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Research Article
Evaluation of Genetic Diversity of Naked Neck and Frizzle Genotypes Based on Microsatellite Markers

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Abstract

Background and Objective: The identification of genetic diversity for heat resistance genotypes, such as naked neck (Na) and frizzle (F) genes, is of great interest for scientists to be discovered along with the native breeds genetic map recognition. Since they represent the most important genotype flocks raised in tropical and semi-tropical areas. The extent of feather (or plumage distribution) and feather shape (straight or curled) divergence between both genotypes (whether homozygous or heterozygous state) morphologically, comparing with normally feather flock, need to be clarified using microsatellite markers technique side by side with productive performance. **Methodology:** According to morphological appearance of feather coverage, a total number of 326 birds, representing 5 genotypes (homozygous naked neck (NaNa), heterozygous naked neck (Nana), homozygous frizzle (FF), heterozygous frizzle (Ff) and normally feathered (nanaff) genotypes) were classified. At sexual maturity, the chickens were individually housed in wire cages located in semi-closed house. Adult body weight, age at sexual maturity, egg number and egg weight were recorded for each genotype throughout the first six month of laying cycle. At 30 weeks of age, egg quality characteristics were examined. Forty birds were randomly assigned (8 birds/genotype) to assess cell mediated immunity through PHA-P injection in wattles. Blood samples were collected from the wing vein. The DNA was purified by successive extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1), respectively. A total of 20 microsatellite markers were selected based on the degree of polymorphism reported in the literature. The PCR amplification was carried out in 25 µL reaction volumes, gels were stained in ethidium bromide and DNA bands were visualized on UV-transilluminator. Data of SSR analyses were scored on the basis of the presence or absence of the amplified products for each primer. The similarity coefficients were then used to construct a dendrogram by Unweighted Pair-Group Method with Arithmetical Average (UPGMA). **Results:** The productive results revealed that the introducing Na and F genes in chicken breeds raised under hot weather significantly improved most of egg production and eggshell quality traits. Moreover, significantly higher cell mediated response was found in naked neck and frizzle genotypes particularly, in homozygous manner compared to normally feathered genotype. The results revealed that the microsatellite markers had 83 alleles with an average of 4.2±0.24 alleles per locus. It could be observed that polymorphism ranged from 25-100% with an average of 64.7% for all markers. A remarkable extensive genetic diversity was seen among the studied genotypes. Genetic distance as a pair-wise comparison of different genotype ranged from 0.14 (NaNa-Nana) to 0.41 (Nana-FF). Both naked neck genotypes and frizzle sibs located in a separate sub-cluster resulted in a clear distinction between the two major genes. **Conclusion:** It was concluded that the evaluation of genetic diversity among chicken genotypes carrying Na or F based on the studied microsatellite markers was efficient and gained consistent results.

Key words: Microsatellites, genetic diversity, major genes, biodiversity, naked neck gene, frizzle gene

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The ecotype of birds varies according to the ambient temperature and available facilities for the animal welfare, some species are acclimatized to survive in both temperate and hot climate with a well productive profile and those got a reputable productivity and livability eminence in tropical areas and therefore, it is soon expanded in many hot climatic countries around the world. The conservation of fowl species received a tremendous concern from the biologists, geneticists and breeders. Microsatellite markers considers the main tool for identifying the genetic divergence of these genotypes by applying molecular tools for conservation and developing poultry genetic resources. The prestigious major genes affecting feather coverage of chicken, particularly naked neck (Na) and frizzle (F), are well known for its effects on heat tolerance[1,2]. These major genes are also believed to confer resistant to diseases and greatly enhance immune status under hot environmental conditions. Many hot-climate regions in Asia, Africa and South America have native breeds carrying both Na and F in a single manner or in combination. Incorporating naked neck and frizzle genes in breeding programs was adopted to improve productive performance and adaptability of chickens under hot ambient temperatures[1-3].

