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# Genetic Relationship among Chicken Populations of India Based on SNP Markers of Myostatin Gene (GDF 8)

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**Abstract:** The data on ten SNP markers of Myostatin gene (GDF-8) was generated on nine breeds/populations of indigenous poultry and Red Jungle Fowl (RJF). The SNPs were five in promoter region, one each in intron 1 and 2 and three in exon 1. The data was analyzed to find out the genetic relationship among the indigenous chicken populations. PCR-RFLP was carried out to genotype the populations at seven SNPs while three were genotyped by SNaPshot method using automated DNA sequencer as no restriction sites were found at the SNP sites. ANOVA revealed 18% of the variation among the populations. The correspondence analysis separated out Punjab Brown, Red jungle fowl and Nicobari populations from the rest of the indigenous chicken populations. It is postulated that the populations of Punjab Brown and Nicobari showed the recent common ancestry with Red jungle fowl.

**Key words:** Chicken, SNP, PCR-RFLP, F-Statistics, genetic distance, correspondence analysis

## Introduction

India has 15 different breeds of chicken that have been documented primarily based on morphometric attributes (Acharya and Bhat, 1984). The morphometric and production variation within the breeds is quite large which makes the differentiation among populations obscure. However these breeds/populations are important genetic resource owing to their adaptive traits and socio-cultural practices of local communities rearing them. These indigenous chicken populations have two important distinguishing features based on the purpose for which these chickens are utilized. The game populations are Chittagong, Danki, Aseel, Ghagus and Kalasthi while other populations constitute the normal birds primarily reared for meat and egg production. The game birds have distinctive musculature compared to other birds. Red Jungle Fowl is considered to be the progenitor of all the present day domestic birds and in India inhabits the hill terrain all along the lower Himalayan ranges from Uttarakhand to Arunachal Pradesh. The RJF birds are still in wild and protected under wild life protection act.

Myostatin, GDF8 is a member of TGF-β superfamily of secreted growth and differentiation factors. This is one of important candidate genes for growth and development and thus has potential applications in animal husbandry. GDF8 is a negative regulator of muscle cell development (Lee and Mc Pherron, 1999). And it has been demonstrated that Myostatin-null mice exhibit a 2-3 fold increase in the skeletal muscle mass due to hyperplasia and hypertrophy. Mutations in Myostatin gene were also associated with double muscling phenotype in cattle (Mc Pherron *et al.*, 1997; Grobet *et al.*, 1997). In chicken, Zhiliang *et al.*, 2004 observed significant association of different genotypes with skeletal muscle growth (fat, body weight and muscle parameters). Zhiliang *et al.* (2002) studied Myostatin gene in different chicken populations and identified seven SNPs in promoter and untranslated regions.

The phylogenetic analyses of different populations in a species are carried out using several type of markers which may be dominant (RAPD) or codominant (Microsatellites and SNPs). The microsatellites are the preferred choice for the population genetic studies owing to high polymorphism while SNPs are biallelic. The SNPs are however markers of choice for any gene based study. This study was undertaken to find out population genetic parameters and construction of a gene tree of ten Indian populations of chicken based on Myostatin gene.

## Materials and Methods

**Populations:** Ten indigenous chicken populations were selected for the present study. Among the selected populations, Aseel, Danki, Kalasthi and Chittagong are reared for game purpose (fighter/game birds) due to their well built musculature. The other group of chicken such as Ankleshwar, Daothigir, Kadaknath, Nicobari and Punjab Brown are reared for meat and egg purposes. Kadaknath breed is unique in terms of pigmentation in all body parts including abdominal body parts-a condition known as Fibromelanosis and the tribals believe that these birds have medicinal value. We also included the Red Jungle fowl in the study which is considered to be the progenitors of indigenous chicken populations and all modern day chicken breeds.

