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Genetic variability determination in a long-term selected Rhode Island Red chicken strain using microsatellite markers

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ABSTRACT

Genetic improvement needs the existence and precise estimation of genetic variability, and microsatellites are a molecular marker of choice for its assessment. Rhode Island Red (RIR) is a brown-egger chicken breed. A RIR population, selected on the basis of 40-week part-period egg production for 30 generations, maintained as a closed flock to develop multi-colored strains for backyard farming, were studied to determine genetic variability and heterozygosity using microsatellite markers. Genomic DNA from 76 randomly selected birds was analyzed at 10 microsatellite loci, alleles were separated on 3.4% MetaPhor Agarose, sized using Quantity-One software and analyzed by POPGENE v1.32. Nine loci demonstrated polymorphism resolving 30 alleles, the average number of alleles/ locus being 3.00 ± 1.41. Allele numbers varied from two to six and size ranged from 102 to 320bp. The average polymorphic information content and Nei's heterozygosity were 0.3134 ± 0.064 and 0.4119 ± 0.2575, respectively, suggesting these loci to be moderately polymorphic and informative. The observed (N_a) and effective number (N_e) of alleles and Shannon's index averaged 3.0000 ± 1.4142, 2.0324 ± 0.9416 and 0.7342 ± 0.4649, respectively. N_e was less than N_a at most loci, indicating the prevalence of heterozygosity at these loci. Mean expected heterozygosity was higher than mean observed heterozygosity at most loci, indicating that the population was not in Hardy-Weinberg equilibrium but was under the influence of some forces such as selection, etc. The same was also demonstrated by Chi-square and G-square tests. It may be inferred that long-term selection for 40-week part-period egg production has resulted in Hardy-Weinberg disequilibrium at the studied microsatellite loci, thereby suggesting a probable association between these microsatellite loci and layer economic traits in RIR chicken, and this might be useful in marker assisted selection for egg production in future.

Key words: heterozygosity, microsatellites, Rhode Island Red chicken

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Introduction

India ranks 3rd in egg and 4th in chicken meat production (PRABAKARAN, 2015). The per capita availability of eggs is around 57 per head per year (DAHD, Annual report 2013-14) against the ICMR recommendation of a minimum of 180 eggs per head per year (BORAH and HALIM, 2014). In order to provide nutritional and livelihood security, it is important to develop good chicken genotypes for backyard poultry production. Rhode Island Red (RIR) chicken is a brown egger breed and is preferred for development of multi-coloured strains suitable for backyard poultry production. In 1980, the Central Avian Research Institute, Izatnagar, imported 10,400 fertile eggs of Rhode Island Red (RIR) chicken from the USA (DAS et al., 2015a) and hatched them at the institute hatchery. RIR birds were then subjected to selection on the basis of part-record egg production for 30th generations and have been maintained as a closed flock since then, along with a random bred control population. The performance of the RIR selected strain is analyzed periodically. Development of commercial strains through long-term selection experiments depends upon genetic variability in the population. Various factors, such as genetic drift, inbreeding, natural selection, etc. may influence the response to selection (REDDY et al., 2004), which are reduced after continuous selection over generations because of the exhaustion of additive genetic variance and fixation of alleles. It is therefore required to estimate genetic variability precisely using either phenotypic or genetic markers. Advances in molecular genetics techniques have provided opportunities for assessment of genetic variability at the DNA level, complementing the traditional breeding procedures with selection based on DNA/ molecular markers. Molecular markers are simply identifiable DNA sequences, found at specific locations in the genome and are transmitted from one generation to the next by the standard laws of inheritance. Genetic markers are also helpful in tracking the inheritance of linked segments of the genome in pedigrees. In poultry, various molecular markers have been used for estimation of genetic variability and relatedness (PARMAR et al., 2007).

Microsatellite markers have been recommended as the marker of choice for biodiversity studies (FAO, 2011).They are regarded as the most convenient tool for determination of heterozygosity and genetic distances, as they are numerous and randomly distributed throughout the chicken genome, they show a higher degree of polymorphisms, follow co-dominant inheritance (ABEBE et al., 2015) and are ideal for deciphering genetic variability (ZHOU et al., 2008). Therefore, the present investigation was undertaken to evaluate the genetic heterogeneity and population structure of a long-term selected pure strain of RIR chicken, using microsatellite markers.

