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Study of genetic variation of myostatin (MSTN) and calpastatin (CAST) genes in two native Iraqi sheep by PCR-RFLP technique

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Abstract

The study aimed to research the genetic variation of the Awassi and Naimi sheep breeds using the two genes myostatin (MSTN) and calpastatin (CAST). Blood samples were collected from 100 animals of the two breeds, and then DNA was extracted using a commercial kit. We used the PCR and RFLP techniques to determine genotypes and allele frequencies. The results showed that the MSTN and CAST genes are polymorphic. The MSTN gene has allelic frequencies (M and m) of 0.81, 0.19, and 0.76, 0.24 in the Awassi and Naimi breeds, respectively. The frequencies of the genotypes MM, Mm, and mm in the Awassi breed were 0.70, 0.19, and 0.11, but in the Naimi breed, they were 0.67, 0.13, and 0.20, respectively. Moreover, the number of alleles observed (Na), the effective number of alleles (Ne) and observed (Ho), and expected (He) heterozygosity were found to be 3, 2.30, 0.24, and 0.35 in the Awassi breed and 2, 1.62, 0.17, and 0.26 in the Naimi breed, respectively. The allelic frequencies (M and N) of the CAST of the Awassi and Naimi breeds are 0.86, 0.14, and 0.88, 0.12, respectively. The frequencies of the genotypes MM, MN, and NN in the Awassi breed were 0.94, 0.04, and 0.02, respectively, while for the Naimi breed, they were 0.95, 0.02, and 0.03, respectively. Also, the Na, Ne, Ho, and He were found to be 2.8, 1.72, 29.6, and 28.57 in the Awassi breed and 1.10, 1.23, 0.17, and 0.15 in the Naimi breed, respectively. According to the chi-square of MSTN and CAST genes, both breeds were not in Hardy-Weinberg equilibrium balance.

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Introduction

Genetic improvement programs play an important role in increasing production and reproduction in sheep through studying the associated genes related to growth, meat quality, milk production, and others (1). Recently, researchers in animal breeding and genetics have begun to study genes that influence meat yield and quality, such as the *myostatin* (*MSTN*) and *calpastatin* (*CAST*) genes (2), two of the most common genes in the study of meat characteristics and quality (3). Molecular studies of the *myostatin* (*MSTN*) and *calpastatin* (*CAST*) genes demonstrate that there is a structural polymorphic (4). Identifying crucial genes affecting several economic traits may provide significant opportunities for future improvement and selection programs, especially marker-assisted selection (MAS) (5). The *myostatin* (*MSTN*) gene is called a specialized growth factor 8 (*GDF-8*) and is part of the family of growth factors known as (*TGF-β*) (6). This includes negative body mass regulation and skeletal muscle growth (7). The *MSTN* gene inhibits skeletal muscle growth, and if a mutation in the gene encoding *myostatin* occurs, it changes its inhibitory role and increases muscle mass (8). The *MSTN* gene was discovered in 1997 in mice and plays a negative role in the growth and development of skeletal muscles (9). The *MSTN* gene in the sheep (*Ovis arise*) genome is located on chromosome 2 and contains 3 exons and 2 introns (10). Sheep *myostatin* mediates the expression of muscle fiber control genes and

practically stops muscle growth by preventing the proliferation of myoblasts; the *myostatin* gene has been studied more in cattle and less in sheep (11). In exon 3 of the sheep *MSTN* gene, three genotypes were first reported by (12). The *calpastatin* (*CAST*) gene is essential in growth parameters and carcass characteristics (13). The *CAST* gene is located in the sheep genome on chromosome 5 and contains 29 exons separated by introns (14). The CAST is one of the promising markers for sheep meat quality and growth rate; the *calpain* activity is inhibited by *CAST*, which affects the regulation of birth weight, growth rate to weaning, and postmortem meat tenderness (15). In 1998, a polymorphism in the *CAST* gene was discovered in the ovine using the PCR-RFLP technique, and two different alleles (M and N) were found (16). Palmer (17) selected the *CAST* gene to study the quality of meat in sheep, using molecular genetics techniques such as PCR-RFLP, the *CAST* gene is an important gene for studying genetic variation in farm animals (18). Sheep are the most suitable agricultural animals adapted for grazing in dry and challenging environmental conditions (19). Iraqi sheep belong to Asian fat-tailed sheep and include four breeds: Awassi, Naimi, Arabi, and Karadi (20). Awassi sheep are multi-purpose animals and comprise 60% of the native sheep population (21). They are used to produce meat, wool, and milk and are the most common breed of small ruminant in Iraq (22). The appearance characteristics of Naimi sheep are similar to Awassi sheep, except that their size is smaller, and they have a significant ability to tolerate a lack of food and water (23). Naimi sheep comprise 18% of the native sheep population (24).

