1. INTRODUCTION:

Arthropods represent a major life form on the earth. Nearly about one million of these have been discovered by now, comprising 75% of all the recorded animal species. Arthropods can be found in all ecosystems (Behura et al., 2006). This truly demonstrates the immense ecological adaptation of them and their extraordinary adaptable life forms in diverse environments. Arthropods form a major group of disease vectors with mosquitoes, flies, black flies, sand flies, lice, fleas, ticks and mites transmitting a number of important diseases. Pathogens transmitted by arthropod (insect) vectors are some of the most dangerous and unpredictable on earth. The impact of such vectors increased as the human population grew. Many such vectors are haematophagous, which feed on blood at some or all stages of their lives.

Vector borne diseases affect two third of the world’s population and cause mortality in millions every year. The cost of combating these diseases and loss of productivity caused due to these diseases has crippled the economy of the endemic countries. Diseases transmitted by insects are of incalculable importance in global public health, threatening human life with vector borne parasitic infections like malaria. Malaria remains one of the major killers of humans worldwide, threatening the lives of nearly half of the world’s population. It thrives particularly in the tropical areas of Asia, Africa, and Central and South America, where it strikes millions of people (NIAID, 2007).

Malaria is not a new phenomenon of India; some of the earliest references to this fever are mentioned in the Atharva Veda which is believed to have been composed about 1500 B.C. The early Egyptians wrote about it on papyrus, and the
famous Greek physician Hippocrates described it in detail. The name malaria is derived from the Italian, “mal aria,” or bad air. In 1880, the French scientist Alphonse Laveran discovered the real cause of malaria, the single-celled *Plasmodium* parasite. Ronald Ross, a British officer working in the Indian Medical service, was the first to demonstrate that malaria parasite could be transmitted from infected patient to other persons through mosquitoes. In further work with bird malaria, Ross showed that mosquitoes could also transmit malaria parasite from bird to bird. This necessitated a sporogonic cycle (the time interval during which the parasite developed in the mosquitoes). This was a marvelous discovery indeed that had resolved the controversy transmission of malaria. For his discovery, Ross was awarded the Nobel Prize in 1902. Almost 20 years later, scientists working in India and Italy discovered that *Anopheles* mosquitoes are responsible for transmitting malaria. Malaria has been a significant factor in virtually all of the military campaigns involving the United States. In World War II and the Vietnam War, more persons died due to malaria than to bullets (NIAID, 2007).

Malaria is caused by a single-celled protozoan parasite from the genus *Plasmodium* (Fig.1). More than 100 different species of *Plasmodium* exist. They produce malaria in many types of animals and birds, as well as in humans. Four species of *Plasmodium* commonly infect humans. Each one has a distinctive appearance under the microscope, and each one produces a somewhat different pattern of clinical symptoms. Two or more species can live in the same area and can infect a single person at the same time. *Plasmodium falciparum* is responsible for most malaria deaths, especially in Africa. The infection can develop suddenly and
produce several life-threatening complications. With prompt and effective treatment, however, it is almost always curable. *Plasmodium vivax*, the most geographically widespread of the species, produces less severe symptoms. Relapses, however, can occur for up to 3 years, and chronic disease is debilitating. Once common in temperate climates, *P. vivax* is now found mostly in the tropics, especially throughout Asia. *Plasmodium malariae* infections not only produce typical malaria symptoms but also can persist in the blood for very long periods, possibly decades, without ever producing symptoms. A person with asymptomatic (no symptoms) *P. malariae*, however, can infect others, either through blood donation or mosquito bites. *P. malariae* has been wiped out from temperate climates, but it still persists in Africa. *Plasmodium ovale* is rare, can cause relapses, and generally occurs in West Africa. The malaria parasite typically is transmitted to people by mosquitoes belonging to the genus *Anopheles*. In rare cases, a person may contract malaria through contaminated blood, or a fetus may become infected by its mother during pregnancy. Because the malaria parasite is found in RBCs, it can also be transmitted through blood transfusion, organ transplant, or the shared use of needles or syringes contaminated with blood. Malaria also may be transmitted from a mother to her fetus before or during delivery (“congenital” malaria).

According to WHO (2013), there are 97 countries and territories with ongoing malaria transmission, and 7 countries in the prevention of reintroduction phase, making a total of 104 countries and territories where malaria is presently considered endemic (Fig. 2). Accordingly is estimated that 3.4 billion people are at risk of malaria globally. WHO estimates that 207 million cases of malaria occurred globally in 2012.
Figure 1: Life Cycle of Malaria Parasite
Figure 2: Distribution of Malaria across the world (Source: WHO, 2013)
(uncertainty range 135–287 million) and 627 000 deaths (uncertainty range 473 000–789 000). Most cases (80%) and deaths (90%) occurred in Africa, and most deaths (77%) were in children under 5 years of age (WHO, 2013).