Materials and methods

Blood samples were collected from 76 randomly selected Rhode Island Red (RIR) pullets, maintained at the Experimental Layer Farm of the institute. Genomic DNA was isolated from 0.1ml of venous blood by the Phenol-Chloroform extraction method (KAGAMI et al., 1990). Having assessed the quality of DNA using 0.7% horizontal Agarose gel electrophoresis, and its purity and quantity by NanoDrop spectrophotometer (NanoDrop Technologies, USA), the DNA samples with an intact band and optical density ratio (260/280nm) within the normal range, 1.7-1.9, were subjected to PCR analysis. A panel of ten informative microsatellite markers (Table 1) with a known association with egg production traits in various chicken breed (CHATTERJEE et al., 2008; CHATTERJEE et al., 2010a; ARYA, 2012) were selected, having been synthesized from M/s Xcelris Genomics Labs Ltd., Ahmedabad (India), and screened for use in the present study.

PCR reactions were carried out in a 25 µL reaction mix prepared by mixing 5 µL of 5X GoTaq® Flexi buffer (Promega, U.S.A), 1.5 µL of 25 mM $MgCl_2$ (Promega, U.S.A), 0.5 µL of 10 mM dNTPs (Thermo Fisher Scientific Inc., U.S.A.), 1 µL (10 pM) each of forward and reverse primers, 0.15 µL (5U/µL) GoTaq® DNA polymerase (Promega, Madison, U.S.A) and 50 ng genomic DNA as a template in nuclease free water. Amplification was carried out in a programmable thermal cycler (PTC-200, M.J. Research, USA) using the PCR programme consisting of initial denaturation at 94 °C for 5 min. followed by 30 cycles of (i) denaturation at 94 °C for 1 min. (ii) annealing at optimized temperature for each microsatellite primer pair for 45 s and (iii) extension at 72 °C for 45 s followed by final extension at 72 °C for 5 min. and then 4 °C for forever. The amplified PCR products were initially checked using 1.5% Horizontal submarine Agarose gel electrophoresis. Having confirmed the successful amplification of all the samples, final resolution was done on 3.4% MetaPhor Agarose gel electrophoresis, along with molecular size marker (ASIF et al., 2008), and documented to resolve microsatellite alleles for further genotyping.

Molecular sizes of various alleles at different microsatellite loci were determined using Quantity One® software through the GelDoc-2000 system (BioRad U.S.A.).The observed alleles in each sample at each microsatellite loci and its probable genotypes were recorded. The number of observed alleles per microsatellite locus, and allelic and probable genotypic frequencies were calculated as described in DAS et al. (2015b).

Table 1. Amplification condition, allele size and allelic frequencies of microsatellite markers in a selected pure strain of RIR chicken population

MS loci		Primer sequence	Chromosomal location	25 mM $MgCl_2$	T (°C)	Allele sizes (bp)	Allele frequency
ADL0020	F	5'GCACTCAAAAGAAAACAAT	1	3.0 µL	55	126,118,110, 102	0.013,0.053, 0.783, 0.151
	R	5'TAGATAAAAATCCTTCCCTT					
ADL0023	F	5'CTTCTATCCTGGGCTTCTGA	5	1.5 µL	61	202, 188, 176	0.105, 0.158, 0.737
	R	5'CCTGGCTGTGTATGTGTTGC					
ADL0102	F	5'TTCCACCTTTCTTTTTTATT	30	1.5 µL	48	120, 112, 110	0.289, 0.408, 0.303
	R	5'GCTCCACTCCCTTCTAACCC					
ADL0176	F	5'TTGTGGATTCTGGTGGTAGC	2	1.5 µL	55	220, 214, 206, 202	0.053,0.243, 0.474, 0.230
	R	5'TTCTCCCGTAACACTCGTCA					
ADL0210	F	5'ACAGGAGGATAGTCACACAT	E 30	1.5 µL	52	132, 124	0.053, 0.947
	R	5'GCCAAAAAGATGAATGAGTA					
MCW0007	F	5'AGCAAAGAAGTGTTCTCTGTTCAT	1	1.5 µL	62	302, 275, 262	0.105, 0.586, 0.309
	R	5'ACCCTGCAAACTGGAAGGGTCTCA					
MCW0014	F	5'AAAATATTGGCTCTAGGAACTGTC	6	1.5 µL	60	192, 176	0.138, 0.862
	R	5'ACCGGAAATGAAGGTAAGACTAGGC					
MCW0041	F	5'CCCATGTGCTTGAATAACTTGGG	2	1.5 µL	57	164	1.000
	R	5'CCAGATTCTCAATAACAATGGCAG					
MCW0069	F	5'GCACTCGAGAAAACTTCCTGCG	26	1.5 µL	55	232, 221, 210, 194,184, 172	0.026, 0.059, 0.198, 0.138, 0.408, 0.171
	R	5'ATTGCTTCAGCAAGCATGGGAGGA					
MCW0103	F	5'AACTGCGTTGAGAGTGAATGC	3	1.5 µL	55	298, 276	0.263, 0.737
	R	5'TTTCCTAACTGGATGCTTCTG					

