4. METHODOLOGY

4.1. Test material

The ten selected multivoltine (MV) silkworm races (Table 4.1.) were screened for bacterial toxicity viz., LPS (Lipopolysaccharide) administration. Based on the LD50 values 3 tolerant and 3 susceptible races would be selected for further study. In present study each experiment was repeated in triplicate and the data was recorded.

Table 4.1. List of Selected silkworm races of *Bombyx mori*

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Race/Accession No.</th>
<th>Race /Accession Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BMI-0001</td>
<td>Pure Mysore</td>
</tr>
<tr>
<td>2</td>
<td>BMI-0017</td>
<td>Nistari</td>
</tr>
<tr>
<td>3</td>
<td>BMI-0009</td>
<td>Kollegal Jawan</td>
</tr>
<tr>
<td>4</td>
<td>BMI-0014</td>
<td>OS-616</td>
</tr>
<tr>
<td>5</td>
<td>BMI-0056</td>
<td>MY1(SL)</td>
</tr>
<tr>
<td>6</td>
<td>BMI-0036</td>
<td>PMX</td>
</tr>
<tr>
<td>7</td>
<td>BMI-0034</td>
<td>AP12</td>
</tr>
<tr>
<td>8</td>
<td>BMI-0006</td>
<td>Hosa Mysore</td>
</tr>
<tr>
<td>9</td>
<td>BMI-0035</td>
<td>A13</td>
</tr>
<tr>
<td>10</td>
<td>BMI-0004</td>
<td>TamilNadu White</td>
</tr>
</tbody>
</table>
Table 4.2. Descriptors of the selected races

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Race /Accession No.</th>
<th>Race /Accession Name</th>
<th>Descriptors*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BMI-0001</td>
<td>Pure Mysore</td>
<td>Thermo tolerant/Disease resistant</td>
</tr>
<tr>
<td>2</td>
<td>BMI-0017</td>
<td>Nistari</td>
<td>Thermo tolerant /Highly tolerant</td>
</tr>
<tr>
<td>3</td>
<td>BMI-0009</td>
<td>Kollegal Jawan</td>
<td>Thermo tolerant /Highly tolerant</td>
</tr>
<tr>
<td>4</td>
<td>BMI-0014</td>
<td>OS-616</td>
<td>Thermo tolerant</td>
</tr>
<tr>
<td>5</td>
<td>BMI-0056</td>
<td>MY1(SL)</td>
<td>Thermo tolerant</td>
</tr>
<tr>
<td>6</td>
<td>BMI-0036</td>
<td>PMX</td>
<td>Thermo susceptible / Disease susceptible</td>
</tr>
<tr>
<td>7</td>
<td>BMI-0034</td>
<td>AP12</td>
<td>Thermo susceptible</td>
</tr>
<tr>
<td>8</td>
<td>BMI-0006</td>
<td>Hosa Mysore</td>
<td>Thermo susceptible</td>
</tr>
<tr>
<td>9</td>
<td>BMI-0035</td>
<td>A13</td>
<td>Thermo susceptible</td>
</tr>
<tr>
<td>10</td>
<td>BMI-0004</td>
<td>TamilNadu white</td>
<td>Thermo susceptible/susceptible</td>
</tr>
</tbody>
</table>

* Descriptors from CSGRC Annual reports (1997-2009)

4.1.1. LPS Administration and LD50 Calculation

The ten MV silkworm races are maintained at Central Sericultural Germplasm Resources Centre, Hosur were selected for the study. The silkworm races were reared as per the standard rearing procedure (Krishnaswamy, 1978). The 5th instar 4th day larvae were treated with an LPS (E.coli 0111:B1) by intravenous injection of 20µl of saline containing 100µg of LPS in 0.75% NaCl solution. In control larvae saline only administrated. From the selected races the 50% mortality (LD50) values were calculated and evaluated (Takahiro and Kato, 1999; Ishii et al., 2008; Genome Pharmaceuticals Institute co., 2000).
4.2. Sample collection

