Introduction and Review of Literature

Mosquito (Family: Culicidae) and mosquito-borne disease have been threatening human and animals for more than a century. The major mosquito vectors span the *Culex*, *Aedes*, and *Anopheles* genera. *Culex* is the major vector of filariasis and Japanese encephalitis, *Aedes* of dengue, and *Anopheles* of malaria [Hemingway *et al.*, 2006]. There are 444 formally named species and 40 unnamed members of species complexes recognized as distinct morphological and genetic species of *Anopheles* in the world [Harbach, 2004]. The *Anopheles* species is rich within the Oriental Region and occupies a wide variety of ecological niches [Reid, 1968; Foley *et al.*, 2007]. In India, 58 species was described, out of which six have been implicated to be main malaria vectors, namely - *Anopheles culicifacies*, *An. dirus*, *An. fluviatis*, *An. minimus*, *An. stephensi*, and *An. sundaicus*. In Northeast India, 23 species has been described [Nagpal and Sharma, 1987] where *An. dirus*, *An. fluviatilis* and *An. minimus* are the primary vectors [Dev *et al.*, 2006].

Although drug and vaccine development for malaria have received increased attention since 2000, there is no major initiative to improve vector control [Hemingway *et al.*, 2006]. Insecticides used for malaria control globally have included – organochlorine (OC), organophosphate (OP), carbamate, and pyrethroid (PY). However, use of chemicals on a vast and increasing scale has led to the widespread development of resistance as a result of selection for
certain genes [WHOPES, 2006]. By 1992 more than 55 different species of anopheline mosquitoes were found to be resistant to one or more of the commonly used insecticides [WHO, 1992].

Survey, habitat preference and general physiology of Anopheles

The Ministry of Health and Family Welfare, Government of India (Annual Report 2003-2004) recorded that malaria does not affect areas situated at 5000 ft. above sea level; and about 85% of malarial patients belonged to below poverty line (BPL) and residents of slum area. The incidence rate (number of cases per 1000 population) was highest in Arunachal Pradesh followed by Orissa, Mizoram, Goa, Meghalaya, Tripura, Jharkhand, West Bengal, Rajasthan and Chatisgarh.

Anopheline habitat mainly occurs within 180 - 2450 meters elevation above the sea level [Devi and Jauhari, 2008]. The habitats identified had some key environmental variables which determined their occurrence and relative abundance, but variations in habitat preference was observed among the various species of anopheline larvae [Herrel et al., 2001]. Usually, maximum number of larvae has been found in mountainous streams and pools (permanent conditions) having clean natural breeding habitats and shady along with moderate vegetation at a water depth of 0.1–0.5 m than other temporary habitats such as rice-fields, ditches, rock holes and irrigation channels and stream margins [Kengluecha et al., 2005; Minakawa et al., 1999; Herrel et al.,
2001; Devi and Jauhari, 2008]. *Anopheles* larvae utilize only a narrow water segment, between the water surfaces, since the larva lack a siphon. They are programmed for four larval instars and during the fourth instar, 80-90% of the growth and biosynthesis takes place. If they pupate they give rise to small or large imagoes depending on the species, while the dead larvae provide additional food for the fourth instar (the sexual differences in growth and biosynthesis become expressed in fourth instar). Hormonal factors suppress glycogen synthesis, thus favoring lipogenesis to some degree of obesity which is necessary for their oviposition. Further, flights range up to 17 km indicating their typical migratory behavior [Briegel, 2003].

Anopheline species abundance and habitat in the Himalayan region had been extensively studied [Devi and Jauhari, 2008; Bhat, 1998]. The survival of immature and emergence of pupae into adults had good correlation to the altitude and temperature of the breeding habitat [Devi and Jauhari, 2008]. Prevalence of *An. lindesayi*, *An. aconitus*, *An. annularis*, *An. culicifacies*, *An. fluviatilis*, *An. jeyporiensis*, *An. nigerrimus*, *An. maculatus*, *An. minimus*, *An. pallidus*, *An. pulcherrimus*, *An. splendidus*, *An. subpictus*, *An. stephensi*, *An. theobaldi*, *An. vagus* and *An. varuna* has been documented by Devi and Jauhari [2008]. Some abundant species recorded were *An. maculatus*, *An. vagus*, *An. sinensis*, *An. kochi*, *An. philippinensis* and *An.dirus* [Bhat, 1998; Dutta et al., 1992].
Biology of insecticide resistance

