



Investigating genetic heterogeneity using microsatellite markers after long term selection for egg production in Rhode Island Red chicken

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ABSTRACT

Genetic heterogeneity was investigated using 24 microsatellite markers and genomic DNA of 24 randomly selected birds from the selected and control lines of RIR chicken maintained at ICAR-Central Avian Research Institute, Izatnagar. The microsatellite alleles were determined on urea-PAGE, recorded using GelDoc system and the samples were genotyped. The complete genotypic data set was analyzed using POPGENE software. The observed heterozygosity (H_o) means were 0.6306 ± 0.3901 and 0.6528 ± 0.4345 in the selected and control line, respectively. Explicitly the control line contained more H_o mean and thus the more diverse than the selected population. The expected heterozygosity (H_e) ranged from 0.5053 (*MCW0059*) to 0.8421 (*MCW0004*) with mean of 0.7066 ± 0.020 in the selected line, and from 0.2899 (*MCW0059*) to 0.9130 (*ADL0136*) with mean of 0.7095 ± 0.030 in the control line. The H_o mean was less than the H_e mean in each population; the Chi square and G-square tests revealed significant deviations of almost all the loci from the Hardy-Weinberg equilibrium. The selected and control line populations had the corresponding genetic identity and genetic distance of 0.5264 and 0.6418 as original measures, and 0.5528 and 0.5928 as unbiased measures. The phylogenetic analysis revealed their moderate genetic diversity reflecting 29.64 to 32.09% common inheritance. This present investigation thus estimated genetic heterogeneity using a set of microsatellite markers after long term selection for egg production in RIR chicken and could be useful in the study of population dynamics under selection pressure.

Keywords: Genetic identity and distance, Hardy-Weinberg disequilibrium, Heterozygosity, Microsatellites, Phylogeny, RIR chicken

Rhode Island Red (RIR) chicken germplasm was brought from the USA in 1980 and subjected to genetic improvement through selective breeding at ICAR-Central Avian Research Institute (CARI, Izatnagar), India (Das *et al.* 2015a). The population got well adopted, acclimatized and genetically improved over last 33 years covering 29 generations of selection and is being maintained as selected line along with a random bred control population (Das *et al.* 2015b). Selection was practiced based on 40-weeks part-period egg production along with an independent culling level for egg weight at 28th weeks of age (Das *et al.* 2015b) and consequently changed the genetic properties of the population since the selected and control populations demonstrated significant differences in the production performances (Anonymous 2011). It was postulated that faster genetic progress in the population could be possible

using genomics data (Albers and Van Sambeek 2002), which may impact on layer breeding in the future. Chicken genome scanning extensively uses microsatellites in assessing genetic diversity (Tadano *et al.* 2007), marker-assisted selection (Ramadan *et al.* 2014) and quantitative trait loci (QTL) analysis (Wardecka *et al.* 2003). The present study was carried out to investigate genetic heterogeneity using microsatellite markers after long term selection for egg production in the selected and control lines of RIR chicken.

MATERIALS AND METHODS

Twenty four birds randomly chosen from the selected and control line of RIR chicken maintained at experimental layer farm of ICAR-Central Avian Research Institute (CARI, Izatnagar) were investigated. Genomic DNA was extracted using whole blood sample through phenol:chloroform extraction procedure, followed by quality and purity checking (Das *et al.* 2015ab). The samples showing intact DNA band and OD 260/280 nm between 1.7 and 1.9 were used. PCR-ready-DNA samples were prepared at a concentration of 50 ng/μl. FAO-recommended primers of 24 microsatellite markers (Table 1) were

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Table 1. Panel of microsatellite markers with map position and optimized annealing temperature (T_a)

