Materials and Methods
3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Experimental Animals

The *Garra gotyla* ray-finned fish of the family Cyprinidae was chosen as the experimental animal for the present study. For validation of the developed markers 6 other fish species were also used i.e. *Garra para lissorhynchus, Garra litaninsis vishwanath, Barilius bendelisis, Schizothorax richardsonii* and *Tor putitora*. Samples were collected from different locations in the wild for the present investigation. The geographical distribution, habitat, collection site and number of samples used are described in Table 3, Fig. 2 and 3.

Main test fish was a coldwater fish, commonly known as *Garra gotyla* (Fig 1). Taxonomically it belongs to-

- **Kingdom**: Animalia (Linnaeus, 1758)
- **Phylum**: Chordata (Bateson, 1885)
- **Subphylum**: Vertebrate
- **Class**: Actinoptergi
- **Division**: Teleostei
- **Order**: Cypriniformes
- **Family**: Cyprinidae
- **Genus**: Garra
- **Species**: Garra gotyla (Gray 1830)

**Fin formulae:** D-iii 7-8; A ii 5; P I 14; V i 8
DNA from fin tissue of *Garra gotyla* was the starting material for laboratory analysis. DNA of each individual was extracted separately as per established protocols. For isolation, identification and validation of microsatellite markers for *Garra gotyla*, DNA was isolated from each mentioned individuals. A partial genomic library containing microsatellite repeats was constructed using 350-750bp insert DNA of *Garra gotyla*. 28 microsatellite markers were developed from this partial genomic library. For library construction genomic DNA of *Garra gotyla* was isolated and subjected to Restriction digestion with Mbo I enzyme. Insert DNA was prepared. The insert DNA was ligated to BamHI digested and CIAP treated pUC 19-plasmid vector. The same ligated product was used to transform competent cells (JM 109) and competent cells were plated on Ampicillin, X- Gal, IPTG containing LB agar plates. The plates were incubated at $37^\circ$C overnight. 3000 positive colonies received in the form of white colonies. Insert DNA was confirmed through plasmid DNA isolation, RE digestion (BamH I and Hind III) colony PCR and colony hybridization. 72 recombinant clones were confirmed to be containing the repeat motif. Plasmid DNA was isolated from these selected recombinant clones and sequenced. Finally 52 positive motifs were characterized through sequencing. PCR amplification of the loci was done by designing primers from flanking region; genotyping for polymorphism was checked using Genepop, Cervus, GDA and Microchecker. As a result, 28 loci were identified as polymorphic in *Garra gotyla*. All the sequences containing microsatellite repeats were submitted in the NCBI GenBank with following accession no. (*HQ288484 to HQ288526* and *JF268657 to JF268665* Table 11).
Table -3 : Collection data and distribution of the fishes under study.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Species</th>
<th>Known Geographical Distribution</th>
<th>Collection Site</th>
<th>Latitude/ Longitude</th>
<th>No. of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Garra gotyla</em></td>
<td>Ganga and Brahmaputra drainage in India and also known from Pakistan</td>
<td>Gola River, Ranibagh, Uttarakhand</td>
<td>29°, 18’, 02’’N 79°, 32’, 02’’E</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Alaknanda River Srinagar Garhwal, Uttarakhand.</td>
<td>30°, 13’, 11”N 78°, 46’, 47”E</td>
<td>20</td>
</tr>
<tr>
<td>2.</td>
<td><em>Garra para lissorhynchus</em></td>
<td>Known from Chindwin drainage of Manipur</td>
<td>Lokchao River, Moreh, Manipur.</td>
<td>24°, 15’, 00”N 94°, 19’, 00”E</td>
<td>9</td>
</tr>
<tr>
<td>3.</td>
<td><em>Garra litanensis</em></td>
<td>Known from Chindwin drainage of Manipur</td>
<td>Lokchao River, Moreh, Manipur.</td>
<td>24°, 15’, 00”N 94°, 19’, 00”E</td>
<td>9</td>
</tr>
<tr>
<td>4.</td>
<td><em>Barilius bendelisis</em></td>
<td>Ganga and Brahmaputra drainage in India and also known from Nepal and Pakistan.</td>
<td>Gola River, Ranibagh, Uttarakhand.</td>
<td>29°, 18’, 02’’N 79°, 32’, 02’’E</td>
<td>9</td>
</tr>
<tr>
<td>6.</td>
<td><em>Tor putitora</em></td>
<td>Ganga-Brahmaputra and Chindwin drainage in India. Also known from Bangladesh, Nepal, Pakistan and Afghanistan</td>
<td>Kosi River, Ramnagar, Uttarakhand.</td>
<td>29°, 24’, 56’’N 79°,08’,01”E</td>
<td>9</td>
</tr>
</tbody>
</table>
Figure 1 Garra gotyla; Figure-2 and Figure-3 is collection site for *Garra gotyla*. Figure-2: Collection site at Alaknanda River Srinagar Garhwal, Uttarakhand, India. Figure-3: Collection site at Gola River, Ranibagh, Uttarakhand, India.
The instruments, chemicals, buffers, enzymes and molecular kits used for this present study are listed below:

3.1.2 Equipment and Instrument

(i) UV-Vis Spectrophotometer, UV-1, Thermo Scientific England.
(ii) Gel Doc system, AP 410209 Alpha Imager, Alpha Innotech, USA.
(iii) Vacuum Concentrator, Concentrator plus, Eppendorf AG, Germany.
(iv) Water Circulatory System, E-100, Lauda, Germany.
(v) UV-Crosslinker, Model Uvitech, France.
(vi) Laminar air flow, UTS-607, Unitech Sales Delhi, India.
(vii) Orbital Shaker Incubator, REMI, Remi Electronic Ltd. INDIA.
(viii) Thermocycler, Gen Amp PCR System 9700, ABI, USA.
(ix) Centrifuge, Model 5430 R, Eppendorf AG, Germany.
(x) Autoclave, ABS-98, Macro-Scientific, India.
(xi) Electrophoresis set (horizontal) & power supply, Biometra, Germany.
(xii) Vertical Gel electrophoresis set & power supply, Hoeffer, USA.
(xiii) Micropipettes, Nichipet, Japan.
(xiv) Bacteriological Incubator, Mac India Ltd, India.
(xv) ULT Freezer -80° C, Model-DW-86L628, 388, Haier, China.
(xvi) Water Bath (circulatory), Thermo-circulator, Labtech.

