MATERIALS & METHODS
CHAPTER 3

MATERIALS AND METHODS

I. COLLECTION OF SAMPLES

Sites of collections

A total of 591 samples of *Labeo dero* were collected from the commercial catches from ten different geographical locations of India (Table 3.1, Fig.2) from ten rivers namely, Beas (Pathankot, Punjab, 32° 66'N 75° 40'E), Satluj (Bhakra Nangal, Punjab, 31° 23'N, 75° 30'E), Ganga (Ajetpur, UP, 29° 58'N, 78° 10'E), Yamuna (West Yamuna canal, Yamuna Nagar, Haryana, 30° 09'N, 77° 21'E), Kosi (Ramnagar, Uttaranchal, 29° 24'N, 79° 07'E), Gerua (Katarniaghat, UP, 32° 19'N, 75° 30'E), Tons (Rewa, MP, 24° 31'N, 81° 17'E), Jiabharali (Bhalukpong, Arunachal Pradesh 27° 28'N, 94° 15'E), Mahanadi (Sonepur, Orissa, 20° 50'N, 83° 56'E) and Godavari (Warangal, AP. 18° 57'N, 79° 06'E).

Tissue samples

**Blood** The blood samples collected through caudal puncture with needle and syringe, were fixed in 95% ethanol in 1:5 (blood: ethanol) ratio and stored at 4°C till use.

**Liver** The ventral side of the fish was cut open and a piece of liver was taken out. Any organ or viscera adhering to the liver was removed. Liver sample was wrapped in aluminum foil on which code number of the fish was written. The wrapped tissues were immediately immersed in liquid nitrogen (LN₂) (-196°C), in cryocans.
Muscle A small white muscle piece was removed after removing the skin above it, using surgical blade and wrapped by aluminium foil labeled with fish number. It was directly immersed in liquid nitrogen and transported to lab.

Table 3.1– Sample size, location, year of collection of *Labeo dero* from different rivers in India.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>River system</th>
<th>River / Reservoir</th>
<th>Location Site</th>
<th>Location (lat. &amp; log.)</th>
<th>Year</th>
<th>Sample size (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Indus</td>
<td>Beas</td>
<td>Pathankot, Punjab</td>
<td>32° 66’N 75° 40’E</td>
<td>April, 2003</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Satluj</td>
<td>Nangal, Punjab</td>
<td>31° 23’N 75° 30’E</td>
<td>September, 2000</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>April, 2003</td>
<td>36</td>
</tr>
<tr>
<td>2</td>
<td>Ganges</td>
<td>Ganga</td>
<td>Ajetpur, Uttarachal</td>
<td>29° 58’N 78° 10’E</td>
<td>March 2001</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>October, 2003</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kosi</td>
<td>Ramnagar, U.P.</td>
<td>29° 24’N 79° 07’E</td>
<td>April, 2003</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>November, 2003</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gerua</td>
<td>Katmania Ghat, U.P.</td>
<td>32° 19’N 75° 30’E</td>
<td>November, 2003</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yamuna</td>
<td>Tajewala, Haryana</td>
<td>30° 09’N 77° 21’E</td>
<td>September, 2000</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>March – April, 2001</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tons</td>
<td>Rewa, M.P.</td>
<td>24° 31’N 81° 17’E</td>
<td>April, 2004</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>Brahmaputra</td>
<td>Jiabharali</td>
<td>Bhaluk Pong, Arunachal</td>
<td>27° 28’N 94° 15’E</td>
<td>March, 2003</td>
<td>56</td>
</tr>
<tr>
<td>4</td>
<td>Mahanadi</td>
<td>Mahanadi</td>
<td>Sonepur, Orissa</td>
<td>20° 50’N 83° 56’E</td>
<td>May, 2004</td>
<td>33</td>
</tr>
<tr>
<td>5</td>
<td>Godavari</td>
<td>Godavari</td>
<td>Warrangal, A.P.</td>
<td>18° 57’N 79° 06’E</td>
<td>October, 2002</td>
<td>02</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>591</td>
</tr>
</tbody>
</table>

Total
Figure 2 – Sample collection sites (*) of Labeo dero during present study.
Transportation and storage of tissues

The blood was transported from the field in 95% ethanol and stored in the laboratory at 4°C. The liver and muscle tissues were transported to the lab, immersed in LN2 in cryocans and in laboratory were stored at -80°C until analysis.