Microsatellite Loci	FP/RP	Primer sequence (5'–3') [#]	Chromosomal map position	T_a (°C)
MCW 0041	FP	5'CCCAATGTGCTTGAATAACTTGGG3'	(2, locus 5) ^a , C3 ^h	57.0
	RP	5'CCAGATTCTCAATAACAATGGCAG3'		
MCW 0043	FP	5'TGACTACTTTTGATACGCATGGAGA3'	E1 ^j	52.0
	RP	5'CACCAAGTAGACGAAAACACATT3'		
MCW 0044	FP	5'AGTCCGAGCTCTGCTCGCCTCATA3'	C12 ^g	63.0
	RP	5'ACAGTGGCTCAGTGGGAAGTGACC3'		
MCW 0048	FP	5'CGTATAGGAGGGTTTCTGCAGGGA3'	C4 ^j	55.0
	RP	5'AAGGAGGAACGCACCGCACCTTCT3'		
MCW 0049	FP	5'AGCGGCGTTGAGTGAGAGGAGCGA3'	(C6, E1) ^j	63.0
	RP	5'TCCCCAACCCGCGGAGAGCGCTAT3'		
MCW 0050	FP	5'GGTGTCCGCACCCCGGAGCTTCTT3'	-	58.5
	RP	5'GCAGCATCGCGCAGCACCGCGGAT3'		
MCW 0051	FP	5'GGAACAAGCTCTTTCTTCTTCCCG3'	(2, locus 3) ^a	50.5
	RP	5'TCATGGAGGTGCTGGTACAAAGAC3'		
MCW 0059	FP	5'AAGTGCCTTTGCTATCCTGATTGG3'	(C1, E2) ^{hij}	50.5
	RP	5'AACTCCTATTGTGCAGCAGCTTAT3'		
MCW 0071	FP	5'TATTTACCCACGGGGACGAATAC3'	2 [#]	58.5
	RP	5'AGGGTGCTGAGAGCTGCCAATGTC3'		
MCW 0075	FP	5'CGTCAAGCCAGATGCTGATGAGTG3'	-	63.0
	RP	5'ATTCCAACCAGAAGTTTGACTCGC3'		
MCW 0001	FP	5'ACTGTCACAGTGGGGTCATGGACA3'	E3 ^c	55.0
	RP	5'ACACGTCCTGTGTACATGCCTGT3'		
MCW 0002	FP	5'TCCAGAGACAGTTGCTCCACATTC3'	C3 [#]	57.0
	RP	5'GCAAGTTAGTTATTGTAGGGGCTC3'		
MCW 0004	FP	5'GGATTACAGCACCTGAAGCCACTA3'	E2 ^c , 3 ^g	57.0
	RP	5'AAACCAGCCATGGGTGCAGATTGG3'		
MCW 0005	FP	5'ACCTCTGCTGGCAAATAAATTGC3'	4 ^f , C11 ^h , E5 ⁱ	55.0
	RP	5'TCACTTTAGCTCCATCAGGATTCA3'		
MCW 0014	FP	5'AAAATATTGGCTCTAGGAACTGTC3'	6 ^{ef} , E11 ^d	60.0
	RP	5'ACCGGAAATGAAGGTAAGACTAGC3'		
MCW 0016	FP	5'ATGGCGCAGAAGGCAAAGCGATAT3'	E2 ^b	57.0
	RP	5'TGGCTTCTGAAGCAGTTGCTATGG3'		
ADL 0102	FP	5'TTCCACCTTTCTTTTTTATT3'	C30 ^{hij} , E29 ⁱ	46.5
	RP	5'GCTCCACTCCCTTCTAACCC3'		
ADL 0136	FP	5'TGTCAAGCCCATCGTATCAC3'	4 ^g , C6 ^h , (C10E5) ^{ij}	52.0
	RP	5'CCACCTCCTTCTCCTGTTCA3'		
ADL 0158	FP	5'TGGCATGGTTGAGGAATACA3'	(C30E29) ^c , (E29C9) ^f	52.0
	RP	5'TAGGTGCTGCACTGGAAATC3'		
ADL 0171	FP	5'ACAGGATTCTTGAGATTTT3'	E43 ^c	46.5
	RP	5'GGTCTTAGCAGTGTGTTT3'		
ADL 0172	FP	5'CCCTACAACAAAGAGCAGTG3'	E8 [#]	46.5
	RP	5'CTATGGAATAAAATGGAAAT3'		
ADL 0176	FP	5'TTGTGGATTCTGGTGGTAGC3'	C2 ^j , E6 ^c	55.0
	RP	5'TTCTCCCGTAACACTCGTCA3'		
ADL 0210	FP	5'ACAGGAGGATAGTCACACAT3'	E30 ^c , 11 ^g	52.0
	RP	5'GCCAAAAAGATGAATGAGTA3'		
ADL 0267	FP	5'AAACCTCGATCAGGAAGCAT3'	(C3E6) ^c	55.0
	RP	5'GTTATTCAAAGCCCCACCAC3'		

FP/RP: Forward/Reverse Primer. [#]Available at <http://poultry.mph.msu.edu>. ^aWardecka *et al.* (2002), ^bCheng *et al.* (1995), ^cWimmers *et al.* (1999), ^dvan Marle-Koster and Nel (2000), ^eHillel *et al.* (2003), ^fRomanov and Weigend (2001), ^gJing-Ting *et al.* (2007), ^hBabar *et al.* (2012), ⁱPandey *et al.* (2002), ^jChatterjee *et al.* (2010).

synthesized (Custom Oligos, 0.01 mM) from M/s Genetics Biotech Asia Pvt. Ltd., New Delhi and optimized for annealing temperature (T_a) (Table 1) using gradient PCR.

