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Assessment of Genetic Variability and Population Structure of Five Rabbit Breeds by Microsatellites Markers Associated with Genes

Tarik S.K.M. Rabie

Department of Animal Production, Faculty of Agriculture, Suez Canal University. Ismailia, 41522. Egypt.

*Corresponding author's e-mail: Tarik.rabie@agr.suez.edu.eg; ORCID: 0000-0001-9678-2323

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ABSTRACT

The present study was intended to estimate the specific genetic variants by using nine genetic markers among five rabbit breeds (New Zealand White, California, Chinchilla, Flander, and Babion) in Egypt. A total of 128 animals were used (19-35 rabbits per breed). A total of 97 alleles were detected across the breeds and the average number of alleles per locus was 2.16±0.11. Five private alleles were present in Babion breed, where the locus INRACCDDV0023 had two private alleles of 293 and 297 base pairs with allele frequencies of 0.4 and 0.1, respectively. The INRACCDDV0036, INRACCDDV0304, and INRACCDDV0241 loci had private allele for each (185bp (freq: 0.24), 197 (freq: 0.47), and 137bp (freq: 0.26), respectively). The mean of H_e values ranged from 0.35±0.06 to 0.49±0.07. The average of the polymorphic information content was 0.41 (ranged from 0.298 at INRACCDDV0211 to 0.599 at INRACCDDV0036 locus). To estimate the genetic deviation of the five rabbit breeds, two parameters were evaluated: genetic differentiation (F_{ST}), and genetic distance. The F_{ST} values varied from 0.029 (INRACCDDV0036) to 0.785 (INRACCDDV0022). The similarity matrix showed that the Chinchilla breed was distinct from other breeds. In addition, among the nine loci, the Hardy-Weinberg equilibrium was highly significant for five loci. Therefore, the rabbit breeds are good reservoirs of allelic diversity that is the major basis for genetic improvement. Consequently, the breeders need a formal conservation plan for such breeds that are in danger of extinction in near future.

Key words: Genetic diversity, Microsatellite marker, Production performance, Rabbits

INTRODUCTION

Rabbits are phylogenetically closer to humans than to rodents. The rabbits' genetic map is still very limited to only one partial map (Korstanje et al., 2001, 2003). During the last 20 years, only markers detectable by conventional biochemical, immunological, and morphological methods have been used for linkage studies in the rabbit (Korstanje, 2000). Moreover, information about genetic variation help to design successful methodologies for the protection and restoration of natural populations. Previously, few efforts were initiated to conserve the available superior germplasm of the rabbits in Egypt (Grimal et al., 2012; Rabie, 2012; El-Aksher et al., 2017; Badr et al., 2016). The discovery of microsatellites in transcripts and regulatory districts of the genome empowered logic scientific enthusiasm for finding their conceivable biological functions. microsatellite markers play a significant role in the guideline of transcription regulation, association of chromatin, the cell cycle and genome size (Li et al., 2004; Gao et al., 2013). Also, several reports indicated that microsatellites are common in various proteins and the frameworks engaged with their genesis may be related to the rapid evolution of proteins (Huntley and Golding, 2000; Katti et al., 2000). In this way, the aim of the current study was to utilize the microsatellite markers to estimate the genetic variations among five rabbit breeds in Egypt.

MATERIALS AND METHODS

Ethical approval

The experiment was carried out according to the National Regulations on Animal Welfare and Institutional Animal Ethics Committee.

Animals

The present experiment was conducted at the laboratory of biotechnology, Animal Production & fish resources Department, Faculty of Agriculture, and the biotechnology research institute, Suez Canal University to identify the genetic variant between five rabbits' breeds in terms of detection of genetic diversity between New Zealand White (NZW, n=35), California (Cal, n=35), Flander (F, n=19), Chinchilla (Ch, n=19), and Babion (B, n=20), with a total number of 128 animals ranged between 19-35 animals per breed.

Blood samples and DNA extraction

A total of 128 individual blood samples representing the five rabbit's breeds were randomly 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 in a tube treated with K3-EDTA (FL medical, Italy) and stored at -20°C until DNA extraction. Genomic DNA was extracted using Quick-gDNA MiniPrep (Zymo Research, USA) to provide superior performance and high purity and yield of extracted DNA. The quality of extracted DNA was examined by NanoDrop® ND-1000 UV-Vis Spectrophotometer enabling highly accurate analyses with remarkable reproducibility.

