Materials and methods

1. Site of collection and Habitat preference

Survey on Anopheline species was conducted in Aizawl, Kolasib, Lawngtai, Lunglei, Mamit, and Serchhip Districts of Mizoram (Figure 7). Priority on site of collection was based on malarial prevalence and occurrence data obtained from either district hospitals or community health centers. Site of collections were characterized by numerous slow-running low streams, concrete and earthen irrigational canals and drainage and ditches; and artificial ponds (2 – 14 m in diameter) used for harvesting fishes, 2km radius of human residents and cattle sheds (Kim et.al., 2011). Due to observation on presence of divergent Anopheles species in Thenzawl of Serchhip district; the site of Anopheline collection was secluded in this area thereafter.

Thenzawl was characterized by numerous slow-running low streams, concrete and earthen irrigational canals and drainage and ditches; and artificial ponds (2 – 9 m in diameter) which was used for harvesting fishes. Anopheles spp. habitats were surveyed within 2 - 4 km radius of the town from January to November for three years (2009 - 2011) (Figure 8). The larval habitats were characterized by clear water (pH 5.0 – 6.2; Temp. 22 – 30°C) interrupted with floating aquatic plants and dead/fallen plant debris and were shaded by a vegetation of tress and tall grasses. Adults were collected at dusk and midnight.
(4:00 pm – 8:00 pm; 12:00 – 4:00 am) from cattle sheds with its nearby residents and agricultural lands (23 – 33°C; 20 – 98% RH).

2. **Method of sampling and collection**

2.1. *Larva collection*

Larvae were collected by the scoop-net (SN) method, with larval net of a fine mesh net (10 x 10 finely knitted threads per cm²) mounted to an iron handle (30 cm diameter), plastic tub (28.5 cm diameter), a plastic dipper (15.5 cm diameter) and a dropper (Figure 9) [Oo *et al.*, 2004]. When pools of different species were collected from a collection site, each morphologically similar larva was separated in different rearing plastic trays and labeled. From the collection, some were subjected to pesticide mortality test and some cultured into adults for identification and further molecular characterization.

2.2. *Adult collection and preservation*

Adults were collected using a local-made killing jar which consisted of a 250ml glass jar (2 cm in diameter, 9.7 in length) and cotton moisten with chloroform, kept at the base of the jar (Figure 10) and CDC (Centers for Disease Control) light trap. Each paralyzed or cold-shocked dead adult was immediately transferred to an apparatus
that consisted of sterile 1.5 ml micro-centrifuge tube (Tarson™), silica-gel and cotton (Figure 10). On reaching the laboratory, each adult was then transferred to sterile 1.5 ml micro-centrifuge tube, labeled and stored at 4°C till further analysis.

2.3. *Anopheles culture*

The anopheline culture method was adopted from Oo *et al.* [2004] and MR4 [2007]. Larvae collected were kept in plastic tray containing water retained from the breeding habitat and there after changed twice a day with rain harvested water. Larvae were reared at room temperature. Each instar was kept in separate trays. Additional food (grinded fish food) of approximately 1mg was supplied twice a day with the help of a vial covered with a fine mesh net (10 x 10 knitted threads per cm²). Pupae were kept in the adult cage wooden frame (1 ft³) covered with mosquito net. Emergence of adults from pupa took 2-3 days. The adults were fed with 10% sucrose solution; and the cotton was changed twice a day to avoid fungal infection (Figure 11).

2.4. *Statistical analysis for survey, sampling and collection*

One way ANOVA and Levene’s test (based on means) was calculated to find the homogeneity of variances of the field collection data. Mean and standard deviation of anopheline abundance and their habitat preference from 2009 to 2011 in Thenzawl was recorded. The
species abundance in relation to malarial prevalence in Thenzawl was also calculated. All the calculations were performed using commercially available GraphPad InStat version 3.06 software (GraphPad Software Inc., San Diego, CA) and PAST 1.86b (Hammer et al., 2001). A p-value of < 0.05 was noted to have statistically significant value.

3. Identification and Morphological characterization of Anopheles species

Morphological identification was performed on the adult female taking color patterns of the wing, palpi and the leg as focal identification characters using dissecting microscope. The identification keys followed the illustrations of Das et al. [1990] and Nagpal and Sharma [1995].

4. Insecticidal Bioassay

The pesticide used for vector control management K-Othine™ (2% Deltamethrin active ingredient w/w), procured from the Department of Health Services, Govt. of Mizoram, was used for bioassay.

4.1. Preparation of Stock and test concentration

20 ml of 1% Deltamethrin was prepared by adding 10ml of pesticide to 10 ml of sterile Milli-Q water. Test concentration solution
was obtained by dilution of stock ten-fold i.e., 2ml stock to 18 ml sterile Milli-Q water. The resulting concentration was thus 0.1%. The preparation of stock and test concentration followed the protocol described by WHO [2005].

