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Genetic Analysis of Highly Inbred Chicken Using RAPD-PCR and Immunocompetence

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Abstract: The data of G-5 generation of inbred and non-inbred populations of Dahlem Red were utilized in the present study. These populations were originally developed by a regular system of full sib and half sib mating for 5 successive generations. A random mated non-inbred population was also maintained. Consequently, 3 sub populations have been developed from the same base population, namely, a full sib mated group (FS), a half sib mated group (HS) and a non-inbred group (NB). The inbreeding coefficient in the G-5 generation in the FS group and HS groups was 67.2% and 44.9%, respectively. These three sub populations were subjected to PCR-RAPD analysis to observe the genetic distance and similarity between different subpopulations. A total of 41 random primers (decamers) were screened for RAPD analysis, out of which 21 primers amplified the genomic DNA, generated 128 to 4544 bp bands. The Mean Average Percentage Difference (MAPD) varied between 13.22 and 22.20. The MAPD was highest between FS and NB and lowest between HS and FS. The intra-population genetic similarity was highest in FS (0.7139) followed by HS (0.7095) and NB (0.7054) which indicated that FS subpopulation is most uniform. The FS and NB had maximum genetic distance (by both band sharing and band frequency) and FS and HS had lowest genetic distance. The immune response to SRBC was highest in NB group followed by FS and HS groups.

Key words: RAPD-PCR, SRBC titre, inbred populations, Dahlem Red

Introduction
Unique population designs used to address molecular genetics questions in poultry (Lamont, 2003). Inbred lines play an important role in research, because of their ability to provide variation among lines and constancy within lines over time and place (Abplanalp, 1992). Twenty-three highly inbred chicken lines derived from Leghorn, jungle fowl, Fayoumi and Spanish breeds were analyzed (Zhou and Lamont, 1999) at molecular level.

Molecular markers based on the DNA sequences are more reliable and variable. Several DNA based techniques such as polymerase-chain reaction-RFLP (PCR-RFLP), random amplified polymorphic DNA (RAPD) and microsatellite analysis are being adapted to identify breed-specific genetic markers and to associate these markers with quantitative traits and disease resistance (Welsh and McClelland, 1990; Williams *et al.*, 1990). The effectiveness of RAPD in detecting polymorphism between chicken populations and their applicability in population studies and establishing genetic relationships among chicken populations has been reported by Sharma *et al.* (2001). Molecular and immunogenetic characterization of pure lines of White Leghorn was reported by Chatterjee *et al.* (2006). The RAPD-PCR was also used to differentiate the different strains of Nicobari fowl and White Leghorn (Ahlawat *et al.*, 2004). There are three aspects of immunological response, namely, humoral response, cell mediated response and phagocytic index. The humoral response to sheep RBC was used for characterization of different

native breeds of chicken of Andaman (Jai Sunder *et al.*, 2004) and its association with economic traits in pure lines of White Leghorn (Shukla *et al.*, 1996). There are very few reports on molecular and immunogenetic characterization of inbred and non-inbred populations in chicken.

The present study was conducted to estimate genetic relatedness using RAPD-PCR method among inbred and non-inbred populations of Dahlem Red and also to evaluate these populations at immunogenetic level.

Materials and Methods
Genetic stock: The data of G-5 generation of inbred (recurrent) and non-inbred populations of Dahlem Red were utilized in the present study. These populations were originally developed by a regular system of full sib and half sib mating for 5 successive generations. A random mated non-inbred population was also maintained. Consequently, 3 sub populations have been developed from the same base population, namely, a full sib mated group (FS), a half sib mated group (HS) and a non-inbred group (NB). In the FS group and HS groups the inbreeding coefficient achieved in the G-5 generation was 67.2 and 44.9%, respectively. The non-inbred (NB) group is random mated with an adequate sire base to avoid any deliberate inbreeding; in the second group, full-sib (FS), recurrent full-sib mating was practiced with 10 sire families per generation and in the third group, half-sib (HS), recurrent half-sib mating was

Table 1: Status of inbred and non-inbred population of D. Red as regards to Fx and probability of fixation

Generation	FS	HS	Probability of fixation of alleles
G1	0.250	0.125	0.000
G2	0.375	0.219	0.063
G3	0.500	0.305	0.172
G4	0.594	0.381	0.293
G5	0.672	0.449	0.409

undertaken with 10 sire base per generation to bring varying levels of homozygosity in them. Recurrent inbreeding in both FS and HS groups were practiced for 5 subsequent generations and NB group was bred at random every generation and avoiding deliberate inbreeding. The inbreeding coefficient (F_x) achieved in the G-5 generation was 0.672 and 0.449 in the FS and HS groups, respectively. The probability of fixation of alleles in FS group was 0.409 (Table 1). The purpose of development of inbred and non-inbred populations was to make them ideal genetic resources for breeding and molecular genetic studies.