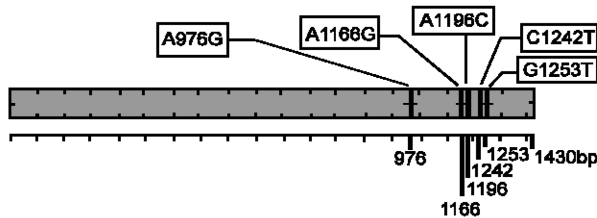


Fig. 1a: Details of SNPs genotyped in promoter region of Myostatin gene

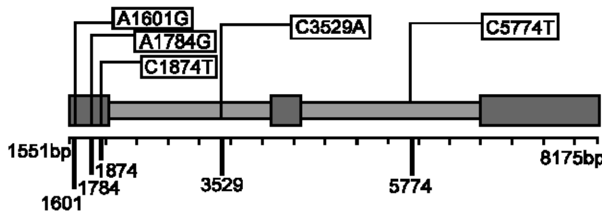


Fig. 1b: Details of SNPs genotyped in exon 1, intron 1 and intron 2 of Myostatin gene

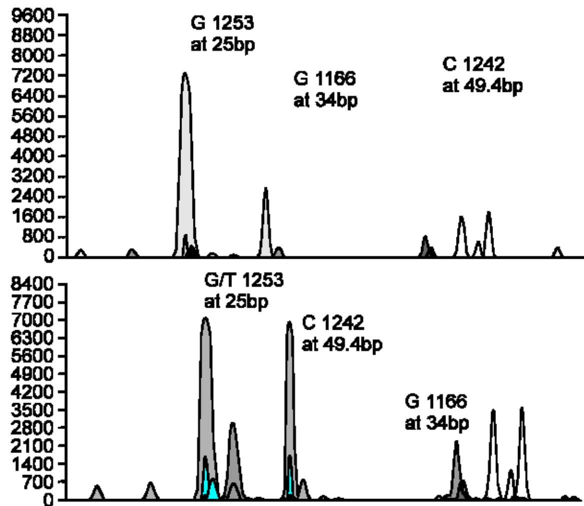


Fig. 2: Genes can views of SNaPshot product run on automated DNA sequencer indicating three SNPs typed (G1166A, C1242T and G1253T) in single PCR product

**Samples:** Twenty four samples each from the ten populations of indigenous chicken were utilized. Genomic DNA was extracted from 0.5 mL of blood collected from wing vein in heparinized vacutainers. The DNA was isolated using the standard laboratory protocols (Sambrook *et al*., 1989).

**Selection and Genotyping of SNPs:** We sequenced myostatin gene in a panel of 24 birds in genomic DNA drawn from diverse populations (DQ912835). Ten SNPs were selected in indigenous chicken which were also available in Chicken VD database. Out of these ten selected SNPs five were in promoter region, one each in intron 1 and 2 and three in Exon-1. Selected SNPs (Fig. 1) were genotyped by PCR-RFLP and SnapShot (Table 1). Restriction enzymes were identified using *NEB cutter Ver 2.0*. PCR products of targeted SNPs were amplified. The PCR reaction was performed in 25 µL reaction containing 50 ng of genomic DNA, 4 pmol of each primer, 1.5 mM $MgCl_2$, 0.2 µM of each dNTP and 1 u Taq DNA polymerase (Sigma) in ABI 9700 GeneAmp Cycler. The amplification was carried out with 5 min denaturation at 94°C, 30 cycles of amplification (94°C for 45 sec, 57-55°C for 45 sec, 72°C for 45 sec) and final extension at 72°C for 10 minutes. The PCR products were checked on 2% agarose for amplification of specific product of expected size and were subjected to restriction digestion by incubating at 37°C overnight after adding 1 unit of the enzyme as per the protocols of the manufacturer (New England Biolabs, USA). The digested samples were run on 2.5% agarose gel at 120v for 30 min. The gels after staining with Ethidium Bromide were scored on gel documentation system. Three SNPs of the promoter region had no restriction site and were genotyped using SNaPshot method (Fig. 2) with ABI 3100 Avant automated DNA sequencer. The allele data was extracted using Gene Mapper Ver 3.0 (Applied Biosystems). The genotype data obtained was subjected to diversity analysis.