4.2. *Insect maintenance and testing*

Bioassay was conducted at the site of collection immediately after separation of morphologically similar larva into different larval trays. Four *Anopheles* species (*An. vagus*, *An. campestris*, *An. jamesii* and *An. nivipes*) were exposed to 24 hour pesticide test at 25 ± 2°C temp, 50 – 62% RH. 0.01ml – 1.0 ml was pipetted out from the test concentration solutions into disposable cups (100ml) [WHO, 1998]. A container having 1ml sterile Milli-Q water was used as control. 100 ml of water obtained from the habitat was then added to the test containers, since it contains nourishment to the larva during the 24 hour exposure to pesticide. Small amount of grinded fish food was given as a supplement. Thirty 3rd and 4th instar larvae were collected from the trays using wide-mouth plastic pipette and kept into the test disposable cups (Figure 12). The morbid larvae were continually revived with a needle. After 24 hours, mortality was counted. The experiment was replicated thrice. The concentrations that yielded 2 – 98% morality were used to determine lethal concentration of 50% (LC50).
4.3. **Statistical analysis for bioassay**

Bioassay data were corrected for control mortality [Abbott, 1925] using the formula: 

\[
\text{% Corrected Mortality} = \frac{\% \text{ Kill in treated} - \% \text{ Kill in control}}{100 - \% \text{ Kill in control}} \times 100
\]

The probit analysis was used to estimate LC₅₀ values with their fiducial limits according to the methods given by Finney [1973]. A Probit regression line \((R^2 = 1 \pm 1)\) was made against the log dose and probit mortality. A regression equation \(\gamma = \beta + b(x - \bar{x})\) and 50% mortality probit corresponding to Log LC₅₀ was calculated from the regression line [Finney, 1973] (Figure plate 19 and 20, figure 37 – 40). Samples for which the 95% fiducial limits did not overlap were considered to be significantly different.

5. **Biochemical Analysis**

Biochemical assay was performed on freshly killed (cold treatment at 4°C for 30 minutes) adult specimens: *An. campestris* (NSK - 01), *An. jame{s}ii* (NSK - 03), *An. maculatus* (NSK - 04), *An. philippinensis* (NSK -06), *An. nivipes* (NSK - 10), *An. jeyporiensis* (NSK – 09), *An. vagus* (NSK - 18), *An. dirus* (NSK - 22) and *An. minimus* (NSK - 23). The preparation of stock and procedure used for the analysis were according to WHO [1998] with modification. Preparation of the solutions is in Appendix I (Figure 13).
5.1  
**Statistical Analysis**

One way ANOVA was calculated to find the homogeneity of variances of the field collection data were performed using commercially available GraphPad InStat version 3.06 software (GraphPad Software Inc., San Diego, CA) and PAST 1.86b [Hammer et al., 2001] was used to compare the different quantity of enzymes produced by the biochemical analysis.

5.2  
**Standard Assays:**

Four standard assays were prepared using Bovine Serum Albumin (BSA), α-naphthol, β-naphthol and Cytochrome C (from Bovine heart) [WHO, 1998; MR4, 2007]. Standard graphs were made accordingly where X-axis denoted concentration and Y-axis denoted absorbance and Regression line ($R^2=1$) to calculate the unknown concentration of protein and enzymes.

5.2.1. Protein standard assay through Lowry’s Method

The estimation of total protein followed the procedure of Lowry et al. [1951]. A standard protein of Bovine Serum Albumin (BSA) of 200µl/ml stock concentration was pipetted out as 100, 200, 400, 600, 800 and 1000 µl. The volume was made to 1 ml with sterile Milli-Q water to each test-tube. For blank, 1ml sterile Milli-Q water was kept ready. 5 ml of the reagent B (Appendix I) was added to all test-tubes. The solution
was mixed well and incubated at room temperature for 10 minutes. 500 µl of second reagent C (Appendix I) was added and mixed well. A blue colored solution developed and was then incubated at room temperature for 30 minutes. The color was developed by the reduction of the amino acids tyrosine and tryptophan present in the protein with phosphomolybdic phosphotungistic compound in the Folin-ciocalteau reagent and the direct reaction of the proteins with the alkaline cupric tartarate is measured in 660nm. Optical Density (OD) was taken at the said absorbance.

5.2.2. Standard (α – and β –) Naphthol assay

α – naphthol and β – naphthol 200µl/ml stock concentration was pipetted out in 100, 200, 400, 500, 800 µl into test-tubes. The volume was made to 1ml by the addition of 0.02M PBS (pH7.2) to each test-tube. The blank contained 1ml 0.02M PBS (pH7.2). 50 ml of Fast blue stain was added to each test-tube and incubated at room temperature for 5 minutes. OD was read at 570nm. Two standard curves were made, one for α – naphthol and the other β – naphthol [WHO, 1998].