Selection of markers and genotyping

Nine microsatellite markers within genes were selected (Table 1) according to Chantry-Darmon et al. (2005). To facilitate, all markers obtained were first tested on the rabbit's genomic DNA for polymorphism, then the PCR reactions were performed in a 25µl final volume containing 6µl of 100 ng of DNA, 6 µl of the PCR Super Mix contained 1.1x buffer (Invitrogen, 10572-014), forward and reverse primers (0.2 – 1uM each), and nuclease-free dH2O to final volume of 25 ul. An Eppendorf thermal cycler was used along with the following P CR profile settings: 5 min at 95^{o}C followed by 35 cycles for 30 sec at 95^{o}C, 45 sec at 53^{o}C, 55^{o}C, 57^{o}C or 59^{o}C annealing temperature, and 60 sec at 72^{o}C, followed by an elongation step at 72^{o}C for 10 min, and finally stop step at 4^{o}C. Subsequently, PCR products were electrophoresed on 1.5% agarose gel containing 0.5% ethidium bromide which viewed under UV light. Therefore, genotyping of the microsatellite markers was done using QIAxcel advanced system.

Statistical analysis

From the data observed from codominant markers, genetic diversity was assessed by calculating the observed (*No*), effective number of alleles (*Ne*), the observed (*Ho*) and the expected (*He*) heterozygosity using GenAlEx 6.5 package (Peakall and Smouse, 2012). The Cervus 3.0.7 program (Kalinowski et al., 2007) was used to assess the polymorphism information content (*PIC*) according to the formula:

$$PIC = 1 - \sum_{i=1}^{n} P_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2P_i^2 P_j^2$$

where P_i and P_j are the frequencies of the i^{th} and j^{th} alleles at a locus with l alleles in a population, respectively and n was the number of alleles.

The F-statistics of pairwise genetic differentiation among the breeds (F_{ST}), reduction in heterozygosity due to inbreeding for each locus (F_{IT}) and the reduction in heterozygosity due to inbreeding within each breed (F_{IS}) were obtained using AMOVA approach as implemented in GenAlEx 6.5. (Peakall & Smouse, 2012). Additionally, deviation from Hardy-Weinberg equilibrium (HW) at each locus in each breed was tested was examined using GENEPOP program (Raymond and Rousset, 1995). To minimize the consequences of genotyping errors, those alleles found in only one type in at least two individuals were private ones. Genetic distances between breeds were calculated based on allelic frequencies (Nei, 1987) and a phylogenetic was constructed with the advantage of the PHYLIP package (Felsenstein, 1993).

Table 1. Characteristics of microsatellite markers used in the present study

Locus	OCU	Accession number	Associated gene symbol	Gene description	PCR Temp[1] (°C)	Map position
INRACCDDV0248	1	AJ874579	PMCH	Pro-melanin concentrating hormone	57	1ql5.1-ql5.2
INRACCDDV0036	3	AJ874398	CD14	Cyclin-dependent kinase inhibitor in the CIP/KIP family	59	3p21prox
INRACCDDV0022	4	AJ874385	ERBB3	Epidermal growth factor receptor3	59	4q11
INRACCDDV0211	5	AJ874545	HAS3	Hyaluronan synthase 3	59	5q14
INRACCDDV0221	7	AJ874555	GPR37	G protein-coupled receptor 37	57	7p21-p12
INRACCDDV0304	10	AJ874626	EGFR	Epidermal growth factor receptor	53	10ql6ter
INRACCDDV0241	14	AJ874574	TIAM1	T cell lymphoma invasion and metastasis 1	55	14q25
INRACCDDV0031	17	AJ874394	CAI2	Carbonic anhydrase 12	57	17q11
INRACCDDV0023	18	AJ874386	CYP2C18	Cytochrome P450 family 2 subfamily C member 18	57	18q31

OCU: Rabbit chromosomes, [1]The optimal annealing temperature in the PCR reaction.