A lot of these native breeds are not genetically documented. However, genetic diversity of indigenous chicken breeds is a valuable resource for genetic improvement and conservation[4-7]. Native breeds and village chicken populations exhibited highly microsatellite and Single Nucleotide Polymorphism (SNP) diversity[8,9]. Microsatellite markers have been shown to be an appropriate tool to estimate the genetic diversity among chicken populations[9-13]. Few attempts were carried out to genetically evaluate native breeds carrying naked neck and frizzle in heterozygous status using microsatellite markers[14-16]. However, little information was known regarding standardizing and characterizing chickens breeds carrying Na and F genes in homozygous or heterozygous status. Therefore, the present study applied microsatellite analysis technique to evaluate the genetic diversity of 5 genotypes (NaNa, Nana, FF, Ff and nanaff) using 20 markers.

MATERIALS AND METHODS

Birds, husbandry and genotypes: A total of 326 birds including 5 genotypes (42 NaNa, 77 Nana, 35 FF, 52 Ff and 120 nanaff) produced from the same origin were used in the current study. At sexual maturity, the chickens were individually housed in wire cages located in semi-closed house. All birds were kept under identical environmental, nutritional and health conditions. The care and handling of birds were in accordance with regulations of animal care committee of Qassim University.

Productive traits: Adult body weight and age at the onset of laying were recorded for each genotype. Egg number and egg weight were determined during the first six month of laying cycle. At 30 weeks of age, an experiment was conducted to determine egg quality characteristics. Also, 40 birds were randomly assigned (8 birds/genotype) to assess cell mediated immunity through PHA-P injection in wattles. Swollen response in wattle was measured 24, 48 and 72 h post injection.

Blood and DNA preparation: Thirty chickens per population were sampled, overall 150 DNA samples were extracted. Blood samples, 3-5 mL per bird were collected from the wing vein using EDTA as an anticoagulation agent and stored at -20°C. The DNA was purified by successive extraction with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1), respectively. The DNA was precipitated first using 0.6 volume of isopropanol, pelleted by centrifugation, then resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH = 8.0). The DNA was reprecipitated by adding two volumes of ethanol in the presence of 0.3 M sodium acetate and pelleted by centrifugation. The pellets were then washed with 70% ethanol, air-dried and finally resuspended in an appropriate volume of TE buffer.

Microsatellite markers: A total of 20 microsatellite markers were chosen based on the degree of polymorphism reported in the literature (Table 1) to identify the genetic diversity among 5 different genotypes of chickens. The microsatellite markers were recommended by the Standing Committee[17].

Simple sequence repeats assay: The PCR amplification was carried out in 25 µL reaction volumes, containing 1X Taq polymerase buffer (50 mM KCl, 10 mM Tris, pH = 7.5, 1.5 mM $MgCl_2$) and 1 unit of Taq polymerase (Pharmacia Biotech, Germany) supplemented with 0.01% gelatin, 0.2 mM of each deoxynucleotide triphosphates (dNTPs) (Pharmacia Biotech, Germany), 50 bp of Sample Sequence Repeats (SSR) primers and 50 ng of total genomic DNA. Amplification was performed in a thermal cycler (Thermolyne Amplitron) programmed for one cycle of 2 min at 94°C and 35 cycles of 30 sec at 94°C, 45 sec at either 42 or 50°C, depending on the melting