Genomic DNA: Blood samples of about 500 µL were collected from the brachial vein of 15 individual birds of either sex of each inbred and non-inbred populations. High quality genomic DNA was extracted by the phenol-chloroform extraction method using the protocol adopted by Hesfer (1997). The pellet of DNA was washed twice with 70% ethanol, air dried and subsequently dissolved in TE buffer (10 mm Tris-hel, ImMEDTA). The quality of isolated genomic DNA was checked by agarose gel electrophoresis. For this purpose, well dissolved DNA samples were resolved on 0.8% agarose (w/v) gel. The concentration of the DNA and its purity were determined by spectrophotometry based on the absorbance at 260 and 280 nm, respectively. The purified DNA from individual as well as pooled DNA from each genetic group was used for further analysis.

RAPD-PCR analysis: RAPD-PCR was carried out with the pooled and the individual genomic DNA samples. A total of 41 random decamer primers of arbitrary sequences of high GC% content, from GENEI, Bangalore and GENETICS, India Limited were used. The amplifications were carried out in a final volume of 50µl reaction mixture containing 15 ng of genomic DNA, 10 ng of the primer, 10 µm of the dNTP's, 1 unit of Taq polymerase and 1X PCR buffer (10 mm Tris-HCl, 1.5 mm $MgCl_2$, 50 mm KCl). The amplification profile consisted of 2 min of initial denaturation at 95°C, annealing at 36°C and extension at 72°C for 60 sec followed by 40 cycles of denaturation at 94°C for 10 sec, annealing at 35°C for 10 sec, extension at 72°C for 2 min and final extension for 5 min at 72°C. A volume of 20 µL of each sample was used for electrophoresis on 1.2% agarose gel run at a constant voltage of 5 V cm^{-1}.

RAPD patterns were visualized and documented using the Gel Documentation system (BioRad, Australia) after staining with ethidium bromide. A 1 kb and 100 bp DNA ladder were used as known molecular size DNA marker.

Recording of data and Statistical analysis: The RAPD patterns were scored for the presence and absence of amplicons. In a binary matrix the presence of a band was recorded as one and the absence as zero. This matrix was used to compute the various parameters namely genetic similarity, genetic distance and Mean Average Percentage Difference (MAPD). RAPD profile of the pooled sample amplification were used for estimation of MAPD between the populations. The RAPD profile from individual sample amplification was used for estimating intra- and inter-population genetic similarity as well as genetic distance based on band frequency. Genetic distance was also computed from band frequency.

Mean Average Percentage Difference (MAPD): Mean Average Percentage Difference (MAPD) was calculated as a measure of inter-population genetic divergence and was expressed in the form of mean described by Gwakisa *et al.* (1994) by using the formula:

Percentage Difference (PD)

$$PD = (Nab/Na+Nb) \times 100$$

Average Percentage Difference (APD)

$$APD = 1/C \ \Sigma \ Pdi$$

Mean Average Percentage Difference (MAPD)

$$MAPD = 1/R \ \Sigma \ Pdi$$

Where:
Nab = The number of fragments that differ,
Na and Nb = The number of fragments in pool a and b
C = The number of inter-population pair-wise comparisons
R = The number of random primers used

Genetic similarity based on band frequency: The within population genetic similarity (WF_i) was estimated using the equation given by Singh and Sharma (2002).

$$WF_{ij} = \frac{1}{N} \Sigma \ V_i$$

Where:
V_i = The proportion of individuals possessing the ith band across all the individuals
N = The total number of bands amplified

The genetic similarity between two populations known as genetic identity index (Yu and Pauls 1993; Zhang *et al.*, 1995) was obtained from the following formula;

$$BF_{ij} = \sum \frac{1}{N} \left[\frac{2(V_i^{(1)} V_i^{(2)})}{(V_i^{(1)})^2 + (V_i^{(2)})^2} \right]$$

Where:

$V_i^{(1)}$ and $V_i^{(2)}$ = The frequency of occurrence of the ith band in group 1 and 2, respectively

N = The total number of bands scored

Genetic distance based on band frequency: An index of genetic distance D_{ij} between two populations was calculated by the following equation.

$$D_{ij} = - \ln (Bf_{ij})$$

Where: (BF_{ij}) = The genetic identity index of two groups

Genetic distance based on band sharing: The genetic distance between the populations were also calculated based on band sharing between the pooled samples RAPD profiles. The genetic distance (D_{ab}) between the population A and B was calculated as:

D_{ab} = $1/N \Sigma 1 - \{N_{ab}/ (N_a + N_b - N_{ab})\}$

N_{ab} = No. of common bands between A and B

N_a = No. of bands in A

N_b = No. of bands in B

N = No. of primers used

Phylogenetic relation: Unweighted pair group method (UPGMA) analysis was performed with Nei's coefficient based on band similarity and a dendrogram was constructed to show the phylogenetic relationship between the different inbred and non-inbred populations. Computer software used were Diversity data base (BioRad) and Quantity One (BioRad).