II. ALLOZYMES ANALYSIS

A. METHODOLOGY

A1. Sample extraction

Tissue samples (liver and muscle) of approx. 10 mg were taken in a tube (1.5ml), homogenized in chilled extraction buffer medium @ 500 mg. ml⁻¹ (Annexure I) and centrifuged (SIGMA Laborzentrifugen GmbH, Osterode) at 10,000 rpm at 4°C for 1 hr. The supernatant was recentrifuged at 10,000 rpm at 4°C for 20 min. and kept on ice till loading on gels.

A2. Electrophoresis

For optimisation for gel concentration, the extracted samples were loaded on 7% and 8% Polyacrylamide Gel (PAGE gels) (Annexure I) and 1 X TBE as running buffer (Annexure I). The gels were run at a constant voltage of 150 V at 4-6°C in a cold handling chamber. The running time depended on the enzyme to be analyzed. After the run, the gels were stained for different enzymes (Whitmore, 1990). The enzyme systems and the staining compositions are given in Annexure I.IV. After the bands appeared with optimum intensity, the gels were washed with distilled water and the staining reaction was stopped with 0.7% acetic acid. The gel concentration on which the allozyme bands were better resolved was used for population studies.
B. Standardisation of Allozyme Electrophoresis

B1 Selection of tissue

- Selection of samples for initial studies
- Selection of tissue for maximum number of scorable allozyme loci

B2 Optimisation of electrophoretic conditions

- Percentage of polyacrylamide gel
- Running buffer
- Volume of extract application
- Electrophoresis running time

A3. Enzyme systems tested

The following 18 enzyme systems were tested (Table 3.2).

Table 3.2 - Enzyme Systems tested for allozyme analysis in *Labeo dero*.

<table>
<thead>
<tr>
<th>Enzyme System</th>
<th>Name</th>
<th>E.C. Number*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPDH</td>
<td>α-Glycerophosphate dehydrogenase</td>
<td>1.1.1.8</td>
</tr>
<tr>
<td>ACP</td>
<td>Acid phosphatase</td>
<td>3.1.3.2</td>
</tr>
<tr>
<td>AK</td>
<td>Adenylate kinase</td>
<td>2.7.4.3</td>
</tr>
<tr>
<td>AAT</td>
<td>Aspartate amino transferase</td>
<td>2.6.1.1</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine kinase</td>
<td>2.7.3.2</td>
</tr>
<tr>
<td>Est</td>
<td>Esterase</td>
<td>3.1.1.1</td>
</tr>
<tr>
<td>GLDH</td>
<td>Glucose dehydrogenase</td>
<td>1.1.1.47</td>
</tr>
<tr>
<td>G6PDH</td>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>1.1.1.49</td>
</tr>
<tr>
<td>GDH</td>
<td>Glutamate dehydrogenase</td>
<td>1.4.1.3</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
<td>1.1.1.27</td>
</tr>
<tr>
<td>MDH</td>
<td>Malate dehydrogenase</td>
<td>1.1.1.37</td>
</tr>
<tr>
<td>ME</td>
<td>Malic enzyme</td>
<td>1.1.1.40</td>
</tr>
<tr>
<td>ODH</td>
<td>Octonol dehydrogenase</td>
<td>1.1.1.73</td>
</tr>
<tr>
<td>PGM</td>
<td>Phosphogluco mutase</td>
<td>5.4.2.2</td>
</tr>
<tr>
<td>GPI</td>
<td>Glucose phosphate isomerase</td>
<td>5.3.1.9</td>
</tr>
<tr>
<td>PGDH</td>
<td>Phosphogluconate dehydrogenase</td>
<td>1.1.1.44</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
<td>1.15.1.1</td>
</tr>
<tr>
<td>XDH</td>
<td>Xanthine dehydrogenase</td>
<td>1.1.1.204</td>
</tr>
</tbody>
</table>

* Shaklee *et al.* (1990)
The liver and muscle samples were tested to maximize the chances of detecting variation at polymorphic loci. For this purpose, four liver and muscle samples each from widely separated geographical sites of collection were chosen randomly, so as to represent the entire range of samples.