Average heterozygosity at each microsatellite marker was calculated according to NEI's (1978) formula:

$$H_i = \frac{2N}{2N-1}(1-\sum_{j=1}^{k} P_j^{\,2})$$

where, P_j is the frequency of j^{th} allele at i^{th} locus with k number of alleles in a population and N is the total number of individuals, assuming that the population was subject to Hardy-Weinberg equilibrium.

Polymorphic Information Content (PIC) at each microsatellite locus was calculated as per BOTSTEIN et al. (1980):

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

where, P_i and P_j are the frequencies of i^{th} and j^{th} alleles, respectively with n numbers of alleles in a population.

The complete genotypic data were subjected to estimation of population genetic parameters, viz., allele frequencies, observed and the effective number of alleles, Shannon's information index, observed and expected heterozygosity, goodness of fit (Chi-square) and likelihood ratio (G-square) tests using POPGENE® version 1.3.1 software (YEH et al., 1999).

Results

Nine out of ten microsatellite loci were found to be polymorphic with the presence of two to six alleles across the population. Out of all the polymorphic microsatellites, MCW0069 was observed to be the most polymorphic marker, showing six alleles of molecular sizes ranging from 172-232bp. A total of 30 distinct alleles were detected in 76 birds at 10 microsatellite markers. The overall allelic frequencies of various microsatellite loci ranged from 0.053 to 0.947.

Nei's average heterozygosity at polymorphic microsatellite loci was estimated as 0.4119 ± 0.2475, which ranged from 0.0997 at ADL0210 to 0.7421 at the MCW0069 locus. The mean PIC values ranged from 0.0947 (ADL0210) to 0.6020 (ADL0176) with a mean of 0.3134 ± 0.064. The PIC values indicated that all the loci, except ADL0102 and ADL0176, were low to moderately polymorphic.

The mean ± SE of the observed and effective number of alleles and Shannon's index were 3.0000 ± 1.4142, 2.0324 ± 0.9416 and 0.7342 ± 0.4649, respectively. The effective number of alleles ranged from 1.1108 (ADL0210) to 3.8778 (MCW0069) (Table 2). The effective number of alleles at each locus was less than the observed number of alleles, indicating the prevalence of heterozygosity at each locus.

Table 2. Measures of genetic variation in a selected pure strain of Rhode Island Red chicken

Microsatellite loci	Nei's H (%)	PIC Values	N_a	N_e	I	H_o	H_e	df	Chi square	G square
ADL0020	0.3612	0.3294	4	1.5655	0.6893	0.1316	0.3636	6	68.642****	66.660****
ADL0023	0.4211	0.3814	3	1.7273	0.7534	0.2105	0.4238	3	88.571****	82.570***
ADL0102	0.6582	0.5845	3	2.9260	1.0864	0.4079	0.6626	3	46.305****	52.272****
ADL0176	0.6606	0.6020	4	2.9462	1.1910	0.3289	0.6650	6	56.104****	59.279****
ADL0210	0.0997	0.0947	2	1.1108	0.2062	0.0263	0.1004	1	46.991****	17.091****
MCW0007	0.5505	0.4752	3	2.2245	0.9133	0.2895	0.5541	3	40.692****	41.228****
MCW0014	0.2381	0.2098	2	1.3126	0.4016	0.1711	0.2397	1	6.511*	5.014**
MCW0041	0.0000	0.0000	1	1.0000	0.0000	0.0000	0.0000	-	-	-
MCW0069	0.7421	0.1451	6	3.8778	1.5246	0.6842	0.7470	15	110.640****	114.805****
MCW0103	0.3878	0.3126	2	1.6335	0.5763	0.5263	0.3904	1	9.411***	14.248****
Mean ± SE	0.4119 ± 0.2475	0.3134 ± 0.064	3.0000 ± 1.4142	2.0324 ± 0.9416	0.7342 ± 0.4649	0.2776 ± 0.211	0.4147 ± 0.2491			