Table 1: Description of 20 microsatellite markers used in the current study

Primers	Chromosomal location	Primer sequence (5'→3') forward and reverse	Annealing temperature (°C)	Allele size range (bp)
MCW0248	1	GTTGTTCAAAAGAAATGCATG	60	205-225
		TTGCTTAACTGGGCACTTTC		
MCW0111	1	GCTCCATGTGAAGTGGTTTA	60	96-120
		ATGTCCACTTGTCAATGATG		
ADL0268	1	CTCCSCCCCTCTCAGAACTA	60	102-116
		CAACTTCCCATCTACCTACT		
MCW0020	1	TCTTCTTTGACATGAATTGGCA	60	179-185
		GCAAGGAAGATTTTGTACAAAATC		
LEI0234	2	ATGCATCAGATTGGTATTCAA	60	216-364
		CGTGGCTGTGAACAAATATG		
MCW0206	2	ACATCTAGAATTGACTGTTCAC	60	221-249
		CTTGACAGTGATGCATTAAATG		
MCW0034	2	TGCACGCACTTACATACTTAGAGA	60	212-246
		TGTCCTTCCAATTACATTCATGGG		
MCW0103	3	AACTGCGTTGAGAGTGAATGC	64	266-270
		TTTCCTAACTGGATGCTTCTG		
LE10166 (POMC)	3	CTCCTGCCCTTAGCTACGCA	60	354-370
		TATCCCCTGGCTGGGAGTTT		
MCW0295	4	ATCACTACAGAACACCCTCTC	60	88-106
		TATGTATGCACGCAGATATCC		
MCW0081	5	GTTGCTGAGAGCCTGGTGCAG	60	112-135
		CCTGTATGTGGAATTACTTCTC		
MCW0014	6	TATTGGCTCTAGGAACTGTC	58	164-182
		GAAATGAAGGTAAGACTAGC		
MCW0183	7	ATCCCAGTGTCGAGTATCCGA	58	296-326
		TGAGATTTACTGGAGCCTGCC		
ADL0278	8	CCAGCAGTCTACCTTCCTAT	60	114-126
		TGTCATCCAAGAACAGTGTG		
MCW0067	10	GCACTACTGTGTGCTGCAGTTT	60	176-186
		GAGATGTAGTTGCCACATTCCGAC		
MCW0104	13	TAGCACAACTCAAGCTGTGAG	60	190-234
		AGACTTGCACAGCTGTGTACC		
MCW0123	14	CCACTAGAAAAGAACATCCTC	60	76-100
		GGCTGATGTAAGAAGGGATGA		
MCW0330	17	TGGACCTCATCAGTCTGACAG	60	256-300
		AATGTTCTCATAGAGTTCCTGC		
MCW0165	23	CAGACATGCATGCCCAGATGA	60	114-118
		GATCCAGTCCTGCAGGCTGC		
MCW0069	26	GCACTCGAGAAAACTTCCTGCG	60	158-176
		ATTGCTTCAGCAAGCATGGGAGGA		

temperature (Tm) value of the primer pair and 1.3 min at 72°C, followed by 20 min at 72°C. After completion of PCR, samples were cooled immediately to 10°C and stored at 4°C until gel separation. A gel-loading solution (5 μL) was added and 10 μL of the total product volume was resolved in 1.5% agarose in 1X Tris-acetate-EDTA (TAE) buffer for 2 h aside, with a 100 bp ladder (Pharmacia, Germany) as the size standard. Gels were stained in ethidium bromide and DNA bands were visualized on UV-transilluminator. The images were photographed by a Polaroid camera (Gel Cam Polaroid camera, Sigma-Aldrich Corp).

Genetic data analysis: Data of SSR analyses were scored on the basis of the presence or absence of the amplified products for each primer. If a product was present in a cultivar, it was designated "1", if absent it was designated "0". Pairwise comparisons among the genotypes based on SSR markers with 25-100% heterozygosity, were used to generate Dice coefficients of similarity[18]. The similarity coefficients were then used to construct a dendrogram by Unweighted Pair-Group Method with Arithmetical average (UPGMA) using NTSYS-PC software version 2.0[19].