3.1.3 Chemicals, enzymes and biological reagents

X-Gal (2 %) 5 bromo-4-Chloro-3-indolyl-β-D-galactopyranoside (Fermantas)

20mg of X-gal dissolved in 1 ml of DMF (Di methyl formamide) to make 2% X-gal solution. It was stored in aluminium foil wrapped polypropylene tube at –20 0C.
**IPTG (25 mg/ml) Isopropyl-β-D-thiogalactopyranoside (Fermantas)**

250 mg of IPG (Mol. Wt. 238.3) was dissolved in 10 ml of sterile water and divided into aliquots to be stored at –20 °C.

**Luria Bertani (LB) Broth (Hi-media)**

Bacto-tryptone (**Himedia**) 10 g

Yeast extract (**Himedia**) 5 g

NaCl (**Himedia**) 10g

The final volume was made up to 1 liter.

**For plates**

Bactoagar (SRL) 18g

Sterilization by Autoclave.

**SOB Media**

Bacto-tryptone (SRL) 20 g

Yeast extract (SRL) 5 g

NaCl (SRL) 0.584 g

KCl (SRL) 0.186 g

The final volume was made up to 1 liter and Sterilization by Autoclave.

**SOC media**

Prepared by adding 0.5 ml of 2M Mg⁺⁺ stock and 2 ml of 1 M glucose (SRL) to 97.5 ml of SOB Medium

**Ampicillin (Himedia)**

50mg of Ampicillin dissolved in 1 ml of sterile water, storage at –20 °C, used 100 μg/ml of culture
Nylon Membrane Biodyne B (Pall life sciences), 0.45 µm, positively-charged Nylon 6, 6 transfer Membrane.

1 M Tris

141.4 g of Tris (SRL) dissolved in 800 ml of double distilled water. The pH was adjusted up to 8 by adding concentrated HCl (Qualigens). In case the solution turned yellow colour, it was discarded. As the pH is time dependant and decreases approximately 0.03 pH units for each 1°C increase in temp, so utmost care was taken to monitor the pH.

0.5M EDTA (pH-8.0)

186.1g of di-sodium EDTA.2H2O (Qualigens) was added to 800 ml of double distilled water. It was prepared by vigorous stirring on magnetic stirrer and pH adjusted to 8.0 with NaOH pellets (~20g of NaOH (SRL) pellets). Then, volume of the sample was made up to 1 liter with distilled water. Finally, the solution was dispensed in to aliquots and sterilized by autoclaving at 121°C and 15lb per sq. in. pressure for 20 minutes in an autoclave.

5M Potassium Acetate

Dissolve 29.442g of potassium acetate (Qualigens) for 60ml of solution. To this added 11.5 ml glacial acetic acid in 28.5 ml double distilled water. The resulting solution is 3 M with respect to potassium and 5M with respect to acetate. It was mixed and let it stand on ice for 10 minutes.

10% Sodium dodecyl Sulphate (SDS)

100g of electrophoresis grade SDS (SRL) was dissolved in 900 ml distilled water and incubated at 68°C. Then, final volume was adjusted to 1000ml.
1N Sodium Hydroxide (NaOH)

40g of Sodium hydroxide (SRL) was dissolved in 1liter of double distilled water.

5x TBE Buffer (50mM Tris, 100 mM EDTA, and 20 mM Boric acid)

Dissolved 54 g Tris, 20ml EDTA (pH 8.0) and 27.5 g boric acid in 900 ml of distilled water and when dissolved completely, the volume was adjusted to 1000 ml. Then it was autoclaved at 120° C and 15 lb per square inches pressure for 20 minutes.

Boric acid (SRL)

Proteinase K (10 mg/ml)

10 mg of powdered proteinase K (Fermantas) was dissolved in 1ml double distilled water.

Phenol pH 8.0 (equilibrated with Tris) (SRL)

Chloroform (molecular grade) (SRL)

Iso-amylalcohol (Qualigens)

Chloroform: Isoamylalcohol

The solution was prepared in the ratio of 24:1.

3M Sodium acetate (pH 5.2)

102.06g of CH₃COONa.3H₂O (SRL) was dissolved in 200ml of doubled distilled water. The pH was adjusted to 5.2 with glacial acetic acid and volume was made up to 250ml. Then the solution was dispensed in to aliquots and sterilized by autoclaving at 121°C and 15lb pressure per sq. in. for 20 minutes in autoclave.
**70 % Ethanol (Merck)**

The solution was made with 70ml absolute ethanol (Merck) and 30ml double distilled water.

**0.8% Agarose gel**

0.8g agarose (SRL) was boiled in 100ml 1X TBE buffer until a clear and transparent solution was formed. The decrease in volume due to evaporation was adjusted with double distilled water.

**1.8% Agarose gel**

1.8g agarose (SRL) was boiled in 100ml 1X TBE buffer until clear and transparent solution was formed. The decrease in volume due to evaporation was adjusted with double distilled water.

**Ammonium persulphate (10%)**

100mg of Ammonium persulphate (SRL) in 1 ml of sterile water, stored at 4°C

**Acrylamide / Bisacrylamide (6%) working solution (Denaturing):**

**Acrylamide / Bisacrylamide (40%) solution (SRL)**  5 ml

TBE Buffer (10%)  10 ml

Urea (SRL)  46 gram

Total volume adjusted to 100 ml

**Formamide Loading Dye**

<table>
<thead>
<tr>
<th></th>
<th>For 2 ml</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formamide (SRL)</td>
<td>1.6 ml</td>
<td>80 %</td>
</tr>
<tr>
<td>EDTA (0.5 M) (Qualigens)</td>
<td>40 µL</td>
<td>10mM</td>
</tr>
<tr>
<td>Xylene Cyanol (Bangalore genei)</td>
<td>2 mg</td>
<td>1mg/ml</td>
</tr>
<tr>
<td>Bromophenol Blue (Bangalore genei)</td>
<td>2 mg</td>
<td>1mg/ml</td>
</tr>
</tbody>
</table>
Tris-EDTA Buffer (TE Buffer pH 7.4)

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris</td>
<td>1 ml</td>
<td>10mM pH (7.4)</td>
</tr>
<tr>
<td>0.5M EDTA</td>
<td>200 µl of EDTA</td>
<td>1mM pH (8.0)</td>
</tr>
</tbody>
</table>

up to 100 ml with double distilled water.

Acrylamide / Bisacrylamide (40%) solution

38gm of acrylamide (SRL) and 2gm of N,N’ methylene bisacrylamide (SRL) were dissolved in 60 ml of distilled water. The mixture was heated to 37°C to dissolve the chemicals. Final volume was adjusted to 100ml with distilled water. Sterilized the solution by filter sterilization (0.45 micron pore size) and adjusted to pH 7.0 and stored in amber colour bottle.

