Appendix I

I. Stock solutions:

1M K$_2$HPO$_4$ (Potassium Phosphate, dibasic): 34.84 g K$_2$HPO$_4$ dissolved in 150 ml sterile milli-Q water. Make final volume to 200 ml.

1M KH$_2$PO$_4$ (Potassium Phosphate, monobasic): 27.22 g KH$_2$PO$_4$ dissolved in 150 ml sterile milli-Q water. Make final volume to 200 ml. Autoclaved and stored at 4°C

1. Potassium Phosphate Buffers:

   1M; pH 7.2:
   - 1M K$_2$HPO$_4$ – 71.7 ml
   - 1M KH$_2$PO$_4$ – 28.3 ml. Autoclaved and stored at 4°C

   1M; pH 6.5:
   - 1M K$_2$HPO$_4$ – 32.95 ml
   - 1M KH$_2$PO$_4$ – 67.05 ml. Autoclaved and stored at 4°C

2. 0.2 M Sodium Acetate Buffer pH 5.0:

   i) 0.2M Sodium Acetate
   - 3.28g of Sodium Acetate dissolved in 150 ml sterile milli-Q water and made up volume to 200 ml with sterile milli-Q water.

   ii) 0.2M Acetic acid:
   - 2.31 ml Glacial acetic acid dissolved in 200 ml sterile milli-Q water.

   iii) 0.2M Sodium Acetate Buffer pH 5.0:
   - 0.2M Sodium Acetate – 35.2 ml
   - 0.2M Acetic acid – 14.8 ml. Autoclaved and stored at 4°C

3. 30mM Naphthyl Acetate(α or β):
   - 0.2793g Naphthyl acetate dissolved in 50 ml Acetone and stored at 4°C.

4. 3% Hydrogen peroxide:
   - 3ml hydrogen peroxide dissolved in 97 ml Sterile Milli-Q H$_2$O and stored at 4°C.

5. TMBZ (3,3’,5,5’-Tetramethyl benzidine) solution:
   - 0.01g TMBZ dissolved in 5ml methanol and stored at 4°C.

6. 5% SDS:
   - 5g SDS dissolved in 100ml Sterile Milli-Q H$_2$O, membrane-filtered and stored at RT.
7. **Reagent A: 2% Sodium carbonate in 0.1 NaOH**:  
0.4g NaOH pellets dissolved in sterile milli-Q water. To this 2g of Na$_2$CO$_3$ was added and the volume made up to 100ml with sterile Milli-Q water.

8. **1% Copper sulphate**:  
0.01 g CuSO$_4$ dissolved in 1 ml sterile milli-Q water.

9. **2% Potassium sodium tartarate**:  
0.02 g Potassium Sodium Tartarate dissolved in 1 ml sterile milli-Q water.

10. **Reagent B**:  
Reagent A – 100 ml  
1% CuSO$_4$ – 1 ml  
2% Potassium Sodium Tartarate – 1 ml Stored at 4°C

11. **Reagent C**:  
Folin phenol colchicine reagent – 20ml  
Sterile milli-Q water – 40ml Stored at 4°C

12. **Protein Standard (200µg/ml)**: 20 mg BSA (Bovine Serum Albumin) dissolved in 100 ml sterile milli-Q water.

13. **Standard α–naphthol (200µg/ml)**: 20 mg α-naphthol dissolved in 0.02 M Phosphate buffer pH 7.2.

14. **Standard β–naphthol (200µg/ml)**: Dissolved 20 mg β–Naphthol + 0.02 M Phosphate buffer pH 7.2.

15. **Standard cytochrome C (Bovine Heart) (200µg/ml)**: 20 mg Cytochrome C dissolved in to 100 ml 0.25 M Sodium Acetate buffer, pH 5.