Haemolymph and midgut was collected separately from each 5th instars 4th day larvae. Sample was collected by cutting an abdominal leg of the larvae in a pre-cooled microfuge tube containing approximately 0.05% of phenylthiourea (PTU) to prevent melanization (Gupta, 1979). PTU was added only for esterase enzyme activity performing vial. Ice-cold marine anticoagulant (MAC; 0.1M glucose, 15 mM Trisodium citrate, 13 mM Citric acid, 0.45 M Sodium Chloride, pH 7.0) was added to the samples and then the haemolymph sample was collected. The midgut was collected by dissecting the larvae. The midgut is grounded and dissolved in extraction buffer (0.607g Tris-Hcl, 2.338g Sodium Chloride, 0.037g EDTA; pH 7.2) and phosphate buffer saline (PBS) containing Sodium Chloride 137 mM, Potassium Chloride 2 mM, Phosphate buffer 10 mM; pH 7.4) for haemolymph, then the samples was transfer to microfuge tube. The haemolymph were centrifuged at 5000 rpm for 5 minutes at 4°C and midgut samples were centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was collected in a separate pre-cooled microfuge tube and stored at-20°C. The protein content was measured according to Lowry et al. (1951) method.

4.3. Measurement of life history traits and Immune functional traits

For each individual race, the following data were collected:

Life history traits data collection:

Larval development rate

The reciprocal of the number of days from hatching to pupation.

Fecundity

The mean number of eggs laid by a female moth was recorded as fecundity from five disease free layings.
Pupation rate/survival rate

The mean number of live pupae obtained to that of larvae retained after III moult in replication and represented in percentage.

Pupal weight

Weight in g measured 24-48 h after the start of pupal stage.

Immune function traits data representation:

Specific activity was expressed by units/ml/mins, coagulation and haemolymph activity (log$_e$ OD units), PO-melanization (OD units), kinetics of esterase Km (mM), Vmax (nmol/min/ml) and phenoloxidase activity Km (mM), Vmax (µM/min/ml), antibacterial activity (log$_e$ ng μL$^{-1}$), hemocyte density (cells per 0.2µL) (Cotter et al., 2004).

4.3.1. Immune functional analysis

Three haemolymph-based immune parameters were then measured for each larva: haemolymph PO activity, antibacterial activity (measured using a lytic zone assay) and haemocyte density. Haemolymph PO activity was measured using the methods of Wilson et al., (2001). In brief, 8 µl of haemolymph were added to 400 µl of ice-cold phosphate-buffered saline (PBS; pH7.4) in a plastic Eppendorf tube and vortexed. Samples were frozen at 20°C to disrupt haemocyte membranes and PO activity was assayed spectrophotometrically with L-Dopa as a substrate. This assay involved adding 100 µl of 20mM L-Dopa to 100 µl of the buffered haemolymph and incubating triplicate samples of the mixture at 20 min at 25°C. PO activity was expressed as the absorbance read at 492nm (Cotter, 2002).

To assess haemocyte density, 10 µl of haemolymph were added to 5µl of Ethylene diamine tetra acetic acid (EDTA) anti-coagulant PBS, and 5 µl of glycerol in a plastic
Eppendorf tube (Cotter, 2002). The contents of the tube were gently mixed and 15µl were pipetted onto a haemocytometer with improved Neubauer ruling. The numbers of haemocytes in five non adjacent squares were counted on each side of the haemocytometer at X400 magnification. The counts for each chamber were summed and averaged to give an estimate of the haemocyte density of each individual.

Antibacterial activity against the LPS was determined using a lytic zone assay. Agar plates containing 10ml of 1% agar with 5 mg/ml in freeze-dried LPS were prepared as described in Kurtz et al., (2000). Holes with a diameter of 2mm were punched in the agar and filled with 70% ethanol saturated with phenylthiourea (PTU), which inhibits melanization of the haemolymph. After the ethanol had evaporated, 1 µl of haemolymph was placed in each well and the plates were incubated at 33°C for 24h. The diameter of the clear zones was calculated.