India contributes approximately two third of all confirmed malaria cases in the South-East Asia Region, with five states Orissa, Chhattisgarh, Madhya Pradesh, Jharkhand and West Bengal. Other highly endemic states include Arunachal Pradesh, Assam, Meghalaya and Tripura. Madhya Pradesh is situated in the central part of India with an area of 308 thousand km2 of which forest cover is on 76,429 km2 (about 25% of the total land area). Madhya Pradesh (population 72.6 Million) along with other states like Orissa (population 42 million), Jharkhand (population 33 Million), and Chhattisgarh (population 25.5 million) contribute for more than 60% of reported (confirmed) malaria cases in India. According to National Vector Borne Diseases Control Program (NVBDCP) epidemiological data for 2010 from predominantly these tribal states with a total population of 173.1 million (out of a total of the country population i. e., 1.21 billion) represent 14.3 % population show persistent malaria transmission with high API (annual parasite incidence), slide positivity rate (SPR) and very high Pf % (Sharma, 2012) (Fig. 3). Madhya Pradesh alone accounts for 6% of the total population of the country but contributes to 8.6% of the total malaria cases. Malaria is prevalent in Madhya Pradesh because of vast tracts of forest with tribal settlement (20% of state population) (Singh et al., 2004; Anon, 2007). The magnitude of the problem can be accessed from an estimate made in 1987, that 54 million individuals of various ethnic origins residing in forested area of India and accounting for 8% of the total population contributed 30% of total malaria cases, 60% of total
falciparum cases and 50% of malaria deaths in the country (Sharma, 1996) (Fig.4). The reasons for such a high diseases prevalence in Madhya Pradesh is mainly due to locations of the villages in the deep forest and is characterized by rocky undulations interspersed with ravines and foothills. Another reason of disease prevalence is innumerable streams which flow into river, Narmada. These streams flow continuously and provide ample breeding sites covered with dense aquatic vegetation for production of number of anopheline species particularly \textit{An. culicifacies} (Singh, 2006).

Mosquitoes belong to the class Insecta, order Diptera, sub-order Nematocerca which contain three subfamilies Toxorhynchitinae, Culicinae and Anophelinae (Coluzzi and Kitzmiller, 1975). Mosquitoes belonging to the genus \textit{Anopheles}, subfamily Anophelinae are the exclusive vectors of human malaria because of their behaviour, physiology and the close relationship with humans (Coluzzi and Kitzmiller, 1975). All types of human malaria are transmitted through anophelines (genus \textit{Anopheles}) only, but not all anophelines are vectors of malaria. To become a malaria vector, the Anopheles mosquito has to be susceptible to malaria sporogony, be anthropophagic and must have enough longevity to become infective to human. The genus Anopheles has 424 species, among these 58 species are recorded from India (ref.) Out of these 58 species six species viz. \textit{An. culicifacies} (rural), \textit{An. stephensi} (urban), \textit{An. fluviatilis} (foothill), \textit{An. minimus} (foothill and forest fringe), \textit{An. bamaii} (formerly known as \textit{dirus}, found in deep forest) and \textit{An. sundaicus} (costal, only in Andman and Nicobar Islands) transmit one of the major diseases of the country i.e. malaria. These are also listed as the primary vectors of malaria. Except \textit{An. stephensi},
Figure 3: Situation of Malaria in India
Figure 4: Situation of Malaria in Madhya Pradesh
all of these vectors exist as species complex that comprise a number of morphologically indistinguishable biological traits and they are commonly known as sibling species or cryptic species or isomorphic species (Subbarao, 1998).

