Discussion and Conclusion

The present study is the first detailed morphological characterization of *Anopheles* species of Mizoram. The majority of the species required morphological identification prior to molecular assays [Harbach, 2004]. Further, correct morphological identification and molecular analysis is also required for vector control and management [WHO, 2012]. However, limitations to morphological identification of species had been identified since it is dependent on characters found in various life stages and it was also noted that the features required for its morphological identification may be lost or damaged at the time of transport to the laboratory. In many cases, each wild-caught female had to produce an egg batch so that an adult progeny from that particular female can be studied [Koekemer *et al.*, 2002]; the process was time consuming and many of the larval forms die before pupation. Therefore, in order to help in confirmation to its species level, COI and ITS genes were targeted using PCR as described later.

It was also the first detailed study on the *Anopheles* and its abundance relating to malarial incidence. A detailed report on the species abundance and prevalence was performed in Thenzawl. The yearly *Anopheles* spp. abundance showed variation which could be due to rise in temperature (mean maximum of 25.3°C in 2009 to 26.3°C in 2011) and annual rainfall (248.03 cm in 2009 to
330.45 cm in 2011) during the three year survey. The present study also recorded that the monthly proportion of total adult *Anopheles* collected gradually increased from February (2.8%) and attained its maximum by May - June (23.1%), or in other words, the pre-monsoon season characterized with on and off rainfall moderated the temperature and humidity and provided adequate means to Anopheline reproduction and breeding; while the population dropped at November (0.85%) (Table 5). This population-shift in the proportion and abundance of *Anopheles* spp. was likely influenced by environmental factors such as the heavy monsoon rains overflowing their larval habitat, occasional cyclone, farming practices such as Jhum cultivation or use of fertilizers and insecticides and interspecific competition (Kim *et al.*, 2011). A total of 218 malaria cases were reported in Community Health Centre, Thenzawl where *P. falciparum* was more prevalent (80.7%) than *P. vivax* (18.3%) with few mixed infections (0.92%). The present study also revealed that the monthly abundance of *An. campestris, An. maculatus, An. jeyporiensis* and *An. nivipes* species had significance with malarial transmission in Thenzawl (Figure 36). Its potentiality had been observed with *An. campestris* [Limrat *et al.*, 2001] and *An. maculatus* [Rongnoparut *et al.*, 1996] in Thailand; *An. nivipes* in North East India [Prakash *et al.*, 2005]; and *An. jeyporiensis* in China [Chow, 1991]. Cryptic species of Barbirostris group had been successfully studied for vector identification due to its implications in diseases (malaria and filariasis) in Thailand [Paredes-Esquivel *et al.*, 2009]; however its status had not been confirmed in Mizoram. Interestingly, the two species of *Anopheles viz.: An. minimus* and *An. dirus* incriminated as malaria vectors of
the state [Das et al., 1982; 1990] were not found in Thenzawl during the survey periods, further, the few species collected from Tuirial showed the limited prevalence of the species and therefore cannot be responsible for the entire malarial cases of Mizoram. Further, An. aconitus incriminated as a natural vector of Plasmodium vivax in central Thailand was reported to have three karyotypic forms Form A, B and C in Thailand [Junkum et al., 2007] but no reports of vector incrimination or forms in Mizoram yet. Similar case is also observed with An. sinensis which is the malarial vector in China [Pan et al., 2012] and Korea [Ree, 2005]. Incubation of the malaria parasite fluctuates with temperature and takes 9 to 30 days to infect [Paaijmans et al., 2011; Jhpiego, 2008]; this statement correlated to the Anopheles species abundance in June and surge of malaria during July in Thenzawl.