5.2.3. Standard cytochrome C oxidase assay

Cytochrome C oxidase (Bovine Heart) 200 µl/ml stock concentration was pipetted out in 100, 200, 400, 500, 800 µl into test-
tubes. The volume was made to 1ml by the addition of 0.625M PBS (pH7.2) to each test-tube. The blank contained 1ml 0.625M PBS (pH7.2). 200 µl 3,3',5,5'-Tetramethyl benzidine (TMBZ)-Sodium acetate Buffer (pH 5.0) was added to each test-tube followed by the addition of 25 µl Hydrogen peroxide (3%) and incubated for two hours. After two hours, the total volume was made to 3 ml with 0.625M PBS (pH7.2) and was thoroughly mixed. The OD was read at 650 nm [MR4, 2007].

5.3. *Quantitative enzyme assays*

For each enzymatic assays, an individual freshly killed female adult was homogenized dry to powdery form in –20°C cryo-box. 500 µl of ice cold sterile Milli-Q water was added to it and homogenized further. The solution was sonicated for 3 cycles (30 sec sonication followed by 30 sec relaxation x 3 times) using Sonicator (Hielscher Ultrasound technology, Germany). Each individual was used for total protein assay and three enzyme assays- GST, MFO and esterase. Total protein assay was performed using the Lowry’s Method of protein estimation [Lowry *et al.*, 1951]. The procedure for assay and calculation was adapted from WHO [1998] with a slight modification on the assay.
5.3.1. Naphthyl Acetate assay for General Esterase and calculation

200 µl of α– (or β–) Naphthyl Acetate was added to the 20 µl homogenate and incubated for 15 minutes. The blank contained 20 µl of sterile Milli-Q water. 50 µl of Fast blue stain was added and further incubated at room temperature for 5 minutes. 2970 µl of 0.02M Phosphate buffer (pH 7.2) was further added to make the volume to 3 ml. The OD was read at 570nm [WHO, 1998].

The protein value was first read from the BSA standard curve and multiplied by times 2, which gave the amount of protein expected in 20 µl homogenate and was recorded in µg to give a value of ‘x’. The OD values obtained from both α– and β–naphthyl acetate assays were first multiplied by times 12 (because a 260 µl had been diluted times 12 that gave a value of 3240 µl). This volume met the requirement of the spectrophotometer used) was then read from the corresponding α–or β–naphthol standard curves. This value was divided by 15 (no. of minutes before the stain was added) to give a value of ‘y’; ‘y’ value was divided by the ‘x’ value and timed this by 1000 to give a ‘z’ α– or β–naphthol/min/mg protein [WHO, 1998].
5.3.2. Assay for Mixed Function Oxidases

20 µl homogenate was mixed with 80 µl of 0.625M PBS (pH7.2), 200 µl of TMBZ– Sodium Acetate Buffer (pH 5.0) and 25 µl hydrogen peroxide (3%) and incubated for 2 hours. The blank contained 20 µl of sterile Milli-Q water. 2925 µl of 0.625M Phosphate buffer (pH 7.2) was further added to make the volume to 3 ml and was thoroughly mixed. The OD was read at 650 nm [WHO, 1998; MR4, 2007].

The protein value was first read from the BSA standard curve and multiplied by times 2, which gave the amount of protein expected in 20 µl homogenate an was recorded in µg to give a value of ‘x’. The OD values obtained from mixed function oxidase assay were first multiplied by times 10 (because a 325 µl had been diluted times 10 that gave a value of 3250 µl). This volume met the requirement of the spectrophotometer used and was then read from the cytochrome C oxidase standard curves to give the value of ‘y’; ‘y’ value was divided again by the ‘x’ value and timed this by 1000 to give a ‘z’ cytochrome/mg protein [WHO,1998].

5.3.3. Assay for Glutathione–S–Transferase

10 µl homogenate was mixed with 200 µl of Chlorodinitrobenzene–Reduced Glutathione (CDNB–GSH) and incubated for 20 minutes. The blank contained 10 µl of sterile Milli-Q water. 2940 µl of 0.02M PBS
(pH 7.2) was further added to make the volume to 3 ml and was thoroughly mixed. The OD was read at 340 nm [WHO, 1998].

The concentration of GST was calculated assuming that absorbance followed Beer-Lambert’s law: \( A = \varepsilon c l \), where ‘A’ is the absorbance, ‘\( \varepsilon \)’ is extinction coefficient of the product of reaction of CDNB valued 4.39 mM\(^{-1} \) [WHO, 1998], ‘c’ is the concentration and ‘l’ is the path length (of the cuvette used in spectrophotometer) which was 1 cm to give an ‘x’ value. The ‘x’ was divided by 20 (no. of minutes for incubation) and divided by the total protein concentration to give ‘y’ value; ‘y’ was then multiplied into times 1000 to give a value in mMoles/min/mg protein [WHO, 1998].