II. **Working solutions**:

1. **Phosphate Buffers**:  
   a) **0.02M pH 7.2 (200 ml)**:  
      1M Phosphate buffer pH 7.2 Stock – 4ml  
      Sterile Milli-Q water – 196ml
   
   b) **0.625 pH 7.2 (200 ml)**: 125 ml  
      1M Phosphate buffer pH 7.2 Stock – 125ml  
      Sterile Milli-Q water – 75ml
   
   c) **0.1M pH 6.5 (200 ml)**:  
      1M Phosphate buffer pH 6.5 Stock – 20 ml  
      Sterile Milli-Q water – 180ml
**The following solutions were prepared fresh 1-2 hours before start of experiment.**

2. 0.06M Naphthyl acetate:
   - 30mM stock (α or β) – 2µl
   - Sterile Milli-Q water – 990µl

3. Fast blue Stain:
   - Fast blue B salt – 0.006 g
   - Sterile Milli-Q water – 600µl
   - Dissolved and then added -
     - 5% SDS – 1400µl

4. 10mM Reduced Glutathione (GSH):
   - GSH – 0.0081g
   - 0.1 M phosphate buffer pH 6.5 – 2.5 ml

5. 63mM Chlorodinitrobenzene (CDNB):
   - CDNB – 0.013g
   - Methanol – 1000µl

6. CDNB – GSH solution:
   - 63mM Chlorodinitrobenzene (CDNB) – 125µl
   - 10mM Reduced Glutathione (GSH) – 2500µl

7. TMBZ – Sodium Acetate Buffer solution
   - TMBZ – 1500 µl
   - 0.2 M Sodium Acetate buffer pH 5.0 – 4500 µl
**Figure 1.** Mechanism of AChE and its interference by OP. (Source: Walsh et al., 2001; Milesen et al., 1998)

**Figure 2.** Metabolism of deltamethrin mediated by subfamilies of CYP6. CYP6M2 produced by An.gambiae [Stevenson et al., 2010], CYP6AA3 by An. minimus [Boonsuepsakul et al., 2008] (Picture adapted from Stevenson et al., 2010).

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**Figure Plate 1**
Figure 3. Detection of polymorphic loci with the RAPD technique. Corresponding chromosomes from B6 and C3H are indicated schematically as horizontal lines. Boxes on each line represent genomic fragments (RAPD loci) that can be amplified with a particular primer. Locus B is polymorphic. An illustration of the gel pattern that would be obtained with amplifiable products from B6 and C3H is shown.

Figure 4. Schematic representation of mitochondrial genome.
Figure 5. Diagramatic representation of ribosomal DNA within the nucleolar organiser region of the eukaryotic genome, the tandem repeats are shown in black boxes, the transcription unit, the non-transcribed spacers and the two internal transcribed spacers (not to scale).

Figure 6. Elements of RNA Secondary Structure.
Figure 7. Map of Mizoram. The figure shows the different districts; and Thenzawl the primary site of collection of *Anopheles* species.

Figure 8. Sites of survey in Thenzawl. The Figure shows the ten sites of collection of *Anopheles* species.
**Figure 9.** Apparatus used for scoop-net (SN) method for collection of *Anopheles* larva. The picture shows larval net, iron handle, plastic tub, a plastic dipper and a dropper.

**Figure 10.** Apparatus used for adult *Anopheles* collection. A: The picture shows a killing jar in the middle and a side-scapped tube used for individual adult. B: CDC (Centers for Disease Control) light trap.
Figure Plate 7

Figure 11. Apparatus used Anopheline culture (A-D) in the laboratory.
Figure 12. Deltametrin (0.1%) bioassay against *Anopheles* larva after 24 hours exposure.

Figure 13. Biochemical analysis of α-naphtyl acetate assay for general esterase.
Figure 14. The clustal W alignment of Ace-1 complete coding sequences of *Anopheles gambiae* (GenBank Accession No. XM321792), *Culex pipiens* (GenBank Accession No. AJ489456) and *Aedes aegypti* (GenBank Accession No. EF209048). The highlighted region shows the sequences selected for AChE primers. Note: sequence of whole coding sequence not shown; * identical nucleotide sequences; - indels (insertions and/or deletions).
Figure 15. Comparison of three DNA extraction procedures on 0.8% agarose gel. The picture shows left: method of Reineke et al. [1998]; middle: Rivero et al. [2004]; and right: Modified procedure of Rivero et al. [2004].