4.4. Biochemical characterization and evaluation of selected silkworm races under biotic stress (LPS)

4.4.1. Esterase (Est) and Prophenol oxidase (Pro-PO) enzyme kinetics

4.4.1.1. Esterase enzyme kinetic analyses

The haemolymph and midgut of control and LPS treated samples from the ten races were assayed for their esterase activity by α and β- Naphthyl acetate with different concentrations viz. 0.2mM, 0.4mM, 0.6mM and 0.8mM using 0.1% Fast blue BB salt as a dye coupler and enzyme assay buffer (0.05M Sodium Phosphate containing 10% Sucrose ; pH 7.4). The reaction was stopped by adding 3.5% of SDS. The developed colour was measured at 600nm in Shimadzu spectrophotometer. From the O.D values, the kinetic activity of Km and Vmax was worked out (Arai et al., 2000; Sparks et al., 1979; Takahiro and Kato, 1999).
4.4.1.2. Activation of Phenoloxidase (PO) from ProPhenoloxidase (Pro-PO)

The enzyme Phenoloxidase (PO) activity was assayed Shimadzu spectrophotometrically by recording the formation of \( o \)-quinones. L-3,4-dihydroxyphenylalanine (L-DOPA) and dopamine were used as diphenolase substrate to measure the PO activity in haemolymph and midgut samples of ten races. The chromogenic nucleophile, 3-methyl 1-2-benzothiazolinone hydrazone (MBTH) was added to the substrates. To measure activity, 100 \( \mu l \) of sample was added addition of 100 \( \mu l \) of substrate for L-DOPA (4mg/ml in FSW) and for dopamine (2mg/ml in FSW) and 1mM MBTH. The reaction was measured at 490nm immediately after the substrates were added. Then it was incubated for an hour at room temperature in the dark before a second reading was taken. PO activity was calculated by substrate the initial readings from measurements made after 60min. Enzyme activities are expressed as the change in optical density (OD) at 490nm (OD\(_{490}\)) according to Saleem Aladaileh et al., 2007; Cotter et al., 2004; Liu et al., 2009; Adamo, 2004.

4.4.1.3. Phenoloxidase activity kinetic analyses

PO activity was measured over time at different concentrations of L-DOPA and dopamine viz., 5, 10, 20, 30, 40, 60mM using haemolymph and midgut samples. The Michaelis-Menten constant (Km) and the maximum reaction velocity (Vmax) were calculated (Saleem Aladaileh et al., 2007 and Cotter et al., 2004).
4.5. Identification of isozyme marker in selected silkworm races under biotic stress (LPS)

4.5.1 Esterase (Est) Isozyme

The samples of haemolymph and midgut of individual ten larvae with and without LPS treated were subjected to electrophoresis under non-denatured conditions (Native-PAGE) on 10% poly acrylamide gel (10% resolving gel in 1.5 M Tris-Hcl, pH 8.8 and 6% stacking gel in 0.2M Tris-Hcl,pH 6.8) in a vertical mini gel electrophoresis system. Electrophoresis was carried out with reservoir buffer (0.025M Tris and 0.192M Glycine;pH 8.3) initially at current 60V and 45 minutes followed by 90V until the tracking dye reached the bottom of the gel. Gels were stained with α or β - Naphthyl acetate substrate separately by the procedure of Simms (1965). The gels were placed separately in a tray containing α or β - Naphthyl acetate esterase substrate (30mg of α or β - Naphthyl acetate,100mg of Fast blue RR salt,1.4g of Sodium phosphate monobasic dihydrate and 0.5g of Disodium hydrogen phosphate; pH 6.5). Then gels were documented and relative mobility (Rf values) was calculated for each bands of esterase from the formula: Rf= Distance of protein migration/ Distance of dye migration (Shi and Jackowski, 1998). Esterase bands were designated as Est1, Est 2, Est 3, Est 4 and Est 5 and based on their substrate α or β and haemolymph and midgut (G) sample and so on from the anodal migration to cathode (Takahiro Shiotsuki and Yusuke Kato., 1999).