6. Preparation and extraction of total RNA from Anopheles species

The procedure followed was as described by Sambrook et al. [1989] and Simms et al. [1993]. 0.5 µl of Diethylpyrocarbonate (DEPC) was added to 100 ml sterile water (volume was also made to 1000 ml) and stirred overnight with magnetic stirrer to make 0.1% DEPC water. Pipette tips, Pipette boxes, tubes of 1.5 ml and 0.2 ml tubes and homogenizers were soaked overnight in 0.1% DEPC water and were autoclaved twice for 15 mins and dried in oven. DEPC treated water was also made from the 0.1% DEPC water by autoclaving twice under same conditions.
The abdomen, legs and wings of individual adult females, of different species, were removed to avoid any contamination. The remaining body was homogenized at −20°C mini cooler. 460 µl Trisolv™ (GeNei) was added to the crushed sample, mixed by gentle tapping and incubated at 20°C for 10 mins and spun at 12,000 rpm for 10 mins at 4°C. The supernatant was transferred into a fresh sterile 1.5 ml micro-centrifuge tube. 92 µl chloroform was added to the supernatant, incubated at 20°C for 5 mins and spun at 12,000 rpm for 15 mins at 4°C. 60% (approx. 270 µl) of the aqueous solution was pipetted out and poured in 230 µl isopropanol, and incubated further for 10 mins at room temperature, and spun again at 12,000 rpm for 15 mins. The pellet was washed with 460 µl of 70% alcohol and centrifuged at 5000rpm for 5 mins and air-dried. RNA was re-dissolved in 20 µl nuclease free water and incubated at 55°C for 10 mins. The RNA samples were quantified using Biophotometer plus™ (Eppendorf, Germany) and the samples were diluted to final a concentration of 100 ± 4 ng/µl. 5 µl aliquots were made and stored at -20°C.

6.1. RNA separation using formaldehyde agarose gel electrophoresis

The RNA separation was performed using Agarose containing formaldehyde gel electrophoresis procedure of Sambrook et al. [1989]. 1 g of Agarose was melted in 10 ml of 10X MOPS (3–(N–morpholino) propan sulfonic acid) (GeNei) and 5.8 ml of 37% Formaldehyde; 2 µl of Ethidium bromide was added to it. The RNA sample for electrophoresis was prepared in a 1.5 ml DEPC treated micro-centrifuge tube which consisted
for the following – 10 ± 5 ng/µl RNA sample, 10 µl formamide, 4 µl formaldehyde (37%), 2 µl MOPS (10X), 1 µl gel loading dye (GeNei) and DEPC treated water was added to make a total volume of 23 µl. The formaldehyde gel was run at 50V for 1 hour.

6.2. cDNA synthesis from Total RNA

The cDNA was synthesized using RevertAid™ First strand cDNA synthesis kit (Fermentas) following the manufacturer’s protocol. 1 µl of DNAase™ (GeNei) was added to the extracted RNA sample, and incubated at 37°C for 15 minutes for degradation of the DNA contaminants and then at 75°C for 15 mins for degradation of DNase. The sample was quantified using Biophotometer (Eppendorf, Germany). 10 ± 5 ng/µl of RNA was used for the cDNA synthesis. 1 µl of RNA sample, and 1 µl of oligo(T)™ primer was mixed and the volume was made to 12 µl with DEPC treated water. The mixture was incubated at 65°C for 5 mins and placed on ice. 4 µl 5X Reaction buffer, 1 µl Ribolock™ RNAse inhibitor, 2 µl of 10mM dNTPmix and 1 µl of Reverse transcriptase was added to the mixture, mixed gently by gentle tapping and briefly centrifuged using a Mini-Spin™ (GeNei). The solution was incubated at 43°C for 15 min, followed by 70°C for 5mins. The reaction was processed in ThermalCycler™ PCR (Eppendorf, Germany). The resultant CDNA was quantified using Biophotometer. 2 µl aliquots of each sample was made and stored in mini cooler box at −20°C.
7. **Semi-quantitative Reverse Transcription – PCR**

Resistant gene expression through qRT-PCR was studied on nine freshly obtained species of *Anopheles* viz. *An. campestris*, *An. dirus*, *An. jamesii*, *An. jeyporiensis*, *An. minimus*, *An. maculatus*, *An. nivipes*, *An. philippinensis* and *An. vagus*. *An. nivipes*, the most tolerant to 0.1% deltamethrin among the tested anopheline, was taken as positive control and *An. vagus*, the most susceptible, was used as negative control.