RESULTS AND DISCUSSION

Productive traits and cell mediated immunity: Traits of productive performance and cell mediated immunity are shown in Table 2. Introducing naked neck gene in both

Table 2: Productive performance and cell mediated immunity for the different genotypes

	Genotypes						
Traits	NaNa	Nana	FF	Ff	nanaff	SEM	Probability
Adult body weight (g)	1291.1[a]	1304.9[a]	1059.9[c]	1178.4[b]	1147.4[b]	15.66	0.0001
Age at sexual maturity (day)	157.5[c]	156.1[c]	154.5[c]	163.9[b]	169.9[a]	3.40	0.0005
Egg numbers	109.4[ab]	114.8[a]	99.5[b]	109.1[ab]	87.6[c]	6.42	0.0001
Egg weight (g)	46.4[ab]	48.0[a]	45.9[b]	46.3[ab]	46.6[ab]	0.88	0.01
Haugh unit	77.4[ab]	78.2[a]	77.9[ab]	77.3[ab]	75.7[b]	0.75	0.01
Shell thickness (mm)	0.380[ab]	0.386[a]	0.373[b]	0.374[b]	0.37[b]	0.006	0.01
Breaking force (kg cm^{-2})	4.01[b]	4.33[a]	3.85[c]	3.99[b]	3.78[c]	0.12	0.005
Wattle swollen response							
24 h post injection	0.66[a]	0.32[b]	0.51[a]	0.30[b]	0.28[b]	0.06	0.0005
48 h post injection	0.29	0.26	0.31	0.20	0.21	0.03	NS
72 h post injection	0.23[ab]	0.18[ab]	0.25[a]	0.18[ab]	0.12[b]	0.03	0.01

[a,b,c] Means within the same row with different letters are significantly differed, NS: Non-significant

Table 3: Alleles number and polymorphic percentage (Mean±SE) for the microsatellite markers

Primers code	Chromosome location	No. of alleles	Polymorphism (%)
MCW0248	1	4	75.0
MCW0111	1	3	66.7
ADL0268	1	3	66.7
MCW0020	1	4	75.0
LEI0234	2	5	80.0
MCW0206	2	3	66.7
MCW0034	2	4	25.0
MCW0103	3	3	33.3
LE10166	3	3	33.3
MCW0295	4	4	75.0
MCW0081	5	5	40.0
MCW0014	6	4	75.0
MCW0183	7	5	60.0
ADL0278	8	3	33.3
MCW0067	10	4	50.0
MCW0104	13	6	83.3
MCW0123	14	4	100.0
MCW0330	17	5	80.0
MCW0165	23	7	100.0
MCW0069	26	4	75.0
Average		4.2±0.24	64.7±4.93

genotypes significantly ($p<0.0001$) increased adult body weight compared with normally feathered counterparts. On the other hand, the chickens carrying F gene in homozygous state had the lighter body weight. The superiority of Na gene on body weight under hot environmental conditions was well established[1]. All naked neck and frizzled genotypes reached sexual maturity earlier than normal plumage hens. With respect to egg production traits (number and weight), several researchers confirmed that both Na and F genes improved egg mass when compared with normal hens under high ambient temperature and this trend was more consistent in heterozygous naked neck genotype[1,20-22]. In terms of egg quality measurements, the current results revealed that the heterozygous naked neck (Nana) genotype significantly ($p<0.001$) improved Haugh unit and shell thickness compared to normally feathered one. Also, both Na and F genes significantly ($p<0.005$) increased breaking strength of eggshell compared to normal genotype (nanaff). However, FF and nanaff genotypes showed almost the same eggshell strength. Concerning cell mediated immunocompetence, both naked neck and frizzle genotypes in homozygous manner (NaNa and FF) had significantly ($p<0.0005$) higher performance compared to normal genotype (nanaff) after 24 h of PHA-P injection. Similar trend was observed at the later time of cell mediated response test[2]. In general, many reports referred that the presence of Na or F gene increased cell mediated response[23-27].