Insecticide resistance, by definition, is an inherited characteristic that allows an insect to survive a dose of a pesticide that would normally prove fatal and according to WHO [1957], "The developed ability in a strain of insects to tolerate doses of toxicant which would prove lethal to the majority of individuals in a normal population of the same species". Management of resistance can help avoid resistance development in vector populations, slow the rate of resistance development, and cause resistant vector to "revert" to a more susceptible level [Brogdon and McAllister, 1998]. To prolong the effectiveness of the currently available insecticides and thereby enhance vector control, it is vital to detect the emergence of resistance at an early stage so that appropriate action can be taken. Traditionally, detection has been based on insecticide susceptibility tests, accompanied by biochemical assays to identify the underlying resistance mechanism [WHO, 2005]. DDT and deltamethrin used in the present study belongs to Organochlorine (OC) and Synthetic Pyrethroids (PY) insecticide, respectively. The primary target of OC is the sodium channels of nerve sheath and PY appears to be acting at virtually every part of the insect nervous system: on sensory neurons, interneurons, motor neurons and on neurosecretion [Devonshire et al., 1992]. It has been shown that DDT prolongs the inward sodium current while suppressing the increase in potassium permeability. A combination of these effects leads to the prolonged falling phase; increases the negative after-potential, and leads, in turn, to repetitive activity. The treated insects rapidly become hypersensitive to external stimuli.
and develop tremors of the body and appendages. After a period of violent motion they fall on their backs and the continuous leg movement eventually becomes more spasmodic and finally by paralysis [Brogdon and McAllister, 1998].

Biochemical resistance to insecticide has two major forms (this excludes behavioral resistance): First, the target-site resistance occurs when the insecticide no longer binds to its target; alterations of amino acids responsible for insecticide binding at its site of action, allowing the insecticide to be less effective or even ineffective [Hemingway and Ranson, 2000]. Targets of pesticide are acetylcholinesterase (AChE) receptors in nerve synapses, and the sodium channels of the nerve sheath. Second, detoxification enzyme-based resistance occurs when enhanced levels or modified activities of General esterases (GE), Glutathione S- Transferase (GST), or Mixed Function Oxidases (MFO) prevent the insecticides from reaching its site of action. Esterases are one of the significant enzymes for insecticide detoxification in insects and comprise six families of proteins belonging to \( \alpha/\beta \) hydrolase fold superfamily [Brogdon and McAllister, 1998]. OP, carbamate and PY’s contain carboxylester and phosphotriester bonds that are subjected to be degraded by esterase enzymes [Fournier et al., 1992]. The MFO complex involves a reductase and one or more cytochrome P\(_{450}\); requiring NADPH as cofactor [Devonshire et al., 1992]. The cytochrome P\(_{450}\) belong to a vast superfamily; they activate insecticides by conversion of phosphorothioates (P=S) to phosphate (P=O). This can result in increased potency for inhibition of
acetylcholinesterase by three or four orders of magnitude. The cytochrome P450 oxidases (also termed oxygenases) metabolize insecticides through O-, S-, and N-alkyl hydroxylation, aliphatic hydroxylation and epoxidation, aromatic hydroxylation, ester oxidation, and nitrogen and thioether oxidation. The P450 monooxygenase are generally the rate-limiting enzyme step in the chain. These enzymes are important in adaptation of insects to toxic chemicals in their host plants [Schuler, 1996] and are also involved in the biosynthesis of ecdysone, juvenile hormone, and pheromone components [Feyereisen, 1995].