The insecticidal bioassay and biochemical assays had found that most Mizoram anophelines were susceptible species to 0.1% Deltamethrin. When an enzyme was overproduced the cause of resistance may be considered to be sequestration rather than metabolism; with the increased enzyme levels acting as a means of holding the pesticide and preventing it from reaching the target site within the insect [WHO, 1998]. Spraying melathion for controlling agricultural pests by farmers [Anonymous, 2012] and IRS (Insecticide Residual Spray) of DDT was known since 1970s while use of ITN (Insecticide treated bed-nets) was observed only from 1990s [Anonymous, 2009]. This fact could be the reason behind many Anopheline species being slightly tolerant to pesticides; this was clearly observed in the bioassay and biochemical results of
An. vagus, one of the most commonly available species, found in areas where pesticide (DDT or melathion) spray by health department personnel or famers or ITNs was minimum [Anonymous, 2009; 2012], may have encountered less resistant selection pressure within its population. Meanwhile An. nivipes, An. jeyporiensis and An. maculatus usually collected nearby human residents and agricultural fields may have developed slight tolerance against the pesticides (Table 6). The overall analysis has shown that most Anopheline species of Mizoram are still susceptible, at present, to 0.01% deltamethrin. Biochemical assays were used to establish the mechanism involved in resistance, to measure changes in resistant gene frequencies in field populations under different selection pressures. The level of resistance conferred was then roughly proportional to the increase in the quantity of enzyme produced. However, they were not complete substitutes for the standard susceptibility tests which were used to measure resistance [WHO, 1998]. The biochemical assay showed that the enzyme production was the least in An. vagus (Table 7) while it was highest for An. nivipes and therefore found congruent with the results of bioassay.

The biochemical assays were only a simple method for detecting the quantity of the resistant enzymes; for example, the assay performed for mixed function oxidase measured the heme content of the insect, the majority of which was associated with cytochrome P450 in non-blood fed insects, was only a rough means of titrating P450 content and not P450 activity per se. However, its results validated the study of mRNA expression profile in the different
Anopheles species and thereby characterized the resistant and susceptible populations. These findings presented a striking contrast in the in vivo expression of mutagenized AChE and CYP6 genes and the qualitative modification of the enzyme production of either AChE or CYP6 or both may have conferred insecticide resistance. Highly expressed mutant acetylcholinesterase gene (Ace1) had been observed in many organophosphate insecticide resistant Anopheles species [Alou et al., 2010; Dabire et al., 2009]. Mizoram had observed its use in agriculture for many years and its Anopheles species may have evolved due to selective pressure since expression of Ace1 was observed in all its natural populations when compared to that of expression of CYP6 gene (Figure 42; Table 9). Further, the use of deltamethrin, a synthetic pyrethroid, newly developed for vector control could have been the reason why all species were not able to amplify the gene. Molecular mechanism of insecticide resistance inferred by the CYP6 genes: CYP6AA2 and CYP6F1 in the Anopheles species have revealed genetic diversity [Villatte et al., 2000]. Some studies suggested that overproduction of P450 decreased the fitness of individuals by metabolizing hormonal endogenous molecules [Cuany et al., 1990]; this statement seemed logical to explain the observations made in the present study. In the light of this statement, studies have also revealed that amino acid substitutions due to pesticide resistance may render lesser effect to the fitness of the individual as long as these substitutions were fixed in population; but once fixed it could facilitate overproduction of the gene as a form of genetic succession and therefore become stable [Taylor and Feyereisen, 1996]. Therefore the present
study, specifically to the expression of CYP6 gene, aroused the need to further investigation – since differential expression of CYP6 genes was observed in species, could the gene be lost due to absence of selection in that species which could not express? A total of 28 CYP6 genes had been identified in resistant An. gambiae through PCR and annotation of the genome [Ranson et al., 2002]. Several PCR methods had revealed that the mRNA of several CYP6 was constitutively overproduced in pyrethroid resistant strains: CYP6P3 and CYP6M2 by An. gambiae s.s field-caught populations [Djouaka et al., 2008; Muller et al., 2008; Mitchell et al., 2012]; CYP6P7, CYP6AA3 and CYP6P8 in An. minimus of Thailand [Rodpradit et al., 2005; Budriang et al., 2011; Duangkaew et al., 2011]; CYP6P9 and CYP6P4 in An. funestus [Amenya et al., 2008; Wondji et al., 2009; Matambo et al., 2010]. The CYP6 genes used in the present study: CYP6AA2 was expressed earlier in resistant An. minimus of Thailand [Rongnoparut et al., 2003] and CYP6F1 in Cx. quinquefasciatus [Kasai et al., 2000]. Based on the findings of the present study, could a specific subgene of CYP6 reflect its relative importance in resistance mechanism? Or is it because CYP6 are under the influence of specific gene regulating mechanisms that influence the expression of its specific subgene and therefore the resistance mechanism? Finally, it remains unclear how many different P450s participate in resistance in a given strain, and how many amino-acid substitutions of importance in resistance will be found in P450s. However, there are many sophisticated and more advanced molecular tools that can enable us to answer these questions. In any event, it is only then that we will be able to consider seriously the possibilities of monitoring accurately each resistant allele.
of P450 and of managing their spread in wild populations of agricultural pests or vectors of disease. Therefore, the expression profiling of CYP6 needed further examination through isolation of the CYP protein, synergic studies and real time (RT) – PCR, which may facilitate in determining the expression status of the gene and its role in resistance mechanism in Anopheles species of Mizoram.