Microsatellite markers and polymorphism: All microsatellite loci used are located on autosomal chromosomes (Table 1). The results of polymorphism percentage and the number of detected alleles are shown in Table 3. It is of interest to summarize that the microsatellite markers had 83 alleles with an average of 4.2±0.24 alleles per locus. The number of amplification bands per primer varied between 3 and 7. The MCW0165 primer recorded the highest alleles (= 7), while six primers out of 20 recorded the lowest alleles (= 3). The current results revealed that the selected microsatellite markers to be a tool for genotype diversity were reliable and informative because the average of allele number per locus was 4.2 loci. Fathi *et al.*[13], Ya-Bo *et al.*[28], Shahbazi *et al.*[29] and Nassiri *et al.*[30] suggested that microsatellite loci used in studies of genetic distance should have more than 4 alleles per locus in order to reduce the standard errors of distance estimates; consequently the microsatellite markers used in the present study were suitable for genetic diversity analysis. In agreement with the study of Fathi *et al.*[13], the used markers in the present study seem to have discriminative power in separation of Saudi native chicken breeds. The average genetic diversity in chicken based on the pooled DNA analysis was 47%[31], while based on individual records was 51%[8].

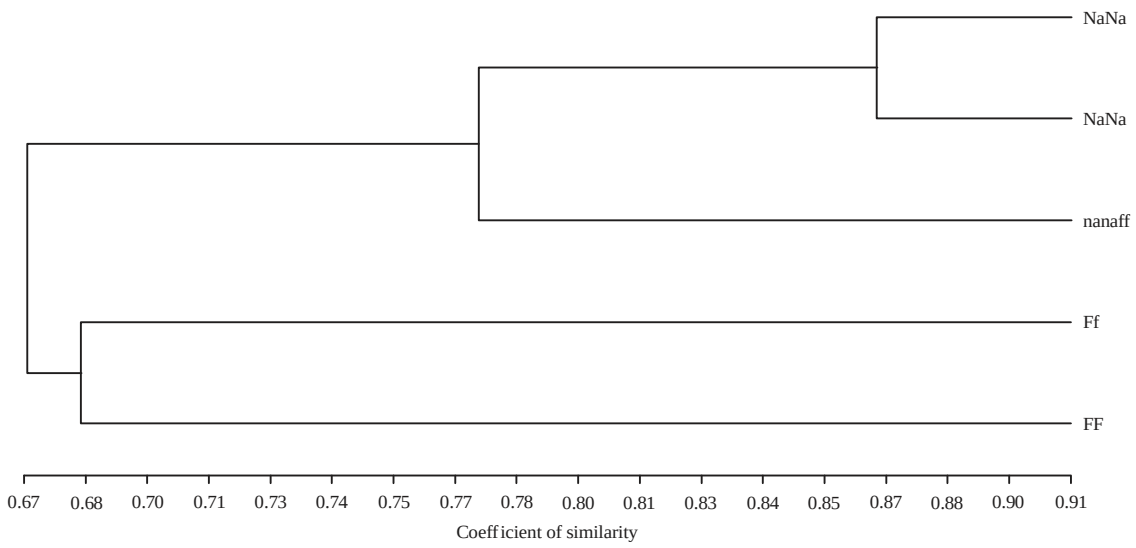

Fig. 1: Dendrogram constructed from similarity coefficients of the studied genotypes

Table 4: Genetic distance matrix of the studied genotypes

Genotypes	NaNa	Nana	FF	Ff	nanaff
NaNa	-				
Nana	0.14	-			
FF	0.32	0.41	-		
Ff	0.27	0.36	0.32	-	
nanaff	0.25	0.21	0.27	0.37	-

NaNa: Homozygous naked neck, Nana: Heterozygous naked neck, FF: Homozygous frizzle, Ff: Heterozygous frizzle, nanaff: Normally feathered