**Acetylcholinesterase** (*AChE*) terminates synaptic transmission by hydrolyzing the neurotransmitter acetylcholine. It is the only cholinesterase in insects [Toutant, 1989] and possesses a substrate specificity that is intermediate between that of vertebrate acetylcholinesterases and butyrylcholinesterases [Toutant, 1989; Corbett, 1974]. Insect *AChE* is composed of a single molecular form which is a glycosylated dimer, attached to a membrane via. a glycolipid anchor [Chaabihi *et al*., 1994]. Insecticides (OP and Carbamate) targets *AChE* and inhibit enzyme activity by phosphorylating the serine residue within the active site gorge [Corbett, 1974]; blocking the transmission of nerve impulses through irreversible inhibition of the enzyme at cholinergic synapses, causing paralysis or death (Figure 1) [Milesen *et al*., 1998; Walsh *et al*., 2001]. Cross-resistance to carbamate and OP can arise by insensitive *AChE* mechanism due to the glycine to serine substitution resulting from a single point mutation in the *Ace-1* gene [Weill *et al*., 2003].
Cytochrome P450 binds molecular oxygen and receives electrons from NADPH to introduce an oxygen atom into the substrate and to form water with the other oxygen atom:

$$\text{Substrate}(S) + \text{NADPH} + H^+ + O_2 \rightarrow (O) \text{NADP}^+ + H_2O.$$ 

The electrons necessary for this reaction are transferred from NADPH on the ‘substrate – PP450’ complex by an NADPH cytochrome P450 reductase, however, this reaction can also be stimulated by cytochrome b5 [Zhang and Scott, 1996]. This protein has absorption at 450 nm when reduced and saturated with CO, hence its name [Omura and Sato, 1964]. The P450s certainly constitute one of the most important superfamily of proteins, considering the large number of forms. To cope with such a diversity it was necessary to adopt a nomenclature based on sequences homologies of P450 [Nelson et al., 1993]. This nomenclature, now universally accepted, designates all gene members of the P450 super-family with a CYP prefix, followed by a numeral for the family, a letter for the subfamily, and a numeral for the individual gene. All members of a family share more than 40% identity at the amino-acid sequence level, and members of a subfamily share more than 55% identity [Berge et al., 1998]. They are ubiquitous enzymes, found from bacteria to mammals and are involved in endogenous metabolism as well as in the metabolism of xenobiotics. In insects, these activities are also essential for the synthesis and the degradation of the steroid moulting hormones and juvenile hormones and also in the metabolism of pheromones [Berge et al., 1998], and also for the adaptive mechanisms of insects to the toxic chemicals synthesized by their host plants [Schuler, 1996]. Its activity can be involved in the
metabolism of virtually all insecticides, leading to an activation of the molecule or, more generally, to a detoxification [Wilkinson and Brattsten 1972]. Its involvement in pyrethroid resistance has been demonstrated by synergistic studies with the monooxygenase inhibitor, piperonyl butoxide (PBO), and detection of increased heme levels in resistant mosquitoes [Vulule et al., 1999; Hargreaves et al., 2000]. In insects, a link between insecticide resistance and increased P450 activity, especially the cytochrome P450 monooxygenase (CYP6) families [Nikou et al., 2003] has been demonstrated (Figure 2). However, the expression profile of all the subfamily of CYP6 may differ during different developmental stages of Anopheles [Nikou et al., 2003].