Taq DNA polymerase (3U/µl) (Bangalore genei)

dNTPs Master mix (Bangalore genei)

The mixture was prepared with 2.5 mM each of dATP, dTTP, dCTP and dGTP and stored at -20°C in aliquots (Bangalore Genei Pvt. Ltd., Bangalore).

Template DNA

DNA sample was dissolved in TE buffer to the final concentration of 50ng/µL.

The chemicals used for the present study were procured from the following firm:

Fermantas: Restriction endonucleases (Bam HI, Mbo I, Eco RI and Hind III) Proteinase K, Rnase, pUC19 vector, M13 Forward sequencing Primer, M13 Reverse Sequencing primer, X-Gal, IPTG, Calf Intestine Alkaline Phosphatase, 174 DNA/Hinf I digested, GeneRuler™1kb DNA ladder, GeneRuler™100 bp DNA ladder and Lambda DNA EcoRI/HindIII Marker.

Hi-media: Luria Agar, Luria Broth, Agar powder and Ampicillin.
SRL: Agarose, Sodium Laurayl sulphate, Sodium hydroxide, Calcium chloride, Magnesium chloride, Magnesium sulphate, Chloroform, Boric acid, Tris (hydroxymethyl) amino methane (Tris buffer), Polyvinyl Pyrollidone, Ethidium bromide (Etbr), N,’N,’N,’N,’ Tetramethylthelyenediammine (TEMED), Ammonium per sulphate (APS), N,N-Methylenebisacrylamide, Acrylamide, Tryptone, Yeast extract, D-Glucose (Dextrose), Malic acid and Phenol.

Bangalore Genei: dNTPs, 10X Taq buffer with 15mM MgCl$_2$, 25mM MgCl$_2$, Taq DNA Polymerase.

Qualigens: Ethylene diammine tetra acetic acid (EDTA), Formaldehyde, Formamide, Acetic acid, Tri sodium citrate, Isopropanol and Glycerol, Glacial acetic acid.

Merck: Ethanol

Roche: DIG easy Hyb solution and Anti-Digoxigenin-AP, Fab fragments.

Pall life sciences: Biodyne B, 0.45 µm, positively-charged Nylon 6, 6 Transfer Membrane.

3.1.4 Molecular biology kits

1) Roche Germany: DIG Nucleic Acid Detection Kit, DIG Oligonucleotides Tailing Kit, 2$^{nd}$ Generation.

2) Novagen, Germany: DNA Ligation Kit.

3) Qiagen, Germany: PCR Purification Kit, Gel Elution Kit and QIAquick PCR Purification Kit.


3.1.5 Buffer and stock solutions:

i) Reagents for Genomic DNA Isolation

Cell Lysis Buffer 100mM NaCl, 50 mM Tris (pH 8.0),
100 mM EDTA (pH 8.0), 1% SDS

Phenol/Chloroform/Iso-amylalcohol mixed to a ratio of 25:24:1

TE Buffer 100 mM Tris (pH 8.0), 10 mM EDTA
ii) Agarose Gel Electrophoresis Reagents

TBE buffer  
45 mM Tris-borate (pH 7.6), 1 mM EDTA

6X Loading dye  
10mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% Xylene cyanol FF, 60% glycerol (Qualigens) and 60mM EDTA

Ethidium Bromide 10mg/ml, Final working concentration used at 0.5 µg/ml

iii) Alkaline Lysis Buffers (For Plasmid DNA Preparation)

a) Solution I (This solution was autoclaved and stored at 4°C)

Glucose (SRL)  
50 mM

Tris-Cl (pH 8.0)  
25 mM

EDTA (pH 8.0)  
10 mM

b) Solution II (This solution was prepared fresh and used at room temperature)

NaOH (SRL)  
0.2N (freshly diluted from 10N stock)

SDS (SRL)  
1.0% (w/v)

c) Solution III  
5 M Potassium acetate (60.0 ml) (SRL), Glacial acetic acid (11.5 ml) (Qualigens), Water (28.5 ml)

iv) Southern Hybridization Reagents

Neutralizing solution  
1.0M Tris HCl (pH 7.4), 1.5 M NaCl

20X SSC (pH 7.4)  
3M NaCl, 300mM Tri-sodium citrate (Qualigens)

Pre-hybridization solution  
5X SSC, 1X Denhart’s solution, 1µg/ml poly A, 0.5% SDS (SRL)

5X Denhart’s solution  
1% (w/v) BSA (SRL), 1% (w/v) Ficoll (SRL), 1% (w/v) PVP (SRL)
Denaturation solution 1.5M NaCl, 0.5M NaOH
Malic acid buffer (pH-7.5) 0.1 M Malic acid (SRL), 0.15 M NaCl
Washing Buffer (pH-7.5) 0.1 M Malic acid (SRL), 0.15 M NaCl, 0.3%, (w/v) Tween 20 (SRL)
Blocking Solutions Dilute 10X blocking Solution (1:10) with Malic acid buffer
Antibody Solution Dilute Anti-Digoxigenin-AP (Roche) (1:5000) in blocking solution
Detection Buffer (pH-9.5) 0.1M Tris-HCL, 0.1M NaCl
NBT/BCIP solution Dissolve 200µl of NBT/BCIP stock (Roche) solution in 10ml of detection buffer.
Low stringency buffer 2X SSC, 0.1% SDS
High stringency buffer 0.1X SSC, 0.1% SDS

3.1.6 Oligonucleotides used: Following are the list of oligonucleotide used in this study:

Table -4: Oligos used as probes for DIG labeling reaction and Colony PCR

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Name</th>
<th>Sequence (5’to 3’)</th>
<th>Length (mer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NRC-1M</td>
<td>GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>NRC-2M</td>
<td>ACACACACACACACACACACACACACACACACACACACACACACACACACACACACACACACACACACAC</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>NRC-3M</td>
<td>ATATATATATATATATATATATATATATATATATAT</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>NRC-4M</td>
<td>CTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>NRC-5M</td>
<td>CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG</td>
<td>27</td>
</tr>
</tbody>
</table>
Table-5 : Oligos used in Colony PCR and sequencing of Plasmid DNA

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Name</th>
<th>Sequence (5’to 3’)</th>
<th>Length (mer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M13F</td>
<td>GCCAGGGTTTTCCAGTCACGA</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>M13R</td>
<td>GAGCGGATAACATAATTTCACACAGG</td>
<td>24</td>
</tr>
</tbody>
</table>