RESULTS AND DISCUSSION

Genetic markers polymorphism

All microsatellite loci typed were polymorphic. The number of alleles per locus, polymorphic information content, expected and observed heterozygosity across all the breeds used are presented in table 2. A total of 97 alleles were detected across the breeds. The typical range of alleles per locus discovered over loci and breeds was 2.16 ±0.11 alleles. The highest number was four alleles and was detected in INRACCDDV0023 and INRACCDDV0036 loci. However, the lowest number was two alleles and was detected in INRACCDDV0022 and INRACCDDV0221 loci. These findings were consistent with Tian-Wen et al. (2010) who reported the average number of alleles was 6.63 and ranged from 2.86 to 9.92. Moreover, Xin-Sheng et al. (2008) found that the average number of alleles was 4.5 (ranged from 3 to 6 alleles) in Wan line Angora rabbits.

Interestingly, Grimal et al. (2012) found an average number of 5.41, ranged from 2 to 12 alleles, with the highest number for INRACCDDV0087 and the lowest for INRACCDDV0105. Also, El-Aksher et al. (2016) reported the average number of alleles for Moshtohor line rabbits was 6.75. Allele frequencies across microsatellite loci were different (Figure 5) that it was due to the differences in the distribution of the allele frequency for each allele size among the breeds. The highest allele frequency was 0.846 for the INRACCDDV0023 with the allele sizes of 211 bp in Chinchilla. The highest allele frequency in NZW and Flander rabbits was 0.842 and 0.763 for INRACCDDV0211 marker with the allele size of 206 bp, respectively. Moreover, the highest allele frequency in California breed was 0.757 for the INRACCDDV0304 marker with allele size of 304 bp (Figure 1).

Finally, the Babion rabbits have the highest allele frequency as 0.50 for the markers INRACCDDV0221 and INRACCDDV0241 with allele sizes of 117 bp and 150 bp, respectively. These results are in line with Xin-Sheng et al. (2008) who revealed that allele frequencies ranged from 0.98 to 0.412 for SOL44 marker, and from 0.049 to 0.48 for SAT13 marker.

Genetic relationships among rabbit genotypes

The results indicated that the Chinchilla breed is distinctive from other breeds (Figure 2). Interestingly, the equality of both California and NZW is presented (Table 3). Galal et al. (2013) concluded that there was a low genetic variation within each of the four rabbit genotypes (APRI line, NZW, Baladi Black, and Gabali breeds) based on biochemical markers. In order to evaluate the genetic variation within breeds, total number of alleles, number of alleles per locus, private alleles, expected heterozygosity (*He*, estimated by Nei, 1978) and observed heterozygosity (*Ho*) have calculated.

Observed and expected heterozygosity across breeds

The observed (*Ho*) and expected (*He*) heterozygosity and the polymorphic information content (*PIC*) for each marker over the examined breeds are displayed in table 2. The wide parameters used to measure the genetic diversity across and within the populations is *He* or the gene diversity as defined by Nei (1973). The *Ho* in all microsatellite markers was higher than *He* at all rabbits' breeds. The means of *He* values were ranged from 0.35±0.06 to 0.49±0.07. The *Ho* for different markers averaged 0.58±0.05 and ranged from 0.06 (INRACCDDV0022*)* to 0.99 (INRACCDDV0036*)*. The overall mean of *He* was 0.422±0.03 and ranged from 0.37 at INRACCDDV0211 to 0.66 at INRACCDDV0036. These results in full agreement with Ben Larbi et al. (2014) who realized that *Ho* ranged from 0.3 to 0.53 across 36 loci used in twelve rabbit populations. The distinguished results might be due to the number of markers and/or the number of populations that used. Similarly, to the obtained results, Xin-Sheng et al. (2008) found that the highest heterozygosity was 0.721 at locus SOL33, and the lowest level of heterozygosity was 0.63 when different markers were used.

From this point, it was clear that although the microsatellites used were different from other studies, the obtained heterozygosity values were closed. The *PIC* might be used to ascertain the heterozygosity and the alleles' numeral in the population. The *PIC* average is 0.41 with the values ranging from 0.298 at locus INRACCDDV0211 to 0.599 at locus INRACCDDV0036. These values were dissimilar with those of Schwartz et al. (2007) who found the lowest *PIC* was 0.27 at locus SOL33 and the highest *PIC* value was 0.70 at locus SAT16.