Bioassay along with biochemical assays will provide more information on insecticide resistance status and their possible mechanisms [WHO, 1998]. However, if the resistance ratio obtained by bioassays showed slower increase when compared to that of biochemical assay, it would be because of more sensitivity of the biochemical assays [Enayati and Ladonni, 2006]. Further, resistance confirmed by bioassays would indicate that the gene frequency of the examined mosquito population was at such level that no resistance management strategies would be placed to success [Brogdon and McAllister, 1998]. Numerous vector species were reported to have developed resistance to insecticides used in public health [Elissa et al. 1993, Vulule et al. 1994, Hargreaves et al. 2000, Hemingway and Ranson, 2000]. Larval bioassay conducted in An. stephensi and An. culicifacies showed tolerance to 0.03mg/L deltamethrin and 0.08% permethrin which revealed that GE, MFO and GST
were about three times as much in the field strains than as those in susceptible strains indicating the involvement of these enzymes in pyrethroid resistance [Ganesh et al., 2003; Enayati and Ladonni, 2006]. Metabolism of DDT (OC) mediated by insect GST was possible through increased production of DDT-dehydrochlorinase [Herath et al., 1988]. Elevated GST levels were observed in DDT-resistant An. dirus [Prapanthadara et al., 1996] and in An. gambiae [Hemingway et al., 1998]. Four different amplicon were identified for insect esterase involved in insecticide resistance throughout the world [Fournier et al., 1992; Brogdon and McAllister, 1998]. Due to ancient gene duplication, the esterase genes - estα and estβ appeared to pre-date Culex speciation [Hemingway and Karunaratne, 1998]. In resistant An. stephensi, esterase overproduction was a common mechanism of resistance [Enayati et al., 2003] and three malathion metabolizing esterases were biochemically purified and kinetically characterized as standard “B” esterases. However, they had little or no activity with the general naphthyl acetate enzyme substrate [Hemingway, 2000]. Elevated levels of β-esterases and mixed function oxidases played a role in detoxification of permethrin and DDT in the resistant An. arabiensis population of Tanzania [Kulkarni et al., 2010] and Zimbabwe [Munhenga et al., 2008]. Examination of insecticide resistance was carried out by Perera et al. [2008] in Sri Lanka and high heterogeneity of populations of Anopheles spp. (An. culicifacies, An. subpictus, An. nigerrimus and An. peditaeniatus) was observed. Biochemical assay also revealed elevation of monooxygenases in An. nigerrimus, An. peditaeniatus and An. vagus populations while An. culicifacies and An. subpictus populations exhibited high levels of insensitive
acetylcholinesterase. The development of physiological resistance to deltamethrin due to increased over-expression of monooxygenase in An. minimus was observed [Chareonviriyaphap et al., 2003]; meanwhile esterase and GST activity could not provide conclusive evidence. Elevated levels of cytochrome P450 monooxygenase associated with PY resistance was also observed in several mosquito genera – An. stephensi, An. subpictus, An. gambiae [Brogdon et al., 1997; Hemingway et al., 1991; Vulule et al., 1994], Culex quinquefasciatus [Kasai et al., 1998] and C. pipiens pallens [Gong et al., 2005].

Biochemical and molecular methods can detect resistance mechanisms in individual insects; therefore, we can confirm resistance with the use of few individuals. Identification of resistance mechanisms help in determining the cross-resistance spectrum, facilitates the choice of alternative insecticides, and allows detailed mapping of geographical areas with resistant populations [WHO, 1998]. Molecular information on resistance mechanisms therefore, can be incorporated into resistance diagnostic procedures, for example, detecting the point mutations that cause target-site resistance or changes in detoxification enzyme specificity [Hemingway et al., 1991]. Recently, several PCR-based detection methods have been developed to detect target site resistance [Hemingway et al., 1998; Hemingway and Karunaratne, 1998; Brogdon and McAllister, 1998]. In addition, a clear understanding of the molecular basis of resistance to insecticides in mosquitoes will aid the
development of new alternatives to the existing control measures [Hemingway and Ranson, 2000].

**Resistant allele studies through Polymerase Chain Reaction**

*AChE* in insects, encoded by the *Ace* gene, is the key enzyme of the cholinergic system because it regulates the level of acetylcholine and terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter [Aldridge, 1950]. Its irreversible inhibition by organophosphate (OP) compounds, which phosphorylate or carbamylate the active-site serine of the enzyme, leads to the accumulation of acetylcholine in the synapses. This inhibition in turn leaves the acetylcholine receptors permanently open, resulting in the death of the insect. In various insect species, the molecular changes at the target site of *AChE* that are responsible for resistance, or insensitivity, to OP and Carbamate (CB) insecticides have been identified [Aldridge, 1950]. Smissaert [1961] described the first case of *AChE* with a reduced sensitivity to pesticides and since then, resistance-modified *AChEs* have been described in many insect species [Menozzi *et al.*, 2004; Nabeshima *et al.*, 2004; Weill *et al.*, 2004; Corbel *et al.*, 2007].

Sequencing of the genes encoding *AChE* in resistant strains showed that the modifications arose from point mutations and combinations of several point mutations in the same protein, were found in several alleles, where they induced higher levels of resistance [Mutero *et al.*, 1994]. Most mutations were
identical in several species, suggesting that a low number of mutations can actually provide resistance [Mutero et al., 1994; Nabeshima et al., 2004; Corbel et al., 2007]. The molecular basis of insecticide resistance due to insensitive AChE conferred by Ace1 has been described in some mosquito species such as An. gambiae, Culex pipiens pipiens, Cx. p. quinquefasciatus [Weill et al., 2002; 2003; 2004; Corbel et al., 2007] and Cx. tritaeniorhynchus [Nabeshima et al., 2004; Corbel et al., 2007]. At least five point mutations in the acetylcholinesterase insecticide-binding site have been identified that singly or in concert act resulting in varying degrees of reduced sensitivity to OPs and carbamate insecticides [Mutero et al., 1994]. Ace gene had also been used as a diagnostic marker to discriminate the 2 subspecies of Cx. Pipiens, Cx. quinquefasciatus and their hybrids; investigation of polymorphism in the gene would contribute to better understanding between medically important taxonomic groups (Bourguet et al., 1998).