For the enzyme systems studied, muscle samples did not provide any additional loci or better resolution of any locus, in comparison to that observed in liver. Therefore, the liver was chosen as the optimum tissue.

Other conditions, that were found optimum, are given in table 4.1. Extract application volume (μl) and electrophoresis running time (min.) were optimized for individual enzymes, after other parameters have been optimized. The optimized parameters were used to resolve electrophoretic patterns of different enzymes for samples from all the localities.

A4. Genotyping

1. Designation of loci

Loci were designated on the basis of distance of migration, which in turn depends on net charge and molecular weight of a particular protein. Higher the molecular weight, lesser is the migration. Highest molecular weight locus was designated as locus 1 of that enzyme, lower to that is 2 and so on.

2. Designation of alleles

At a particular locus, most common allele is designated as 100 and correspondingly based on rate of migration; other alleles were designated in reference of that allele.
3. Genotyping of individuals

Individuals were genotyped at a particular locus based on number and pattern of bands at that locus. This is dependant on the subunit structure of enzyme system being studied, e.g. mono-, di-, tri- or tetra meric.

Monomeric: In the homozygous condition, an equivalent version of the enzyme is produced by both of the chromosome pair, i.e. a "double dose". When these loci produce different allelic products in the heterozygous condition, the dose is equivalent for each version but each as half of the double dose of the homozygous condition. Thus expected ratio of expression of the two products in the heterozygosity is 1:1 (Whitmore, 1990).

Dimeric: Homomers of dimeric enzymes are comprised of two subunits. Homozygotes express a single zone of activity because all gene products have combined identical entities regardless whether the subunits are products of the same locus or its pair in a diploid (Whitmore, 1990).

Heterozygotes express homomers and heteromers. The quantity of heteromer would be twice that of either homomer if the subunit has combined randomly. The expected ratio of the three kinds of products would be 1:2:1. The heteromer would be expected to have an electrophoretic mobility equidistant between that of the two homomers (Whitmore, 1990).

III. MICROSATELLITES ANALYSIS

A. ISOLATION OF GENOMIC DNA

Total genomic DNA from ethanol preserved blood was extracted by the modified procedure of Ruzzante et. al. (1996).

A1. Lysis of ethanol fixed blood cells

Approximately 50 µl fixed blood cells were centrifuged at 10,000rpm for 20 min at 4°C in a 50 ml centrifuged tubes. Then, cells pellet was washed with 1.0 ml high T.E. buffer (Annexure II) twice with centrifugation at 10,000 rpm for 20 min at 4°C. Washed blood cells were incubated in 0.5 ml incubation buffer (Annexure II) at 37 °C for overnight.

A2. Purification and Precipitation of DNA

After incubation, sample was extracted with tris-satured phenol: chloroform: isoamyl alcohol (25: 24: 1 v/v/l) by centrifugation at 10,000 rpm for 10 min at 20°C. The supernatant was extracted again with chloroform: isoamyl alcohol (24:1 v/v) by centrifugation. Precipitation of DNA was done by adding one tenth volume of 3 M sodium acetate (pH 5.2) and 2.5 times ice cold absolute ethanol. Then, DNA pellet was obtained through centrifugation at 10,000 rpm for 10 min at 4°C and washed with 70% ethanol. After drying, pellet was suspended in TE (pH 8.0) (Annexure II) and stored at 4°C.
A3. RNase Treatment

RNase treatment was done by adding 1μl RNAase (Annexure II) to DNA solution and incubated at 37°C in water bath for 2 hours and stored at 4°C.