****P≤0.0001; ***P≤0.001; **P≤0.01; *P≤0.05; Nei's H, PIC, N_a, N_e, I, H_o, H_e and df denote estimates of Nei's unbaised heterozygosity, Polymorphic Information Content, Observed number of alleles, Effective number of alleles, Shannon's index Observed heterozygosity Expected heterozygosity and degrees of freedom.

The observed and expected heterozygosities, chi-square and likelihood ratio tests results are presented in Table 2. The mean ± SE of observed *(H_o)* and expected *(H_e)* heterozygosity were 0.2776 ± 0.211 and 0.4147 ± 0.2491, respectively. H_o and H_e for individual locus varied from 0.0263 (ADL0210) to 0.6842 (MCW0069) and 0.1004 (ADL0210) to 0.7470 (MCW0069), respectively. The mean H_e was higher than the mean H_o which indicated that the population was not in Hardy-Weinberg equilibrium, but under the influence of some forces, such as selection for some economic traits that might be associated with microsatellite loci. Since the mating plan was formulated using the pedigree records of at least three generations, the chances of inbreeding were negligible. The results of Chi-square test and G-square tests also revealed significant differences between H_o and H_e frequencies, demonstrating that the population was in Hardy-Weinberg disequilibrium at all loci, which might be due to the influence of external forces. Since the studied population was continuously being selected for part-period egg production and also small in size, this might be the reason for it being in Hardy-Weinberg disequilibrium.

Discussion

It is well-known that neutral markers are used to determine genetic diversity. However, this investigation assessed the impact of long-term selection on egg production associated microsatellite profiles. The present observation of nine polymorphic microsatellite loci out of ten, with an average allele number per locus of 3.000 ± 1.4142 was more than in the report by ARYA (2012), who observed nine polymorphic loci out of 47 microsatellite loci using 3.4% MetaPhor Agarose in a high and low egg production sub-population of White leghorn chicken, but comparable to that of DESHMUKH et al., (2015), who reported 17 polymorphic loci out of 25 microsatellite loci in different chicken breeds using 3.4% MetaPhor Agarose. The allele number and average number of alleles per locus in the present study were lower than earlier reports in various native and exotic chicken breeds (PANDEY et al., 2002; VIJH and TANTIA, 2004; PANDEY et al., 2005; BAO et al., 2008; CHATTERJEE et al., 2010b). The wide variation in numbers and sizes of alleles in the pure selected strain of RIR chicken studied could be attributed to the differences in the genetic architecture of the genomes analyzed, and also may be due to loss or fixation of alleles during its long-term selection.

The polymorphic information content (PIC) and Nei's average heterozygosity estimates are suitable parameters for investigating genetic variation. Polymorphic information content (PIC) is a good index of genetic diversity evaluation and represents the degree of informativeness of a marker (PARMAR et al., 2007). According to VANHALA et al., (1998), a PIC value of >0.5 indicates a high degree of polymorphism, a value of <0.25 indicates a low degree of polymorphism, and a value between 0.25 and 0.50 is indicative of a moderate degree of polymorphism at a particular locus. In the present