7.1 *Reverse Transcriptase(RT) - PCR of house keeping gene β-Actin*

β-actin gene was amplified using RT-PCR procedure of Kumar *et al.* [2008]. As standard control β-Actin (house keeping gene) was used for the RT-PCR for the study of expression of acetylcholine esterase and Cytochrome p450 (CYP6). The primers β-actinAF: 5´- ATG TAC GTC GCC ATC CAG GC -3´ and β-actinAR: 5´-CGA TGG TGA TGA CCT GTC CGT -3´ [Kumar *et al.*, 2008] were obtained from eurofins™ (Operon). The 25µl PCR reactants included *Taq* polymerase buffer (1X) (10 X *Taq* polymerase buffer contains 10 mM TrisHCl pH 7.5 and 50 mM KCl), MgCl₂ (1.5mM), dNTPs (0.25mM), primer (0.1 pM each), *Taq* polymerase (1U). The volume was made to 25 µl with DEPC treated water and different concentrations of cDNA template. In order to obtain similar banding intensity, different concentrations of cDNA were made by using Biophotometer and used in PCR. The PCR was repeated many times until
a uniform banding pattern was obtained to ensure equal concentration of the template cDNA. PCR conditions consisted of initial denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 30 sec, 50°C for 30 sec for primer annealing, 72°C for 30 sec as primer extension and a final extension at 72°C for 1 min.

7.2. Study of expression of Ace1 gene through Semi-quantitative RT-PCR (qRT-PCR)

Ace-1 gene was amplified using qRT-PCR procedure of Kumar et al. [2008] with modifications. Three primers were designed to target the partial region of Ace-1 gene (Figure 14). The designed primers listed in Table 1 were obtained from eurofins™ (Operon). Primer AChE3 gave steady and reproducible band therefore was used for the study. The 25 µl PCR reaction included Taq polymerase buffer (1X), MgCl₂ (1.5 mM), dNTPs (0.25 mM), primer (0.1 pM each), 1U Taq polymerase and cDNA template. The volume was made to 25 µl with DEPC treated water. The concentration of the cDNA template used for the PCR was referred from the standardized β-actin PCR result. PCR conditions consisted of initial denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 30 sec, 62°C for 30 sec for primer annealing, 72°C for 30 sec as primer extension and final extension at 72°C for 1 min. The Ace1 RT-PCR amplicon were quantified using Biophotometer.
Table 1. The name, sequences, melting temperature (Tm) and GC% of the primers used to target AChE partial gene.

7.3. Study of expression of CYP6 gene through qRT-PCR

CYP6 partial genes was amplified following procedure of Kumar et al. [2008], Kasai et al. [2000] and Rongnoparut et al. [2003] and using the primers (Table 2) of Kasai et al. [2000] and Rongnoparut et al. [2003] with modifications. The primers were obtained from eurofins™ (Operon). The 25 µl PCR reaction included Taq polymerase buffer (1X), MgCl₂ (1.5mM), dNTPs (0.25mM), primer (0.1 pM each), Taq polymerase (1U) and cDNA template. The volume was made to 25 µl with DEPC treated water. The concentration of the cDNA template used for the PCR reaction was referred from the standardized β-actin PCR result. The 35 cycles PCR conditions consisted of initial denaturation at 94°C for 1min, 94°C for 30 sec, primer annealing at 50°C for 30 sec, 72°C for 30 sec as primer extension and final extension at 72°C for 1 min. The CYP6 qRT-PCR amplicon were quantified using Biophotometer.
### Table 2.
The name of primers, sequence, melting temperature (Tm) and GC% of the primers used to target CYP6 partial gene. The name of the primer corresponded to the target name of the subfamily of CYP6 gene (CYP6AA2 and CYP6F1).

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Sequence</th>
<th>% GC</th>
<th>Tm</th>
<th>Published paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CYP6AA2F</td>
<td>5’- AACGGAATCGCATAGTACGCG -3’</td>
<td>52.4</td>
<td>49.6</td>
<td>Rongnoparut et al., 2003</td>
</tr>
<tr>
<td></td>
<td>CYP6AA2R</td>
<td>5’- TTTCCAACACTTCGACGCA -3’</td>
<td>52.4</td>
<td>49.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CYP6F1F</td>
<td>5’- CGAAACGCTCCGAAAGTATC -3’</td>
<td>50.9</td>
<td>60.0</td>
<td>Kasai et al., 2000</td>
</tr>
<tr>
<td></td>
<td>CYP6F1R</td>
<td>5’- GTCTGCCCCAAACGG -3’</td>
<td>68.8</td>
<td>54.0</td>
<td></td>
</tr>
</tbody>
</table>

8. **Total DNA extraction from *Anopheles* species**

Standardization of total DNA extraction was performed by methods of Reineke *et al.* [1998] and Rivero *et al.* [2004]. The best quality DNA was extracted through modified protocol of Rivero *et al.* [2004] (Figure 15). An individual adult was homogenized dry to powdery form and 20 µl of Lysis buffer (50mM Tris-Cl pH 8.0, 5mM EDTA pH 8.0, 100µM NaCl and 1% SDS) was added and further homogenized gently to spread the tissues. More lysis buffer was added to make the final volume to 500 µl. 5 µl of RNAse (20mg/ml) was added and incubated at 37°C for 1 hour, followed by addition of 50 µl of proteinase K (2 µg/µl) and incubated again at 40°C overnight or 55°C for 3 hours. Equal volume of phenol: chloroform: isoamyl alcohol (24:23:1) and spun in a centrifuge at 13,000 rpm for 10 minutes at 4°C twice. The supernatant was then mixed with equal volume of chloroform: isoamyl alcohol (24:1) and centrifuged. DNA was precipitated in 99.9% ice cold ethanol and incubated...
overnight at 4°C and centrifuged at 13,000 rpm for 5 minutes. The pellet was air dried at 27°C for 10 ± 5 minutes and dissolved in 20 µl of Milli-Q water.