4.5.2 Phenol Oxidase (PO) Isozyme

The samples of haemolymph and midgut of individual ten larvae with and without LPS treated were subjected to electrophoresis under non-denatured conditions (Native-PAGE) on 6% poly acrylamide gel (6% resolving gel in 6g Tris-Hcl, pH 8.8 and 3% stacking gel in 0.125M Tris-Hcl, pH 6.8) in a vertical mini gel electrophoresis system. Electrophoresis was carried out
with reservoir buffer (0.025M Tris and 0.192M Glycine; pH 8.3) initially at current 60V and 45 minutes followed by 90V until the tracking dye reached the bottom of the gel. After electrophoresis the gels were stained with a solution containing 5 mM MBTH in 0.1 M PBS (pH 7.4) and diphenolase substrates, L-DOPA (20mM) or Dopamine (10mM) (Newton et al., 2004).

Then gels were documented and relative mobility (Rf values) was calculated for each bands of esterase from the formula: Rf= Distance of protein migration/ Distance of dye migration (Shi and Jackowski, 1998). Esterase bands were designated as PO1, PO2 and PO3 and based on their diphenolic substrates L-DOPA or dopamine and haemolymph and midgut sample (G) and so on from the anodal migration to cathode (Saleem Aladaileh et al., 2007; Kohji Yamamoto et al., 1999; Pang et al., 2009).

4.6. **Antibacterial peptide marker identification in selected silkworm races under biotic stress (LPS).**

SDS- polyacrylamide gel electrophoresis was performed using standard methods on the 8cm mini gels (Laemmli, 1970). 50-100 µg of protein samples was electrophoresed on 15% acrylamide gels of 1mm thickness. Electrophoresis was carried out with an initial volt of 35 for 30 mins followed by 75 V for 3 hrs until the tracking dye reached the bottom of the gel. Gels were stained with 0.2% Coomassive Brilliant Blue R-250 in 40% methanol and 10% acetic acid for 1 hour and destained overnight with the destaining solution containing 30% methanol and 10% acetic acid. The gels were scanned using Biovis gel document for the analysis of banding pattern and molecular weight estimation (Fairbanks et al., 1971).
4.7. Immune marker identification in selected silkworm races under biotic stresses (LPS)

4.7.1. Coagulation assay

Making a calibration curve: A 2% solution of Amaranth Red was made up in Insect Ringer solution (IRS) (128 mM Sodium chloride, 18 mM Calciam Chloride, 1.3 mM Potassium Chloride, 2.3 mM Sodium bicarbonate), and a second solution was made up in Insect Ringer supplemented with LPS (0.5 mg/ml). 5 µl of Amaranth solution was diluted in 25, 50, 100, 200, 300 and 400 µl in Insect Ringer or LPS (0.5 mg/ml in Ringer), respectively. A 5 µl aliquot of these dilutions was added to 195µl LPS/Ringer and the optical density at 520nm (OD520) was measured photometrically on a plate reader. The OD was plotted against dilution volume in order to construct standard curves for LPS/Ringer dilutions (Haine et al., 2007).

4.7.2. Measuring clotting in vivo

Experiments were assigned to two treatments: ‘Ringer’ and ‘LPS’. Ten MV silkworm races were anaesthetized for 30 seconds with CO₂ then injected with 5 µl Amaranth in Ringer, or Amaranth in Ringer with LPS, respectively. After a set period of time (5, 15, 30, 45 and 60 minutes after injection), individuals were briefly CO₂ anaesthetized and a 5 µl sample of haemolymph was taken from an anterior leg. Three consecutive 5 µl samples were taken from each individual. Each sample was added to 195 µl of Ringer/LPS solution in a 96-well plate and the OD520 measured. The mean OD520 for the three measures for each individual was converted to dilution of the dye in insect haemolymph, based on the standard curves, in order to obtain a relative measure of in vivo coagulation between treatments (Haine et al., 2007).
4.8. Antigen –Antibody Agglutinations/aggregations/Laminarin

Ten silkworm races are tested against LPS antigens by slide agglutination and by microagglutination/aggregation.