Mayr in 1942 coined the word sibling species to denote morphologically similar but reproductively isolated population within a taxon. Many terms are used when describing groups of closely related and/or cryptic species, which may or may not be distinguishable on the basis of morphology alone. The isomorphic nature of such species occurs because speciation does not always involve morphological change. Sibling species are cryptic sister species, i.e., each other's closest relatives which are difficult or impossible to distinguish morphologically (Bickford et al., 2006). The term species complex is used to describe groups of closely related species, and is usually used to describe more recently derived species that have few if any morphological differences among the members of the complex, such as the *Anopheles quadrimaculatus* Complex (Reinert et al., 1997). The term species group is used to describe groups of species whose members are more distantly related and often at least some of the members are distinguishable based on morphological characters, such as the Nearctic members of the *Anopheles maculipennis* Group (Porter and Collins, 1996). Cryptic species complexes are common in mosquitoes (Walton et al., 1999), including genus *Anopheles* (Collins and Paskewitz, 1996). Because the members of anopheline species complexes often differ in ecological or behavioural characteristics that affect their ability to transmit disease (Collins and Paskewitz, 1996), the ability to accurately identify cryptic species is essential for implementing effective vector control. This is best illustrated using the "Anophelism without malaria" problem that
hindered control of the disease in Europe during the early 1900s until it was discovered that the primary vector, *An. maculipennis*, was actually a group of isomorphic species (i.e., they had been dealing with *An. maculipennis s.l.*), whose members differed in their potential to transmit *Plasmodium* parasites, the causative agents of malaria (Walton *et al.*, 1999). Since then, particularly in regions where malaria is endemic, many cryptic species complexes have been discovered within the genus *Anopheles* (Collins and Paskewitz, 1996; Besansky, 1999).

Among all six primary anopheline vectors, *Anopheles culicifacies* (Diptera: Culicidae) is a principal malaria vector in rural, periurban and tribal settings (Mishra *et al.*, 2012), its contributing to almost 70% of malaria cases in India. *An. culicifacies* has a wide distribution that extends from Ethiopia, Yemen and Iran in the west via Afghanistan, Pakistan, India, Bangladesh, Myanmar and Thailand, to Laos and Vietnam in the east. To the north it is found in Nepal and southern China, and in the south in Sri Lanka. This major vector species is widely distributed and present as a species complex comprising five members i.e. A, B, C, D and E, on the Indian subcontinent (Subbarao, 1988) (Fig. 5). While species B is widespread in many Asian and South Asian countries such as Iran, Cambodia, China and Thailand, species A, C and D are mainly confined to northern India. Species E is the new addition to this complex and has been reported in southern India. *An. culicifacies* is the main vector of rural malaria (Subbarao, 1988).

It is a small to medium sized mosquito with *Culex* like sitting posture. It is a zoophilic species, when high densities build up relatively large numbers feed on men,
Figure 5: Distribution of *Anopheles culicifacies* Complex in India (Source: Subbarao, 1991)
rests during daytime in human dwellings and cattle sheds. Biting time of each vector is determined by its generic character, but can be readily influenced by environmental conditions. Most of the vectors, including *An. culicifacies*, start biting soon after dusk. Biting starts much earlier in winter than in summer but the peak time varies from species to species. It breeds in rainwater pools and puddles, borrow pits, river bed pools, irrigation channels, seepages, rice fields, wells, pond margins, sluggish streams with sandy margins. Extensive breeding is generally encountered following monsoon rains.

Various methods and techniques have been used for identifying sibling species ranging from crossing experiments, cytogenetics, isoenzymes, hydrocarbon profile, DNA probes, rDNA-PCR, mt DNA-PCR and RAPD-PCR as given below.

**Crossing experiments:** Crossing experiments have been carried out in order to ascertain mating between sibling species in nature and to identify sibling species of *An. culicifacies* complex (Miles, 1981, Mahmood *et al.*, 1984, Subbarao *et al.*, 1988 and 1993).

**Cytogenetics:**

**Polytene chromosomes:** Cytogenetical techniques’ involving karyotyping of polytene chromosome was one of the earliest tools for the study of anopheline genetics.

**Mitotic Karyotyping:** This approach involves the use of metaphase chromosomes of brain cells in 3rd or 4th instar larvae therefore semi gravid females are not required.

**Isoenzymes:** Isoenzymes or alloenzymes are different molecular forms of an enzyme coded by different allelic forms of a gene. Therefore, after electrophoresis the enzyme activity can be visualized on a gel and the species can be identified.
**Hydrocarbon profiles:** This approach involves the analysis of cuticular component through Gas Liquid Chromatography (GLC). The profiles of each isomorphic species can be identified from varying retention times of the cuticular hydrocarbon. This technique has many advantages over that of established polytene chromosome and mitotic karyotypic identification. The material could be stored and analysed in any condition and both sexes and all life stages can be identified. However, this approach does involve expertise, sophisticated equipment and more importantly is very time consuming.

**DNA based methods:**

**DNA probes:** Probes are short and specific stretches of DNA or RNA strands that are radiolabelled or non-radiolabelled and that can hybridize with complementary single strand nucleic acid stretches.