The PCR reactions were carried out in 25 µl reaction mix prepared by gently mixing 2.5 µl of 10× Taq buffer with MgCl₂, 2.5 mM of each dNTP, 0.8 µM of each primer,

0.75 U of Taq DNA polymerase and 50 ng of template DNA into nuclease-free water. The PCR amplification was carried out in a programmable thermal cycler (PTC 200, MJ Research, USA) following the thermal cycles of initial denaturing at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at optimized

annealing temperature for each primer pair for 45 sec and extension at 72°C for 45 sec, followed by a final extension at 72°C for 5 min and then 4°C forever.

The probable molecular sizes of the PCR-amplified products were determined through 1.4% horizontal agarose gel electrophoresis. Approximate 10 µl of the PCR product was loaded along with 5 µl of 100 bp DNA ladder (Bangalore Genei, India), and run at 2–5 volts/cm for 60 min. The products onto the gel were then examined/photographed under UV-transillumination. The microsatellite alleles were identified through running the PCR-amplified products on vertical denaturing polyacrylamide gel electrophoresis (6% urea-PAGE) (Das *et al.* 2015ab). The electrophoresis was carried out for 15–30 min without loading product to ensure its smooth running when loaded. Approximate 10 µl of each PCR product was mixed with 6× loading dye. The product-mix was denatured at 95°C for 10 min, snapped immediately on ice for 10 min, and immediately loaded along with 4 µl of 100 bp DNA ladder (Bangalore Genei, India). The electrophoresis was then carried out at 5–6 volts/cm for 4–4½ h followed by silver staining (Beidler *et al.* 1982). The microsatellite alleles were identified according to their molecular sizes determined using Quantity One software on GelDoc 2000 (BioRad, USA). All the samples were then genotyped. The

complete genotypic data were then analyzed using POPGENE software version 1.32 (Yeh *et al.* 1999).

RESULTS AND DISCUSSION

The estimated observed (H_o) and expected (H_e) heterozygosities, the goodness of fit (Chi-square) statistics evaluating overall discordance of genotype frequencies and the likelihood ratio (G-square) statistics contrasting the observed and expected genotype frequencies at each microsatellite locus are presented in Table 2. The H_o represents the number of individuals heterozygous per locus. The estimated H_o demonstrated the optimum range in the selected and control line populations in conformity to the earlier reported range (0.00 to 0.91) across nine microsatellite markers in eight chicken lines (Vanhala *et al.* 1998). Out of 24 microsatellite loci, four loci (*MCW0014*, *MCW0049*, *MCW0059*, *MCW0071*) across the lines and one locus (*MCW0041*) in the control line demonstrated H_o of zero indicating that no individual was heterozygous on these loci. Four loci (*MCW0041*, *MCW0050*, *ADL0176*, *ADL0210*) in the selected line and three loci (*MCW0050*, *MCW051*, *ADL0176*) in the control line demonstrated $H_o > 0.5 > 0$, indicating that individuals less than 50% were heterozygous at these loci, which might be due to the isolation with the subsequent loss of unexploited genetic potential. Another

Table 2. Estimated observed and expected heterozygosities along with Chi-square and G-square statistics at various microsatellite loci in the selected and control line populations of RIR chicken