3.2 METHODS

3.2.1 Extraction and purification of Genomic DNA

Total genomic DNA was isolated from the fin tissue of the sampled individuals using standard proteinase K digestion followed by extraction with phenol: chloroform and precipitation with ethanol following Sambrook et al. (2001). 50mg of fin tissue were chopped in a 2ml of sterile Eppendorf tubes. To the tissues 500µl of cell lysis buffer and 5µl of proteinase K (20mg/ml) was added to the tissue, mixed well and incubated in a water bath at 37°C for overnight. After overnight incubation an equal volume of phenol (Tris saturated and equilibrated at pH 8.0) was added to each tube, mixed gently and centrifuged at 10000 rpm at 4°C for 10mins. The upper aqueous phase of the tube was transferred to a new sterile tube and an equal volume of phenol and chloroform: Isoamyl alcohol (24:1 v/v) was added, mixed gently and centrifuged at 10,000 rpm at 4°C for 10 mins again. The upper aqueous phase was collected in a new sterile tube and to the tubes chloroform: Isoamyl alcohol (24:1 v/v) was added equally, mixed gently and centrifuged at 10,000 rpm at 4°C for 10 mins. The aqueous phase containing DNA was precipitated with 2.5 volume of ice cold ethanol, pelleted at 10,000 rpm for 10mins at 4°C. The supernatant was discarded and the pellet was washed with 70% ethanol and dried at room temperature. The DNA pellet was resuspended in 100 µl TE buffer (pH-8.0).
The isolated DNA was also given RNaseA treatment for 1 hour at 37°C. To the DNA samples, 2µl of the RNase Stock solution (10mg/ml) was added and incubated at 37°C for 1hr. Treated samples were then extracted with phenol-chloroform extraction as described earlier.

### 3.2.2 Checking the Quality of DNA

The quality of DNA was checked by running the DNA samples in 0.8% agarose gel. Only those DNA samples were analyzed further which showed intact band in the gel. The samples which showed shearing were discarded and the DNA was again isolated from those samples.

### 3.2.3 Quantification of DNA

Quantification of the genomic DNA was determined by spectrophotometric method. An absorbance of 1 at 260nm corresponds to 50 µg of double stranded DNA per ml of solution. 5 µl of DNA sample was added to 995 µl of double distilled water. Mixing was done by inverting the tubes several times and spinning at room temperature. The absorbance was taken at 260nm and 280nm by taking double distilled water as blank. The ratio of OD\(_{260}\)nm / OD\(_{280}\)nm provides an estimate of the purity of nucleic acid therefore the absorbance was taken at 260 nm and 280 nm (Glasel, 1995). In pure preparations of DNA/RNA samples OD\(_{260}\)/OD\(_{280}\) ratio value should be between 1.8 and 2.0. Accordingly purity of each samples were verified.

The Concentration of DNA was calculated by using the following formula:

\[
\text{DNA concentration (µg/ml)} = \text{OD}_{260} \times \text{Dilution factor} \times \frac{50 \, \mu \text{g DNA/ml}}{1 \, \text{OD}_{260} \, \text{unit}}
\]

Whereas DNA is diluted 200 times with double distilled water so dilution factor is 200.
Figure-4: Construction of a partial genomic library and development of microsatellite markers from microsatellite enriched genomic library.
Figure-5: Construction of partial microsatellite containing library.
Bacterial colonies containing cloned segments of insert DNA

Nylon membrane

Bacterial cells were transferred to highly negatively charged nylon membrane

Cells were transferred, denatured and crosslinked to nylon membrane

Probes were added to filter

Hybridization on filter

Blue colour precipitate confirming clone containing gene of interest.

Master plate

Colones containing gene of interest.

**Figure-6**: Nucleic acid hybridization to find the gene of interest.
3.2.4 Insert DNA preparation

10µg of genomic DNA was digested with restriction enzyme Mbo I (Fermentas) which cut the DNA at specific site (GGATCC) and produce the sticky end (GATC).

The reaction set up was as follows:

Reaction Mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Sample (10 µg)</td>
<td>4.0 µl</td>
</tr>
<tr>
<td>10X Buffer</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>Sterile water</td>
<td>38.5 µl</td>
</tr>
<tr>
<td>Mbo I Enzyme (10 Units/µl)</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>50.0 µl</td>
</tr>
</tbody>
</table>

The DNA and enzyme mixture was incubated at 37°C for 4 hours. The resulting fragments were separated in 1.2% agarose gel. Around 350 to 750bp DNA fragments were excised from the gel, purified with NucleoSpin-Extract-Kit (Macherey-Nagel). The absorbance was checked of the eluted product and quantification is done using spectrophotometer as explained earlier.

3.2.5 Vector DNA preparation

Vector preparation was carried out using pUC19 plasmid which contained BamH I restriction site in multiple cloning sites (MCS) (Fig. 8). This restriction endonucleases (RE) was selected due to its cutting sites (GATC) which was complementary to Mbo I (used for insert DNA preparation) cutting site (Fig. 7).

10µg pUC19 plasmid was digested with 50 units of Bam HI enzyme.

The reaction set up was as follows:

Reaction Mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X RE Buffer</td>
<td>10.0 µl</td>
</tr>
<tr>
<td>Plasmid vector (10 µg)</td>
<td>66.0 µl (Stock conc. 0.151µg/µl)</td>
</tr>
<tr>
<td>Sterile water</td>
<td>19.0 µl</td>
</tr>
<tr>
<td>Bam HI Enzyme (10 Units/µl)</td>
<td>5.0 µl (50 Units)</td>
</tr>
<tr>
<td>Total volume</td>
<td>100.0 µl</td>
</tr>
</tbody>
</table>
The reaction mixture was incubated at 37°C for 4 hours. The digested product was checked in 1% agarose. The digested samples were extracted with phenol: chloroform and the DNA were recovered by standard precipitation (Sambrook et al 2001) and dissolved in 110 µl of 10mM Tris-Cl. Initially 20µl of the DNA preparation was stored (to be used as one of the control in transformation reaction). And the remaining 90 µl of linearized plasmid DNA was dephosphorylated.

### 3.2.6 Dephosphorylation of linearized vector DNA

Dephosphorylation of the linearized vector DNA was done using Calf intestine alkaline phosphotases (CIAP). Alkaline phosphates removes the 5’terminal phosphate residue from both termini of the linearized plasmid DNA (Seeburg et al., 1977; Ullrich et al., 1977) thus suppressing the self-ligation and circularization of the plasmid DNA.

To 90µl of linearized pUC19 plasmid DNA 0.5 unit of CIAP was added.