Estimation of humoral response to SRBC titre: Each pullet received an intravenous injection of 0.1 mL of 0.5% suspension of packed Sheep Red Blood Cells (SRBC) in normal saline at 52 week of age of the birds. Five days later, the blood was collected from wing vein of each bird in the individual test tubes. Sera were collected after two hours of incubation at room temperature and kept at -40°C. The total antibody titre was determined by haemagglutination test performed in the microtitre plates. From individual serum sample two fold serial dilutions was carried out in NSS and equal amount of 2% SRBC suspension was added in each well. The serum was not added in the control well. The plates were incubated at 37°C for about 1 h. The reciprocal of highest dilution showing 50% agglutinations was expressed as titre (n). The titre was transformed into $\log_2^{(n+1)}$ for further analysis.

Results

In the FS group, the inbreeding coefficient achieved in the G-5 generation was 67.2% and the probability of fixation of alleles was 0.409. The inbreeding coefficient was 44.9% in HS group in the same generation (Table 1).

Screening of primers: A total of 41 random primers (decamers) were screened for RAPD analysis, out of which 21 primers generated polymorphic amplification pattern. These primers were utilized in the subsequent analysis with individual DNA samples of the HS, FS and NB subpopulations. The sequence as well as GC contents of the 21 random primers along with the number of bands amplified and size ranges of the bands are presented in Table 2. A total of 111 polymorphic bands were amplified. The band size ranged between 128 and 5467 bp. The primer 103 produced highest number of polymorphic bands (24), while primer 100, 112, 75 and 69 produced lowest (1) number of polymorphic band.

RAPD profile: The pooled samples were amplified to observe Mean Average Percentage Difference (MAPD) (Table 3) and genetic distance based on band sharing (Table 4). The individual samples were amplified to observe the inter- and intra-population genetic similarity and genetic distance using band frequency.

Mean Average Percentage Difference (MAPD): The MAPD between the populations ranged between 13.22 and 22.20. The Mean Average Percentage Difference (MAPD) was highest between FS and NB and lowest between HS and FS (Table 3).

Genetic similarity: The genetic similarity was calculated as intra population and inter population genetic similarity index using band frequency in the individual RAPD profile (Table 3). The intra population genetic similarity ranged from 0.7054 (NB) to 0.7139 (FS). The FS subpopulation was the most homogenous one with genetic similarity of 0.7139.

The inter-population genetic similarity varied from 0.6868 (between NB and HS) to 0.7812 (between HS and FS). The populations HS and FS were found to be the closest with the highest inter-population genetic similarity (0.7812) and the lowest similarity was between NB and HS (0.6868).

Genetic distance: Genetic distance between the populations was calculated based on band frequency in the individual sample as well as band sharing in the pooled sample (Table 4). The genetic distance between the populations varied between 0.2693 and 0.3856 when based on band frequency and between 0.2180 and 0.3266 when based on band sharing. In both the cases the genetic distance was lowest between HS and FS and highest between FS and NB.

Table 2: Sequence of primer, percentage of GC content, number of polymorphic bands and their size range from the RAPD primer

Primer name	Sequence 5'→ 3'	GC (%)	No. of polymorphic bands	Size range (bp)	
				Minimum	Maximum
93	TGGCGTCCTT	60	7	128	1946
94	TCGGCGGTTC	70	4	250	3282
96	CTCGCTATCC	60	2	318	2324
100	TCGGCACGCA	70	1	239	2221
103	CCCCGGTAAC	70	24	156	4229
110	AAGGGCGAGT	60	20	154	4113
112	CCAGCCGAAC	70	1	195	2359
128	TGTGCCCGAA	60	4	289	2825
129	TCTCCGCAAC	60	3	327	2495
137	CCCCCTATCA	60	4	245	4544
168	ACCGTTCCAG	60	4	444	4000
171	GTCGTTCCTG	60	4	284	2275
172	AAAGGGGTCC	60	4	357	1781
75	CATTGGGGAG	60	1	387	2959
79	GGCACGTAAG	60	3	270	1566
40	ACAACGCCTC	60	2	282	1989
50	GGGACGTTGG	70	10	237	3015
71	AGCGTCACTC	60	4	290	3161
73	CAGCGACTGT	60	5	236	2832
77	GTCCGTACTG	60	3	277	2469
69	TCGCCCCAAA	60	1	484	5467
Total			111		