Similarly, Xin-Sheng et al. (2008) found the *PIC* average was 0.642 (ranged from 0.559 to 0.705). Moreover, another range of *PIC* (0.60 - 0.86) was obtained by El-Aksher et al. (2016). Accordingly, the microsatellite markers that utilized could propose their adequacy in the linkage mapping programs and genetic polymorphism studies in rabbits (Schwartz et al., 2007; Hongmei et al., 2008; Tian-Wen et al., 2010).

Table 2. Variability parameters for the microsatellite markers

Locus	Na	Ne	I	*Ho*	*He*	Nm	F	F_{ST}	F_{IS}	F_{IT}	*PIC*
INRACCDDV0022	0.80	0.79	0.27	0.02	0.20	0.068	0.92	0.785	0.922	0.983	0.372
INRACCDDV0248	2.80	2.58	0.98	0.88	0.61	7.469	-0.47	0.032	-0.448	-0.401	0.560
INRACCDDV0023	2.20	1.75	0.63	0.53	0.40	0.737	-0.32	0.253	-0.329	0.008	0.421
INRACCDDV0221	2.00	1.73	0.61	0.64	0.42	5.096	-0.51	0.047	-0.546	-0.474	0.319
INRACCDDV0036	3.20	2.90	1.10	0.99	0.65	8.508	-0.54	0.029	-0.536	-0.493	0.599
INRACCDDV0211	2.00	1.63	0.56	0.56	0.38	3.431	-0.44	0.068	-0.491	-0.390	0.298
INRACCDDV0031	2.00	1.48	0.51	0.41	0.32	0.712	-0.26	0.260	-0.258	0.069	0.335
INRACCDDV0304	2.20	1.80	0.65	0.58	0.42	1.440	-0.37	0.148	-0.402	-0.195	0.406
INRACCDDV0241	2.20	1.79	0.65	0.58	0.42	4.402	-0.37	0.054	-0.407	-0.331	0.364
Overall mean ± SE	2.16±0.11	1.83±0.11	0.66±0.04	0.58±0.05	0.42±0.03	3.541±1.025	-0.35±0.05	0.186±0.081	-0.277±0.153	-0.136±0.155	0.408

Na: Number of different alleles, Ne: Number of effective alleles, I: Shannon's information index, *He*: expected heterozygosity. *Ho*: observed heterozygosity, F_{IS}: heterozygosis deficit, F_{ST}: population variation, F_{IT}: heterozygosity due to inbreeding, Nm: Gene flow, F: Fixation index, PIC: Polymorphic information content, SE: Standard error.