4.8.1. Qualitative assay: Slide Agglutination Tests

The control aliquots of two adjacent suspensions were added with the drops of saline on a slide. Then a drop of antiserum (LPS treated) to one suspension was added in one suspension and followed by examine for agglutination (clumping) of the suspension (with antiserum) and clearing of the saline. Slide agglutination was read macroscopically after 3 minutes of mixing room temperature (NSM, Agglutination test, 2010; Myrick and Ellner, 1982).

4.8.2. Quantitative assay

Appropriate positive and negative serum controls were included with all tests, as well as controls for diluents and antigen. Dilutions of antisera ranged from 1:20 to 1:120 by making appropriate two fold dilutions in 0.5ml of 0.85% saline containing 0.2% of Safranin O and then adding 0.5ml of the appropriate antigen to each series of Biochip or Therasaki plates. The microtiter plates were sealed with plastic covers and incubated at 37˚C. The plates were then placed in a refrigerator at 4˚C for 2 hr before being read (Gaultney et al.,1971 ;Amanai et al.,1991).

Reading the pattern of agglutination in microtiter plates:

Nonagglutinated antigen sedimented as a sharply defined, round button in the microtechnique. Agglutinated antigen was either completely dispersed and did not form sediment or, if it settled, formed poorly defined sediment with a crenated edge that was easily differentiated from the button of nonagglutinated antigen. The titer of the agglutination reaction in microtiter plates was determined by observing the highest dilution of antiserum that permitted
sedimentation of a round button of nonagglutinated antigen. The end points were thus designated by a negative reaction rather than by a positive one. The purpose of chose this procedure because the end point of nonagglutinated antigen was easier to define as a round button than was the dispersed or poorly sedimented agglutinated antigen. The difference between agglutinated and nonagglutinated antigen is easier to read. Addition of Safranin 0 to diluents enhances visibility of nonagglutinated antigen by making the button bright red. The buttons are easily observed at lower dilutions, but as the end point of titration is approached the button becomes small, and experience is required to determine the precise titer in microtiter plates.

4.9. Identification of immune genes associated with tolerant restricted patterns in selected silkworm races.

4.9.1. List of gene associated with stress and immune related functions

- Esterase- exonic gene for functional associated stress
- Esterase-intronic gene for structural associated stress
- Attacin gene-Antimicrobial gene
- Cecropin gene-Antimicrobial gene

4.9.2. Identification of genomic contig of selected genes

*Bombyx mori* esterase was already purified, characterized and N-terminal sequence determined as ESPRVTVKHGTLSKPKTYSGYEYFLQ. The N-terminal sequence of esterase protein was blast (tblastn) searched with *B.mori* .EST database of Tokyo University ‘Silkbase’ ([http://www.ab.a.u-tokyo.ac.jp/silkbase](http://www.ab.a.u-tokyo.ac.jp/silkbase)). The esterase genomic contig was BLAST searched in genomic contig. Similarly the antimicrobial genes viz., attacin and cecropin genomic
organization was identified by NCBI (http://www.ncbi.nlm.nih.gov). The gene sequence was BLAST searched with B.mori genomic DNA database for identification genomic contig homologous sequence to corresponding gene sequence (Tanaka et al., 2008, Ponnuvel et al., 2008; Hang et al., 2005).

4.9.3. Translated amino acid sequence identification

The amino acid sequences were further analyzed through conserved domain search for the presence of domain in esterase, attacin, cecropin genes using the programme conserved domain search (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

4.9.4. Sequence annotation

Bombyx mori EST of blood esterase and immune proteins genes of B. mori was compared between other lepidopteron insects and grouped into cluster of sequences belonging to the same gene. Alignment of these sequences allowed accurately determining the genomic DNA nucleotide sequences and reconstituting the full-length cDNA. In parallel EST sequences were subjected to BLAST (Blast local alignment search tool) search.