Insect Cytochrome P450 had been assigned to six families: five i.e., CYP6, 9, 12, 18 and 28 are insect-specific and CYP4 has sequence homologies with families in other organisms [Berge et al., 1998]. Hughes and Nei [1988] and Ota and Nei [1994] proposed that duplication events may be followed by a winnowing process whereby some duplicate genes ‘die out’ because of accumulation of deleterious mutations; this ‘evolution by the birth-and-death process’ allowed number of functional genes within a family to remain stable. Multiple duplication and divergence events are thought to have allowed xenobiotic-metabolizing P450s, such as CYP2 and CYP3 in mammals
and CYP6 in insects, to diversify and acquire new functions [Feyereisen, 2006]. Feyereisen [2006] suggested that insect CYP genes formed four major clades – CYP2, CYP3, CYP4 and mitochondrial clade and the ‘founder’ mitochondrial P450 gene probably originated in the CYP2 clade. Further, the CYP6 genes were among the first genes of CYP3 clade to be cloned and characterized from insects and were related to the vertebrate CYP3 and CYP5 families [Feyereisen, 1999]. Genes in CYP6 clade are the most numerous among insect P450 genes, and are often found in large clusters. Considerable evidence linked members of this clade to xenobiotic metabolism and also insecticide resistance, pesticides and natural products.

A total of 28 CYP6 genes have been identified in 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. CYP6AA7, CYP9J40, CYP9J34, and CYP9M10 had been found to overexpress in the permethrin resistant Cx. quinquefasciatus [Komagata et al., 2010; Liu et al., 2011]. CYP6P3 and CYP6M2 was expressed by resistant An. gambiae s.s field-caught populations [Djouaka et al., 2008; Muller et al., 2008; Mitchell et al., 2012]; CYP6P7, CYP6AA3 and CYP6P8 may possibly metabolize different classes of pyrethroid insecticides in An. minimus [Rongnoparut et al., 2003; Rodpradit et al., 2005; Budriang et al., 2011; Duangkaew et al., 2011] and their homology models was later developed by Lertkiatmongkol et al. [2011]. CYP6P9 and CYP6P4 were
also over expressed in pyrethroid resistant *An. funestus* [Amenya *et al.*, 2008; Wondji *et al.*, 2009; Matambo *et al.*, 2010].

**Phylogenetic studies**

**Phylogenetics** is the science of estimating the evolutionary past, in molecular phylogeny, based on the comparison of DNA or protein sequences. The idea of representing these hypotheses as trees probably dates back to Darwin, but the numerical calculation of trees using quantitative methods is relatively recent [Sneath and Sokal, 1973], and their application to molecular data even more so [Zucherlandl and Pauling, 1965]. In the age of rapid and rampant gene sequencing, molecular phylogeny has truly come into its own, emerging as major tool for making sense of overwhelming genomic data.

A phylogeny is an evolutionary tree that shows how different species are related to each other [Baldauf, 2003; Harrison and Langdale, 2006]. One of the fundamental applications of phylogenies is in classification [Whitfield, 2003; Hibbett *et al.*, 2007; Freitas and Brown, 2004; Seago *et al.*, 2011]. Phylogenies help us systematically classify organisms in an evolutionary framework and are less prone to errors and individual biases [Sidow and Thomas, 1994; Baldwin *et al.*, 1997; Zhang *et al.*, 2012]. Thus phylogenies can be used to compare and contrast molecular systematics with morphology based classification [Wagner, 1989; Lounibos *et al.*, 1998; Lewis, 2001; Seago *et al.*, 2011]. Phylogenies are an important component in the field of historical biogeography [Kelly *et al.*,]
Furthermore they are useful in studying species evolution (budding speciation, sympatric vs. allopatric) and in identifying cryptic species [Wilkerson et al., 1993; 1995; Lounibos et al., 1998; Besansky, 1999; Lehr et al., 2005; Oliver et al., 2007; Paredes-Esquivel et al., 2009]. The study of evolution of behavioral and morphological characters in a phylogenetic context (character evolution) is also an exiting application of phylogenetics [Steinke et al., 2004; Millanes et al., 2011; Ritz et al., 2012]. Gene evolution too can be studied in a phylogenetic framework (molecular evolution) [Robe et al., 2005; Friedrich and Tautz, 1997; Lio and Goldman, 1998; Foley et al., 1998]. Therefore, phylogenies can be built using molecular data or morphological data, but in recent times molecular data are increasingly being used.

