The chicken growth hormone gene of intron I region from three varieties of Kadaknath breed of poultry was amplified by PCR and a 770 bp product was obtained. The products from each variety (Jet black, Golden and Pencilled) were digested with *Msp I* (5 U), which recognizes the 5′-C↓CGG-3′ sequence. The RFLP pattern revealed restriction fragments of 529, 373, 241 and 156 bp sizes, which indicated the presence of two restriction sites. In total, three RFLP patterns was observed at two restriction sites. The genotypic frequencies obtained for different varieties were tested for equilibrium using chi-square test. The differences among genotypes for all the three varieties were found to be non-significant, which indicated that population was in Hardy-Weinberg equilibrium. The alleles A and A2 were not observed in all the three varieties of Kadaknath breed of poultry. The overall allelic frequencies for A1 and A3 alleles were 0.375 ± 0.016 and 0.625 ± 0.020, respectively for all the three varieties. The highest allelic frequency (0.700 ± 0.011) was obtained for A3 allele in Pencilled variety and lowest (0.300 ± 0.022) for A1 allele was also observed in Pencilled variety. The phylogenetic consensus tree grouped all the three varieties into one cluster. The highest genetic distance (0.0748) was observed between Jet black and Pencilled and smallest (0.0051) between Jet black and Golden varieties. The genetic differences among all the three varieties based on phylogenetic tree were negligible; which indicates non-significant differences among each other. Thus it can be concluded that all the three varieties belonging to the Kadaknath breed of poultry have almost similar genetic base.

**Keywords**: cGH gene polymorphism, genetic distance, indigenous chicken, phylogenetic

**IPC Code**: Int. Cl. 8 C12N15/10, 15/16

**Introduction**

Genetic characterization can be done using cytogenetic, biochemical and recently developed molecular methods. However, cytogenetic and biochemical methods are less sensitive, because of lower degree of polymorphism. A new class of markers called ‘molecular markers’, capable of detecting genetic variation at the DNA sequence level, has been developed as a consequence to tremendous progress in the field of recombinant DNA technology. A molecular marker is an identified genomic site and marker alleles represent polymorphism at DNA sequence level. Various types of molecular markers, such as traditional restriction fragment length polymorphs (RFLPs)1,2, minisatellites, also known as variable number of tandem repeats (VNTR)3, microsatellite4,5, random amplified polymorphic DNA (RAPD)6,7, have been in use. These markers can be used to distinguish breeds/strains/genetic groups on the basis of their genetic composition or loci under study.

Growth is a complex physiological process that exists from conception until maturity in animals and birds; consequently, accurate measurement of entire growth phase cannot be performed easily. Thus, simplified and practical measures used to evaluate growth of chickens are body weight and weight gain. The chicken growth hormone (cGH), a polypeptide hormone synthesized in and secreted by pituitary gland, is highly polymorphic which involved in a wide variety of physiological functions such as growth, body composition, egg production, ageing and reproduction8,9 as well as immune responsiveness10. Using RFLP-PCR technique, the presence of genetic variants in growth hormone gene can be characterized and association of such alleles with traits of economic importance can be studied. The knowledge of polymorphism can also be useful in phylogenetic analysis as well as in design of breeding programmes11. In addition, growth hormone plays an
important role in innate and acquired immune systems. In particular, it has been shown to affect thymulin excretion and growth of thymus, the proliferation of lymphoid cells, the activity of phagocytic cells and haemopoiesis. Chicken growth hormone gene has been used as a candidate gene for marker assisted selection for improved performance. Despite the importance of native chicken in tribal/rural areas, information is lacking on their genetic make up with respect to performance, adaptability, resistance to diseases, genetic variability and genetic relationships. Therefore, objective of the present study was to evaluate the genetic variability among three different varieties of indigenous Kadaknath breed of poultry, found in Jhabua District of western Madhya Pradesh, India.