Reaction set up was as follows:

<table>
<thead>
<tr>
<th>Reaction Mixture</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>10X CIAP buffer</td>
<td>10 µl</td>
</tr>
<tr>
<td>Linearized pUC19 DNA</td>
<td>90 µl</td>
</tr>
<tr>
<td>Calf intestine alkaline phosphotases (1U/µl)</td>
<td>0.5 µl (0.5 U)</td>
</tr>
<tr>
<td>Total volume</td>
<td><strong>100.5 µl</strong></td>
</tr>
</tbody>
</table>

The reaction mixture was incubated at 37°C for 30 mins. The CIAP was inactivated by heating the reaction mixture at 65°C for 30mins in the presence of 5mM EDTA. The reaction mixture was cooled to room temperature and then extracted once with phenol and once with phenol: chloroform. DNA was recovered by standard precipitation with ethanol and by centrifugation at 10000
rpm for 10 mins at 4°C. The pellet was washed with 70% ethanol at 4°C and centrifuge again. The precipitated DNA was dissolved in 50 µl TE (pH 8.0). Concentration of the linearized plasmid DNA was estimated using spectrophotometer as described earlier.

3.2.7 Ligation

The 350-700bp size insert DNA was ligated with CIAP treated pUC19 vector in the molar ratio of 3:1 using DNA ligation kit (Novagen). One control reaction was also performed containing linearized plasmid DNA without insert DNA for assessing if linearized plasmid was self ligating.

The reaction set up was as fellows:

Reaction Mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X ligation Buffer</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>100mM DTT</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>10mM ATP</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Plasmid vector (50 ng)</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Insert DNA (150 ng)</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>3.5 µl</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>1.0 µl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>10.0 µl</strong></td>
</tr>
</tbody>
</table>

The reaction mixture was incubated at 16°C for 8 hours. After incubation the enzyme is inactivated by heating the mixture at 70°C for 10mins. The ligation efficiency was assessed by agarose gel electrophoresis of ligation reaction product. The ligation mixture was used directly for transformation.
Figure-7: Restriction site for enzyme BamHI.

Figure-8: pUC19 plasmid vector with its multiple cloning sites (MCS) regions.
3.2.8 Competent cell preparation

Competent cells were always prepared fresh following Sambrook et al. (2001). JM109 strain of *E. coli* was used for competent cell preparation following calcium chloride method (Sambrook et al., 2001). Single bacterial colony was picked from freshly prepared LB agar plate of *E. coli* JM109 and inoculated in 50ml LB broth, incubated in Orbital shaker (200rpm) at 37°C overnight. 1ml of overnight grown culture was inoculated further in 100ml LB broth and incubated at 37°C. The growth of the culture was monitored at different intervals at 600nm using UV-Vis spectrophotometer. The culture was harvested when OD$_{600}$ reached to 0.35. The culture was cooled for 10 minutes at 4°C and then cells were recovered by centrifugation at 4100rpm for 10 minutes at 4°C. The supernatant was removed and the cell pellet was resuspended in 30ml of ice-cold MgCl$_2$-CaCl$_2$ solution (80mM MgCl$_2$, 20mM CaCl$_2$). Again the cells were recovered by centrifugation at 4100rpm for 10 minutes at 4°C. The cell pellets were resuspended by swirling in 2ml of ice cold 0.1M CaCl$_2$ and aliquoted as 160μl cells in each 10 tubes and stored in -80°C after adding 40μl Glycerol.

3.2.9 Transformation

Frozen Competent cells prepared by above method, were thawed on ice for 5 minutes. A total of 4μl ligation reaction was dispensed into 200μl of the cells in a 1.5ml Eppendorf tube. A positive and negative control was also included to measure the efficiency of transformation and to eliminate the possibility of contamination. The positive control contains 200μl competent cells and 1 μl intact pUC19 (50ng/ μl) and negative control contains 200μl competent cells only (no plasmid DNA). Tubes were then stored on ice for 30 minutes. The cells were then subjected to heat-shock treatment by putting in a pre-heated 42°C circulatory water bath for exactly 90 seconds without shaking. Tubes were then
immediately placed on ice for 2 minutes. Then, 800µl of SOC medium was added to each transformation reaction. The tubes were then transferred to an incubator shaker set at 37 ºC and incubated for 45 minutes before plating on LBA/IPTG/X-Gal/Ampicillin plates. The transformants were collected by centrifugation for 1 minute at room temperature and then gently resuspend the cell pellet in fresh 100µl SOC and mixed properly. The transformed cells were then spreaded gently over the surface of the agar plate. The plates were then incubated at 37ºC for overnight. After incubation the positive clones were identified in the form of white and blue colonies (Fig. 17 & 18).

3.2.10 Colony Hybridization

Colony Hybridization is the screening of a library with a labeled probe (radioactive, bioluminescent, etc.) to identify a specific sequence of DNA, RNA, enzyme, protein, or antibody. Hybridization reactions are specific; probes will bind only to sites that have complimentary sequences. This specificity allows one to find a specific sequence in a complex mixture full of similar sequences. Microsatellite DNA is identified in libraries of cloned DNA by colony hybridization to oligonucleotide probes containing simple sequence repeat (SSR) DNA, a common procedure in molecular biology laboratories.

First of all before screening the recombinant clone for microsatellite detection using colony hybridization the oligonucleotides were enzymatically labelled at their 3’end (Oligonucleotides tailing) with terminal transferases by incorporation of a single digoxigenin-labelled dideoxyuridine-triphosphate (DIG ddUTP, Fig 9) and determination of the yield of DIG-labelled oligonucleotides were also done for checking the labelling efficiency.

3.2.10.1 Oligonucleotides tailing: DIG labelled Tailing of oligonucleotide was done using DIG Oligonucleotides Tailing Kit, 2nd Generation (Roche, Germany).
The reaction set up was as follows:

Reaction Mixture:

- 50 pmole oligonucleotide in distilled water 9 µl
  (To a final concentration of 10pmole/ µl)
- Reaction buffer 4 µl
- CoCl₂ solution 4 µl
- DIG-dUTP solution 1 µl
- dATP solution 1 µl
- Terminal transferases 1 µl
- Total volume 20 µl

Reaction mixture was mixed and centrifuged briefly. Incubated at 37°C for 15mins and then place on ice. The reaction was stopped by adding 2 µl of 0.2 M EDTA (pH 8.0).

3.2.10.2 Determination of labelling efficiency for colony hybridization

Determination of the yield of DIG-labelled oligonucleotides is most important for optimal and reproducible hybridization results. The preferred method for quantification of labelled probes is the direct detection method.

Dilution series

Comparison was done with a series of dilution of test labelled oligonucleotide to a series of dilutions of the control labelled oligonucleotide (vial 6 of the DIG oligonucleotide tailing kit).