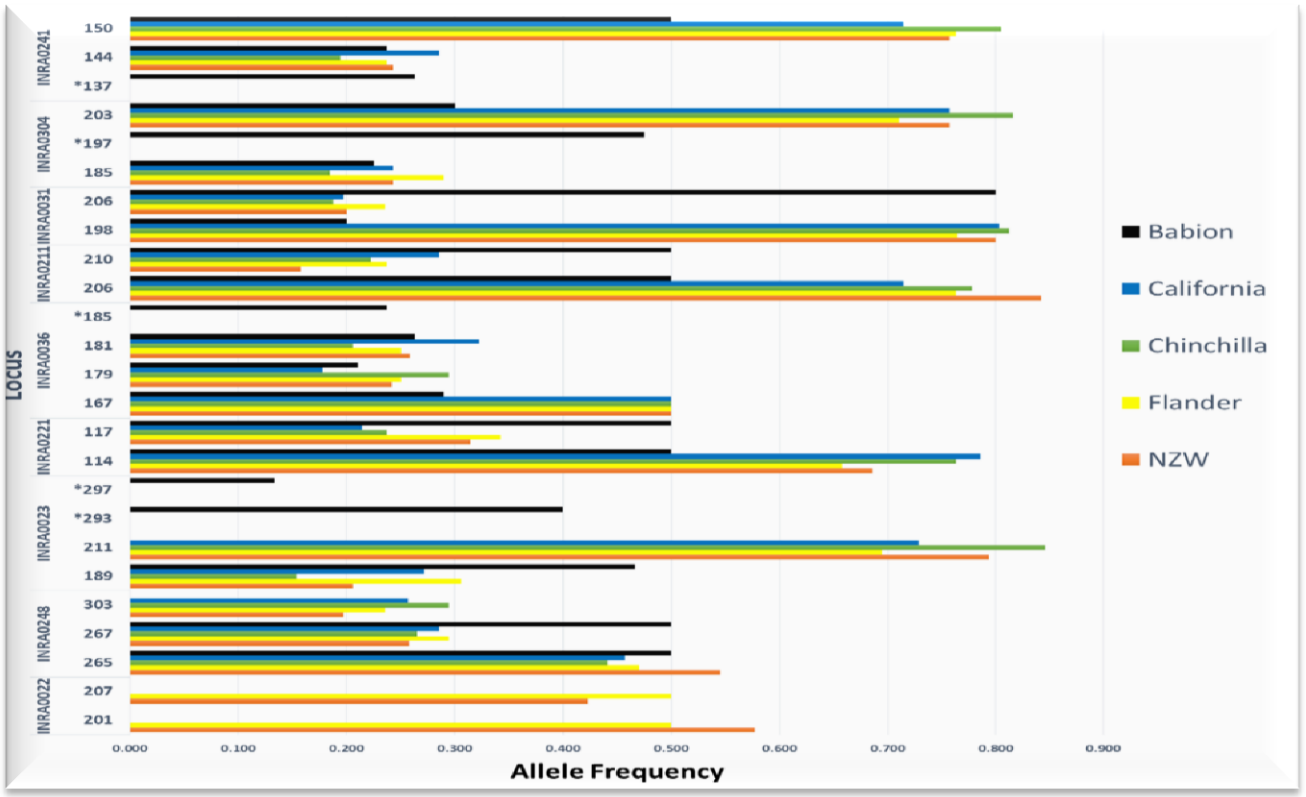

Figure 1. The allelic size and allele frequency per locus for each rabbit breed. *Private allele; NZW: New Zealand White

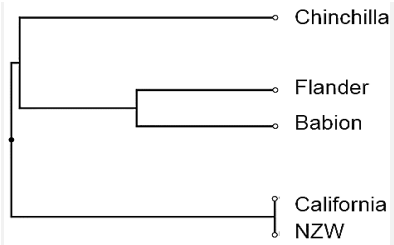

Figure 2. Cladogram developed by NJ cluster analysis showing the coefficient of genetic similarities among the rabbit breeds based on microsatellite markers within gene analysis. NZW: New Zealand White

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Table 3. Genetic distances among the different rabbit breeds

Rabbit breeds	Chinchilla	New Zealand White	Babion	Flander	California
Chinchilla	0	0.235	0.5	0.529	0.235
New Zealand White		0	0.382	0.353	0
Babion			0	0.265	0.382
Flander				0	0.353
California					0

Hardy-Weinberg Equilibrium and private alleles over the studied breeds

Among the nine loci, the Hardy-Weinberg equilibrium (HW) was highly significant differentiated (P≥0.001) for five loci, but not significant with four loci (Table 4). Although, INRACCDDV0241 locus was highly significant for Babion, it was not significant for NZW, Flander, and Chinchilla. Instead, the INRACCDDV0022 locus was highly significant in NZW, it was significant in Chinchilla, California, and Babion breeds (Table 4). Moreover, all the microsatellite loci in this examination were polymorphic, showing that the loci were appropriate for the genetic investigation of lab rabbits in Egypt. Private alleles were likewise present in five alleles and were realized in Babion breed (Figure 3). The locus INRACCDDV0023 had two private alleles at 293 and 297 bp with allele frequency 0.4 (freq: 0.4), and 0.1 respectively. The locus INRACCDDV0036, INRACCDDV0304, and INRACCDDV0241 had private allele for each (185 bp (freq: 0.24), 197 (freq: 0.47), and 137 bp (freq: 0.26), respectively (Figures 1 and 3). In contrast, Grimal et al. (2012) did not reach any private allele for the locus INRACCDDV0241 with four Egyptian breeds and Spanish New Zealand White breed. Increasing the numbers of individuals sampled has two effects, one is to increase the integer of private alleles in the samples, thereby increasing the accuracy of the evaluations of gene flow (Slatkin, 1985).