**Statistical analysis:** The analysis of molecular variance (ANOVA) was carried out using the software ARLEQUIN version3.0. Several genetic distances perform well for reconstruction of phylogenies when the populations are of the same species or taxa and are very closely related. Nei's Standard Genetic distances were estimated using the POPGENE software (Yeh *et al*., 1999) The Cavalli-Sforza and Edwards (1967) chord distance was calculated. The method involved the transformation of data into angular distance$\theta$. The populations in this genetic distance are conceptualized as existing as points in a m-dimensional Euclidean space which are specified by m allele frequencies (m being total number of alleles in both populations.) $\theta$ as the angle between these points

where $$\cos\theta_i = \sum_{i}^{mi} \sqrt{x_{ij}\, y_{ij}}$$

and $x_i$ and $y_i$ are the frequencies of the ith allele in populations X and Y respectively. From this a chord distance in the space between the two points measured along a straight line was calculated as

$$Dc = (2/\prod r)\sum_{j=1}^{r} \sqrt{2(1-\cos\theta)}$$

We utilized the genetic distance matrices for preparation of genetrees using UPGMA (Sneath and Sokal, 1973)

Table 1: SNPs location and genotyping methodology

| Region of the Gene | SNP From to Position of the nucleotide | Primers (product size in Brackets) | Methodology | Enzyme | Genotypes |
|---|---|---|---|---|---|
| | G976A | | PCR-RFLP | Nla III | GG-401,104,50,49 AA-451,104,49 GA-451,401,103,50,49 |
| Promoter | A1166G | For: CAAAATgTTTATTCCTgCTCA | SnaPshot | | |
| | A1196C | Rev:CTAAACAgATCCgggACAgC (605bp) | PCR-RFLP | Sca I | AA-321,283 CC-605 AC-605,321,283 |
| | C1242T | | SNaPshot | | |
| | G1253T | | SnaPshot | | |
| | G1601A | | PCR-RFLP | Aci I | GG-357,189,60 AA-546,60 GA-546,357,189,60 |
| Exon-1 | G1784A | For: TggCATATATAAggCACACCA Rev: gggAgAgCCTgAgAAggAgT (606bp) | PCR-RFLP | Bbv I | GG-279,130,107,90 AA-409,107,90 GA-409,279,130,107,90 |
| | C1874T | | PCR-RFLP | Bbs I | CC-465,140 TT-605 CT-605,465,140 |
| Intron-1 | G3529A | For: AgCAATCAgCTCCAgTAgTCg Rev: gAgCgATgTgTACAggCAgA (1170bp) | PCR-RFLP | Bsp1286 I | GG-571,433,96,70AA-667,433,70 GA-667,571,433,96,70 |
| Intron-2 | T5774C | For: gCTCTgCAgACCATgTgAgA Rev: ggACTgCTgAgAgAgCCTTg (432bp) | PCR-RFLP | Bss S I | TT-432 CC-300,132 CT-431,300,132 |