study, two loci out of nine polymorphic microsatellite loci exhibited a high degree of polymorphism, while 4 loci showed moderate polymorphism and three loci showed a low degree of polymorphism; the mean *PIC* value was 0.313 ± 0.064. These markers could provide enough information for assessment of genetic diversity. OTT (2001) suggested that useful polymorphic loci must have heterozygosity of at least 0.10. In the present study, the heterozygosity at all nine polymorphic microsatellite loci varied from 0.0997 to 0.7421; the mean heterozygosity was 0.4119 ± 0.2475. The heterozygosity and PIC values observed in the present study were lower for most of the microsatellite loci as compared to the results reported earlier in different chicken populations (PANDEY et al., 2002; VIJH and TANTIA, 2004; PANDEY et al., 2005; BAO et al., 2008; CHATTERJEE et al., 2010b), which might be due to the small size of the RIR population analyzed, and it was in an extensive breeding programme, leading to either loss or fixation of some of the alleles in the course of long-term selection. The present findings are in accordance with the report by WIMMERS et al. (2000), who reported lower heterozygosity (0.45) in Aseel native chicken, where the samples were taken from an organized flock and the birds were subjected to selection pressure and subsequent inbreeding.

The effective number of alleles was less than the observed number of alleles at all microsatellite loci in the present study. PANDEY et al. (2002) suggested that if all the alleles at a locus were equally frequent, the population of homozygotes would be the reciprocal of the number of alleles at this locus in the population, and if there were variations in the allele frequencies, the population of homozygotes would be greater than this. A high effective number of alleles was reported by PANDEY et al. (2002) in Aseel, Nicobari, and Miri native chicken breeds and a higher N_e, as well as Shannon's index, were reported by PANDEY et al. (2005) in Ankaleshwar chicken at ADL0020, ADL0023, ADL0102, ADL0176, ADL0210, MCW0007, MCW0014 and MCW0041 loci than those estimated in the present investigation. CHATTERJEE et al. (2010b) reported higher N_a and N_e as well as Shannon's index for ADL0020, ADL0023, ADL0102, ADL0176, ADL0210, MCW0014 and lower for MCW0007 in Kadaknath and Aseel breeds and three different chicken lines than those estimated in the present study. However, on PAGE analysis of amplicons, DAS et al., (2015b) reported the observed and effective number of alleles, as well as Shannon's index as 4, 3.5714 and 1.3322 at ADL0102, 3, 2.3810 and 0.9433 at ADL0176, 5, 3.7895 and 1.4452 at ADL0210, 3, 2.2727 and 0.9503 at MCW0014 and 3, 2.1818 and 0.8877 at MCW0041, respectively, in a pure selected strain of RIR chicken, which are quite comparable to those determined by MetaPhor analysis in the present study.

In the present study, the mean expected heterozygosity (H_e) was higher than the mean observed heterozygosity (H_o) which indicated that the population was not in Hardy-Weinberg equilibrium but was under the influence of some external forces, selection is one of the most probable reasons. Since, the studied population was continuously being selected for part-period egg production, parents were selected to avoid inbreeding and

mated, and it was also small in size, so this might have been the reason for it being in Hardy-Weinberg disequilibrium. PANDEY et al. (2005) reported observed and expected heterozygosity estimates in Ankaleshwar at ADL0020 as 0.850 and 0.640, at ADL0023 as 0.333 and 0.804, at ADL0102 as 0.410and 0.709, at ADL0176 as 0.816 and 0.740, at ADL0210 as 0.342 and 0.527, at MCW0007 as 0.692 and 0.675, at MCW0014 as 0.539 and 0.624 and at MCW0041as 0.300 and 0.369, respectively. CHATTERJEE et al. (2010b) reported higher observed and expected heterozygosity estimates than those estimated in the present study at ADL0020 (0.74 and 0.76), ADL0023 (0.91 and 0.79), ADL0102 (0.59 and 0.76), ADL0176 (0.90 and 0.80), ADL0210 (0.99 and 0.62), MCW0007 (0.83 and 0.49) and MCW0014 (0.72 and 0.67) in Kadaknath and Aseel breeds, and three different chicken lines. Comparable findings were reported by DAS et al. (2015b) in a pure selected strain of RIR chicken, where the observed heterozygosities at various loci were mostly lower than the expected heterozygosity, with estimates as 0.8000 and 0.7579 at ADL0102, 0.2000 and 0.6105 at ADL0176, 0.1667 and 0.7681 at ADL0210, 0.0000 and 0.5895 at MCW0014 and 0.3333 and 0.5652 at MCW0041, respectively and DESHMUKH et al. (2015) estimated higher observed and expected heterozygosity estimates in RIR chicken for ADL0176 (0.571 and 0.561), MCW0007 (0.546 and 0.416), MCW0014 (0.250 and 0.431) and MCW0069 (0.154 and 0.492), respectively**.** The significant differences between H_o and H_e frequencies shown by Chi-square and G-square tests also demonstrated that the population was under Hardy-Weinberg disequilibrium at all microsatellite loci.