The test Labelled oligonucleotide (100pmol/22µl) was diluted to a starting concentration of 2.5pmol/µl and then dilution was prepared as described in the table 6.
Table-6: Dilution series of labelled and control oligonucleotide were prepared as described in the table:

<table>
<thead>
<tr>
<th>Tube</th>
<th>Oligo (µl)</th>
<th>From Tube</th>
<th>Dilution buffer (Vial10) (µl)</th>
<th>Dilution</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>Original (2.5pmole/ µl)</td>
<td>48</td>
<td>1:25</td>
<td>100 fmol/ µl</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>1</td>
<td>7</td>
<td>1:3.3</td>
<td>30 fmol/ µl</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>1</td>
<td>18</td>
<td>1:10</td>
<td>10 fmol/ µl</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>2</td>
<td>18</td>
<td>1:10</td>
<td>3 fmol/ µl</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>3</td>
<td>18</td>
<td>1:10</td>
<td>1 fmol/ µl</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

Procedure: From tube 2-6, 1µl spot of the labelled test oligonucleotide and the labelled control oligonucleotide was applied to the nylon membrane. Nucleic acid was fixed to the membrane by cross linking with UV-light for 5 minutes. Now the membrane was transferred into a plastic container with 20ml washing buffer, the container was incubated at 20°C for 2 minutes with continuous shaking. After washing step, membrane was incubated for 30 minutes in 10ml of blocking solution and after that membrane was again incubated for another 30 minutes in 10ml of antibody solution. The Membrane was now washed two times in washing buffer for 15 minutes each. Now the membrane was equilibrated with 10ml of detection buffer for 5 minutes. Then the membrane was placed in a plastic transparency film and covered with 10 ml of detection buffer containing 200µl of NBT/BCIP stock solution (Roche, Germany) and incubated for 8 hours. After colour precipitate was clearly visible the reaction was stopped by changing its solution with 20ml of TE-buffer. Finally, the blot with positive colour was photographed using digital camera.
Figure-9: Non-radioactive oligonucleotide tailing and detection (adapted from DIG oligonucleotide tailing kit 2\textsuperscript{nd} generation, Roche applied sciences, Germany)
3.2.10.3 Colony hybridisation and Screening of Positive clones

Using sterile toothpick bacterial colonies were transferred one by one onto the grided agar plate containing ampicillin in duplicate (200 colonies per plate). Agar plates containing colonies were pre-cooled at 4°C for 30 minutes. The master plates were incubated at 37°C. After growth of the colonies a highly positive charge nylon membrane (Bio-dyne B, 0.45µm, Pall Life Science) was placed on the surface of each master plate for 1min, lifted and placed on a separate precooled agar plate for 1 minute for a replica plate. The replica plate was also incubated at 37°C for future plasmid DNA isolation. Then the nylon membrane was kept on a glass petridish with colonies upward containing denaturing solution (0.5M NaOH, 1.5M NaCl) and incubated for 15 minutes. After incubation membrane was air-dried briefly on a new dry sheet of whattman paper. Membrane disc was now transferred into a new plastic film containing 1ml neutralization solution (1.5M NaCl, 1.0M Tris-HCL, pH 7.4) and incubated for 15 minutes. Air-dry step was again repeated. Now the membrane disc was transferred to a plastic film containing 2X SSC and incubated for 10 minutes. The membrane containing the colonies was cross-linked by illuminating the disc with UV light for 5 minutes in UV-Cross-linker (Uvi Tech, France). Simultaneously oligonucleotides tailing with DIG-dUTP was done using Roche oligonucleotide tailing kit. The white colonies were screened by using (CA)n/(GT)n, (GA)n/(CT)n, (GAA)n/(CTT)n and (CCA)n/(GGT)n probes.

Membrane discs were now placed in a sealable hybridization bag and the membrane was prehybridized at 42°C in 20 ml of DIG Easy Hyb (Roche, Germany). 15ml of DIG Easy Hyb containing denatured labelled probe (25ng/ml) was hybridised with the membrane in a closed container at 42°C for 1hr. Membrane was then washed twice with Low Stringency Buffer (2x SSC,
0.1% SDS) at room temperature for 15 minutes each. Again the membrane was washed twice with High Stringency Buffer (0.1X SSC, 0.1% SDS) at 68ºC for 15 minutes each. Membrane was washed briefly with washing buffer (0.1M Malic acid, 0.15M NaCl and 0.3% Tween 20) and the reaction was blocked in 30ml of blocking solution (Roche, Germany) for 30 minutes. The membrane was placed in 20ml of diluted (1:10000) antibody solution (Roche, Germany) after blocking. Then the washing step was again repeated twice with washing buffer to remove unbound antibody. The membrane was now equilibrated with 20ml of detection buffer (0.1M NaCl 0.1M Tris-HCL) for 5 minutes. Then the membrane was placed in a plastic transparency film and covered with 10 ml of detection buffer containing 200µl of NBT/BCIP stock solution (Roche, Germany) and incubated for 1-16 hours. After colour precipitate was clearly visible the reaction was stopped by changing its solution with 20ml of TE-buffer. Finally, the blot with positive colour was photographed using digital camera for future reference and plasmid DNA was isolated from the positive colonies.

3.2.11 Plasmid DNA isolation

3.2.11.1 Preparation of cells

The plasmid DNA was isolated following conventional method (Sambrook et al 2001). The colonies identified as positive in colour detection from hybridization were picked up from replica plate using autoclaved loop and placed in 15 ml LB broth containing ampicillin. The cell in the LB broth was grown in an Orbital shaker pre set at 37ºC with agitation for overnight. After overnight incubation the culture was transferred into a 15ml tube and the bacteria are recovered by centrifugation at 4000rpm for 10 mins at 4ºC. The medium was removed gently leaving bacterial pellet as dry as possible.
3.2.11.2 Lysis of cells

The bacterial pellet was resuspended in 200 µl of ice-cold alkaline lysis solution I by vigorous vortexing, and the suspension was transferred to a 1.5 ml Eppendorf tube. 400 µl of freshly prepared alkaline lysis solution II was added to each bacterial suspension. Contents of the tube were mixed by inverting the tubes rapidly five times, and then tubes were stored in ice. 300 µl of ice-cold alkaline lysis solution III was added to each tube and tubes were inverted several times so that lysis solution dispersed through the viscous bacterial lysate. Tubes were then stored in ice for 5 minutes. The bacterial lysate were then centrifuge at 10000 rpm for 5 minutes at 4°C. 600 µl of the supernatant was transferred to a fresh Eppendorf tube. An equal volume of phenol:chloroform was added to each tube. The organic and aqueous phase was mixed by vortexing and the emulsion was centrifuged at 10000 rpm for 2 minutes at 4°C. The upper aqueous layer was transferred to a fresh Eppendorf tube.