Materials and Methods

DNA Isolation
The blood samples of randomly selected unrelated Kadaknath birds of both sexes were collected from the breeding tract. Two ml of venous blood was collected from the wing vein of each bird in EDTA vacuette tubes (Greiner Labortechnik, Austria). The samples were transported in ice and subsequently stored at –35°C. Chicken genomic DNA was isolated according to the method described earlier with minor modifications. Incubation with proteinase-k and sodium dodecyl sulphate (SDS) was followed by phenol-chloroform extraction.

Chicken Growth Hormone (cGH) Gene Primers
The following pair of primers specific to growth hormones gene was used in the present study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 1 forward</td>
<td>5’ATC CCC AGG CAA ACA TCC TC 3’</td>
</tr>
<tr>
<td>Primer 2 Reverse</td>
<td>5’CCT CGA CAT CCA GCT CAC AT 3’</td>
</tr>
</tbody>
</table>

Polymerase Chain Reaction (PCR)
The intron I region of chicken growth hormone gene was amplified using a reported set of primer. PCR was carried out in a final reaction volume of 25 μL in MJ Research thermal cycler. Each reaction volume contained 2.5 μL of 10×PCR buffer, 0.50 μL (10 mm each) of dNTPs, 1μL of each primer (10 p/μL), 0.3 μL of Taq polymerase, 3.0 μL of template DNA and 16.7 μL of sterile HPLC water. The reaction mixture was subjected to initial denaturation of 95°C for 4 min followed by 30 cycles of 94 °C for 30 sec, annealing at 62°C for 120 sec and extension at 72°C for 90 sec. Final extension was given for 5 min at 72°C. The reactions were carried out in 200 μL thin walled PCR tubes.

Restriction Endnuclease (RE)
The Restriction Enzyme MspI was used to cut the amplified growth hormone gene.

Restriction Site

| 5’ … C ↓ C G G … 3’ |
| 3’ … G G C ↑ C … 5’ |

Restriction Digestion
The PCR products were digested with MspI using the method described earlier. The PCR product (10 μL) from each tube of cGH was digested with 5 units of MspI in manufacturer’s recommended assay buffers in a final reaction volume of 25 μL. The reaction mixture was incubated at 37°C for at least 3 h in MJ Research thermal cycler.

Agarose Gel Electrophoresis
The electrophoresis of the enzyme digested PCR products (5 μL of PCR product mixed with 1μL of gel loading dye) from each tubes was done on 1.5-2.0% agarose gel (depending on the expected size of amplified product) containing ethidium bromide at constant voltage 80 V for 2 h at 37°C using 0.5×TBE along with 100 bp ladder as molecular size marker. The amplified product was visualized as a single compact band under UV light and documented by Syngene’s gel documentation system. The bands sizes were judged by Syngene Gene Tool software comparing with molecular size markers and images were recorded.

The phylogenetic tree was constructed using either presence or absence of restriction sites and also from genotypic frequencies in the cGH motifs of growth hormone gene.

Statistical Analysis

Calculation of Gene and Genotypic Frequencies
Genotypic frequencies of different PCR-RFLP patterns were estimated from the combination of various RFLP alleles generated based on presence or absence of one or more restriction sites. Different genotypes were identified on the basis of different
patterns. Gene frequencies were calculated from genotypic frequencies. The allele frequencies were calculated using standard methods. The Chi-square ($\chi^2$) test for goodness of fit was used to find out difference among various genotypes and tested for Hardy-Weinberg equilibrium.

**Phylogenetic Analysis**

To study the genetic variation within and between the three varieties of Kadaknath breed of poultry, the allele frequencies data were subjected to phylogenetic analysis using program in the PHYLIP package. The data were subjected to bootstrapping to create 600 multiple data set. The genetic distances obtained were subjected to neighbour-joining analysis and construction of dendogram was done with appropriate option of PHYLIP.