4.9.5. Identification of exons and introns

Exons and introns were predicted using Spidey programme (Wheelan et al., 2001), a specific tool for mRNA and genomic alignment. Spidey takes as input a single genomic sequence and a set of mRNA accession numbers or FASTA sequences. The data were examined carefully to get the percent identity per exon, the number of gaps per exon, the overall percent identity, the percent coverage of the mRNA, presence of an aligning or non-aligning poly – A tail, number of splice donor sites, the presence or absence of splice donor and
acceptor sites for each exon, and the occurrence of an mRNA that has a 5′ or 3′ end (or both) that does not align to the genomic sequence.

The data input of genomic DNA from *B. mori* and mRNA sequence pertaining to esterase exon & intronic region gene and attacin and cecropin gene. The nucleic acid sequences were analyzed for the presence of exons and introns by spidey programme (a tool for mRNA and genomic alignments) (http://www.ncbi.nlm.nih.gov/spidey/spideyweb.cgi). The BLAST alignments are sorted by score and then assigned into windows by a recursive function which takes the first alignment and then goes down the alignment list to find all alignments that are consistent with the first (same strand of mRNA, both the mRNA and genomic coordinates are non-overlapping and linearly consistent). On subsequent passes, the remaining alignments are examined and put into their own non-overlapping, consistent windows, until no alignments are left. All windows are retained at this point and they go on to the next step one by one until the requested number of models has been generated. Because the windows are non-overlapping, and because each window should contain most of a gene model, Spidey is able to generate accurate models without mixing up exons from adjacent genes. All parameters are default unless otherwise specified. All processing is done at the rate of one mRNA sequence at a time. The first step for each mRNA sequence is a high-stringency BLAST against the genomic sequence. The resulting hits are analyzed to find the genomic windows.

4.9.6. Primer designing

The forward and reverse gene specific primers were designed for available gene sequences of esterase, attacin, cecropin genes using the programme of primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/). Two pairs of primers were designed for esterase intronic and exonic region (Table 4.3.). The primers were designed for exonic regions of attacin,
cercropin (Table 4.4.). Primer 3 is a complete rewrite of the original PRIMER programme (primer 0.5), written by Steve Lincoln, Mark Daly and Eric Lander (Tanaka et al., 2008, Ponnuvel et al., 2008; Hang et al., 2005).

4.9.7. DNA Quantification and PCR reaction

4.9.7.1. Genomic DNA isolation

Moths were collected from the controlled rearing condition and DNA was isolated separately from individual moths from each ten MV races using standard phenol chloroform method. Genomic DNA was isolated from the moths briefly; Moth was ground in liquid nitrogen, added lysis buffer (pH 7.5) containing 200 mM Tris-HCL, 25mM EDTA, 300 mM Nacl and 2% SDS) and proteinase k (100 µg/ml) was added to the ground tissue and incubated at 37°C overnight. The DNA was extracted through saturated phenol and phenol-chloroform-isoamylalcohol (24:24:1) and then Chloroform. The extracted DNA was precipitated with ethanol in the presence of 3M Sodium acetate. The precipitated DNA was again washed with 70% ethanol and dissolved in Tris-EDTA buffer (pH 8.0). RNA contamination was removed with RNAase A (100µg/ml) treatment and DNA was re-extracted with Phenol-Chloroform method. The extracted DNA was dried, dissolved in TE buffer and diluted to 10ng/µl through serial dilutions and quantified on 0.8% agarose gel against uncut lambda DNA (10ng/µl) as a standard (Nagaraja and Nagaraju, 1995).
Table 4.3. Details of esterase genes and primer sequences, restriction enzymes utilized in the study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>cDNA accession number</th>
<th>Contig accession number</th>
<th>5' → 3' Primer sequence</th>
<th>Contig binding location</th>
<th>Anneling temperature</th>
<th>Product size</th>
<th>Restriction enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esterase Intronic region</td>
<td>EST clone fbpv0006 retrieved from database tBlastN</td>
<td>BAAB01009617</td>
<td>CTCATCCACACCGTTCTCAA</td>
<td>792bp</td>
<td>56˚C</td>
<td>763bp</td>
<td>HindIII, Dral, EcoRI, BamHI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CACGGGTGGAAGAGAGAGC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Esterase Exonic region</td>
<td></td>
<td></td>
<td>AGTCCACAACCACCAGTCTTT</td>
<td>2806bp</td>
<td>532bp</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>TGTTGAAATAATCCTCTAGACGAAGG</td>
<td>3338bp</td>
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</table>