The phylogenetic analysis performed with RAPD-PCR and COI in the present study was done in accordance with the traditional trend of similar morphological characteristic relatedness. To assess the magnitude of the evolutionary correlation between similar homologous phenotypic traits [Wagner, 1989] and between different traits, various models [e.g. Lewis, 2001; Seago et al., 2011] had been applied to infer ancestral states but not for phylogeny reconstruction per se. Keeping all these in view, the objective of molecular phylogenetic analysis was to draw a broader outline of evolutionary affinity among different groups of the anophelines and how it could help in the identification and description.

The cluster analysis inferred by morphological character profile (Figure 44) had revealed Anopheline species of closely similar taxa grouped in their respective series as Neocellia and Myzorhynchus series following the description made by Harbach et al. [2004]. Grouping could not be deduced for Pyretophorus and Myzomyia series since a single species was analyzed, however its closely linked affinity was shown. Further, the tree had displayed
species that belonged subgenus *Cellia* and subgenus *Anopheles* formed single cluster, supporting the morphological character-based classification.

RAPD markers has been widely used in the determination of population structure without prior knowledge of DNA sequences and it gives a good resolution of genetic differences, however they are known to be dominant and quantitative [Bardakci, 2001]. However, the loss of information per locus can be easily balanced by using a high number of loci [Jain et al., 2010]. Therefore in the present study, a total of 1203 loci produced by eleven RAPD primers were used for RAPD profiling. The technique had indeed revealed the genetic diversity among the *Anopheles* population; but could not reveal the shared derived characteristics of the species which was especially observed in the case of *An. maculatus* and *An. jeyporiensis* (Figure 45). The previous displayed its similarities with *An. vagus* rather than its closely related taxa in Neoellia series. *An. jeyporiensis* could not form a cluster within its subgenus *Cellia*. The overall tree topology displayed paraphyletic clustering within the subgenus *Cellia*. It is noteworthy to mention that RAPD-PCR had been used for the study of intraspecific diversity as observed in the cryptic species of *An. albitarsis* [Wilkerson et al., 1993; 1995], *An. gambiae* [Wilkins et al., 2006], *An. cruzi* [Calado et al., 2006] *An. fluviatilis* [Dezfouli et al., 2002].

The 699bp COI sequence used in the present study satisfies the required sequence length for DNA barcoding where a minimum criterion was 500bp [Frezal et al., 2008]. The obtained from Tajima’s test \( \chi^2 = 4.90, P = \)
0.02686 with 1 df) showed that the COI sequences evolved significantly faster across the lineages. The differences in most interspecific sequence are synonymous (p<0.005) except one case of non-synonymous, causing a codon change in *An. jamesi*vs.*nivipes*. The COI sequences were free of ambiguities and no intraspecific variation was observed in nucleotide composition. This suggests that the *Anopheles* species are composed of one consensus type. This finding is observed in figure 48, where intraspecific species formed a monophyletic clusters. Similarly, low rate evolutionary divergence rate within member species belonging to Myzorhynchus, Pyretophorus series observed monophyletic clusters in figure 48. The net evolutionary divergence of COI nucleotides scored the lowest between Myzomyia–Neocelia series scored the lowest of $0.023 \pm 0.006$, suggesting close affinity between the groups, as was observed in the phylogenetic tree. This close affinity was proved from the earlier studies by Swain *et al.* [2010] among Myzomyia and Neocelia series.