**Results and Discussion**

**PCR Amplification of Chicken Growth Hormone (cGH)**

In the present study, the intron I region of chicken growth hormone gene was amplified by set of primers earlier used by Kuhnlein et al. They reported the PCR product size of 756 bp, whereas in the current experiment, it was found to be 770 bp on 1.5 percent agarose. The difference of about 14 bp in the PCR fragment suggests the possibility of insertion/duplication of the sequence, which can only be confirmed by sequencing. Similar difference of 14 bp was also reported in White Leghorn chickens for intron I region of growth hormone gene. However, no report was available on growth hormone gene polymorphism in Kadaknath breed of poultry.

**Restriction Fragment Length Polymorphism (RFLP) of cGH**

The PCR products were digested with MspI (5 U), which recognizes the 5′-C↓CGG-3′ sequence. The following three different RFLP patterns were observed (Fig. 1).

(i) 529, 373, 241 and 156 bp
(ii) 529 and 241 bp
(iii) 373, 241 and 156 bp

The RFLP pattern revealed the restriction fragments of 529, 373, 241 and 156 bp sizes. This indicated the presence of two restriction sites at 373 (site A) and 529 bp (site B) position in 770 bp amplicon. The first one is probably the same as that reported by Kuhnlein et al at 373 bp position, producing two fragments of 373 and 397 bp. Restriction patterns with 529, 373, 241 and 156 bp fragments in the present study also revealed the presence of additional restriction site (site B) at 529 bp. A difference of 14 bp in size of PCR product could be due to the size difference in original fragment (756 vs 770 bp). The exact size, sequence and position of restriction site could only be confirmed by sequencing. If two sites are accepted (373 and 529 bp), there is possibility of ten different restriction patterns. However, in the present study, only following three RFLP patterns were observed.

**RFLP Pattern–I**

This pattern with 529, 373, 241 and 156 bp fragment is possible when site A was present on one allele and site A as well as site B on another allele.

```
      529 241  
A1 A3
      373 156 241  
    +, + 373, 156, 241
```

**RFLP Pattern–II**

This pattern with 529 and 241 bp fragments is produced when site B was present on both the alleles.

```
      529 241  
A1A1
      529 241  
    -, + 529, 241
```
**RFLP Pattern–III**

The third RFLP pattern with 373, 241 and 156 bp fragments is generated when both the restriction sites were present on both the alleles.

\[
\begin{align*}
373 & \quad \downarrow \quad 156 & \quad \downarrow \quad 241
\end{align*}
\]

\[A_3A_3\]

\[
\begin{align*}
373 & \quad 156 & \quad 241
\end{align*}
\]

\[+ , + 373, 241, 156\]

Pipalia\(^{18}\) studied the PCR-RFLP of intron I of chicken growth hormone gene in BNT, BWLH and WLH chickens. He reported restriction fragment of 529, 373, 241 and 156 bp sizes digested with restriction endonuclease \(MspI\), which generated 3 different RFLP patterns. Thus, the results reported in the present study for PCR-RFLP of cGH in three varieties of Kadaknath breed of poultry are in agreement with the previous studies.

**Genotyping and Allelic Frequencies**

The genotypic frequencies were calculated by considering presence of various RFLP patterns in three varieties of Kadaknath breed of poultry and are presented in Table 1. In all the three varieties of Kadaknath breeds studied, the frequency for genotype \(A_1A_3\) was found the highest (0.455-0.600), followed by \(A_3A_3\) (0.314-0.409). However, the lowest frequency was observed for genotype \(A_1A_1\) (0.111-0.143). Moreover, genotype \(A_1A_1\) was found absent in variety Pencilled. Among the varieties, the highest frequency was observed in Pencilled for \(A_1A_3\), Jet black for \(A_1A_1\) and Golden for \(A_3 A_3\).