Microsatellite locus	Selected line					Control line				
	H_o	H_e	df	Chi square	G-square	H_o	H_e	df	Chi square	G-square
<i>MCW0041</i>	0.3333	0.5652	3	6.956	7.647	0.0000	0.6377	3	29.022***	26.515***
<i>MCW0043</i>	0.8000	0.7368	6	11.714	12.968*	0.8333	0.8406	15	28.889*	25.083*
<i>MCW0044</i>	1.0000	0.7536	6	34.000***	30.890***	1.0000	0.8116	10	34.000***	32.250***
<i>MCW0048</i>	1.0000	0.7246	6	15.600*	18.789**	1.0000	0.7386	6	17.714**	17.163**
<i>MCW0049</i>	0.0000	0.7536	6	44.952***	35.328***	0.0000	0.5217	3	30.933***	23.134***
<i>MCW0050</i>	0.3333	0.7246	6	15.600*	18.998**	0.2000	0.6105	3	11.413**	14.748**
<i>MCW0051</i>	0.6667	0.5072	3	2.567	3.681	0.1667	0.5652	3	13.527**	16.499***
<i>MCW0059</i>	0.0000	0.5053	1	11.221***	14.547***	0.0000	0.2899	1	15.439***	11.969***
<i>MCW0071</i>	0.0000	0.6737	3	24.381***	23.360***	0.0000	0.6737	3	24.381***	23.360***
<i>MCW0075</i>	1.0000	0.7536	6	34.000***	30.890***	1.0000	0.8632	15	47.000***	33.935**
<i>MCW0001</i>	1.0000	0.8406	15	57.000***	39.888***	1.0000	0.8000	6	22.000**	21.148**
<i>MCW0002</i>	1.0000	0.7789	6	28.000***	26.297***	1.0000	0.7536	10	34.000***	26.428**
<i>MCW0004</i>	1.0000	0.8421	15	28.000*	26.297*	1.0000	0.7246	10	34.000***	26.428**
<i>MCW0005</i>	0.8333	0.7971	10	25.786**	26.559**	0.8800	0.8000	10	28.844**	22.161*
<i>MCW0014</i>	0.0000	0.5895	3	25.697***	21.325***	0.0000	0.5895	3	25.697***	21.325***
<i>MCW0016</i>	1.0000	0.6667	6	34.000***	26.428***	1.0000	0.7246	6	15.600*	18.789**
<i>ADL0102</i>	0.8000	0.7579	6	21.214**	20.607**	1.0000	0.8116	10	34.000***	32.250***
<i>ADL0136</i>	1.0000	0.7101	6	13.300*	17.430**	1.0000	0.9130	36	62.750**	41.981
<i>ADL0158</i>	0.6667	0.6232	6	11.758	10.269	0.8333	0.8696	21	55.467***	37.845*
<i>ADL0171</i>	0.6667	0.6812	3	2.530	3.109	1.0000	0.7971	10	22.500*	23.252**
<i>ADL0172</i>	1.0000	0.7536	6	34.000***	30.890***	1.0000	0.6377	3	11.000*	15.614**
<i>ADL0176</i>	0.2000	0.6105	3	11.413**	14.748**	0.1667	0.5072	3	14.067**	16.696***
<i>ADL0210</i>	0.1667	0.7681	10	52.619***	36.950***	0.6667	0.7065	6	12.387	14.120*
<i>ADL0267</i>	0.6667	0.8406	21	47.143***	37.504*	1.0000	0.8406	15	26.333*	26.704*
Mean±SE	0.6306±0.08	0.7066±0.02				0.6528±0.089	0.7095±0.03			

H_o , H_e and df denote observed heterozygosity, expected heterozygosity and degree of freedom, respectively. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

four loci (*MCW0051*, *ADL0158*, *ADL0171*, *ADL0267*) in the selected line and one locus (*ADL0210*) in the control line demonstrated $H_o > 0.75 > 0.5$. Most of the loci (*MCW0001*, *MCW0002*, *MCW0004*, *MCW0005*, *MCW0016*, *MCW0043*, *MCW0044*, *MCW0048*, *MCW0075*, *ADL0102*, *ADL0136*, *ADL0172*) across the lines; and few (*ADL0158*, *ADL0171*, *ADL0267*) in the control line demonstrated $H_o > 0.75$. As of most of the loci demonstrated higher H_o probably due to the existence of large number of heterozygous alleles, the results could indicate low level of inbreeding and were quite comparable to the estimates reported for *MCW0005*, *MCW0014*, *MCW0016* in RIR chicken (Vanhala *et al.* 1998). The H_e estimates genetic diversity and represents the frequency of heterozygotes expected for a population to be in Hardy-Weinberg equilibrium. The present H_e ranged from 0.5053 (*MCW0059*) to 0.8421 (*MCW0004*) in the selected line, and 0.2899 (*MCW0059*) to 0.9130 (*ADL0136*) in the control line; the ranges might be compared to the available reports (Chatterjee *et al.* 2010, Vanhala *et al.* 1998).