An effective DNA based identification system required efficient target sequence that enabled species-level identification; and species boundaries should be congruent with the morphological taxonomic work [Cywinska *et al.*, 2006]. The limitations of morphological identification was observed in many Anopheline species in the present study, for example, *An. sinensis* vs.*An. peditaeniatus* has only one unique difference, a fringe spot on vein 5.2 (present in *sinensis* and absent in *peditaeniatus*); *An. philippinensis* vs. *An. nivipes* (pre-sector dark mark on vein 1: extends beyond humeral dark mark of costa in *nivipes* and does not extend in *philippinensis*) [Das *et al.*, 1990 and Nagpal and
Sharma, 1995], such unique character is also present in Barbirostris group. These unique and delicate characters can be lost during handling and transport to laboratory. The RAPD-PCR profile between An. sinensis vs. An. peditaeniatus and philippinensis vs. An. nivipes gave 65% and 67% genetic similarity (Jaccard’s coefficient) respectively; morphological Euclidean distance gave 1.23 and 2.24 character–based similarity; while COI gave a significant 7.2 % and 10.8 % nucleotide difference (p-distance). The comparison of the trees produced from morphology, RAPD-PCR and COI gene, suggests that RAPD-PCR was not efficient in species identification and description of Anopheline species because of improper clustering. Lehr et al. [2005] had also observed incongruence in the clusters of RAPD-PCR profile and COI gene of Anopheles. Though COI was described as the most slowly evolving region of all mitochondrial protein-coding genes by Howland and Hewitt [1995], this gene solved the purpose of molecular phylogeny of the anophelines since the phylogeny inferred from the COI sequences were unequivocally supported by analysis of morphological characters as described by Christophers [1924], Sallum et al. [2002] and Harbach et al. [2004]. It, therefore, supported the proposed utilization of the gene for the barcode of animals by Hebert et al. [2003a].

The ten Anopheles species in study of ITS sequence analysis has been incriminated as primary vectors in Southeast Asia [Ma et al., 2006; Alam et al., 2010; Tikar et al., 2011; Sarma et al., 2012a; 2012b]. An. philippinensis was found predominant in Mizoram and species differentiation based on female
wing characters proved to be incorrect in 74% specimens [Sarma et al., 2012b]. Hence, in the present study is very useful for proper identification of Anopheles species of Mizoram through ITS2 marker. The ClustalW results showed the expected better alignment in the Cellia subgenera individually as well as dissimilarity when all the 10 species were considered simultaneously. The multiple sequence alignments depicted extremely high divergence and the sequence mid-region had more conserved sites than towards the 5'- or 3'-ends.

The ITS2 sequences were free of ambiguities and no intraspecific variation was observed either in length or nucleotide composition. This suggests that the Anopheles species in the studied areas are composed of one consensus type. This finding is further proved from the earlier studies on An. nivipes and An. philippinensis [Sarma et al., 2002a], An. subpictus [Ma et al., 2006], An. annularis, An. nivipes and An. philippinensis [Alam et al., 2010] and An. baimaii [Prakash et al., 2006]. But, Kaura et al. [2010] reported intragenomic variation in An. subpictus populations collected from North India. The presence of genetic homogeneity within the same species, as observed here in incriminated vectors, may be due to contiguous forest type and lack of ecological barriers, which are also the likely reasons for its universally efficient vectorial status [Ma et al., 2006; Alam et al., 2010; Tikar et al., 2011; Sarma et al., 2011a, 2011b]. Concerted evolution (gene conversion and unequal crossing over) maintain homogeneity of all rDNA copies [Dover, 1981]. Mutations rapidly spread to all members of the gene family even if there are arrays located on
different chromosomes [Tautz et al., 1988]. In ITS2, this can lead to fixed interspecific differences and intraspecific homogeneity. The efficiency of homogenization of rDNA is usually high [Liao, 1999]. Differences in the complementary bases in the ITS2 sequences arise due to substitutions in the form of transitions and transversions (ts/tv). There were a number of indels and base substitutions accounting for both the length and sequence variabilities.

ITS2 sequences of Anopheline species aligned as per the taxonomic classification of the six groups (Figure 50). There seems to be huge interspecific variations, which proves that ITS2 sequence can be a good marker to differentiate the Anopheles species of Mizoram. ITS2 region of Anopheles species are rich in GC content and low in Neocellia followed by Myzomyia and Pyretothorus. In Neocellia series, MFE was high and number of RNA secondary structure were low and vice-versa in Pyretothorus. DNA sequences of the ITS2 of the ribosomal RNA (rRNA) transcription unit have proven useful in resolving phylogenetic relationships of closely related taxa and in distinguishing species in mosquitoes due to their relatively rapid evolution rates [Ma et al., 2006; Li and Wilkerson, 2007; Alam et al., 2007; Sarma et al., 2011a, 2011b]. Numerous studies have utilized the ITS12 region for phylogenetic comparisons because it has been reported that the ITS12 is more informative than other gene sequences [Coleman, 2003]. The potential to predict the folding structure (providing signals to guide the ribosomal coding regions) has enhanced the role of ITS in phylogenetic studies, since it is
important to guide reliable sequence alignment based on secondary structures [Michot et al., 1999].