Table 2: Population wise Allele Frequencies of ten selected SNPs

| Locus | Allele | Ankleshwar | Aseel | Chittagong | Danki | Daothigir | Kadaknath | Kalasthi | Nicobari | PBrown | RJF |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NlaIII | A | 0.479 | 0.875 | 0.583 | 0.417 | 0.688 | 0.688 | 0.646 | 0.938 | 0.854 | 0.891 |
| | G | 0.521 | 0.125 | 0.417 | 0.583 | 0.313 | 0.313 | 0.354 | 0.063 | 0.146 | 0.109 |
| SNaP2 | G | 1.000 | 1.000 | 0.979 | 0.958 | 1.000 | 1.000 | 1.000 | 0.729 | 0.750 | 0.891 |
| | A | 0.000 | 0.000 | 0.021 | 0.042 | 0.000 | 0.000 | 0.000 | 0.271 | 0.250 | 0.109 |
| ScaI | A | 0.938 | 0.813 | 0.792 | 0.500 | 0.417 | 0.521 | 0.792 | 0.438 | 0.354 | 0.500 |
| | C | 0.063 | 0.188 | 0.208 | 0.500 | 0.583 | 0.479 | 0.208 | 0.563 | 0.646 | 0.500 |
| SNaP4 | C | 0.917 | 0.917 | 0.875 | 0.979 | 0.688 | 1.000 | 0.896 | 0.458 | 1.000 | 0.938 |
| | T | 0.083 | 0.083 | 0.125 | 0.021 | 0.313 | 0.000 | 0.104 | 0.542 | 0.000 | 0.063 |
| SNaP5 | G | 0.833 | 0.792 | 0.958 | 0.875 | 1.000 | 0.958 | 0.750 | 0.375 | 0.958 | 0.563 |
| | T | 0.167 | 0.208 | 0.042 | 0.125 | 0.000 | 0.042 | 0.250 | 0.625 | 0.042 | 0.438 |
| AciI | A | 0.521 | 0.604 | 0.333 | 0.458 | 0.521 | 0.229 | 0.646 | 0.667 | 0.521 | 0.750 |
| | G | 0.479 | 0.396 | 0.667 | 0.542 | 0.479 | 0.771 | 0.354 | 0.333 | 0.479 | 0.250 |
| BbvI | C | 0.938 | 0.958 | 0.938 | 1.000 | 0.875 | 0.813 | 0.979 | 0.125 | 0.458 | 0.000 |
| | T | 0.063 | 0.042 | 0.063 | 0.000 | 0.125 | 0.188 | 0.021 | 0.875 | 0.542 | 1.000 |
| BbsI | G | 0.479 | 0.354 | 0.625 | 0.417 | 0.396 | 0.521 | 0.313 | 0.396 | 0.479 | 0.453 |
| | A | 0.521 | 0.646 | 0.375 | 0.583 | 0.604 | 0.479 | 0.688 | 0.604 | 0.521 | 0.547 |
| Bsp 1286I | G | 0.917 | 0.896 | 0.854 | 1.000 | 0.792 | 0.938 | 0.938 | 1.000 | 0.625 | 0.969 |
| | A | 0.083 | 0.104 | 0.146 | 0.000 | 0.208 | 0.063 | 0.063 | 0.000 | 0.375 | 0.031 |
| BssSI | G | 0.563 | 0.688 | 0.583 | 0.750 | 0.729 | 0.479 | 0.771 | 0.479 | 0.708 | 0.625 |
| | A | 0.438 | 0.313 | 0.417 | 0.250 | 0.271 | 0.521 | 0.229 | 0.521 | 0.292 | 0.375 |

and neighbour joining algorithm (Saitou and Nei, 1987). The correspondence analysis was carried out using GENETIX software (Belkhir *et al.*, 2004).

## Results and Discussion

The selected SNPs were genotyped in all the nine indigenous populations and Red Jungle Fowl. The allele frequency was calculated for each loci and population. Locus Snap 2 of the promoter region was less polymorphic with G allele fixed in Ankleshwar, Aseel, Daothigir, Kadaknath and Kalasthi populations. Similarly for locus Snap 4 allele C was fixed in Kadaknath and Punjab brown. Loci NlaIII, ScaI, Aci I and BssSI were found to be polymorphic in all the ten populations (Table 2).

**Analysis of Molecular variance:** The analysis of molecular variance revealed 18% variation among populations and this was statistically significant (Table 3). The fixation index of population differentiation was 0.18. The Pairwise population differentiation ($F_{ST}$) was estimated (Table 4) and tested for its significance between the pair of populations. The permutation test revealed that all the $F_{ST}$ values were statistically significant except between Ankleshwar-Kalasthi, Ankleshwar-Aseel, Ankleshwar-Chittagong and Kalasthi-Aseel populations.