**Random Amplified Polymorphic DNA (RAPD) polymerase chain reaction**

RAPD-PCR utilizes short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR, where a single oligonucleotide of random sequence is employed and no prior knowledge of the genome subjected to analysis is required [William et al., 1990]. It amplifies anonymous fragments of DNA from any genome. The profile of amplified DNA primarily depends on nucleotide sequence homology between the template DNA and oligonucleotide primer at the end of each amplified product. Nucleotide variation between different sets of template DNAs will result in the presence or
absence of bands because of changes in the priming sites (Figure 3). The size distribution of amplified fragments varies among species. Closely related taxa have similar fragment distributions, while distantly related ones are more divergent. Therefore, amplification products from the same alleles in a heterozygote differ in length and will be detected as presence and absence of bands in the RAPD profile [Bardakci, 2001]. Thus, RAPD-PCR distributions contain considerable phylogenetic information [Espinasa and Borowsky, 1998]. However, there are several disadvantages that must be taken into account when using the technique. It is not possible to distinguish whether a DNA segment is amplified from a locus that is heterozygous or homozygous. RAPD markers are therefore dominant [Bardakci, 2001]. This most easily counteracted drawback reduces the information provided by each locus. Because each primer can amplify several loci and there are many commercially primers, the loss of information per locus can be easily balanced by using a high number of loci [Jain et al., 2010]. RAPD-PCR provides a very versatile and widely applied biotechnology technique in entomology. This method 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. This technology had been used for the study of genetic diversity of Anopheles species and identification – viz. 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].
Phylogeny construction using conserved regions - mitochondrial cytochrome oxidase 1 (CO I) gene and Internal Transcribed Spacer 2 (ITS2) rDNA

Using conserved molecular sequences, it is possible to define and diagnose molecular operational taxonomic units (MOTU) that have a similar extent to traditional 'species'. Any barcoding system should aim to acquire data for at least a nuclear and an organellar gene from single specimens for study of evolution patterns and taxonomic and systematic studies, thus, barcode system is likely to become universal [Blaxter, 2004]. The existence of numerous combined nuclear and mitochondrial gene datasets provides an opportunity to examine across a broad array of insect groups; both of these two types of data show patterns of nucleotide substitution that characterize nuclear and mitochondrial genes [Lin and Danforth, 2004].

Mitochondrial genes have been for many years the most commonly used source of data for studies of insect molecular phylogeny, biogeography [Morgan et al., 2009] and/or phylogeography [Simon et al., 1994]. Mitochondrial genes are viewed as advantageous for phylogenetic analysis for several reasons. First, mitochondrial genes are generally easier to amplify than nuclear genes and conserved mitochondrial primers are widely available [Simon et al., 1994]. Second, mitochondrial genes lack non-coding regions (i.e., introns) that are common in single-copy nuclear genes; third, mitochondrial genes are clonally inherited (through the maternal lineage) and non-recombining, making
recombination, paralogy, and heterozygosity (heteroplasmy in mitochondrial genes) less of a problem for phylogenetic analysis [Lin and Danforth, 2004]. Fourth, mitochondrial genes are generally thought to evolve at higher rates than nuclear protein-coding genes. In insects, mitochondrial genes are estimated to evolve 2–9 times faster than nuclear protein-coding genes [DeSalle et al., 1987].