A4. Quantification of DNA

Concentration of DNA was determined through gel electrophoresis in 0.7% agarose with ethidium bromide incorporated and dissolved in 0.5 X TAE buffer. The gels were run at 70 V/cm and visualized under UV florescence. The concentration of total DNA was determined by comparing with the known quantity of DNA and adjusted to 25 ng DNA / μl suspension by serial dilutions and stored at 4°C, to be used as template for PCR.

B. METHODOLOGY FOR AMPLIFICATION OF MICROSATELLITE LOCI

B1. Polymerase chain reaction conditions

- Template DNA concentration

  For PCR of microsatellite loci, 25-50 ng of total DNA was used as template DNA, in a total reaction volume of 25μl.

- Reaction mix

  The amplification reaction mix contained 1X PCR buffer (10 mM Tris-HCl, pH9.0, 50 mM KCl; 0.01% gelatin), 2.0 mM MgCl$_2$, 0.2 mM each dNTP, 5 pmoles of forward and reverse primer each, 1.5 U of Taq Polymerase and template DNA.

  Reaction conditions

  Amplification reaction was performed in a MJ Research PTC 200 thermocycler with the following conditions:
(i) Initial denaturation at 94°C for 5 minutes (1 cycle),
(ii) Denaturation at 94°C for 30 seconds, annealing at temperature for 30 seconds and
elongation at 72°C for 1 minute (for 25 cycles)
(iii) Final elongation at 72°C for 4 minutes (1 cycle) and
(iv) Soak at 4°C.

Following amplifications, the PCR products were stored at 4°C and analyzed within 24
hours.

B2. Polyacrylamide Gel Electrophoresis and Staining

The amplified products were analyzed by polyacrylamide gel
electrophoresis on 6-10% non-denaturing polyacrylamide (19:1) gels with 1 X TBE
as gel running buffer (Annexure II). The gels were run for 4-5 hrs at 10V/cm at 4-6°C.
The bands were visualized by staining with silver stain (Silver Staining Kit,
Amersham Pharmacia Biotech USA) and their images were stored in the computer.
The DNA size marker (Msp I cut pBR322 DNA) was run on both sides and in the
middle of the gel to determine the size of the amplified products. For resolving the
PCR products for cross species amplification, the PCR products were analyzed on
10% PAGE gels.

C. STANDARDIZATION OF PCR AND PAGE CONDITIONS

C1. Optimization of PCR and PAGE conditions

- Total DNA Concentration

  The initial tests were done using 50 ng, 25 ng and 10 ng DNA per PCR reaction.
• **Annealing temperature of primers**

The annealing temperatures were based on the composition of the forward and reverse primer sequences. $T_m$ was calculated individually for both the primers.

$$T_m = \frac{2(A+T) + 4(G+C)}{}$$

where $T_m =$ Melting temperature of primer

$A+T =$ the sum of the nitrogenous base Adenosine and Thymine

$G+C =$ the sum of the nitrogenous base Guanine and Cytosine

Annealing temperature of a primer was calculated 3-10 °C below the melting temperature of the primer.

• **Primer Concentration**

5 pmoles each of forward and reverse primer was used.

• **Electrophoresis**

a. **Gel Concentration:**

The amplified products were initially run on 10% PAGE. According to the size of the amplified product 6%, 8%, 10% gels were used.

b. **Sample Volume:**

Different volumes loaded on the gels and according to the resolution the sample volume was standardized.

c. **Running time:**

Initially all the gels were run for 5 hrs on 10% non-denaturing polyacrylamide (19:1) gels using 1X TBE as running buffer and then standardized according to the product size.
D. STRATEGY FOR MICROSATELLITE ANALYSIS

D1. Identification of Microsatellite markers for population studies

• Collection of microsatellite/ primer sequences

Available microsatellite information in closely related species (upto order/family level) were collected from ASFA, recent issues of related journals and Genbank through Internet was reviewed till 2003. The available and designed microsatellite primer sequences are given in Annexure III.