All markers exhibited a varied percentage of polymorphism among the five studied genotypes. However, there were 56 polymorphic loci out of 83 loci. It could be observed that polymorphism ranged from 25% for MCW0034 marker to 100% for MCW0123 and MCW0165 markers with an average of 64.7±4.9% for all markers. In agreement with the findings of present study, Shahbazi *et al.*[29] suggested that the number of polymorphic microsatellite loci should also be at least 15-20 to improve the discriminating ability of the dendrogram analysis. Additionally, Rosenberg *et al.*[32] stated that at least 12-15 highly variable microsatellites should be genotyped in at least 15-20 individuals per hypothesized-population to achieve 90% of the clustering success. The results revealed that the percentage of polymorphic loci was higher in normally feathered genotype (nanaff) (90%) than that of heterozygous naked neck (Nana) genotype (60%). The residual genotypes were intermediate (heterozygous frizzled Ff (85%), homozygous naked neck NaNa (75%) and homozygous frizzle FF (65%)). The maximum number of polymorphic loci were detected in nanaff genotype (18) followed by Ff (17), NaNa (15), FF (13) and Nana (12). However, the great genetic polymorphism found in the studied genotypes was resulting from phenotypic variations in feather structure and distribution. The high genetic diversity in these genotypes is important for genetic resource conservation and further breeding programs.

Genetic diversity and similarity among naked neck and frizzled genotypes: The genetic distance matrix among naked neck and frizzled genotypes of chickens is shown in Table 4. A remarkable extensive genetic diversity is seen among the studied genotypes. It could be observed that the genetic distance as a pair-wise comparison of different genotype ranged from 0.14 (NaNa-Nana) to 0.41 (Nana-FF). Genetic distance was the highest between Ff and nanaff (0.37). The higher genetic distance between both genotypes of frizzle appearance may be due to a lot of modifying genes associated with homozygous frizzle (FF) chickens[23]. These modifiers could alter the genetic material of FF and let it far from Ff genotype. However, the high genetic diversity found among these genotypes is of interest for conservation of genetic resource under high environmental circumstances.

Dendrogram constructed from coefficient of similarity illustrating clusters for the different genotypes is given in Fig. 1. Two main clusters were identified. The first cluster included frizzled genotypes, while the second one divided into two sub-clusters. One of them included the naked neck genotypes, while the second incorporated the normally feathered type. As expected both naked neck genotypes and frizzle sibs located in a separate sub-cluster resulting in a clear distinction between the two major genes. Higher similarity (0.86) was recorded between both naked neck genotypes

(homozygous and heterozygous). This high figure of similarity (0.86) suggested that the 2 genotypes of naked neck may be genetically derived from the same genetic origin. Similarity was more pronounced between normally feathered genotype and both genotypes of naked neck than that of both frizzled genotypes. In congruent with findings of present study, Granevitze *et al*.[8] and Hillel *et al*.[31] stated that the existence of multiclustered populations referred to high polymorphic situation.

CONCLUSION

In conclusion, evaluation of genetic diversity among chicken genotypes carrying Na or F genes using 20 microsatellite markers studied in the present study was efficient and gained reliable results. Conservation of naked neck and frizzle chicken breeds is of interest to improve productive performance in further breeding programs under high ambient temperature regions.

SIGNIFICANCE STATEMENT

The study discovered that the naked neck genotypes (NaNa and Nana) were inherited from the same origin with the normally feathered genotype, in contrary with the frizzle genotypes which were inherited from other heterogeneous sub-cluster. This emphasize the morphological structure of the feather where both genotypes (naked neck and normal feathered) are identical in the plumage shape and differed only in the extent or distribution of feather on the body. While frizzle genotype not only differed with the normally plumage in feather amount and extent, but also varied in the feather structure with a curvy shape (curled) against the body surface. This fact will help the breeders in hybridization programs to avoid the genetic segregations. In addition, use of normally feathered native/local breeds mating with naked neck genotypes when introducing the heat tolerant genes into tropical region was more beneficial than introduction of frizzle genotypes which need more generations and wasting more time and money to be prevalent.

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