The general attributes of mtDNA still make it one of the premier marker systems for analysis of population genetic (e.g., measurement of gene flow and population subdivision, estimation of female effective population size) and phylogenetic (e.g., reconstruction of relationships, estimation of divergence times) questions [Gerber et al., 2001]. The Consortium for the Barcode of Life (CBOL) has so far accepted mitochondrial encoded cytochrome oxidase 1 (COI) as the default DNA barcode region for vertebrates and insects and promotes its use in as many other clades as possible. A 658-bp region (the Folmer region) of the mitochondrial cytochrome c oxidase subunit I (COI) gene was proposed as a potential ‘barcode’ [Hebert et al., 2003b]. Genomic approaches to taxon diagnosis exploit diversity among DNA sequences to identify organisms. In a very real sense, these sequences can be viewed as genetic ‘barcodes’ that are embedded in every cell [Hebert et al., 2003b].

Cytochrome c oxidase I gene (COI) does have two important advantages (Figure 4). First, the universal primers for this gene are very robust, enabling recovery of its 59-end from representatives of most; if not all, animal
phyla [Zhang and Hewitt, 1997]. Second, COI appears to possess a greater range of phylogenetic signal than any other mitochondrial gene [Frezal et al., 2008]. In common with other protein-coding genes, its third-position nucleotides show a high incidence of base substitutions, leading to a rate of molecular evolution that is about three times greater than that of 12S or 16S rDNA [Knowlton and Weigt 1998]. COI is also useful to assign unknown species to species and enhance the discovery of new species and facilitate identification [Hebert et al., 2003a], when morphological traits do not clearly discriminate species [Kumar et al., 2007]. Further, the dwindling pool of taxonomist signals the need for a new approach to taxon recognition [Hebert et al., 2003b]. COI was successfully employed for the taxonomic arrangement of subgenus Cellia [Mohanty et al., 2009], Neocellia Series [Morgan et al., 2009] and Myzomyia series [Chen et al., 2003] and reveals cryptic species [Lounibos et al., 1998; Besansky, 1999; Lehr et al., 2005; Paredes-Esquivel et al., 2009].

Ribosomal DNA is used to answer systematics and phylogenetic questions in a wide variety of organisms. In mosquitoes, each transcriptional unit is made up of an external transcribed spacer, an 18S subunit, an internal transcribed spacer one (ITS1), a 5.8S subunit, an internal transcribed spacer two (ITS2), and a 28S subunit [Hwang, 2007; Kampen, 2005] (Figure 5). The functional regions that produce the ribosomes are highly conserved, while at the same time, there are transcribed and non-transcribed spacer regions. These regions have high interspecific variability and intraspecific homogeneity, making them useful for study of relationships of closely related species.
complexes and solving taxonomic problems by virtue of its fast evolution [Proft et al., 1999; Alvarez and Wendel, 2003; Alam et al., 2007; 2008]. These sequences also resolve the phylogenetic relationships among recently diverged taxa, contributing to polymorphism aiding in molecular taxonomic studies [Koekemoer et al., 1998; Zhang and Hewitt, 2003]. This fact was observed in the study of An. funestus complex [Koekemoer et al., 1998], An. subpictus [Chandra et al., 2010], An. fluviatilis S and An. minimus C species [Singh et al., 2006], An. melanoon [Nicolescu et al., 2004] and An. nivipes and An. philippinensis [Sarma et al., 2012] where morphological characters were not able to give conclusive identification system.

As one of the most popular phylogenetic markers for eukaryota, ITS2 locus was proposed to be used as a universal DNA barcode for identifying plant species [Casimiro et al., 2004; Yao et al., 2010; Liu et al., 2012] and as a complementary locus for COI to identify animal species [Yao et al., 2010]. This locus suggested a high degree of predictability across eukaryotes and its secondary structure could be used for comparison at species and generic level [Coleman, 2009]. In Protists the compensatory base changes (CBC) in the 30 bp highly conserved region of Helix III of ITS-2 correlate with the extent of sexual compatibility, therefore, CBC region of the ITS was suggested as universal DNA Barcode marker for describing the species [Coleman, 2009]. The similar proposal was also for Fungi [Schoch et al., 2012] because of its highest probability of successful identification for the broadest range in the genus.
Helical regions generally define the RNA secondary structure and are short, less than 10 base pairs in length forming stems and loops: hairpins, bulges, and internal loops (Figure 6). Secondary structure of RNA is important for the function of the RNA and is shown to be conserved in functional domains. Further, the secondary structure with the minimum free energy (MFE) is predicted to be the most stable secondary structure for the strand. For in-depth analysis, calculation of secondary structure and determination of structural conservation is essential. Structural energy, GC content, total base pairs, and number of loops are the various parameters known to stabilize the RNA secondary structure and it is shown that eukaryotic groups share the same overall secondary structure [Banerjee et al. 2007a].