Only *MCW0059* locus demonstrated expected heterozygosity less than 50% in the control line. Loci *MCW0014*, *MCW0016*, *MCW0041*, *MCW0048*, *MCW0050*, *MCW0051*, *MCW0071*, *ADL0176* across the populations; *MCW0043*, *MCW0059*, *ADL0136*, *ADL0158*, *ADL0171* in the selected line; and *MCW0004*, *MCW0049*, *ADL0172*, *ADL0210* in the control line demonstrated $H_e > 0.75 > 0.5$. Loci *MCW0044*, *MCW0075*, *MCW0001*, *MCW0002*, *MCW0005*, *ADL0102*, *ADL0267* across the lines; *MCW0049*, *MCW0004*, *ADL0172*, *ADL0210* in the selected line; and *MCW0043*, *ADL0136*, *ADL0158*, *ADL0171* in the control line demonstrated $H_e > 0.75$. Most of the loci demonstrated higher H_e probably due to the existence of large number of heterozygous alleles indicating low level of inbreeding. The present estimates were quite comparable to the estimates reported for *MCW0005*, *MCW0014*, *MCW0016* in RIR chicken (Vanhala *et al.* 1998). The estimated means of the H_o and H_e in the selected and control line might be compared to the reports in different chicken populations (Hillel *et al.* 2003, Pirany *et al.* 2007, Rajkumar *et al.* 2008, Chatterjee *et al.* 2010). The differences attributed in different studies might be due to their different genetic base or differences in breed/line/strain and also different loci studied. Among the present populations, the control line had more average H_o and thus was more diverse than the selected line, which could be due to the reasons that the control line was not subjected to any selection. The present populations demonstrated that the H_o mean was less than the H_e mean indicating that both of the populations were not in Hardy-Weinberg equilibrium but under the influences of some forces like selection for some economic traits which might be associated with microsatellite loci. Notably, the selected population was under the selection for egg production for 29 generations. Though, the loci *MCW0001*, *MCW0002*, *MCW0004*, *MCW0005*, *MCW0016*, *MCW0044*, *MCW0048*, *MCW0075*, *ADL0102*, *ADL0136*, *ADL0172* across the populations; *MCW0043*, *MCW0051*, *ADL0158* in the selected line; and *ADL0171*, *ADL0267* in

the control line demonstrated $H_o > H_e$ apparently. The tests of Chi-square and G-square in both of the populations revealed significant differences between the observed and expected frequencies for almost all the loci except *MCW0041*, *MCW0043*, *MCW0051*, *ADL0158*, *ADL0171* in the selected line, and *ADL0136*, *ADL0210* in the control line, thereby revealed significant deviations from Hardy-Weinberg equilibrium for these loci, which might be due to the influence of some extraneous forces as the control population was small and selected line was under selective mating for the last 29 generations. Besides, a variety of other causes including population subdivision and genetic drift (Lawson and Kofron 1989), presence of null alleles and wrong genotyping (Vanhala *et al.* 1998) etc. might play a residual role into the deviations from Hardy-Weinberg equilibrium.

The estimated Nei's original (Nei 1972) measures of genetic identity and genetic distance between the two populations were 0.5264 and 0.6418, respectively. The corresponding estimates of Nei's unbiased (Nei 1978) genetic identity and genetic distance were 0.5528 and 0.5928. Nei's genetic distances among different chicken genotypes were reported and estimated using most of the markers of the present panel (Chatterjee *et al.* 2010, Rajkumar *et al.* 2008). The genetic distance gives the extent of gene differences between the populations (Ojango *et al.* 2011). The high degree of genetic distance between the selected and control line populations might be due to the fact that the selected line has undergone 29 generations of selection for egg production and might have resulted in accumulation of some microsatellite alleles and loss of some others over generations. The high genetic identity observed between the two lines might be due to reason that both the lines had been developed from same genetic base.

In animal diversity studies, hierarchical procedures are called phylogenetic analysis for which measures of the genetic distances are used to construct a dendrogram, also called phylogenetic tree (Ojango *et al.* 2011). A phylogenetic tree constructed based on the Nei's original and unbiased measures of genetic distance (D_A) using unweighted pair group method (UPGMA) modified from NEIGHBOR procedure of PHYLIP Version 3.5 showed moderate genetic diversity between the two populations, thus summarized their evolutionary relationships and categorized them into two distinct genetic groups. The UPGMA cluster analysis revealed one node which was further diverged into the selected and control line populations. The branch length between these populations was moderate, and was indicative of their divergence period. The data was simulated with 1000 permutations using bootstrapping to generate a confidence of 95%, however, none of the bootstraps replicates produced tree containing ties. Bootstrapping was done to provide confidence statements about grouping of the lines as revealed by the dendrogram and hence tested the validity of the clusters obtained. Higher percentage bootstrap value obtained in the present study revealed higher confidence in the grouping

(Ojango *et al.* 2011).

Based on the findings, it is concluded that the RIR-control line chicken was more diverse than the selected line. These chicken lines were not in Hardy-Weinberg equilibrium but were under the influences of some forces like selection favouring some production traits. These chicken lines had high genetic identity due to common genetic base and genetic distances resulted due to selection, and were separated a few generations back from a common genetic base. Investigation using present set of microsatellite markers could be useful in the study of population dynamics under selection pressure.

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