**Genetic distance:** The genetic distances between populations provide a relative estimate of the time that must have elapsed since the populations/breeds

Table 3: Results of ANOVA carried out population wise

| Source of variation | d.f. | Sum of squares | Variance components | Variation (%) |
|---|---|---|---|---|
| Among Populations | 9 (p-1) | 162.824 | 0.33437 Va | 17.99 |
| Within Populations | 486 (2n-p) | 740.620 | 1.52391 Vb | 82.01 |
| Total | 495 | 903.444 | 1.85828 | |
| Fixation Index | $F_{IS}$ : 0.18 | | | |

Table 4: Population differentiation using $F_{ST}$ values (above diagonal-significance)

| | Ankleshwar | Aseel | Chittagong | Danki | Daothigir | Kadaknath | Kalasthi | Nicobari | PBrown | RJF |
|---|---|---|---|---|---|---|---|---|---|---|
| Ankleshwar | 0 | - | - | + | + | + | - | + | + | + |
| Aseel | 0.0529 | 0 | + | + | + | + | - | + | + | + |
| Chittagong | 0.0167 | 0.0707 | 0 | + | + | + | + | + | + | + |
| Danki | 0.0660 | 0.1010 | 0.0602 | 0 | + | + | + | + | + | + |
| Daothigir | 0.1103 | 0.0775 | 0.0705 | 0.0569 | 0 | + | + | + | + | + |
| Kadaknath | 0.0922 | 0.1071 | 0.0315 | 0.0670 | 0.0705 | 0 | + | + | + | + |
| Kalasthi | 0.0295 | 0.0038 | 0.0727 | 0.0529 | 0.0727 | 0.1242 | 0 | + | + | + |
| Nicobari | 0.3264 | 0.2878 | 0.3261 | 0.3413 | 0.2541 | 0.2990 | 0.2974 | 0 | + | + |
| PBrown | 0.2048 | 0.1572 | 0.1579 | 0.1679 | 0.0867 | 0.1135 | 0.1792 | 0.1986 | 0 | + |
| RJF | 0.3139 | 0.2738 | 0.3196 | 0.3256 | 0.2648 | 0.2785 | 0.2855 | 0.0805 | 0.1415 | 0 |

(-) non significant (+) significant

Table 5: Matrix of Genetic Distances (above diagonal-Dc) and (below diagonal-Ds)

| | Ankleshwar | Aseel | Chittagong | Danki | Daothigir | Kadaknath | Kalasthi | Nicobari | PBrown | RJF |
|---|---|---|---|---|---|---|---|---|---|---|
| Ankleshwar | - | 0.056 | 0.040 | 0.109 | 0.152 | 0.115 | 0.040 | 0.484 | 0.298 | 0.455 |
| Aseel | 0.030 | - | 0.076 | 0.124 | 0.119 | 0.120 | 0.020 | 0.403 | 0.233 | 0.389 |
| Chittagong | 0.016 | 0.039 | - | 0.101 | 0.083 | 0.068 | 0.080 | 0.473 | 0.208 | 0.460 |
| Danki | 0.036 | 0.051 | 0.034 | - | 0.161 | 0.115 | 0.077 | 0.53 | 0.293 | 0.515 |
| Daothigir | 0.066 | 0.045 | 0.045 | 0.035 | - | 0.125 | 0.123 | 0.445 | 0.190 | 0.451 |
| Kadaknath | 0.050 | 0.055 | 0.022 | 0.036 | 0.043 | - | 0.142 | 0.475 | 0.158 | 0.392 |
| Kalasthi | 0.020 | 0.009 | 0.041 | 0.030 | 0.044 | 0.066 | - | 0.433 | 0.281 | 0.424 |
| Nicobari | 0.278 | 0.217 | 0.286 | 0.289 | 0.215 | 0.240 | 0.235 | - | 0.36 | 0.121 |
| PBrown | 0.140 | 0.097 | 0.106 | 0.106 | 0.063 | 0.071 | 0.117 | 0.166 | - | 0.241 |
| RJF | 0.215 | 0.168 | 0.228 | 0.221 | 0.186 | 0.179 | 0.184 | 0.051 | 0.090 | - |

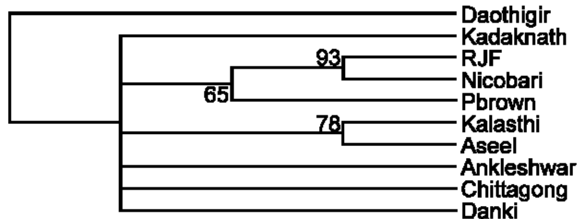


Fig. 3: Cavalli S'forza Edward chord distance (Dc) with 1000 bootstraps over loci and utilizing NJ algorithm for the construction of genetree