However, ITS region often varies by insertions or deletions within an individual, making sequencing very difficult [Elbadri et al., 2002]; ITS sequences are also very difficult to align as they tend to evolve by insertion and deletion rather than substitution, making the secondary steps of phylogenetic reconstruction problematic [Zhang and Hewitt, 2003].

**Status of Malaria and Anophelines in Mizoram**

Mizoram (92.15–93.29°E and 21.58–24.35°N) belonging to the Indo-Burma region, is located in north-eastern India, has average rainfall of 2793.67 mm p.a., average relative humidity of 57% and a moderate climate. The main agricultural practice of the state is Jhum or shifting cultivation and the primary
pest management is use of organophosphate insecticide: Malathion [Dept. of Agriculture, Govt. of Mizoram through verbal contact]. The state undergoes conversion to secondary habitats at rapid rates due to human land use [Raman, 2001].

The results of GIS projects Mizoram as a possible malaria prone/high-risk region within northeast India due to climate change in 2050 [Bhattacharya et al., 2006]. War against malaria started since 1957. Therefore, malaria transmission by Anopheles vector (Diptera: Culicidae) within the region is no less significant than other parts of the world [Bhattacharyya et al., 2010]. Further, report of Directorate of National Vector Borne Disease Control Program, New Delhi, stated the mortality rate in Mizoram on account of malaria was had a high 17% in 2006 and 2009 when compared to other states of the country. The record of the Mizoram State Vector Borne disease control program (MSVBDCP) stated malaria was highest in Lunglei district (42.2%) followed by Aizawl-west (33.95%) and Serchhip district (14.7%) within 2003 – 2007. Towards vector control and management of Malaria disease MSVBDCP, funded by the World Bank (The Global Fund to Fight AIDS, Tuberculosis and Malaria project), has been setting up Full Therapeutic Depot (FTD) and Accredited Social Health Activist (ASHA) in all towns and villages to help malaria patients get treatment on time. The entire state is targeted for two rounds of an organochlorine, 1% DDT spray per annum (IRS) and distribution of 1% K-othrine\textsuperscript{TM}, a synthetic pyrethroid (Deltamethrin 2.5% active ingredient v/v) treated bed-nets (ITN) in rural areas [Mizoram Govt. Health Department sources].
Very few information on Anopheline prevalence, geographical distribution and relative density of potential vector populations had been described from Mizoram. The first Anopheline survey was done by Das and Baruah [1982] and indicated the incrimination of *An.minimus* and *An. dirus* as malarial vectors. The survey carried out by Nagpal and Sharma [1987] reported the presence of *An. barbirostris*, *An. nigerrimus*, *An. aconitus*, *An. annularis*, *An. karwari*, *An. kochi*, *An. maculatus*, *An. majidi*, *An. nivipes*, *An. philipinensis*, *An. theobaldi*, *An. vagus*, *An. maculatus var. willmori* and *An. kuchingensis*, but no record of and *An. dirus*. From Tlabung, south of Mizoram, *An. baimaii* (*An. dirus* Complex) has been incriminated as a vector of human *Plasmodium* [Das et al., 1990; Sarma et al., 2012a].

The undertakings of the MSVBDCP towards malarial eradication had focused on the human treatment, IRS and ITNs [Anonymous, 2009]; while neglecting effective and efficient surveillance system: entomological component *viz. Anopheles* habitat and seasonal abundance, adult population prevalence and their role in malaria transmission. Therefore, the present study was conducted to study the prevalence and abundance of *Anopheles* species and their habitat preference; to establish the baseline susceptibility status against a commonly used synthetic insecticide; quantitative estimation of resistance enzymes (GE, MFO and GST); to study of the expression of cytochrome P450 and acetylcholinesterase; and to study the phylogenetic relationship between the species.