Table 4.4. Details of attacin, cecropin genes and primer sequences, restriction enzymes utilized in the study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Contig accession number</th>
<th>5' → 3' Primer sequence</th>
<th>Contig binding location</th>
<th>Anneling temperature</th>
<th>Product size</th>
<th>Restriction enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Attacin</td>
<td>NM_001043541</td>
<td>GTCATCAACCGGAATGACTA</td>
<td>517bp</td>
<td>58˚C</td>
<td>148bp</td>
<td>PstI, EcoRI, HindIII,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAAGGAGAATCCANCATTG</td>
<td>665bp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cecropin</td>
<td>DQ233467</td>
<td>ACTGTTCGACATCGCCTCTCA</td>
<td>20bp</td>
<td>65˚C</td>
<td>200bp</td>
<td>EcoRI, HindIII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GACAGCTGGTCAGCCTTGA</td>
<td>220bp</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
4.9.7.2. PCR condition and separation of amplified product

The polymerase chain reaction was done in an MJ Research Thermal Cycler, PTC200, using 20µl reaction mixture containing 50-100ng of genomic DNA as template, 2.0 µl of 10X PCR buffer (GeNei), 0.2mM dNTPs (GeNei), 1.5mM MgCl₂ (GeNei), 66ng of forward and reverse primer each and 0.3U of Taq DNA polymerase (GeNei). The PCR reaction was performed with two sets of primers for each gene (esterase and antimicrobial peptide genes viz., attacin and cecropin) (Table 4.3. & Table 4.4.). The PCR schedule was 94°C for 3 mins followed by 30 cycles of 94°C for 30s, 56-60°C for 30s, 72°C for 2 min and a final extension of 7 min at 72°C.

The PCR products were resolved on 1.5% agarose gel in Tris-Acetic acid EDTA buffer (TAE) containing (0.04M Tris acetate and 0.001M EDTA; pH 8.0) and electrophoresis was carried out with a constant voltage of 80V in parallel with DNA standard markers. Gel was stained with Ethidium bromide (0.5µg/ml) and photographed with gel documentation (Kohji Yamamoto et al., 2000; Ponnuvel et al., 2008; Hong et al., 2005; Tanaka et al., 2008).

4.10. Polymorphism identification through Restriction digestion (RFLP)

- The restriction digestion was carried out in 20µl of volume in a sterile microfuge tube, the reaction mixture contains 5µl of PCR product viz., Esterase or Attacin or Cecropin gene amplified DNA, 10X RE buffer, 10 to 15 units of restriction enzymes viz., EcoRI, HindIII, BamHI, Pst I and Dral and nuclease free water to a final volume of 20 µl.
- The digestion mixture was incubated in a waterbath at 37°C for an hour and followed by inactivated by 65°C for 20 minutes. The restricted pattern was analysed by 0.7% agarose gel in Tris Boric acid EDTA (TBE ; pH 8.0) containing
5µg/ml Ethidium Bromide (Etbr) with gel loading buffer (0.25g Bromophenol blue, 0.25 g Xylene cyanol, 0.1 M EDTA, 40 g Sucrose).

- The electrophoresis was carried out in a sub-marine electrophoresis (BioTech) apparatus at 90V at 37°C and photographed with gel documentation.
- The presence and absence of RFLP bands was scored and the cluster groups were constructed using isoforms and RFLP datum to associate the markers with tolerant and susceptible races (Chatterjee et al., 1993; Johnson et al., 2010; Mohandas et al., 2004)