• Designing of primers for microsatellite sequences and custom synthesis of primers

Wherever microsatellite sequences were available but not the primer sequence for a particular locus, the primers for microsatellite sequences were designed through softwares PRIMER3 (Rozen and Skaletsky, 1998) and DNASIS and custom synthesised for use.

D2. Cross-species amplification of microsatellite loci in Labeo dero using microsatellite primers of related species

Microsatellite Primers Tested

For identification of additional microsatellites in Labeo dero, twenty four primers from Cyprinus carpio, 5 from Catla catla, 5 from Barbus barbus, 5 from Barbodes goniotus, 6 from Labeo rohita, 10 from Campostoma anomalum and 7 from Pimphales promelas were tested for cross species amplification (Table 3.3). These belong to the same family Cyprinidae as that of L. dero. Primer sequences of each microsatellite locus are given in Annexure III.
Table 3.3 - Selected microsatellite sequences and primers for cross species amplification in *Labeo dero*.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Donor species</th>
<th>No. of primer pairs tested</th>
<th>Loci/Primer</th>
<th>Genebank Accession No.</th>
<th>Status</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Cyprinus carpio</em></td>
<td>21</td>
<td>MFW1, 2, 6, 7, 9, 11, 14, 15, 16, 17, 18, 19, 20, 23, 24, 26, 28, 29, 30, 31, 32</td>
<td>AY169249-50</td>
<td>A</td>
<td>Crooijmans <em>et. al.</em>, 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>Cc80 and 72</td>
<td>AB043469</td>
<td></td>
<td>Yue <em>et. al.</em>, 2002</td>
</tr>
<tr>
<td>2</td>
<td><em>Barbus barbus</em></td>
<td>5</td>
<td>Barb37, 54, 59, 62, 79</td>
<td>AY45378-82</td>
<td>A</td>
<td>Chenuil <em>et. al.</em>, 1999</td>
</tr>
<tr>
<td>3</td>
<td><em>Catla catla</em></td>
<td>5</td>
<td>CcarG1, G2, A12, C3 and A7</td>
<td></td>
<td>A</td>
<td>Naish and Skibinski, 1998</td>
</tr>
<tr>
<td>4</td>
<td><em>Barbodes goniotus</em></td>
<td>5</td>
<td>Bgo 80, 81, 82, 83 and 84</td>
<td>AJ291680-84</td>
<td>A</td>
<td>McConnell, 2000</td>
</tr>
<tr>
<td>5</td>
<td><em>Labeo rohita</em></td>
<td>6</td>
<td>R1, 2, 3, 5, 6 and 12</td>
<td>AY507518-22,24</td>
<td>B</td>
<td>Das, 2000</td>
</tr>
<tr>
<td>6</td>
<td><em>Campostoma anomalum</em></td>
<td>10</td>
<td>Ca3, 5, 6, 8, 10, 11, 12, 15,16 and 17</td>
<td>AF277575,77, 78,80,82-84, 87-89</td>
<td>A</td>
<td>Dimososki <em>et. al.</em>, 2000</td>
</tr>
<tr>
<td>7</td>
<td><em>Pimphales promelas</em></td>
<td>7</td>
<td>Ppro48, 80,118, 126,132,168 and 171</td>
<td>AY254350-254357</td>
<td>A</td>
<td>Bessert and Guillermoorti, 2003</td>
</tr>
</tbody>
</table>

Total tested 62

Status A: Microsatellite Primer sequences available in literature
Status B: Only microsatellite sequences were available in literature, the primers were designed through PRIMER3 and DNASIS software.
D3. Identification of primers giving polymorphic microsatellite loci in *Labeo dero*

The selected primer pairs were tested on ten individuals of *Labeo dero* through PCR and PAGE and primers amplifying distinct bands were identified. The amplified products were categorized as scorable and unscorable at a particular locus, as having one / two distinct bands at a particular locus and not distinct / more than two, respectively. The monomorphic locus has only one band in all individuals while polymorphic loci showed one/two bands in an individual. The primers that gave distinct polymorphic bands were then used in studying the population of *L. dero* collected from different river systems of India for genetic differentiation.