The study objective is to determine the genetic variation of the *MSTN* and *CAST* genes in two Iraqi sheep breeds using the PCR-RFLP technique.

Materials and methods

Ethical approve

This study was conducted from 30/9/2022 to 25/2/2023 in the Laboratory of Physiology and genetic engineering in the Department of animal production techniques at the Al-Musiab Technical College/Al-Furat Al-Awsat Technical University and its location in Babylon, Iraq, with the ethical approval of the Institutional Animal Care and use committee No. 1116 in 9/15/2022.

Animals and blood collection

The study was conducted in the city of Babylon, placed in central Iraq, on two native sheep breeds, the Awassi breed (50 animals) and the Naimi breed (50 animals), from both sexes (males and females). Blood samples were collected 10 ml from the jugular vein using an EDTA tube. The samples were stored at -20°C until DNA extraction.

The DNA Extraction

DNA was extracted from the blood using a unique extraction kit from the company (Geneaid, USA), and accordance with the instructions in the kit. The quantity and quality of the extracted DNA were determined using gel electrophoresis at a concentration of 1% agarose.

PCR Amplification

Amplification of the PCR was performed with a final volume of 20 µl, which contains [master mix (Ampliqon, Denmark) 10 µl, 5µl (50 ng) DNA template, 2.5 µl of PCR buffer (10X), 1.0 mM $MgCl_2$, 0.5 mM dNTPs, 1.0 unit Taq DNA polymerase), 2 µl RFLP primer (forward and reveres), 3 µl of DNA sample and 5 µl DNase free water)]. Each of the genes was amplified using two pairs of primers. Primer sets recommended by Tolee *et al*. (25) for the *MSTN* gene and Palmer *et al*. (16) for the *CAST* gene were utilized (Table 1).

All PCR reactions were performed by the Bio-Rad T100 thermocycler (Bio-Rad T100, USA) the following way: initial denaturation at 95°C for 5 min, followed by 30 cycles consisting of denaturation at 95°C for 30 secs, annealing at 58°C (*MSTN*) and 62°C (*CAST*) for 45 secs, extension at 72°C for 1 min, and final extension at 72°C for 7 min (26,27). After this step, the PCR products were electrophoresed on a 2 % agarose gel for 60 minutes at a voltage of 85 volts. After staining with ethidium bromide, the bands obtained were visualized under UV transillumination (LABY, India).

Table 1: The genes, location on the chromosome, primers, PCR fragments length, and restriction enzymes of the *MSTN* and *CAST* genes in sheep breeds are indicated.

Genes/ Ch.	Primers	Size (bp)	Restriction Enzymes	References
MSTN/ (2)	F: 5'CCG GAG AGA CTT TGG GCT TGA3' R: 5'TCA TGA GCA CCC ACA GCG GTC3'	337	HaeIII (BsuRI)	(16)
CAST/ (5)	F:5'TGG GGC CCA ATG ACG CCA TCG ATG3' R:5' GGT GGA GCA GCA CTT CTG ATC ACC3'	622	MspI (HpaII)	(14)

Restriction fragment length polymorphism (RFLP) analysis

The RFLP technique was used to determine the genotype and genetic variation of the animal's genome, which were analyzed for both genes. The digestion reactions were performed in a final volume of 20 µl, containing 10 µl of PCR product, 5 µl ddH_2O, 4 µl 10X buffer, and 0.5 µl HaeIII (BsuRI) enzyme (Thermo Fisher Scientific, USA) for the

MSTN gene (9), and 0.5 µl MspI (HpaII) restriction enzyme (Thermo Fisher Scientific, USA) for the *CAST* gene (17). PCR products were incubated at 37 ºC for 14-15 hours using a thermocycler (Biometra, Germany). After digestion, the study samples were run to a 2% agarose gel electrophoresis concentration at 85 volts for 60 minutes. The gel was stained with ethidium bromide, measured using the 12-line (100-1000 bp) ladder (Life Science Company), and visualized under UV transillumination (LABY, India).