Genotypic frequencies obtained for three different varieties of Kadaknath breed of poultry, were tested for equilibrium using chi-square test. The differences among genotypes for all the three varieties were found to be non-significant (Table 1), which indicated that population was in Hardy Weinberg equilibrium. Similar findings were also reported by Pipalia\(^{18}\) in BNT, BWLH and WLH breeds of poultry. He reported a non-significant difference among genotypes of three different genetic groups, which indicated that population was in Hardy Weinberg equilibrium. As all populations were in genetic equilibrium, it can be concluded that alleles at this locus have remained free from any factors changing gene and genotypic frequencies.

Based on genotypic frequencies, allelic frequencies were calculated for each variety and are presented in Table 1. The allele frequencies were calculated by considering presence or absence of restriction sites at different alleles. An allele with absence of both the sites was designated as ‘\(A\)’ allele, with presence of only one site at 529 bp as ‘\(A_1\)’ allele, with presence of only one site at 373 bp as ‘\(A_2\)’ and presence of two restriction sites at 529 and 373 bp as ‘\(A_3\)’ allele. The allele \(A\) and \(A_2\) were not observed in all the three varieties of Kadaknath breed of poultry. The overall allelic frequencies of \(A_1\) and \(A_3\) alleles were 0.375±0.016 and 0.625±0.020, respectively for all the three varieties of Kadaknath breed of poultry. The highest allelic frequency (0.700±0.011) for \(A_3\) allele was obtained in variety Pencilled, followed by Golden (0.636) and Jet black (0.586). Similarly, the highest frequency (0.414) for \(A_1\) was observed in Jet black.

<table>
<thead>
<tr>
<th>Table 1—Distribution of cGH/(MspI) RFLPs genotypic frequencies and allelic frequencies in three varieties of Kadaknath breeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Genotypic frequencies</td>
</tr>
<tr>
<td>No.</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>Number of birds</td>
</tr>
<tr>
<td>Cal. (\chi^2) Value</td>
</tr>
<tr>
<td>(B) Allelic frequencies</td>
</tr>
<tr>
<td>No.</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

Figure in parenthesis indicates position of restriction site
\(^\wedge\)-Indicate genotypes, NS-Non-significant
followed by Golden (0.364) and Pencilled (0.300). However, Pipalia\textsuperscript{18} reported higher frequency of A\textsubscript{1} allele in BNT, BWLH and WLH breeds of poultry as compared to the present study.

Genetic Divergence

The objective of the present study was to evaluate genetic distances and phylogenetic relationship among all the three varieties (Jet black, Golden and Pencilled) of Kadaknath breed of poultry. The phylogenetic tree was constructed using the presence and absence of restriction site and/or genotypic frequencies in the cGH motif of growth hormone gene. The phylogenetic consensus tree, constructed using the boostrapped data and neighbour-joining method, grouped all the three varieties into one cluster (Fig. 2). The Nei’s genetic distance (Da) values between Jet black and Golden, Jet black and Pencilled and Golden and Pencilled varieties were found to be 0.0051, 0.0748 and 0.0730, respectively (Table 2). The highest genetic distance (0.0748) was observed between Jet black and Pencilled and smallest (0.0051) between Jet black and Golden varieties. All the three varieties of Kadaknath breed had small genetic distance between each other. The genetic differences among all the three varieties based on phylogenetic analysis were negligible, which indicated non-significant differences among each other. Thus, it can be concluded that all the three varieties belongs to the Kadaknath breed of poultry having almost similar genetic base.

Pipalia\textsuperscript{18} studied the phylogenetic relationship between BNT, BWLH and WLH chickens by conducting phylogenetic analysis and constructing the phylogenetic trees, using the presence and absence of restriction sites and/or genotypic frequencies in the cGH1 and cGH2 motifs of growth hormone gene. He reported that WLH was at equidistance from BWLH and BNT and having almost similar genetic base. Thus, the results reported for phylogenetic analysis in the three varieties of Kadaknath breed of poultry in the present study were found in close agreement with the results of Pipalia\textsuperscript{18}.

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References