Table 3: Mean Average Percentage Difference (MAPD) using pooled samples, inter population and intra-population genetic similarity using individual samples

Genetic	Groups		
	NB	HS	FS
NB	0.7054	0.6868	0.7723
HS	22.12	0.7095	0.7812
FS	22.20	13.22	0.7139

Above diagonal: Inter population genetic similarity, Below diagonal: Mean Average Percentage Difference (MAPD), Diagonal: Intra-population genetic similarity

Table 4: Genetic distance based on band frequency of individual samples and band sharing of pooled samples

Genetic	Groups		
	NB	HS	FS
NB	--	0.3003	0.3266
HS	0.3789	--	0.2180
FS	0.3856	0.2693	--

Above diagonal: Genetic distance using band sharing, Below diagonal: Genetic distance using band frequency

Phylogenetic relation: Cluster analysis of the RAPD pattern (Fig. 1) of the three subpopulations revealed that both the inbred populations (FS and HS) belong to the same cluster, while the NB subpopulation is further away in the phylogenic tree.

Humoral immune response to SRBC: The humoral immune response to SRBC of all the subpopulations differed significantly (p<0.05) from each other (Table 5). The immune response to SRBC was the highest in NB group (8.79) followed by FS (7.60) and HS (6.23) groups.
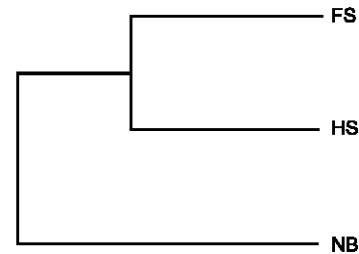

Fig. 1: Cluster analyses of different pure lines of White leghorn based on Interpopulation genetic similarity

Discussion

The data of G-5 generation of inbred and non-inbred populations of Dahlem Red were utilized in the present study. These populations were originally developed by a regular system of full sib and half sib mating for 5 successive generations. A random mated non-inbred population was also maintained. Consequently, 3 sub populations have been developed from the same base population, namely, a full sib mated group (FS), a half sib mated group (HS) and a non-inbred group (NB). The primary interest of this program was to evolve ideal genetic resources, with variant homozygosity and inbreeding coefficients, for molecular analyses. In the FS group, the inbreeding coefficient achieved in the G-5 generation was 67.2% and the probability of fixation of alleles was 0.409. The inbreeding coefficient was 44.9% in HS group in the same generation (Table 1).

In the present study the focus was on determining the genetic similarity and variability between the inbred and

non-inbred populations of Dahlem Red as well as immune response to SRBC in the FS and HS. Since the lines belong to the same breed, characterization at the molecular level was necessary to unravel the genetic variation between them as a result of recurrent inbreeding.

Screening of primers: A total of 41 random primers (decamers) were screened for RAPD analysis, out of which 21 primers generated polymorphic amplification pattern. These primers were utilized in the subsequent analysis with individual DNA samples of the HS, FS and NB subpopulations. The sequence as well as GC contents of the 21 random primers along with the number of bands amplified and size ranges of the bands are presented in Table 2. A total of 111 polymorphic bands were amplified. The band size ranged between 128 and 4544 bp. The primer 103 produced highest number of polymorphic bands (24), while primer 100, 112, 75 and 69 produced lowest (1) number of polymorphic band. These results demonstrate the potential of RAPD-PCR as an efficient tool for detecting the polymorphism amongst the lines of a breed.

RAPD profile: The pooled samples were amplified to observe Mean Average Percentage Difference (MAPD) (Table 3) and genetic distance based on band sharing (Table 4). The individual samples were amplified to observe the inter- and intra-population genetic similarity and genetic distance using band frequency.

Mean Average Percentage Difference (MAPD): The MAPD between the populations which is an indicator of the genetic divergence ranged between 13.22 and 22.20. The Mean Average Percentage Difference (MAPD) was highest between FS and NB and lowest between HS and FS (Table 3). The MAPD between different inbred (HS and FS) populations and non-inbred population in the present study was lower than between the pure line populations of White Leghorn (Chatterjee *et al.*, 2006) and higher than between different strains of Nicobari fowl (Ahlawat *et al.*, 2004), which might be due to different strains/genetic groups and different sets of primers utilized in different experiments.