ITS2 repeat was used as the covariate, with RNA secondary structures as the dependent variable. A highly significant effect of the covariate was observed in all the Anopheline species. This showed that RNA secondary structures depended on the copy number of repeats, but more importantly that the relationship between ITS2 repeat and RNA secondary structures was altered with folding structures which provide signals to guide the ribosomal coding regions. GC content is also known to influence structural energy [Banerjee et al., 2007a]. Frequency of the bases is an important measure for sequence conservation analysis [Banerjee et al., 2007b]. In general, the ITS2 sequences of Anopheles species in Mizoram have high numbers of internal loops suggesting that the non-complimentary domains are high in spacer regions. When the repeats are minimal in occurrence and copy numbers, the structure becomes highly stable having a low minimum free energy as evidenced in An. vagus despite being the longest bp in length.

Among the 10 ITS2 secondary structures constructed, 2 species belonged to type I, 3 species were of type II, 2 species were of type III, and 3 species were of type IV. Type I, III, IV and Type III, IV structures were observed in Neocellia and Pyretophorus series. In contrast, type II was only seen in the series Myzomyia. The domain structure varied among species and could be classified into: a) domain I in An. subpictus divided into 5 sub-domains; b)
domain II elongated and divided into 2-6 sub-domains in all Anopheline species except An. subpictus, An. jeyporiensis and An. vagus and was reduced in the latter two species; c) domain III divided into 2 sub-domains in An. annularis, An. vagus, An. philippinensis and An. varuna whereas reduced in An. subpictus and An. jeyporiensis; d) domain IV divided into 5 sub-domains in An. jeyporiensis and reduced in An. maculatus, An. nivipes and An. varuna; e) domain V divided into 2 sub-domains in An. nivipes and reduced in An. jamesii; and f) domain VI divided into 5 sub-domains in An. vagus and reduced in An. jamesii (Figure 49). ITS2 exhibited the highly conserved four domain model in fruit flies [Schlotterer et al., 1994] and six-helicoidal ring-model structure found in yeast, insects, and vertebrates [Aguilar and Sanchez, 2006].

While the ITS2 region presents a dramatic range of length variations among Anopheles species, its size remains relatively heterogenous within each of the major groups [Kampen, 2005; Marrelli et al., 2005; Paredes-Esquivel et al., 2009; Sarma et al., 2012a]. This is unlike as observed in corals, where there was homogeneity in size within major groups [Chen et al., 2004]. An. vagus had the longest ITS2 regions, but possesses low repeats and polynucleotide microsatellites. In An. jeyporiensis, An. varuna and An. jamesii possess significantly longer ITS2 regions than others due to the occurrence of microsatellites. In contrast, shorter ITS2 regions found in An. maculatus though it possesses all types of microsatellites and highest repeats. Different compositions of microsatellites were also observed in the six groups of three series of Cellia indicating that ITS2 microsatellites evolved independently in the different lineages of Anopheles genera. Although there are very less
conserved regions between the *Anopheles species of Mizoram*, all of them retain certain common folding patterns that are required for its functions such as processing and maturation of certain ribosomal units [Hancock *et al.*, 1988; Yeh and Lee, 1990; van der Sande *et al.*, 1992]. The genetic differentiation within the genus *Anopheles* may be greater than that found between other genera in the family Culicidae [Wesson *et al.*, 1992].

In Mizoram, the origin and molecular nature of *Anopheles* species is poorly understood, despite the region having high malarial incidence and rich biodiversity. The present study, a diagnostic PCR assay for distinguishing the Cellia subgenera members of *Anopheles* species was developed based on the interspecific ITS2 variation. PCR based COI and ITS2 protocol provides a means for vector ecologists, malaria epidemiologists and control personnel to accurately identify members of the subgenera *Cellia* and a better understanding of their genomic status in Mizoram.

To conclude *An. campestris*, *jeyporiensis*, *maculatus* and *nivipes* were suspected malarial vectors of Mizoram. However, its confirmation requires molecular confirmation. The susceptible species to 0.01% deltamethrin is *vagus*; while tolerant species is *nivipes*. The expression of CYP6 gene need further molecular investigation perhaps through synergid studies of the cytochrome P450 protein and Real time PCR of the gene. RAPD-PCR is not an efficient molecular marker to conclude taxonomic classification however COI and ITS2 gene solved the purpose of molecular taxonomy and phylogeny.