3.2.11.3 Recovery of Plasmid DNA

Nucleic acid was precipitated from the supernatant by adding 600 µl of Isopropanol at room temperature. The precipitated nucleic acid was collected by centrifugation at 10000 rpm for 5 mins at room temperature. Supernatant was removed and the pellet was allowed to dry by inverting the tubes on a paper towel. DNA pellet was then washed with 1ml of 70% ethanol and again centrifuged at 10000 rpm for 2 minutes at room temperature to recover the DNA. The DNA pellet was dissolved in 100µl of TE (pH 8.0). The isolated DNA was also given Rnase A treatment for 1 hour at 37°C. To the DNA samples, 2µl of the Rnase Stock solution (10mg/ml) was added and incubated at 37°C for 1hr. DNA solution was then stored at -20°C.
3.2.12 Confirmation of the recombinant clone using RE digestion and colony PCR

The isolated plasmids were confirmed through RE digestion. The recombinant plasmid have MCS region on both sides of the insert DNA. Two Restriction enzymes were selected one each from the two MCS of the recombinant plasmid. The recombinant plasmid was digested with Eco RI and Hind III.

Reaction set up was as follows:

<table>
<thead>
<tr>
<th>Reaction Mixture</th>
<th>1Rx</th>
<th>55 Rx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double distilled water</td>
<td>16.64 µl</td>
<td>915.0 µl</td>
</tr>
<tr>
<td>10X buffer Tango</td>
<td>2.00 µl</td>
<td>110.0 µl</td>
</tr>
<tr>
<td>R.E. Enzyme Hind III (10U/µl)</td>
<td>0.36 µl</td>
<td>19.0 µl</td>
</tr>
<tr>
<td>R.E. Enzyme Eco RI (10U/µl)</td>
<td>0.36 µl</td>
<td>19.0 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>20.0 µl</td>
<td></td>
</tr>
</tbody>
</table>

19µl of the reaction mixture was added to each aliquoted tube and 1 µl plasmid DNA was added separately to each aliquoted tube. The tubes were then incubated overnight in a water bath pre set at 37°C. The RE digested products were then check in 1% agarose gel along with size standard marker (Lambda DNA EcoRI/HindIII Marker), intact pUC19 plasmid and Bam HI digested pUC19 plasmid (Fig. 26).

3.2.12.1 Secondary screening of the recombinant clone using colony PCR

Insert DNA were also confirmed with PCR amplification of plasmid DNA using M13 F/R primers. Reaction set up was as follows:
Reaction Mixture

<table>
<thead>
<tr>
<th>Component</th>
<th>1Rx</th>
<th>95 Rx</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X amplification buffer</td>
<td>1.0 µl</td>
<td>95.0 µl</td>
</tr>
<tr>
<td>dNTP's (10mM)</td>
<td>1.0 µl</td>
<td>95.0 µl</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>1.0 µl</td>
<td>95.0 µl</td>
</tr>
<tr>
<td>Forward sequencing Primer (M13 F) (5 µM)</td>
<td>0.5 µl</td>
<td>47.5 µl</td>
</tr>
<tr>
<td>Reverse sequencing Primer (M13 R) (5 µM)</td>
<td>0.5 µl</td>
<td>47.5 µl</td>
</tr>
<tr>
<td>Taq polymerase (1U/µl) (1U/sample)</td>
<td>1.0 µl</td>
<td>95.0 µl</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>4.0 µl</td>
<td>380.0 µl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>9.0 µl</strong></td>
<td></td>
</tr>
</tbody>
</table>

9 µl of the master mix is aliquoted in each 95 pcr tubes. Then 1 µl of isolated plasmid (50 ng/µl) is added to each tube except one which was control. Control tube has all the components except plasmid DNA. PCR tubes were now put in the thermal cycler with the following reaction condition:

**Reaction conditions**

- 94°C 4 mins (Initial denaturation)
- 94°C 1 min (Denaturing temp)
- 50°C 2 mins (Annealing temp)
- 72°C 2 mins (Elongation)
- 72°C 10 mins (Final Elongation)
- Hold at 4°C forever

The PCR products of both test reaction mixture and control reaction were analysed through 1.2% agarose gel with GeneRuler<sup>TM</sup>1kb DNA ladder and GeneRuler<sup>TM</sup>100 bp as a size standard marker (Figure 27 and 28).
Colony PCR which was described earlier was step to confirm that insert was present in the plasmid. But to confirm if those insert DNA have microsatellite repeats, secondary screening of the recombinant clone was also done following the protocol to the protocol described by Sambrook et al 2001 and Wang et al. 2007. For this test positive clones were picked and subjected for colony PCR with three primers (Universal M13 forward and M13 reverse vector primers and one synthesized complementary di- and trinucleotide repeat primer) to screen microsatellite containing inserts.

The reaction set up was as follows:

<table>
<thead>
<tr>
<th>Reaction Mixture</th>
<th>1Rx</th>
<th>95 Rx</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X amplification buffer</td>
<td>1.0 µl</td>
<td>95.0 µl</td>
</tr>
<tr>
<td>dNTP's (10mM)</td>
<td>1.0 µl</td>
<td>95.0 µl</td>
</tr>
<tr>
<td>MgCl$_2$ (25mM)</td>
<td>1.0 µl</td>
<td>95.0 µl</td>
</tr>
<tr>
<td>Forward sequencing Primer (M13 F) (5 µM)</td>
<td>0.5 µl</td>
<td>47.5 µl</td>
</tr>
<tr>
<td>Reverse sequencing Primer (M13 R) (5 µM)</td>
<td>0.5 µl</td>
<td>47.5 µl</td>
</tr>
<tr>
<td>Probe (TG/AC/CT/CAG) (5 µM)</td>
<td>0.5 µl</td>
<td>47.5 µl</td>
</tr>
<tr>
<td>Taq polymerase (1U/µl) (1U/sample)</td>
<td>1.0 µl</td>
<td>95.0 µl</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>5.5 µl</td>
<td>523.0 µl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>10.0 µl</strong></td>
<td></td>
</tr>
</tbody>
</table>

10µl of the master mix was dispensed into 95 pcr tubes. One control reaction was also set which contains all components except template DNA. Using a sterile toothpick, bacterial colony was touched and washed in the aliquoted master mix. PCR tubes were now closed and incubated in a boiling water bath for 10 minutes. Samples were allowed to cool at room temperature
and centrifuged briefly for 1 min. To each tube 1 µl Taq DNA polymerase (1 U/µl) is added. PCR tubes were now put in the thermal cycler with the following reaction condition:

- 94°C 4 mins
- 94°C 1 min
- 50°C 2 mins
- 72°C 2 mins
- 72°C 10 mins
- Hold at 4°C forever

The PCR products of both test reaction mixture and control reaction were analysed through 1.2% agarose gel with GeneRuler™1kb DNA ladder and GeneRuler™100 bp as a size standard marker (Figure 29). Those clones that produce a PCR smear on agarose gel are considered to be containing microsatellite motif (Wang et al., 2007).