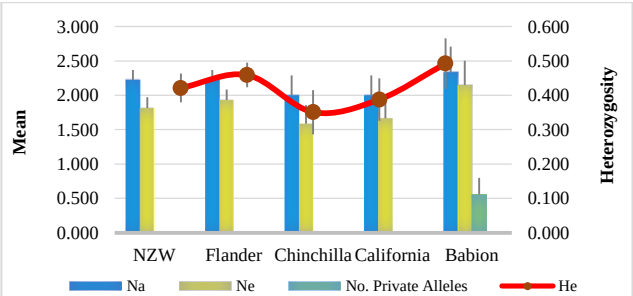

Figure 3. Allelic patterns across five rabbit breeds. Na: number of different alleles, Ne: number of effective alleles, No: number of private alleles, and He: Expected heterozygosity. NZW: New Zealand White

Genetic Variation and breeds diversity

To estimate the genetic variation of the five rabbit' breeds, genetic differentiation (F_{ST}), and genetic distance were evaluated. The negative F_{IS} values observed for all studied locus except the INRACCDDV0022 locus (Table 2) as Tian-Wen et al. (2010) when observed negative F_{IS} values. Contradictory, El-Aksher et al. (2016) attained the F_{IS} with positive values but were closed to zero which indicated low inbreeding within the population. In addition, the negative F_{IS} values would reflect random sampling error or the individual has fewer homozygotes than one would expect by chance at the genome-wide level. The values of F_{ST} for the nine loci are shown in table 2. The F_{ST} values fluctuated from 0.029 (INRACCDDV0036) to 0.785 (INRACCDDV0022).

Other reports showed that the emphatically low F_{ST} (0.0137 and 0.099) (Grimal et al., 2012; Tian-Wen et al., 2010). Additionally, F_{ST} comparisons from entirely unexpected components of the genome will offer bits of knowledge into the demographic history of populations (Holsinger and Weir, 2009). Shannon's Information index (I) averaged 0.66 and ranged between 0.27 (INRACCDDV0022) to 1.1 (INRACCDDV0036). This record is a proportion of strength and it is the likelihood that two individuals randomly represented from an infinitely population will be different species. In addition, Simpson's Index is usually expressed as the reciprocal, so the higher values represent higher diversity which was indorsed by the patterns of the neighbor-joining phylogenetic tree (Figure 4). Moreover, the genetic diversity within individuals (78%) and among breeds (22%) was highly significant (Table 5). In addition, gene flow (Nm) ranged from 0.068 at INRACCDDV0022 to 8.508 at INRACCDDV0036 and averaged 3.541. Slatkin (1985) counted that if the value of Nm >1, the quality trade among populace can avert the effect of genetic drift and diminish the genetic divergence among populaces. In the current study, the obtained Nm was indicating that the gene flow was one of the significant variables impacting the genetic construction of rabbits' populations. The moderately high gene flow likely averts genetic distinctions, which is the purpose behind the watched low genetic differences. That is the motivation behind why the difference within individuals was higher than that among breeds. Along these, the absence of differentiation between many breeds such as NZW and California is credited to gene flow.

It can be expected that gene flow would be constrained, and that reasonable level of genetic structure would be obvious among test from individuals selected from the area isolated by obstructions and separations more than a few kilometers. Be that as it may, investigations dependent on 9 microsatellite loci from 128 rabbits uncovered all chromosomes, therefore this study assumed to be in low to adequate level of genetic diversity as demonstrated by Estes-Zumpf et al. (2010). A past report brief that microsatellite markers utilized in investigations of genetic variation and distances should don't have any less than four alleles in order to curtail the standard errors of estimated distances (Barker, 1994) and that such microsatellite markers should have a *Ho* of somewhere in the range of 0.3 and 0.8 inside the population (Takezaki and Nei, 1996).

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Table 4. Results of Chi-Square test for Hardy-Weinberg equilibrium