E. SEQUENCING OF PCR AMPLIFIED MICROSATELLITE MARKERS

E1. Identification of samples

Individuals with monomorphic genotype of most common allele, that gave dark bands, without any stutter bands were selected. After PCR amplification with primers for particular locus, 4 μl of reaction mix was loaded on polyacrylamide gels with 1X TBE as running buffer, to check for amplification and approximate quantity of PCR product.

E2. Primers for sequencing

The primers used in PCR were used for sequencing on automated DNA sequencer ABI377 according to manufactures instructions.
F. GENOTYPING OF PCR PRODUCT

F1. Calculation of molecular weight of PCR Product

Molecular weight of the PCR product was calculated with regard to the standard molecular weight markers with the software BIOVIS 1D (Expert Vision, Mumbai). The bands having the same molecular weight are taken as the same allele for different individuals.

F2. Assigning of alleles and genotyping

The genotype of allele was designated according to the molecular weight of the PCR product. e.g. If an individual is homozygote for the allele with molecular weight of 100 bp, the individual is genotyped as 100100. In the case of heterozygous individual, the genotyping is done, according to the molecular weight of both the PCR products.

F3. Data Recording

The genotypes of all samples were entered in MS Excel sheets. The data was initially entered according to their loading sequences on gels and then it was sorted so that the data was arranged population wise with the genotype at all loci in their respective columns.

G. STATISTICAL ANALYSIS OF DATA OF BOTH ALLOZYME AND MICROSATELLITE MARKERS

G1. Parameters of genetic variation

To obtain allele frequencies, mean number of alleles per locus, heterozygosity values, expected ($H_{exp}$), observed ($H_{obs}$), polymorphism at 0.95 criteria
(P=0.95) and polymorphism at 0.99 criteria (P=0.99), the genotype data were analysed using software Genetix 4.05.2 (Belkhir et al., 1997).

**Allele frequency**

Frequency of an allele is given by:

\[
P = \frac{2 \, H_0 + H_e}{2N}
\]

Where

- \( H_0 \) = number of homozygotes for that allele
- \( H_e \) = number of heterozygotes for that allele
- \( N \) = number of individuals examined

**Heterozygosity**

The calculation of heterozygosity per locus is

\[
H_L = 1 - \sum x_i^2
\]

Where \( x_i \) is the frequency of the \( i \)th allele at a locus.

**G2. Test for conformity to Hardy Weinberg expectations**

In the present study, the options available in Genepop v.3.3 (Raymond and Rousset, 1995a) were used to determine conformity to Hardy Weinberg expectations of genotype frequencies. The probability test that follow exact Hardy Weinberg test of Haldane (1954) was used to compute the probability of confirmation to Hardy Weinberg expectations. The probabilities were computed for the null hypothesis (\( H_0 \)) the populations follow Hardy Weinberg equilibrium. More powerful, score test (Rousset and Raymond, 1995) was employed calculate the probability, against the specific alternate hypothesis (\( H_1 \)) of heterozygote deficiency or excess, as indicated by positive or negative \( F_{IS} \) value. The software computes exact P-value by complete
enumeration method, when alleles are less than five. For more than five alleles (microsatellites), Markov chain method (Guo and Thompson, 1992) is used with parameters dememorization = 1000, batches = 100 and iteration = 100. The significance level of probabilities was estimated through sequential Bonferroni adjustment of critical level of 0.05 (Lessios, 1992).

G3. Test for Linkage disequilibrium between pair of loci

Probability values obtained from the test to determine linkage disequilibrium between pairs of allozyme and microsatellite loci in each sample was also calculated through option available in software Genepop ver. 3.3, probability test (Raymond and Rousset 1995a). Probability values for linkage disequilibrium between pair of loci were computed within samples and overall samples. The null hypothesis, the loci does not exhibit linkage disequilibrium, was examined at the probability level of 0.05, after adjusting for sequential Bonferroni correction (Lessios, 1992).