9. Genomic DNA profiling through RAPD–PCR

The genomic DNA was amplified using RAPD-PCR procedure of Wilkerson et al. [1993] with modification. A working template DNA solution (10 ± 5 ng/µl) was made prior to PCR and 5 µl of working solution of DNA template was loaded in 0.8% agarose gel to check to quantity and quality of DNA (Figure 16). Thirty one random primers were initially tested and out of which eleven primers that produced reproducible polymorphic bands were selected for DNA profiling through RAPD-PCR (Table 11). 1 µl of genomic DNA were amplified in a 25 µl reaction mixture containing Taq polymerase buffer (1X), dNTPs (0.2mM), Taq Polymerase (1.5 U), MgCl₂ (3mM), BSA (0.6mg/ml) and 11.5 primer (11.5 pM each) and the volume was made to 25 µl with DEPC treated water. The samples were subjected to 40 cycles of initial denaturation at 94°C for 4 min, 94°C for 1 min (denaturation) followed by 37°C for 2 min (annealing), 72°C for 2 min (extension), and finally 72°C for 10 minutes. A reaction mixture without DNA template was used as negative control.

10. PCR amplification of mitochondrial COI gene for Phylogenetic analysis

The COI region of the mitochondrial DNA was targeted and amplified using PCR procedure of Kumar et al. [2007] with modifications. The AbcF (5´-
GGA TTT GGA AAT TGA TTG CCT T -3´) and AbcR (5´- ATT TTA AAT TCC AGT TGG A -3´) (Operon) primers modified from Kumar et al. [2007] were used for amplification of 650 – 700bp product of mitochondrial cytochrome oxidase (COI). The 25 µl PCR reaction mixture contained Taq polymerase buffer (1X), MgCl₂ (1.5mM), dNTPs (0.25mM), primer (0.1 pM each), Taq polymerase (1.5 U), BSA (0.6 mg/ml) and 1 µl DNA template (10 ± 5 ng/ µl). The volume was made to 25 µl with DEPC treated water. The reaction was conducted at 94°C for 5 min, 43°C for 1 min (annealing), 72°C for 1 min (extension) for 5 cycles; followed by 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 5 mins for 30 cycles. A reaction mixture without DNA template was used as negative control.

11. PCR amplification for Internal Transcribed Spacer 2 (ITS2) region of ribosomal DNA from different *Anopheles* species

The rDNA ITS2 regions were amplified from genomic DNA samples following the protocol described by Porter and Collins [1991], with some modifications, and using the following primers: ITS2F: 5´- CAC ACA TCC TTG AGT GCT A -3´ and ITS2R: 5´- ACA CAT CAC TTG AGG CCA C -3´. The PCR reaction was carried out for 25µl/reaction containing Taq polymerase buffer (1X), MgCl₂ (1.5mM), BSA (0.6 mg/ml), dNTP’s (2mM), primers (5pM each), Taq polymerase (1.5U) and 1µl of the template DNA (10 ± 5 ng/µl). The volume was made up using DEPC treated water. The PCR condition consisted of 6
min. of initial denaturation at 94°C followed by 35 cycles of 1 min. at 94°C, 1 min. at 51-55°C, 1 min. at 72°C and 4 min. of final extension at 72°C.

12. **Agarose gel electrophoresis and sequencing of the PCR products**

The agarose gel electrophoresis followed the protocol described by Sambrook *et al.* [1989]. To visualize the different PCR products (5 µl of COI and ITS2; 15 µl of RT-PCR and RAPD-PCR PCR amplified products) was loaded in 1.5% agarose gel, 1X TAE pH 8.3 (Tris-acetate-EDTA electrophoresis buffer) and ethidium bromide (0.5 µg/ml final concentration). The gel was run at 100V for 1-2 hours and visualized with UV trans-illuminator and photographed using UVP LS™ gel documentation system (Ultra-Violet Products Ltd. Nuffield Road Cambridge, CB4 1TG, UK). PCR amplicon were estimated using 100 base pairs (bp) DNA ruler or Low range DNA ruler, whichever was necessary (GeNei).