**rDNA cistron:** The intergenic spacer (IGS) and Internal Transcribed Spacers (ITS 1 and ITS2) within the nuclear ribosomal genome have become very popular targets for addressing taxonomic issues among anophelines. It has been noted that the nucleotide sequence of these spacer regions are often much more polymorphic between species than within species. This makes this region of the genome useful for delineating molecular differences between cryptic species by length or sequence polymorphism.

**mt. DNA:** Mitochondrial DNA has been used in sibling species identification in various insect taxa as well as in some anophelines (Mitchel et. al., 1992; Narang et. al., 1993). However, the mitochondrial genome is frequently utilized in phylogenetic and population genetic studies.

**RAPD:** Random Amplified Polymorphic DNA (RAPD) analysis makes use of a set of primers of 8 - 10 nucleotides whose sequence is essentially random. The random
primers are fixed individually or in pairs in PCR reaction to amplify fragments of genomic DNA from the organism of interest.

Identification of cryptic species of malaria vectors by standard morphological characters is known to be problematic and frequently unsatisfactory (White, 1977). The limitations of morphological taxonomy for resolving species boundaries stimulated the advocacy of multidisciplinary approaches to mosquito systematic (Faran, 1979).

The pioneer work in the field on the basis of morphological difference between sibling species of *An. culicifacies* was done by B.P. Das. It was reported by him that distribution pattern of transparent dots on the spermathecae differs in two sibling species A and B. In species A, transparent dots were restricted to supra and infra basal, infra centro-basal, infra centro-apical up to infra apical regions whereas, in species B, these dots were distributed to almost all areas of spermathecae (Das, 1990).

Morphologic characteristics for identifying various species are largely lacking. However useful morphologic characters occur in the egg stage and can be used to separate sibling species. Usefulness of scanning electron microscopic technique to study the morphology of *Anopheles* groups has been proved for identification of closely related species or geographical trends within species. A discriminate function analysis of egg characteristics of the 5 known species of the *An. quadrimaculatus* Say complex permitted correct classification of 97.7% of the eggs to species. Despite the importance of the *An. culicifacies* complex in disease transmission, eggs of these species have not been examined by SEM. So in the present study, efforts have been
made to describe the egg surface morphology and morphometrics of eggs of *An. culicifacies* in details using scanning electron microscopy.

The study of genetic variations has been estimated by several techniques including morphological studies, cytogenetics, protein electrophoresis and direct measurement of DNA variability. Significant progress has been made in understanding insect diversity by using classical genetic principles. Common visible markers including eye colour, body spots/ stripe or bands and hairs or spines were used as phenotypic markers in studying pattern of dispersal, mating behavior and inheritance of genetic traits in insects (Bartlett *et al.*, 1968; Fay and Craig, 1969; Bond *et al.*, 1970; Bartlett and Butler, 1975). Although the phenotypic markers are found at all time of life span of the organism and can be readily used for studies in field conditions, but they suffer from many practical limitations. The major drawback is that these visible phenotypes are relatively infrequent and often hard to score specially in long time preserved specimens. Also, it is difficult and time-consuming to induce genetic mutations in laboratory populations to develop new phenotypic markers, and sometimes they interfere with the overall fitness of the organism. Furthermore, identification of phenotypic markers must be accompanied with the information as to how the trait is inherited to the offspring before they are used as faithful markers. Because the phenotypic markers are rare, use of these markers in mapping a trait is difficult.

It is difficult to carry out cytological identification of members of *An. culicifacies* complex in disease control programme due to the requirement of highly skilled technical personnel and applicability of this technique to half-gravid female
mosquitoes only. Some alternative methods are available; isozyme analysis of lactate dehydrogenase (ldh) is helpful in differentiation of species A and D from species B and C (Adak et al., 1994) with 94×6% specificity. DNA probe hybridization assay – based on highly repetitive DNA sequences (Gunasekara et al., 1995) was able to distinguish species A from species B/C when single mosquito-extract was diluted to 1/200. However such hybridization assay, which is based on difference in copy number across species, is sensitive to variation in amount of template DNA loaded and can be unreliable (Krzywinski and Besansky, 2003). Identification by cuticular hydrocarbon analysis (Milligan et al., 1986) was not promising due to intraspecific variability in field samples (Subbarao, 1988). Polymerase chain reaction (PCR) and PCR-based assays have emerged as reliable and sensitive method for the differentiation of closely related species or cryptic members of the species complex. The ribosomal DNA (rDNA) regions are usually used for such PCR-based species diagnostic assays (Collins and Paskewitz, 1996). In the present study we report an AS-PCR assay targeting D3domain of 28S-rDNA which differentiates species A and D from species B, C and E. Two allele-specific PCR assays (AD-PCR and BCE-PCR) using sequence differences in the mitochondrial cytochrome oxidase II (CO II) subunit. The AD-PCR assay distinguishes species A and D, whereas the BCE-PCR assay distinguishes species B, C, and E. Thus, with a combination of two PCR assays, namely the D3-PCR/ITS2-RsaI assay, followed by either the AD-PCR or the BCE-PCR assay, it is possible to identify individual specimens of any of the species of this complex. This assay system is the first, and the best available at present to distinguish all sibling species and especially to discriminate non-vector, species B from all the
vector species, A, C, D, and E, of the An. culicifacies complex. Until another DNA-based method involving fewer steps is developed, this assay system can be used in all malaria epidemiologic studies where An. culicifacies is prevalent.