Although, neutral markers are the best way to determine genetic diversity in a population, in the present investigation, microsatellite markers with an association with egg production were employed to assess their role in genetic diversity estimation in a long-term selection experiment. Based on the results of this study, it may be concluded that the explored set of microsatellites was good enough to analyze the genetic architecture of a population. Long-term selection based on 40-week part-period egg production might be associated with the few microsatellite loci studied and it influenced their allelic frequencies resulting in Hardy-Weinberg disequilibrium. The study suggested assessing and evaluating the influence of microsatellite genotypes on egg production traits so as to use them in marker assisted selection programmes for genetic improvement in layer type chickens.

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RAHIM, A., S. KUMAR, R. YADAV, J. DEBNATH, J. KRISHNAN: Uporaba mikrosatelitskih biljega za utvrđivanje genetske varijabilnosti kod dugotrajno selekcioniranih linija crvenih rodajland kokoši. Vet. arhiv 87, 511-522, 2017.

SAŽETAK

Genetsko poboljšanje traži postojanu i preciznu procjenu genetske varijabilnosti. S tom svrhom moguće je koristiti mikrosatelite kao molekularne biljege. Crveni rodajland je pasmina kokoši koja nese smeđa jaja, a ovo je istraživanje provedeno u populaciji koja je kroz 30 generacija selekcionirana na osnovi 40-tjedne proizvodnje jaja. Populacija je održavana kao zatvoreno jato namijenjeno za razvoj višebojnih linija koje bi se držale u dvorišnom tipu uzgoja. Cilj istraživanja bio je utvrditi genetsku varijabilnost i heterozigotnost primjenom mikrosatelitskih biljega. Genomska DNA od 76 slučajno odabranih kokoši analizirana je na 10 mikrosatelitskih lokusa. Aleli su razlučeni na 3,4 % MetaPhor agarozi, a njihova veličina i analiza određeni su pomoću Quantity-One i POPGENE v1.32 računalnih programa. Devet lokusa bilo je polimorfno pokazujući 30 alela, pri čemu je prosječni broj alela/lokusu iznosio 3,00 ± 1,41. Broj alela kretao se od 2 do 6, a veličina u rasponu 102 do 320 bp. Prosječna informativnost polimorfizma i Neieva heterozigotnost iznosili su 0,3134 ± 0,064 te 0,4119 ± 0,2575, što pokazuje da su promatrani lokusi umjereno polimorfni i informativni. Opaženi (N_a) i efektivni broj (N_e) alela te Shannonov indeks imali su prosječne vrijednosti 3,0000 ± 1,4142, zatim 2,0324 ± 0,9416 i 0,7342 ± 0,4649. N_e je bio manji od N_a na većini lokusa upujući na prevalenciju heterozigotnosti na tim lokusima. Na većini lokusa srednja očekivana heterozigotnost bila je veća u odnosu na srednju opaženu heterozigotnost, što je pokazalo da populacija nije bila u Hardy-Weinbergovoj ravnoteži već je bila pod utjecajem nekih sila kao npr. selekcije ili drugo. Isto je potvrđeno Hi-kvadrat i G-kvadrat testovima. Može se zaključiti da je dugotrajna selekcija s obzirom na 40-tjednu proizvodnju jaja rezultirala Hardy-Weinbergovom neravnotežom na istraženim mikrosatelitskim lokusima što upućuje na moguću povezanost između tih mikrosatelitskih lokusa i gospodarski važnih obilježja nesivosti kod kokoši crvenog rodajlanda. Navedeno bi moglo biti korisno u budućoj selekciji potpomognutoj s biljezima za proizvodnju jaja.

Ključne riječi: heterozigotnost, mikrosateliti, crveni rodajland, kokoš