### 3.2.13 Sequencing of the Positive clones

The clones which were found to be positive after secondary screening were sequenced using ABI 3130 Genetic Analyzer (Applied Biosystem). Around 72 sequences were sequenced. All the sequences were checked for, if it contains vector sequence contamination. This was done using a VectorScreen, an online program of NCBI. Those sequences which were found to contain the vector sequence were manually corrected. The vector sequences were removed from those sequences. The sequences were now analyzed for the presence of repeat motifs using Tandem Repeats Finder software (Benson, 1999) with the parameters: match 2; mismatch 7; indel 7
and max period size 500. The sequences were uploaded in Sequin file and submitted to GeneBank NCBI.

3.2.14 Primer designing

Primers were designed from the flanking region of the microsatellite DNA using the PRIMER3 software (Rozen and Skaletsky 2000). PRIMER3 is online software for primer designing. Primers were designed with the following parameters:

Table-7: Range of Primer Size, primer Tm, primer GC%; annealing oligo and monovalent cations concentration.

<table>
<thead>
<tr>
<th>Specification</th>
<th>Min</th>
<th>Opt</th>
<th>Max</th>
<th>Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer Size (bp)</td>
<td>18</td>
<td>20</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Primer Tm</td>
<td>57</td>
<td>60</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>Primer GC %</td>
<td>20</td>
<td>-</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Conc. Of Monovalent cations</td>
<td>50.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annealing oligo conc.</td>
<td>50.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Primer were synthesise by Eurofins (Germany).

3.2.14.1 Standardization of the annealing temperature for the designed primers

The melting temperature of each member of a primer pair is calculated using the equation known as “The Wallace Rule” (Suggs et al., 1981; Thein and Wallace, 1986).
The equation is:

\[ Tm \text{ (in } ^\circ \text{C}) = 2(A+T) + 4(G+C) \]

Where \((A+T)\) is the sum of the A and T residues in the oligonucleotides and \((G+C)\) is the sum of G and C residues in the oligonucleotides.

### 3.2.15 PCR using microsatellite primers (PCR-Genotyping)

Microsatellite loci were amplified by PCR in a 10µl volume containing 50ng DNA, 200µM of dNTPs, 0.5 µM of each primer, 1.5mM MgCl\(_2\), 10mM Tris(pH 9.0), 50mM KCL, 0.01% Gelatin and 0.5U of Taq DNA Polymerase (Bangalore Genei). The PCRs were performed in a Gene Amp PCR system 9700 thermocycler (Applied Biosystem) and the PCR amplifications were consisted of 94°C for 4 mins; followed by 34 cycles of 94°C for 30 secs, 50–64°C for 35 secs (depending on the primer pairs; Table 12), 1 min at 72°C; and a final extension for 10 mins at 72°C. Amplified products were dried on a vacuum concentrator (Concentrator plus Eppendorf), mixed with 2µl of formamide loading dye, heat denatured at 95°C for 5min and then separated on 6% denaturing polyacrylamide gel with 7.5 M urea and 1x TBE. Separation was performed in a vertical gel electrophoresis (Hoeffer, SE 600, Amersham Biosciences) which were further visualized by Ethidium bromide staining. The size of alleles were estimated according to the 174 DNA/Hinf I marker (Fermantas).

### 3.2.16 Statistical Analyses: Analysis of Microsatellite Polymorphism

Twenty eight microsatellite loci (GGM-001, GGM-002, GGM-006, GGM-007, GGM-008, GGM-009, GGM010, GGM011, GGM012, GGM013, GGM014, GGM015, GGM016, GGM018, GGM019, GGM021, GGM023a, GGM024, GGM027, GGM028, GGM034, GGM043b, GGM023b, GGM044,
GGM046, GGM047, GGM048 and GGM045) were amplified using primer pairs, designed for the purpose. The oligo sequences of the forward and reverse primers of each locus are given in the table no 10. The region containing microsatellite is amplified by PCR using primers that flanks the microsatellite. Polymorphisms of microsatellite loci were evaluated in a total sample of 40 individuals, 20 individuals each from two populations, Ranibagh (Gola River, 29°, 18’, 2’’ longitude North and 79°, 32’, 2’’ longitude East), Srinagar Garhwal (Alaknanda River, 30°,13’,11” latitude North and 78°,46’,47” longitude East). Cross-species amplification with primers from the 28 loci was also examined in 5 species (9 individual each) of the family Cyprindae. The species were *Garra para lissorhynchus*, *Garra litanensis vishwanath*, *Barilius bendelisis*, *Schizothorax richardsonii* and *Tor putitora* (Table 13).

For microsatellite genotyping Etbr stained gels were visualized in Alpha Imager, ALPHA INNOTECH, USA. Fragment size was estimated based on φX174 *Hinf* I digest (Fermantas). Since the microsatellites are co-dominant markers, allele frequencies were estimated by direct count. The inbreeding coefficient index (F<sub>IS</sub>), pairwise genetic differentiation (F<sub>ST</sub>), observed heterozygosity (H<sub>O</sub>), expected heterozygosity (H<sub>e</sub>), tests for Hardy-Weinberg and pairwise disequilibrium were calculated by Fisher's exact test using GDA software (Lewis and Zaykin, 1997). To assess the significance of the F-statistics estimates a bootstrapping over loci with 10000 replicates and the confidence interval of 95% was also generated.

Deviations from Hardy–Weinberg equilibrium (HWE) for each locus and genotypic linkage disequilibrium (LD) between all pairs of loci were tested using GENEPOP 3.3 (Raymond and Rousset, 1995; Rousset, 2008). All loci were tested for multilocus genotypic disequilibrium within population samples and
across the entire data set, using the Markov chain method in GENEPOP 3.3. Each locus was tested for evidence of null alleles with Micro-checker (Van Oosterhout et al. 2004). Polymorphism Information Content (PIC) was estimated using allele frequencies in each polymorphic microsatellite locus, using Cervus 3.0 (Marshall et al., 1998 and Kalinowski et al., 2007).