Genetic similarity: The genetic similarity that represents the genetic homogeneity was calculated as intra population and inter population genetic similarity index using band frequency in the individual RAPD profile (Table 3). The intra population genetic similarity ranged from 0.7054 (NB) to 0.7139 (FS). The FS subpopulation was the most homogenous one with genetic similarity of 0.7139. This is obvious because the FS subpopulation was developed by recurrent full-sib mating for 5 generations with the inbreeding coefficient of 0.672 and probability of fixation of alleles of 0.409 and expected to be more homogeneous than either HS and NB, (Table

1). The within population genetic similarity has been reported to range between 0.76 to 0.84 (Sharma *et al.*, 2001) in chicken and 0.73 to 0.93 in quails (Kumar *et al.*, 2000).

The inter-population genetic similarity varied from 0.6868 (between NB and HS) to 0.7812 (between HS and FS). The populations HS and FS were found to be the closest with the highest inter-population genetic similarity (0.7812) and the lowest similarity was between NB and HS (0.6868). This could be due to the fact that HS and FS are inbred populations and developed by 5 generations of recurrent half-sib and full-sib inbreeding from the same base population. However, the lowest genetic similarity between HS and NB might be due to the fact that the random primers explored only a small portion of the whole genome. The inter-population genetic similarity has also been reported in different pure line populations of White Leghorn (Chatterjee *et al.*, 2006) and in quail (Kumar *et al.*, 2000).

Genetic distance: Genetic distance between the populations was calculated based on band frequency in the individual sample as well as band sharing in the pooled sample (Table 4). The genetic distance between the populations varied between 0.2693 and 0.3856 when based on band frequency and between 0.2180 and 0.3266 when based on band sharing. In both the cases the genetic distance was lowest between HS and FS and highest between FS and NB. This might be due to the fact that both the HS and FS subpopulations were developed by 5 generations of recurrent inbreeding and NB by random mating from the same base population. Therefore, due to inbreeding HS and FS have become closure together, while NB and FS are going apart. However, the range of genetic distance by band sharing and band frequency were little different. Earlier workers (Sharma *et al.*, 2001; Chatterjee *et al.*, 2006) have reported a similar analysis using both methods in different chicken breeds and found that the range of genetic distance by band sharing is lower than that by band frequency. Though the magnitude of genetic distance differs by the two methods, the trend of result is the same.

Phylogenetic relation: Cluster analysis of the RAPD pattern (Fig. 1) of the three subpopulations revealed that both the inbred populations (FS and HS) belong to the same cluster, while the NB subpopulation is further away in the phylogenic tree. This could be due to the fact that HS and FS are inbred populations and NB is non-inbred population. The closeness in the inbred populations have occurred due to homozygosity. The cluster analysis in other chicken populations was also observed by earlier workers (Chatterjee *et al.*, 2006; Ahlawat *et al.*, 2004).

Table 5: Comparative performances of the SRBC response in different genetic groups in G-5 generation of Dahlem Red

Trait	FS (N = 45)	HS (N = 43)	NB (N = 149)	Overall mean (N = 237)	$h_s^2 \pm SE$
SRBC titre (log 2 value)	7.60±1.78[b]	6.23±1.54[c]	8.79±1.44[a]	7.73±1.30	0.27±0.19

Humoral immune response to SRBC: The humoral immune response to SRBC of all the subpopulations differed significantly (p<0.05) from each other (Table 5). The immune response to SRBC was highest in NB group (8.79) followed by FS (7.60) and HS (6.23) groups. This might be due to effect of inbreeding. Earlier workers (Shukla *et al.*, 1996; Chatterjee *et al.*, 2006) had also observed significant difference of humoral immune response to SRBC in different pure lines of White Leghorn. The heritability estimate of SRBC titre was moderate (0.27). Chatterjee *et al.* (2006) also obtained moderate heritability of this trait in pure lines of White Leghorn.

Conclusion

The MAPD varied between 13.22 and 22.20 and was highest between FS and NB. The intra-population genetic similarity ranged from 0.7054 (NB) to 0.7139 (FS). The FS subpopulation was found to be most homogeneous with intra-population genetic similarity of 0.7139. The HS and FS were found to be closest and NB and HS were furthest. The genetic distance between FS and NB was highest as obtained by both band frequency and band sharing. The FS and HS belong to the same cluster, while, NB in a different cluster. The immune response to SRBC was highest in NB group (8.79) followed by FS (7.60) and HS (6.23) groups. The immune response to SRBC was found to be moderately heritable.

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