G4. Inter population heterogeneity of allelic and genotypic frequencies

Genetic homogeneity of nine sample sets was determined through an exact test (G based test) that assumes random samples of genotypes (Genepop ver. 3.3, Genotype differentiation test). This test was performed on genotype tables and possible non-independence of alleles within genotypes will not affect test validity (Raymond and Rousset 1995b; Goudet et al. 1996). Standard Bonferroni correction was applied to significant levels, for the simultaneous tests made (Lessios, 1992).

The null hypothesis in all the tests was, the genotype proportions are homogenous across the population. The test was performed and probabilities were computed for each pair of population at each locus and over all loci. The significance
of probabilities was compared to critical probability level 0.05, adjusted for sequential Bonferroni.

**G5. F-statistic**

Wright F (fixation)-statistics describes partitioning of genetic variability in population structure of diploid organism. The index $F_{is}$ refers to Hardy –Weinberg distribution of genotypes of individuals within subpopulations and $F_{st}$ genetic differentiation of sub populations within the total populations. Effectively $F_{is}$ describes whether population of Homozygous (Homozygous allele within individual) in a sample is in agreement with a proportion expected under Hardy –Weinberg model. Where $F_{is}$ is positive in local populations, this could be due to inbreeding and this index is often called as inbreeding coefficient.

**G6. Population differentiation ($F_{st}$)**

$F_{st}$ is an index of a genetic differentiation that describes how much variation in allele frequencies exists between the local populations. It can also be interpreted as variance of allele frequencies among local populations relative to the maximum value possible based on the mean allele frequency across all local populations. Alternative to $F_{st}$ that $R_{st}$ allow for this difference in evolution of alleles in microsatellite loci.

In the present study, Weir and Cockeram’s (1984) estimator analogue of one of the Wright’s statistics $F_{st}$ (Wright, 1951), was calculated with Genetix 4.05.2 (Belkhir et al. 1997) for both allozyme and microsatellite loci. For the genotype data of microsatellite markers, Theta ($\theta$) was calculated over all loci across the samples for each pair of sample. Theta values range from 0 to 1. The population with no genetic differentiation will have value close to 0. The probability of accepting the null
hypothesis that $F_{st}$ does not deviate from zero was calculated through 1000 bootstrap (Genetix 4.05.2, Belkhir et al. 1997). The computed probabilities were compared to critical probability level of 0.05, after sequential Bonferroni correction to pinpoint the pair of samples that exhibit significant genetic differentiation. For visual display of the results, the genetic relationship, UPGMA dendrograms was constructed using pairwise theta value, using genetic data analysis (GDA ver 1.0) software (Lewis and Zaykin, 2001).

G7. Bottleneck analysis

Populations that have experienced a recent reduction of their effective population size exhibit a correlative reduction of the allele numbers ($k$) and gene diversity ($H_e$, or Hardy-Weinberg heterozygosity) at polymorphic loci. For these purpose, probability values for ascertaining significance difference of expected heterozygosity ($H_{exp}$) values at individual loci from that at expected at mutation drift equilibrium ($H_{eq}$) were calculated through the software Bottleneck ver. 1.2.02. (Cornuet et. al., 1998), Bottleneck under infinite allele model (IAM) for allozyme loci. For microsatellite loci, estimates were made under infinite allele model (IAM), stepwise mutation model (SMM) and Two phase model (TPM). For microsatellite loci, TPM is generally recommended model, as all loci may not strictly mutate under SMM.

Many genetic indices have been developed based on the assumptions, among others, that variation is due to point mutation in DNA sequence and their is relatively constant to point mutation. Infinite allele model (IAM) is based on the assumption that all new alleles arise \textit{de novo} and there are an infinite number of
potential alleles at a locus. However, microsatellite allele differs from one another by number of repeats and there are not an infinite number of new alleles. One allele gives rise to another by addition or loss of a repeat. This is called stepwise mutation model (SMM).

Wilcoxon's rank test was used to determine, if a population exhibits a significant number of loci with heterozygosity excess. The null hypothesis for the test was there is no excess heterozygosity.