Statistical analysis

The allele sizes were calculated using UVdoc 99.02 analysis software (UVI Tech, Cambridge, UK) using the virtual gel image produced by the PCR products. Then, to prepare input files for each specific software, use CONVERT version 1.31 (28). To estimate the genotype and allele frequencies, the observed (Na) and effective (Ne) number of alleles, observed (Ho), expected (He) heterozygosities, and Hardy-Weinberg equilibrium were calculated using POPGENE software version 1.32 (29) and ARLEQUIN software version 3.5.2.2 (30).

Results

As shown in figure 1, the quantity and quality of extracted DNA were determined for Awassi and Naimi sheep samples. The results showed that the extracted DNA was good and could be used in the study.

The MSTN gene

After PCR amplification, a 337 bp *MSTN* gene was obtained. Then the PCR products were digested using the HaeIII (BsuRI) restriction enzyme. The allele m was affected by adding the enzyme and split into three pieces, while the M allele was not. Three fragments of 133, 123, and 83 bp were produced by the digestion of the m allele (Figure 2).

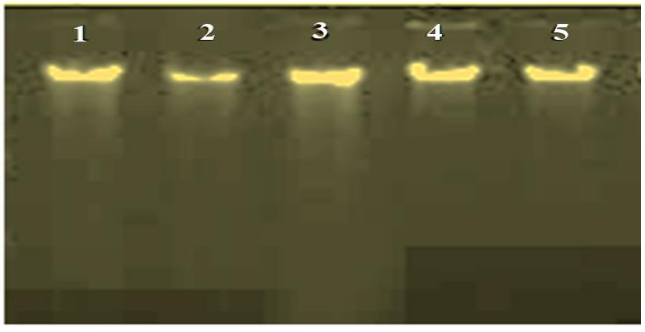

Figure 1: DNA extracted from blood samples of Awassi and Naimi sheep.

Table 2 shows the frequency of different genotypes and alleles in the population of the two Iraqi native sheep breeds, and most of the investigated animals are two native sheep breeds of the MM genotype. The observed frequencies of 0.70 (35 animals), 0.19 (10 animals), and 0.11 (5 animals) of the MM, Mm, and mm genotypes in the Awassi breed; 0.67 (33 animals), 0.13 (7 animals), and 0.20 (10 animals) of the frequencies of the same genotypes in the Naimi breed, respectively. M and m allelic frequencies were found to be 0.19 and 0.81 in the Awassi breed and 0.76 and 0.24 in the Naimi breed, respectively (Table 2).

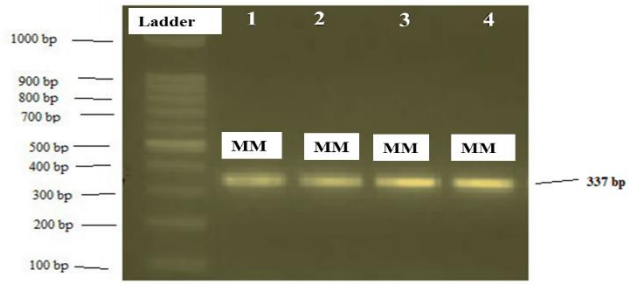

Figure 2: DNA electrophoresis of the *MSTN* gene after digestion with HaeIII (BsuRI) restriction enzyme in the Awassi sheep.

Table 2: The genotypes, number of animals, genotype, and allelic frequencies of the *MSTN* gene of sheep breeds are described

Sheep breeds	Genotype	Number of animals	Observed Genotype frequency	Allele frequency	
				M	m
Awassi sheep	MM	35	0.70	0.81	0.19
	Mm	10	0.19		
	mm	5	0.11		
Naimi sheep	MM	33	0.67	0.76	0.24
	Mm	7	0.13		
	mm	10	0.20		

The observed (Na) and effective (Ne) number of alleles, Shannon index (I), and coefficient of inbreeding (F_{IS}) are presented in the two native sheep breeds (Table 3). The results related to the Awassi breed Na (3), Ne (2.36), Ho (0.24), He (0.35), F_{IS} (0.81), and I (0.471), but the results related to the Naimi breed Na (2), Ne (1.62), Ho (0.17), He (0.26), F_{IS} (0.95), and I (0.526). Respecting $\chi 2$ analysis in the two sheep breeds (Table 3), the value of $\chi 2$ was 3.15 and 2.32 in the Awassi and Naimi, respectively, which showed that the sheep population in our study is not in equilibrium with the Hardy-Weinberg equation.