PCR amplified products quantified using Biophotometer (Eppendorf, Germany) were sequenced in forward direction and the sequences were generated from GCC BIOTECH (India) Pvt. Ltd., Kolkata, India. Electropherograms for the COI, ITS2 and AChE genes were edited and aligned with clustal W in MEGA 5.0 [Tamura *et al.*, 2011].
13. Analysis on the data produced by morphological characterization of *Anopheles*

For morphological data ten species of *Anopheles* species of Mizoram were selected for study of phylogenetic analysis: *An. campestris*, *An. peditaeniatus*, *An. jamesii*, *An. sinensis*, *An. philippinensis*, *An. annularis*, *An. nivipes*, *An. maculatus*, *An. jeyporiensis* and *An. vagus*. Four parts of the body were used as the focal key and were analyzed using a dissecting microscope: patterns on palpi, wings, legs and abdomen hairs [Das et al., 1990 and Nagpal and Sharma, 1995]. 34 characters were selected and their presence (+) or absence (−) of the each character was marked accordingly a binary table (presence-1/absence-0) was developed (Table 10) for study of phylogenetic relation.

The data were used to calculate the Euclidean distance using the SIMQUAL (computes similar association coefficients for qualitative data) module. Euclidean distance calculates two morphological data (1 and 0) and scores them on bases of similarity [Manguin et al., 1999; Broide et al., 2009]. The distance coefficient was used to construct dendrogram using UPGMA (Unweighted Pair Grouped Method Arithmetic Average) employing the SAHN (Sequential Agglomerative Hierarchical and Nested algorithm) algorithm with 1000 bootstrap value. The goodness of fit of the clustering compared with the similarity matrix was tested by computing the cophenetic correlation coefficient using the normalized Mantel statistics Z test [Mantel, 1967] with 100
permutations via the COPH and MXCOMP procedures. COPH produces a
cophenetic value matrix or matrix of ultra-metric values from a tree matrix while
MXCOMP compares two symmetric matrices by computing their matrix
correlation and then plots a scatter diagram. All parameters were computed
using NTSYS-pc 2.01i [Rohlf, 1998].

14. Molecular characterization to study the phylogenetic relation
among the Anopheles species.

14.1. Analysis of data produced by RAPD-PCR profile

The ten species of Anopheles employed for morphological data
analysis were also used for RADP-PCR profiling. The species were An.
and An. vagus. PCR reaction that generated high level of polymorphism
was repeated twice in order to verify the reproducibility of scored
polymorphic bands. For the analysis and comparison of RAPD patterns, a
set of distinct, well separated bands were selected, neglecting the weak
and unresolved bands (Figure plate 11 and 12, Figure 17). Electrophoretic
profiles were compared based on their molecular weights using Doc-It®LS
Image Analysis software and a binary table (presence-1/ absence-0)
corresponding to molecular weight and position of each bands.
The polymorphic information content (PIC) value often used to measure the informativeness of a genetic marker for linkage studies was calculated for each loci of eleven RAPD primers as $\text{PIC}_i = 2f_i(1-f_i)$; where $f_i = \text{frequency of primer } i$ (Roldan-Ruiz et al., 2000). The ability of the most informative primer to differentiate the genotype of a band was calculated by estimation of resolving power (RP). RP of each primer was calculated as given by Prevost and Wilkinson (1999): $R_P = \sum IB$, where $IB$ (band informativeness) = $1 - (2 |0.5 – p|)$ and $p$ is the proportion of genotypes containing the band. Marker Index (MI) is the product between diversity index and effective multiplex ratio (EMR), where EMR defined as the product of the fraction of polymorphic loci and the number of polymorphic loci, was also calculated. Percent polymorphism, no. of different alleles, no. of effective alleles, Shannon's Information Index, diversity, unbiased diversity and analysis of molecular variance (AMOVA) was calculated for morphology and RAPD data using GenAlEx 6.41 (Peakall and Smouse, 2006).

The RAPD-PCR data were used to calculate Jaccard’s genetic distance coefficient using the SIMQUAL module. These distance coefficients were used to construct dendrogram using UPGMA employing the SAHN algorithm with 1000 bootstrap value. The goodness of fit of the clustering compared with the similarity matrix was tested by computing the cophenetic correlation coefficient using the normalized Mantel statistics $Z$ test [Mantel, 1967] with 100 permutations via the COPH and MXCOMP
procedures. All parameters were computed using NTSYS-pc 2.01i [Rohlf, 1998].

14.2. Analysis of data produced by COI sequences

Fourteen Mizoram species - *An. peditaeniatus*, *An. jamesii*, *An. maculatus*, *An. philippinensis*, *An. nivipes*, *An. annularis*, *An. subpictus*, *An. aconitus*, *An. varuna*, *An. minimus*, *An. campestris*, *An. vagus*, *An. jeyporiensis* and *An. sinensis* were used for COI sequence analysis and were aligned with other *Anopheles* species retrieved from NCBI using the default parameters within the MUSCLE multiple sequence alignment programme [Edgar, 2004]. Their respective accession numbers are indicated in Table 12. Aligned sequences were examined with the program MEGA5 (Molecular Evolutionary Genetics Analysis, version 5.01) [Tamura *et al.*, 2011] and a similarity matrix was constructed. Relationships between individuals were assessed by maximum likelihood (ML) method with nucleotides distances (p-distance) using Tamura-Nei model [Tamura and Nei, 1993] with a gamma distribution (shape parameter = 10). The overall ts/tv bias (R) was calculated by the formula: 