Figure 16. The diluted 11 DNA working samples for RAPD Analysis on 0.8% Agarose gel. (DNA bands are hardly visible with naked eye). The order of gel lane is as follows: An. campestris, An. peditaeniatus, An. jamesii, An. sinensis, An. philippinensis, An. annularis, An. nivipes, An. maculatus, An. jeyporiensis and An. vagus of Mizoram anopheline; and the last lane belongs to An. campestris Changmai, Thailand.
Figure Plate 13
**Figure 17.** RAPD–PCR profile of eleven Anopheles species produced by 11 primers. Gel lane is in the order: Low range DNA ruler, *An. vagus*, *An. maculatus*, *An. jamesii*, *An. nivipes*, *An. philippinensis*, *An. jeyporiensis*, *An. peditaeniatus*, *An. sinensis*, *An. annularis* and *An. campestris* of Mizoram; *An. campestris* from Chiang Mai, Thailand and Low range DNA ruler.
Figure Plate 13:  Figure 18 (top left) Anopheles peditaenius (NSK02); Figure 19 (Top right) An. maculatus (NSK02); Figure 20 (Bottom left) An. jeyporiensis (NSK09); and Figure 21 (Bottom right) An. subpictus (NSK11)

Note: All figures show the adult form of female species

Figure Plate 15
Figure Plate 14: Figure 22 (top left) *An. aconitus* (NSK13); Figure 23 (Top right) *An. varuna* (NSK20); Figure 24 (Bottom left) *An. kochi* (NSK21); and Figure 25 (Bottom right) *An. dirus* (NSK22).

Note: All figures show the adult form of female species.
Figure 26. *An. minimus* (NSK23) (Adult Female)

Figure 27. *An. campestris* (NSK01)

Figure 28. *An. jamesii* (NSK03)

**Figure Plate 15:** Figure 26 *An. minimus* (NSK23) portraying the adult form of female species; Figure 27 *An. campestris* (NSK01); and Figure 28 *An. jamesii* (NSK03). Note: Figure 26 shows adult form while Figures 27 to 28 shows larval, pupal and adult forms of the species.

**Figure Plate 17**
Figure 29. *An. philippinensis* (NSK06)

Figure 30. *An. annularis* (NSK07)

Figure 31. *An. nivipes* (NSK10)

**Figure Plate 16:** Figure 29 *An. philippinensis* (NSK06); Figure 30 *An. annularis* (NSK07); and Figure 31 *An. nivipes* (NSK10). *Note:*
Figures 25 to 27 shows larval, pupal and adult forms of the species.

Figure 32. An. sinensis(NSK15)

Figure 33. An. vagus(NSK18)

Figure 34. An. culiciformis(NSK19)

Figure Plate 17: Figure 32 An. sinensis(NSK15); Figure 33. An. vagus(NSK18); and Figure 34. An. culiciformis(NSK19). Note: Figures 28 to 30 shows larval, pupal and adult forms of the species.
**Figure 35.** Graphical representation of total *Anopheles* spp. at Thenzawl, January – November, 2009 – 2011. Species are displayed in their respective voucher nos. NSK 01: *An. campestris*; NSK 02: *An. peditaeniatus*; NSK 03: *An. jamesii*; NSK 04: *An. maculatus*; NSK 06: *An. philipinensis*; NSK 07: *An. annularis*; NSK 10: *An. nivipes*; NSK 15: *An. sinensis*; NSK 09: *An. jeyporiensis*; and NSK 18: *An. vagus*.
Figure 36. Graphical representation of monthly relation of Anopheles spp abundance with malarial prevalence at Thenzawl, January – November, 2009 – 2011.
Figure 37. Regression line drawn against % probitmortality and log dose of *An. vagus*.

Figure 38. Regression line drawn against % probitmortality and log dose of *An. campestris*.