Locus	*df*	New Zealand White			Flander			Chinchilla			California			Babion			Hardy-Weinberg Equilibrium for locus over breed
		ChiSq	Prob	Sig	ChiSq	Prob	Sig	ChiSq	Prob	Sig	ChiSq	Prob	Sig	ChiSq	Prob	Sig	
INRACCDDV0022	1	18.45	0.00	***	8.00	0.00	**	M	-	-	M	-	-	M	-	-	***
INRACCDDV0248	3	12.07	0.01	**	5.58	0.13	NS	4.71	0.19	NS	9.90	0.02	*	M	-	-	***
INRACCDDV0023	1	2.29	0.13	NS	3.48	0.06	NS	0.43	0.51	NS	4.86	0.03	*	3.00	0.01	*	NS
INRACCDDV0221	1	7.35	0.01	**	5.14	0.02	*	1.83	0.18	NS	2.60	0.11	NS	M	-	-	**
INRACCDDV0036	3	21.69	0.00	***	12.00	0.01	**	17.00	0.00	***	31.00	0.00	***	6.00	0.00	**	***
INRACCDDV0211	1	0.67	0.41	NS	1.83	0.18	NS	1.47	0.23	NS	5.60	0.02	*	M	-	-	NS
INRACCDDV0031	1	1.88	0.17	NS	1.61	0.20	NS	0.85	0.36	NS	1.99	0.16	NS	1.25	0.26	NS	NS
INRACCDDV0304	1	3.60	0.06	NS	3.15	0.08	NS	0.97	0.32	NS	3.60	0.06	NS	20.00	0.00	***	NS
INRACCDDV0241	1	3.60	0.06	NS	1.83	0.18	NS	1.05	0.31	NS	5.60	0.02	*	19.00	0.00	***	***

Prob: Probability, ChiSq: Chi-Square, M: Monomorphic, NS: Not significant, ***df***: Degree of freedom, Sig: Significant (* p≤0.05, ** p≤0.01, *** p≤0.001)

Table 5. Analysis of molecular variance in studied generations

Source	*df*	*SS*	*MS*	Est. Var.	%	F-statistic	p-value
Among breeds	4	127.420	31.855	0.598	22	0.226	0.001
Among individuals	123	239.299	1.946	0.000	0	-0.051	0.960
Within individuals	128	276.000	2.156	2.156	78	0.186	0.001
Total	255	642.719	--	2.754	100	--	--

df: degrees of freedom, *SS:* sum of squares, *MS:* mean square, Est. Var: Estimated variance.

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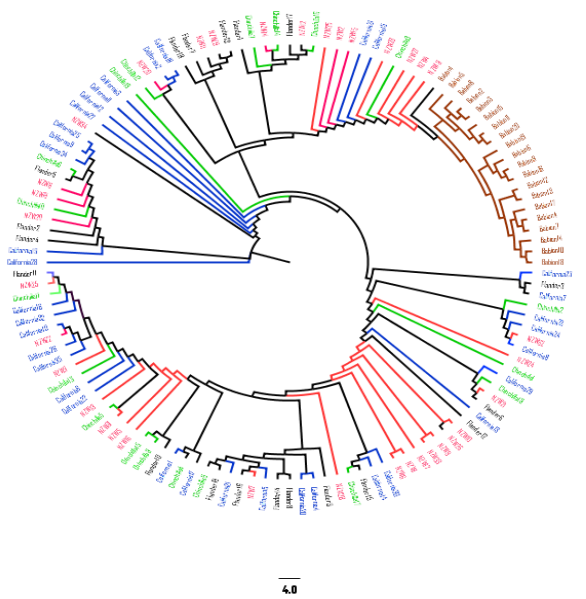

Figure 4. Neighbor-Joining phylogenetic tree based on allele sharing distance for 128 rabbits from different breeds. New Zealand White (red), California (blue), Flander (black), Chinchilla (green), and Babion (brown).

CONCLUSION

One of the main points of the current study was to interpret patterns of differentiation among microsatellite loci taking into account their genome location. The highest number of alleles was identified in INRACCDDV0023 and INRACCDDV0036 loci, which they had two and one private alleles, respectively, located at map position of 18q31 and 3p21prox. Moreover, on chromosome 4 the INRACCDDV0022 locus was highly significant deviated from Hardy-Weinberg equilibrium in New Zealand White breed, but it was not in Chinchilla, California, and Babion breeds. It is essential to note, sampling of loci was not dense enough to recognize all separated chromosome regions or to affirm that the identified alleles of high polymorphic loci did not represent multiple independent information. It is suggested to address these concerns by scanning the genome with a much higher density of markers. Finally, this study emphasized the necessity of biodiversity inquires in rabbits to characterize complex patterns.

DECLARATION

Acknowledgments

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Conflict of interest

There is no conflict of interest.

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