The *CAST* gene

The results illustrated four fragments of 622, 336, and 286 bp sizes. Three genotypes were found (Figure 3); MM (336 and 286 bp), NN (622 bp), and MN (622, 336 and 286 bp).

Table 3: The observed (Na), the effective (Ne) number of alleles, heterozygosity (Ho and He), coefficient of inbreeding (F_{IS}) Shannon index (I), and Chi-square test ($\chi 2$) for Hardy-Weinberg equilibrium of the ovine *MSTN* gene in sheep breeds studies

Sheep breeds	Allele number		Heterozygosity		F_{IS}	I	χ^2
	Na	Ne	Ho	He			
Awassi sheep	3	2.36	0.24	0.35	0.81	0.471	3.15
Naimi sheep	2	1.62	0.17	0.26	0.95	0.526	2.32

Na: number of alleles observed, Ne: effective number of alleles, FIS: coefficient of inbreeding, I: Shannon index, Ho: observed heterozygosity, He: expected heterozygosity, and $\chi 2$: Chi-square test for Hardy-Weinberg equilibrium.

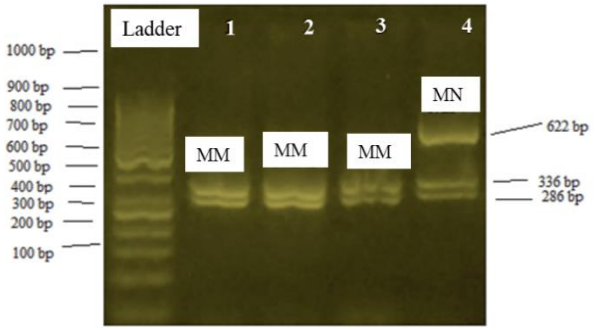

Figure 3: The *CAST* gene was shown after digestion using the restriction enzyme MspI (HpaII) in the Naimi sheep.

The frequencies of the MM, MN, and NN genotypes in Awassi and Naimi sheep populations were 0.94, 0.04, and 0.02 and 0.95, 0.02, and 0.03, respectively. Allelic frequency was 0.86 for the M allele and 0.14 for the N allele in Awassi sheep; however, the corresponding allele frequencies in Naimi sheep were 0.88 (M) and 0.12 (m) (Table 4).

The results of the allele number (Na and Ne), observed (Ho) and expected (He) heterozygosity, coefficient of inbreeding (F_{IS}), Shannon index (I), and chi-square (χ^2) of the *CAST* gene of Awassi and Naimi Iraqi sheep breeds are presented in Table 5. In the Awassi and Naimi sheep breeds, the Na and Ne results were 2.8, 1.10, and 1.72, 1.23, respectively (Table 5). The observed (Ho) and expected (He) heterozygosity values were 29.6, 0.17, and 28.57, 0.15 in the Awassi and the Naimi sheep, respectively (Table 5). The F_{IS} values found in this study, Awassi (0.72) and Naimi (0.79), respectively. The Shannon index (I) was 0.531 and 0.431 for the Awassi and Naimi sheep breeds, respectively. The chi-square analysis showed that the studied *CAST* gene in two native breeds of Iraqi sheep showed that the different herds were not in Hardy-Weinberg equilibrium (Table 5).

Table 4: The genotypes, number of animals, genotype, and allelic frequencies of the *CAST* gene of Awassi and Naimi breeds

Sheep breeds	Genotype	Number of animals	Observed Genotype frequency	Allele frequency	
				M	N
Awassi sheep	MM	47	0.94	0.86	0.14
	MN	2	0.04		
	NN	1	0.02		
Naimi sheep	MM	48	0.95	0.88	0.12
	MN	1	0.02		
	NN	1	0.03		

Table 5: Allele number (Na and Ne), coefficient of inbreeding (F_{IS}), Shannon index (I), Heterozygosity (Ho and He), and Chi-square test (χ^2) for Hardy-Weinberg equilibrium of the *CAST* gene of Iraqi Sheep Breeds

Sheep breeds	Allele number		Heterozygosity		F_{IS}	I	χ^2
	Na	Ne	Ho	He			
Awassi sheep	2.8	1.72	29.6	28.57	0.72	0.531	3.38
Naimi sheep	1.10	1.23	0.17	0.15	0.79	0.431	3.42

Na: number of alleles observed, Ne: effective number of alleles, FIS: coefficient of inbreeding, and I: Shannon index, Ho: observed heterozygosity, He: expected heterozygosity, and $\chi 2$: Chi-square test for Hardy-Weinberg equilibrium.

