery of sequence variation. In addition, the phylogenetic tree was formulated using the Neighbor-Joining method. The optimal tree with a sum of branch length 0.1029 resulted in phylogenetic diversity based on rabbit sequence reads (Figure 1) and the similarity between studied breeds is given in Table 1.

![Phylogenetic tree](image1.png)

**Figure 1. Phylogenetic tree developed by NJ-cluster analysis among the rabbit breeds based on gene sequence data.**

<table>
<thead>
<tr>
<th></th>
<th>Chinchilla</th>
<th>California</th>
<th>Babion</th>
<th>Flander</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chinchilla</td>
<td>100</td>
<td>99.24</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>California</td>
<td></td>
<td>90.45</td>
<td>90.84</td>
<td>100</td>
</tr>
<tr>
<td>Babion</td>
<td>89.45</td>
<td></td>
<td>99.53</td>
<td>97.5</td>
</tr>
<tr>
<td>Flander</td>
<td>89.84</td>
<td>90.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NZW</td>
<td>91.03</td>
<td>92.02</td>
<td>97.02</td>
<td></td>
</tr>
</tbody>
</table>

Although C and NZW breeds have been existing in the same ecosystem for long time, they have 92% similarity to each other. The same similarity percentage (97%) was observed between C and both of B, and F which was close to that of NZW with (97.5%). However, NZW made the highest contribution to the intra-breed diversity between all analyzed breeds. These results indicated that NZW breed lowers the genetic diversity and demonstrated that quantifying the contribution to the intra-breed diversity is important for assessing conservation priorities. Identified breeds that should be given high priority for conservation purposes in terms of genetic diversity based on sequence analysis were F and B breeds.

Moreover, five single nucleotide polymorphisms have been detected (SNP: A>T, and T>A) for B and F located at 185, and 508 bp, C>A and G>C for Ch located at 268, and 919 bp, A>T for B located at 267 bp, and four SNPs between C and NZW located at 880, 886, 899, and 900 bp (Figure 2).

![SNPs](image2.png)

**Figure 2. Single nucleotide polymorphism (SNP) detected between New Zealand white, and California breeds (the arrow shows the SNPs locations).**

In spite of the fact that these SNPs represent an enormous resource for gigantic examinations, variety in recombination frequencies in the genome makes it hard to anticipate what the real hereditary separation is for firmly divided loci. The SNPs found in this study may be in a similar pattern of Carneiro *et al.*
(2014) who found that the SNPs with checked allele frequency are in contrast in wild and residential rabbits and were enhanced for conserved noncoding sites. Furthermore, the highly density segment (CCTCCCT) has been blasted against all sequence reads using BLASTN software ver 2.3.1 (http://www.ncbi.nlm.nih.gov/blast) showing no evidence of similarity or association between them. Accurate high-density linkage maps proved to be the backbone for linkage studies (Daw et al., 2000; Fingerlin et al., 2006) and enhanced high-density linkage maps as of late ended up accessible in mouse (Shifman et al., 2006), and human (Matise et al., 2007). These high-determination maps demonstrated a solid association between recombination problem areas and high density of the 7-nucleotide oligomer “CCTCCCT” either in mouse (Shifman et al., 2006) or human (Myers et al., 2005). Needless, to report that more advanced analysis should be done on both laboratory and bioinformatics analysis levels to get more information and add extra discovered SNPs to the rabbit’s maps, also, Fabuel et al. (2004); and Ruane (2000) emphasized that the conservation decisions for animal genetic resources should take into account not only molecular marker-based genetic diversity but also other factors such as specific traits, productive performance, and future economic interest.

**Maximum Likelihood Estimate (ML) of Transition/Transversion Bias:**

The estimated Transition/Transversion (ti/tv) bias \( (R) \) was 0.67, estimated of substitution pattern and rates are shown in Table 2. The nucleotide frequencies were \( A = 22.12\% \), \( T/U = 21.13\% \), \( C = 26.72\% \), and \( G = 29.99\% \). For estimating ML values, the tree topology was automatically computed resulted in a maximum Log likelihood of -2111.414. Practically, the genomic DNA sequences transitions were occurred at higher frequencies than transversions (Curtis and Clegg 1984; Wakeley 1994, 1996). Thus, the analytic ratio of ti/tv is useful in a set of SNP calls, this ratio is often evaluated separately for both previously discovered and novel SNPs. Moreover, it is not clear which aspects of the evolutionary process and/or analysis that have caused the observed patterns. These results are corresponded to Yang and Yoder, (1998) who reported an expected over correction result of multiple transitions at the same site using the maximum-likelihood method at a low sequence divergence that may have significant implications. Moreover, Wakeley (1996) found a huge variance in confirmed ti/tv at lower divergence levels when examined a limited number of sites for sequences with a high transition bias. Therefore, this underlines the need for more consideration of the multiple factors that affect sequence evolution, therefore more examinations are needed using divergent breeds with a numerous sequence reads.

**Table 2. Maximum Likelihood estimate of substitution matrix**

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>T/U</th>
<th>C</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-</td>
<td>6.23</td>
<td>7.89</td>
<td>10.52</td>
</tr>
<tr>
<td>T/U</td>
<td>6.51</td>
<td>-</td>
<td>12.75</td>
<td>8.83</td>
</tr>
<tr>
<td>C</td>
<td>6.51</td>
<td>10.05</td>
<td>-</td>
<td>8.83</td>
</tr>
<tr>
<td>G</td>
<td>7.76</td>
<td>6.23</td>
<td>7.89</td>
<td>-</td>
</tr>
</tbody>
</table>

Each entry presents the probability of substitution \((r)\) from one base (column) to another base (row). Tamura and Nei model (1993) was used to estimate substitution outline. Wherever, the rates of different transversional substitutions are shown in \textit{italics}, and those of transitional substitutions are shown in \textbf{bold}. Relative values of instantaneous \( r \) should be considered when evaluating them.

**CONCLUSION**

In need to formal conservation plan exists in Egypt for such resources since many have been lost or now face extinction. Besides, policies to protect the local species will require more scientific contributions on the genetic structure in its local dissemination extend. The assumption is that exotic breeds are better. On the other hand, this is not generally the case.

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The objective of this study was to assess the genetic diversity of rabbits in Egypt by using microsatellites.

A rapid population expansion retains genetic diversity within European rabbits in Australia. Molecular Ecology 12: 789-794


The study evaluated the genetic diversity of rabbits in Egypt by using microsatellites. The results showed that the five rabbit breeds used in the study (NZW, F, B, C, and CA) exhibited a high level of genetic diversity. The study also highlighted the importance of conservation programs to maintain the existing genetic diversity of rabbit populations in Egypt.

There is a need for a systematic approach to monitor the genetic diversity of rabbit populations in Egypt and to implement conservation strategies to preserve this genetic diversity.

ABIS15504

The study used a rapid population expansion approach to retain genetic diversity within European rabbits in Australia. This approach involves the use of microsatellites to assess the genetic diversity of rabbits.

The study found that the five rabbit breeds used in the study (NZW, F, B, C, and CA) exhibited a high level of genetic diversity. The study also highlighted the importance of conservation programs to maintain the existing genetic diversity of rabbit populations in Egypt and to implement conservation strategies to preserve this genetic diversity.