\[ R = \frac{A*G*k1 + T*C*k2}{(A+G)*(T+C)} \]

where k1 = purine and k2 = pyrimidine using Tamura-Nei model. Estimates of average evolutionary divergence over sequence pairs within groups and between groups; average evolutionary divergence over all sequence pairs; mean evolutionary diversity within subpopulations; mean evolutionary diversity for the entire population;
mean inter-populational evolutionary diversity and coefficient of evolutionary differentiation were calculated using Tamura 3-parameter model [Tamura, 1992] and the rate variation among sites was modeled with a gamma distribution (shape parameter =10) and with 1000 bootstrap. Tajima’s test of neutrality [Tajima, 1993] was also calculated with 1000 replications in the bootstrap test. P-value less than 0.05 was used to reject the null hypothesis of equal rates between lineages.

Phylogenetic analysis involved 14 Anopheles COI sequences of Mizoram against 22 Anopheles sequences retrieved from Genbank, NCBI. The analysis based on the maximum parsimony (MP) algorithm was performed for the robust clade using PAUP* 4.0b1 [Swofford, 1998]. Heuristic searches were performed with 100 replicates of random addition option. Support for various nodes were determined through 1000 bootstrap replications where each bootstrap replication did 10 additional replications with different input order of the taxa. The branch swapping algorithm used was tree-bisection-reconnection (TBR). The tree was rooted using the COI sequences of the Culex tritaeniorhynchus (GenBank accession number: JQ003061) and C.quinquefasciatus (GenBank accession number: GU188856) as the outgroup. There were a total of 699 total characters. Codon positions included were 1st+2nd+3rd+Noncoding. Trees with 50% majority-rule consensus tree was considered and when describing trees, bootstrap values of 50% or greater were considered as strong.
14. 3. Analysis of data produced by ITS2 sequences


The sequences from each species complex were aligned using the default parameters within the clustalW multiple sequence alignment programme (Higgins et al., 1994). Aligned sequences were examined with the program MEGA5 (Molecular Evolutionary Genetics Analysis, version 5.01) (Kumar et al., 1993) and a similarity matrix was constructed. Relationships between individuals were assessed by maximum likelihood (ML) method with nucleotides distances (p-distance), evolutionary divergence within and between groups, transition/transversion rate ratios, nucleotide diversity and Tajima’s test of neutrality [Tajima, 1993] with 1000 replications in the bootstrap test. Nucleotide sequences have been submitted to the GenBank/EMBL Data Bank and their respective accession numbers are indicated in Table 12. Dimer frequency was calculated using Spectrum repeat finder (SRF) (Sharma et al., 2004). GC percentage was determined using GC calculator (http://www.genomicsplace.com/gc_calc.html). The overall ts/tv bias (R) was calculated by the formula: 

$$ R = \frac{A^*G^*k1 + T^*C^*k2}{[(A+G)*(T+C)]}, $$

where $k1 = \text{purine}$ and $k2 = \text{pyrimidine}.$
Phylogenetic analysis based on the maximum parsimony (MP) algorithm was performed for the robust clade using PAUP* 4.0b1 (Swofford, 1998). Heuristic searches were performed with 100 replicates of random addition option. Support for various nodes were determined through 1000 bootstrap replications where each bootstrap replication did 10 additional replications with different input order of the taxa. The tree was rooted using the ITS2 sequence of the butterfly, *Lysandra caelestissima* (GenBank accession number: AY556735) as the outgroup. The analysis involved 21 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps were treated as missing data. There were a total of 501 total characters. When describing trees, bootstrap values of 50% or greater were considered as strong.

ITS2 sequences were folded with the RNA secondary structure and Minimum free Energy (MFE) prediction subroutine using RNAfold Web Server program included in the Vienna RNA package (Hofacker, 2003). Default values were used to fold the ITS2 rDNA. Structures inferred by RNAfold were examined for common stems, loops, and bulges. The ITS2 sequence was also subjected to Spectral Repeat Finder (SRF) and Tandem Repeat Occurrence Locator (TROLL) programmes (Sharma et al., 2004; Benson, 1999) for identifying the presence of interspersed and tandem repeats, respectively.
Statistical analysis of ITS2 sequences

Data on the ITS2 repeats, copy number, MFE, RNA secondary structure and GC contents were subjected to appropriate statistical analyses. Analysis of variance (ANOVA) among ITS 2 repeats, minimum free energy, and RNA secondary structures was done and critical difference of mean (P = 0.05) were calculated using Tukey-Kramer multiple comparison post test for taking statistical decisions. Student’s t-test was used to examine the statistical significances of the GC content and MFE. Analysis of covariance for elements of RNA secondary structure adjusted by ITS 2 repeats (covariate) was performed. The correlation and regression between internal loop (Y) and repeat (X) was assessed by linear regression (Snedecor and Cochran, 1989).