Discussion

It has been stated that *myostatin* gene polymorphisms are different in sheep, and in this research, all samples showed the two Iraqi native sheep that were polymorphic. These genotyping results are consistent with the polymorphism in the sheep *MSTN* gene previously observed in studies Aiello *et al*. (9), Grochowska *et al.* (31). Also, the results showed polymorphisms of the *MSTN* gene in the sheep breeds, which showed three genotypes: MM, Mm, and mm. These results are similar to those obtained Sahu *et al*. (32), Bozhilova-Sakova *et al*. (33), AL-Barzinji and Ameen (34). Based on the results of allelic frequencies, most Awassi and Naimi sheep individuals possess the M allele more than the m. These results are similar to the results of Grochowska *et al*. (31), Georgieva *et al*. (35). In addition, the frequency of

the MM genotype is higher than the Mm and mm genotypes in individuals of both breeds due to the Inbreeding that happens as a consequence of keeping a small number of rams in the herds, leading to an increase in homozygosity.

Our Na and Ne results for Awassi and Naimi sheep are higher than those reported by Iroanya *et al*. (36), Farhadian *et al.* (37), but lower than Dimitrova *et al.* (38). The obtained value of Ho and He in two native breeds are higher than those acquired by Mahrous *et al.* (39), Farhadian *et al*. (37) and lower than Al-Thuwaini (40), Nei (41). In the two native sheep, the frequency of heterozygous individuals indicated low genetic variation within the *MSTN* gene. This might be because these animals live in small herds with a few rams and genetic drift. Therefore, (F_{IS}) gained was higher than Khederzadeh *et al*. (42). Also, the (I) obtained in this study was elevated than Farhadian *et al.* (37), Putri *et al*. (12) and lower than Dimitrova *et al*. (38). Due to the M allele's higher frequency than the N allele, which results in a decrease in frequency at each locus, there is a difference between the number of effective and observable alleles and the low variation between the two sheep breeds. Shannon's index, which measures biodiversity, was low for two Iraqi breeds, showing that the *MSTN* gene in this study was lowly polymorphism. A positive and high F_{IS} value indicates that inbreeding is one of the essential reasons for the absence of heterozygotes in Iraqi sheep breeds. Low heterozygotes in the studied populations may be related to several variables, including the animal's mating system, sample size, and selection (42). These results are similarly reported by Iovenko *et al.* (43), Saygili and Ozdemir (44), Farhadian *et al.* (37).

The sheep population in our study is not in equilibrium with the Hardy-Weinberg equation. Disequilibrium in the equilibrium position may be the presence of some disruptive factors such as selection, migration, and sample size. It should be noted that the H.W.E. for the *MSTN* gene in these two breeds was similar Mahrous *et al.* (39), Degtyarev *et al*. (45). In this study, results showed that the genetic variation of the two sheep breeds is low due to inbreeding, the small number of animals, genetic drift, and the overlapping and closeness of the areas in which animals live geographically.

The *calpastatin* gene (*CAST*) is one of the most important genes used in the study of genetic variation. In this study, the results for genotypic and allelic frequencies for the two sheep breeds were consistent with those Kolosov *et al.* (46), Uppe *et al.* (47), Ardiclila *et al.* (48). Also, we showed that the observed genotype frequency (MM) and the allele frequency (M) are very high in the two sheep breeds of Awassi and Naimi due to the lack of genetic improvement programs for sheep and the low number of animals.