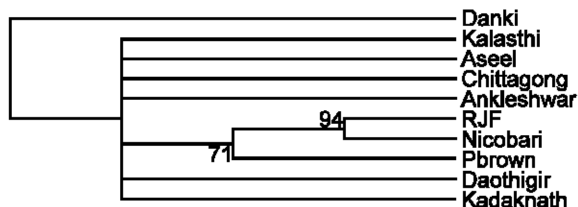


Fig. 4: Nei's standard genetic distance (Ds) with 1000 bootstraps over loci and utilizing NJ algorithm for the construction of genetree

existed as a unified and a cohesive population. The sub populations may have been formed when there was a mating within population coupled with a reduced amount of gene flow between the populations. The amount of genetic distances among the sub populations may be small and can be attributed to the isolation of the populations or a group of individuals having separated from one another over short span of time. The Nei's standard genetic distance Ds was estimated among the populations. Similarly the Cavalli Sforzas' Edward chord distance (Dc) was estimated among the populations (Table 5). Maximum Nei's genetic distance was observed between Nicobari and Danki (0.289) where as minimum genetic distance was between Ankleshwar and Chittagong populations (0.016). Edward's Chord distance revealed minimum genetic distance between Aseel and Kalasthi (0.020) which can be attributed to both breeds/populations being game birds and in geographic contiguity. The maximum genetic distance was between Red jungle fowl and Danki (0.515). This can be explained on the basis that there is likely selection for aggressive behaviour and fighting instinct in Danki birds while all the Red Jungle Fowl birds are in wild.

We utilized two algorithms (Neighbor Joining and UPGMA) for the construction of the dendrograms (Fig. 3 and 4) using bootstrapping over loci. The chord distance dendrogram revealed Daothigir joining the populations

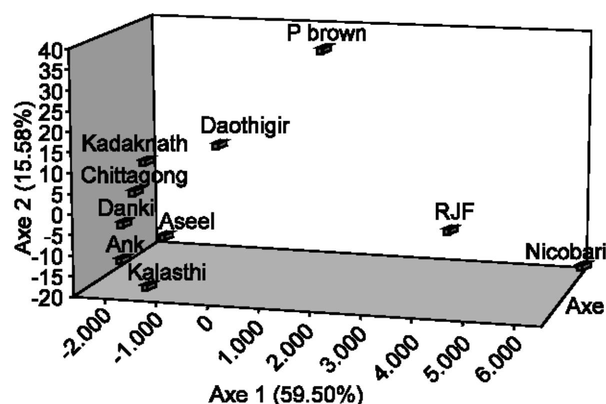


Fig. 5: Three dimensional figure depicting the correspondence analysis based on Myostatin gene

in the last. In Nei's standard genetic distance dendrogram Danki joined the tree in last. RJF and Nicobari joined with one another with a bootstrap value of 93%. This can be explained that the original Nicobari fowls may have separated recently from the RJF in Andaman and Nicobar group of islands. The Aseel and Kalasthi birds came closer to one another and both of them are game birds and might be sharing the common ancestry. The RJF samples were collected in the Himachal Pradesh which is an adjoining region of Punjab and this may be the reason that RJF, Nicobari and Punjab Brown join on one node. Similar results have also been reported using a set of 25 microsatellite loci (Tantia *et al.*, 2006). The results of the UPGMA algorithm was very similar to NJ except that Kadaknath was last to join the genetree. The differences amongst NJ and UPGMA gene tree may be due to the methodology these two different algorithms utilize. The results and the dendrograms using the Nei's Standard genetic distances revealed similar results.

The correspondence analysis revealed that first three dimensions contributed to 84.82% of the total variation explained. The three dimensional plot (Fig. 5) revealed the Nicobari, RJF and Punjab Brown populations to be distinct identities while all the other population came close to one another. The results of the gene tree are very similar to the results obtained from microsatellite data (Tantia *et al.*, 2006). This gives credence to use of SNP markers for the population genetic studies.

## Acknowledgement

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