Figure Plate 19. A regression line for calculation of LC$_{50}$ in the tested *An. vagus* and *An. campestris*; x–axis corresponds to log of dose concentration (in %)

Figure Plate 22
and y-axis corresponds to probit of mortality (in %), following the method of Finney [1973].

**Figure 39.** Regression line drawn against % probitmortality and log dose of *An. jamesii*.

**Figure 40.** Regression line drawn against % probitmortality and log dose of *An. nivipes*.

**Figure Plate 20.** A regression line for calculation of LC₅₀ in the tested *An. jamesii* and *An. nivipes*; x-axis corresponds to log of dose concentration (in %)

**Figure Plate 23**
and y–axis corresponds to probit of mortality (in %), following the method of Finney [1973].
Figure 41. The formaldehyde agarose gel for the separation of total RNA extraction. Lane 1: *An. nivipes* and lane 2: *An. vagus*.

Figure 42. 1.5% agarose gel showing standardized β–actin gene qRT-PCR. The gel lane was shown in the order: 100 bp DNA marker (GeNei), *An. campestris*, *An. dirus*, *An. minimus*, *An. maculatus*, *An. jamesii*, *An. jeyporiensis*, *An. nivipes*, *An. philippinensis* and *An. vagus*.

Figure 43. Gene expression of Resistant Genes through qRT-PCR (200 - 250bp) in 1.5% agarose gel. The figure displays expression of (from top) D: *CYP6F1*; C: *CYP6AA2*; B: *Ace1*; and A: β–Actin. The gel lanes are in

**Figure Plate 25**

Figure 44. A dendrogram displaying complete linkage Euclidean distances using UPGMA method (with 1000 bootstrap value) using 34 morphological characteristics. The figure shows I: Sub genus Cellia and II: Sub genus Anopheles.
Figure 45. Dendrogram developed from pair-wise genetic similarity according to Jaccard's coefficient by UPGMA method (with 1000 bootstrap value) using RAPD-PCR profile. The figure shows I: *Cellia* and II: *Anopheles*.
Figure 46. PCR-amplified COI regions of the Mizoram Anopheles species. Lanes M - 100bp DNA ladder; the lanes (1-14) are An. peditaeniatus (JN596970), An. jamesii (JN596971), An. nivipes (JN596974), An. maculatus (JN596972), An. philippinensis (JN596973), An. annularis (JN832671), An. aconitus (JN832673), An. sinensis (JX988757), An. varuna (JN832675), An. subpictus (JN832672), An. minimus (JN881335), An. campestris (JQ003058), An. vagus (JQ915196) and An. jeyporiensis (JN881334) of Mizoram species; 15-16 are An.campestris(JQ003059), An.barbirostris(JQ003060) from Changmai University, Thailand; and An.gambiae (JQ003061) from South Africa. Note: The GenBank accession numbers are in bold.

Figure 47. PCR-amplified ITS2 regions of the Mizoram Anopheles species of the subgenera Cellia. (lanes M - 100-1000 bp ladder; lanes 1,2,3,7,13 - An. jeyporiensis; lanes 4- An. nivipes; lane 5- An. philippinensis; lanes 6,9 - An. minimus; lane 8 – An. varuna; lane 10 – An. subpictus; lanes 11,15,16 – An.maculatus; lanes 12,17 – An. vagus; lane 14 – An. jamesii; lane 18 – An. annularis).
Figure 48. Molecular phylogenetic analysis by maximum parsimony method based on COI sequence data. The tree was rooted with COI sequence of Culex tritaeniorhynchus (GenBank: JQ003061) and C. quinquefasciatus (GenBank: GU188856).
Figure 49. ITS2 secondary structures of *Anopheles* species of Mizoram.
Figure 50. Molecular phylogenetic analysis by maximum likelihood method based on ITS2 sequence data for 10 *Anopheles* species from Mizoram. The tree was rooted with ITS2 sequence of *Lysandra caelestissima* (AY556735). MFE – minimum free energy (Kcal/mol); RSS – RNA secondary structures (hairpin, internal loop, bulge); GC - GC content (%).