The results of the Na and Ne were higher than the recorded results of Suleman *et al.* (49) and lower than Dimitrova *et al.* (50). This difference between Na and Ne and low variation is due to the higher frequency of the M allele compared to the N allele, which decreases frequency at any locus. The frequency of heterozygous individuals in the two sheep breeds indicates low genetic diversity concerning the *CAST* gene. This interpretation cancels the study because its meaning denies the existence of two breeds but a group of mixed animals. Heterozygote deficiency, because of inbreeding and genetic drift, is a factor in this deficiency. These results are higher than the recorded results of Kirikci (51), Greguła-Kania (52), but lower than Khederzadeh *et al.* (42), Ramadevi *et al*. (53). The F_{IS} values found in this study showed individuals in the two populations of sheep breeds are closely related. The F_{IS} level seems very high in Awassi and Naimi sheep breeds. These results (F_{IS}) in two breeds are higher than Bahrampour *et al.* (54), Azari *et al.* (55). Our results showed that I in the Awassi sheep showed higher than Khederzadeh *et al.* (42); however, for the Naimi sheep, the result was lower. Therefore, this results in the two native sheep being higher than Dimitrova *et al.* (50).

The chi-square analysis showed that the studied *CAST* gene in two native breeds of Iraqi sheep showed that the different herds were not in Hardy-Weinberg equilibrium. This disequilibrium results from the substructure of populations under a severe selection process. The results of the two sheep breed of the study are similar to those of some researchers Tolee *et al.* (25), Dimitrova *et al.* (50).

The parameters for studying the genetic variation of the *CAST* gene of Awassi and Naimi Iraqi sheep breeds are presented, showing a low level of genetic variation because of inbreeding, bottlenecks, and founder effects. The Ho is greater than the He in both breeds, and this indicates that the two sheep breeds are in a state of slow improvement and can be used in animal breeding programs and genetic variation.

Conclusion

We concluded from our study the chance of using the *MSTN* and *CAST* genes polymorphic as molecular markers in genetic improvement and selection programs for growth-related traits, as well as showing that the PCR-RFLP technique is important in the study of genetic variation in Awassi and Naimi sheep breeds.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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دراسة التنوع الوراثي لجينات الميوستاتين والكالباستاتين في اثنين من الأغنام العراقية المحلية باستخدام تقنية أطوال قطع التقييد للتفاعل التضاعفي لسلسلة الدنا

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الخلاصة

هدفت الدراسة الى بحث التنوع الوراثي لسلالتي الأغنام العواسية والنعيمية وباستخدام الجينين الميوستاتين واالكالباستاتين. أظهرت النتائج أن الجينين الميوستاتين والكالباستاتين متعددة الأشكال. بينت النتائج أن الجين الميوستاتين يحتوي على ترددات اليلية M و m إذ بلغت ٠,٨١ و ٠,١٩ و ٠,٧٦ و ٠,٢٤ في الأغنام العواسية والنعيمية على التوالي. كانت تكرارات التراكيب الوراثية MM و Mm وmm في سلالة العواسي ٠,٠٧، ٠,١٩ و ٠,١١ أما في سلالة النعيمي فقد كانت ٠,٦٧، ٠,١٣ و ٠,٢ على التوالي. علاوة على ذلك، عدد الاليلات المشاهدة (Na) والاليلات المؤثرة (Ne) والخلط الاليلي المشاهد (Ho) والمتوقع (He) لتكون ٣، ٢,٣، ٠,٢٤ و ٠,٣٥ في سلالة العواسي و ٢، ١,٦٢، ٠,١٧ و ٠,٢٦ في سلالة النعيمي، على التوالي. كانت نتائج الجين الكالباستاتين الترددات الاليلية M و N في سلالاتي العواسي والنعيمي هي: ٠,٨٦، ٠,١٤ و ٠,٨٨ و ٠,١٢ على التوالي. كانت تكرارات التراكيب الوراثية MM ، MN وNN في سلالة العواسي ٠,٩٤، ٠,٠٤ و ٠,٠٢ على التوالي بينما كانت تكرارات التراكيب الوراثية في سلالة النعيمي هي: ٠,٩٥، ٠,٠٢ و ٠,٠٣ على التوالي. أيضا وجد أن Na ، Ne، Ho و He هي ٢,٨، ١,٧٢، ٢٩,٦ و ٢٨,٥٧ في سلالة العواسي و ١,١، ١,٢٣، ٠,١٧ و ٠,١٥ في سلالة النعيمي على التوالي. وفقا لمربع كاي لم تكن كلا السلالتين في توازن هاردي- واينبرغ.

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