Bionomics is that part of biology which deals with the relationships of a given species and its environment. Basic studies on the bionomics of mosquitoes include the development of immature stages, i.e., eggs, larvae, and pupae as well as the life of the adults under the influences exercised by the environmental conditions. Genetic factors, which govern the basic lines of behaviour and ecological factors, which may produce different type of reactions in a population having the same genetic characteristics.

The larval and adult stages of mosquitoes exist in two different environments, each stage being under the influences of its immediate surroundings. The adaptations of eggs, larvae, pupae and adults to certain environmental patterns constitute the influence of factors controlling seasonal and geographical distribution of the species.

Among the ecological factors, the phenological one is of considerable importance. For example, the ability of mosquitoes to breed in fresh or salt water, or both, is controlled by genetic factors. Host preference seems also to be governed by genetic factors, but intensity of feeding on a certain host may vary from palace to place and even from day to day, not only with the availability of hosts, but also with the changes in meteorological conditions. The duration of development of mosquitoes is dependent mainly on climatic factors. The rate of metabolic processes of insects are largely controlled by temperature, hence certain biological events such as duration of aquatic stages, duration of blood digestion and maturation of ovaries and consequently
the frequency of feeding varies according to temperature. These features underline the importance of careful recording of the environmental factors during such studies.

Different patterns of distribution and density of mosquito species are dependent upon several important environmental and man-made factors. The environmental factors like atmospheric temperature and rainfall, also availability of breeding sites and resting places. Knowledge of breeding, resting and biting habits and longevity of a vector species is therefore essential for organizing anti-vector measures and the evaluation of the impact of such measures. Each species is adapted to live in a particular “niche” in the community. In short, the environmental factors which govern the distribution, abundance and density of mosquito are climate, physical and chemical conditions of habitat, hosts, and enemies and in some cases competition can also play an important role. This study is an effort to assess the impact of above mentioned factors on the mosquitoes.

The discovery of species complexes adds new dimensions to vector control. Members of the complexes are genetically isolated by either pre-mating or post-mating barriers. Hence the genetic structure of each species differs from the other and thus has to be taken into account for all types of control strategies. Failure to recognize sibling species of anopheline taxa may result in failure to distinguish between a vector and a non-vector; hence the assessment of the impact of control measures may be seriously misleading if they are carried out on a morphologically defined taxon which could be a mixture of two or more sibling species (Coluzzi, 1988).

Vector control is an essential component of malaria control but has become less effective in recent years, partly due to poor use of alternative control tools,
inappropriate use of insecticides, lack of an epidemiological basis for intervention, inadequate resources and infrastructure, and weak management (WHO, 1995). Changing environmental condition, the behavioral characteristics of certain vectors and resistance to insecticides have added to the difficulties (WHO, 1995). Ecological changes driven by deforestation, human migration and unmanaged urbanization have increased the densities of human hosts and vector breeding sites in some malarious region (Gratz, 1999; Robert et al., 2003). Knowledge of the breeding habits of the sibling species can help in designing optimal vector control strategies (Surendran and Ramasamy, 2005).

The proposed study aims at the following objectives:

1. **Bioecological characteristics of An. culicifacies sibling species.**

2. **Molecular techniques for differentiation of An. culicifacies sibling species.**

3. **Morphological characteristics of An. culicifacies sibling species.**

4. **Cytotaxonomic examination for sibling species in the taxon An. culicifacies.**
Possible outcome of the study:

The proposed study would be highly useful in identifying the sibling species of *An. culicifacies* complex based on their morphological, bioecological and molecular patterns using different molecular markers and tools. The data generated may provide useful information about the various species specific characteristics present in the sibling species of *An. culicifacies*. In conclusion this information will be helpful in better understanding of sibling species complex occurring in these areas and also the patterns of disease prevalence. Such information may also be helpful in developing of keys for sibling species identification and also to develop newer approaches for their control strategies.