Introduction

Malaria is a protozoan disease prevalent in the tropical and some temperate areas. It can be prevented and treated, but is still responsible for the death of about 881,000 persons every year, 90% of whom are from the African continent and about 85% of these deaths occur in children below the age of five (WHO malaria report, 2013). In India, along with malaria other vector-borne diseases such as dengue, Japanese encephalitis (JE), chikungunya, lymphatic filariasis and kala-azar etc. have considerable impact, in terms of morbidity and mortality. However, the major burden of vector-borne diseases in India comes from malaria. Malaria is a complex disease affected by various factors influenced by human activities and environmental conditions such as flood, drought and other disasters. This creates favorable conditions in which mosquitoes can thrive, leading to increased probability for transmission of malaria. According to the National Vector Borne Disease Control Programme (NVBDCP) in India, more than 95% of the population lives at risk of malaria infection. In South East Asia, India is one of the largest countries offering numerous geographical variations and regional ties to the surrounding areas. The Southeast Asia is estimated to contribute about 2.5 million malaria cases to the ever increasing global malaria burden and of this; about 76% cases are contributed by India alone. The vast geographic and seasonal variation in India provides many possible ways for malaria parasite to affect disease severity, its transmission and response to drugs (Kumar et al., 2012).

India harbors about 58 anopheline species, out of which a good number of species, i.e., nine species of Anopheles genus belonging to Cellia subgenus have been designated as vectors of human malaria parasites. Among these, six species (An. minimus, An. fluviatilis, An. culicifacies, An. dirus, An. stephensi, An. sundaicus) are considered as the primary vectors, while three (An. annularis, An. nivipes/An. philippinensis and An. varuna) are designated as secondary vectors (Dash et al., 2007).

All these mosquito species in India except An. stephensi have been characterized as species complexes with a number of morphologically indistinguishable sibling species which vary for their role in malaria transmission (Subbarao, 1998). A
number of studies have been carried out on *An. stephensi*, but till now there is no evidence indicating its existence as a species complex. However, three ecological variants of *An. stephensi* have been reported from many regions. Earlier these ecological variants were considered as races (Sweet *et al.*, 1938) and as subspecies (Puri, 1949). But later they were designated as variants not a subspecies (Rutledge *et al.*, 1970). These three ecological variants, *i.e.* ‘type’, ‘intermediate’ and ‘mysorensis’ can be characterized by egg morphometrics (Rao *et al.*, 1938; Subarao *et al.*, 1987; Sweet and Rao, 1937). The ‘type’ form is an efficient vector of malaria in urban areas. It is largely the ‘type’ form that is responsible for malaria outbreaks in urban areas. It is a thermophilic species and has a longer flight range and maintains a high degree of contact with the human population. In rural areas, it is predominantly a zoophilic species and rests outdoors in cattle sheds, barracks, poorly constructed houses and breeds in fresh water ponds, stream beds, seepage canals and wells. The variety ‘*mysorensis*’ is largely zoophilic and has no role in malaria transmission (Chakraborty *et al.*, 1998; Ghosh *et al.*, 2008; Sharma *et al.*, 1993). The ‘*intermediate*’ is typically recorded in rural and peri-urban localities, but its role in malaria transmission is not known. The existence of ecological variants is further evident by Y–chromosome variation (Saguna, 1992), spiracular index (Nagpal *et al.*, 2003) and frequencies of inversion polymorphism in urban and rural populations in the range of its distribution (Mahmood and Sakai, 1984). However, results of cross-mating experiments were variable ranging from infertility to reduced fertility (Rutledge *et al.*, 1970; Sweet *et al.*, 1938) as opposed to full compatibility between populations.

Malaria today is totally different from malaria in the past and is becoming more severe year after year due to increased complexities such as insecticide resistance in vector and drug resistance in parasite. Under the continued assault of insecticides, vector mosquitoes have developed resistance creating the problem of controlling malaria at the vector level. In India insecticide resistance has been reported in *An. stephensi* from Delhi, Goa, Haryana, Rajasthan and Karnataka. Due to the problem of increasing resistance in the mosquitoes, innovative vector control strategies are being developed as the conventional methods such as indoor residual spraying and insecticide-treated nets are effective but operationally difficult and logistically demanding.
The problems of parasite resistance to antimalarial drugs (Meshnick, 1998) and the spread of insecticide-resistance in vector populations (Collins & Paskewitz, 1995) have prompted the search for alternative, sustainable methodologies to control malaria. It has been suggested that the manipulation of vectors through the introduction of genes conferring refractoriness to the parasite (Collins & Besansky, 1994) could be one such approach. The wide scale release of genetically modified mosquitoes to render vector populations refractory to malaria parasite infection and improved sterile insect techniques are being considered for *An. gambiae* and *Aedes aegypti* (Alphey et al., 2002; Cristophides, 2005; Collins et al., 2000; James, 2000; Windbichler et al., 2011), with the potential to be rolled out to other mosquito vectors if they prove successful. This can be achieved by releasing the transgenic mosquitoes with parasite inhibiting genes in the environment (Atkinson and Michel, 2002). Considerable progress has been made towards the identification of mosquito refractory mechanisms (Atkinson and Michel, 2002; Feldman et al., 1998; Vernick et al., 1995) although the study of possible mechanisms for introducing refractory genes into natural populations of vectors has not advanced much beyond the theoretical stage (Kidwell and Wattam, 1998).

Whether using conventional methods or genetic-based methods of vector control, comprehensive knowledge and understanding of the biology, distribution, genetic population structure and gene flow regime of the vector species is essential for effective vector control. This understanding is critical in the determination of the spatial and temporal scale required for such gene introductions. Focal gene introductions would be unsuitable for populations experiencing little or no gene exchange, as the genes of interest would not spread. Determining genetic structure can help to understand heterogeneities in disease transmission due to genetically distinct vector populations and to predict the spread of genes of interest, such as those involved in insecticide resistance or refractoriness (Ndo et al., 2010). Such information may also allow a more rational management of insecticides to defer the development of resistance and could help explain the role of vectors in geographical variation in malaria transmission. The population genetic study can enable not only the identification of parasite origins but also routes of population movement, and therefore the likelihood of successful elimination within a given region with respect to parasite
A wide variety of genetic markers are available for population genetic study of malaria vector. These include traditional tools and classical genetic markers to highly polymorphic markers. Out of these, microsatellites are the most important because they have the potential to provide contemporary estimates of migration, have the resolving power to distinguish relatively high rates of migration from panmixture, and can estimate the relatedness of individuals (Selkoe and Toonen, 2006). In a biological/evolutionary context they are useful as markers for parentage analysis. They can also be used to address questions concerning degree of relatedness of individuals or groups. From there we can move up to the genetic structure of subpopulations and populations (using tools such as F-statistics and genetic distances). They can be used to assess demographic history (e.g., to look for evidence of population bottlenecks), to assess effective population size ($N_e$) and to assess the magnitude and directionality of gene flow between populations. In addition, they are favored because they found in large numbers and evenly spaced in the genome. Microsatellites were selected for the present genetic analysis because in addition to above said qualities, they are selectively neutral and found to be variable even in populations which have low levels of allozyme and mitochondrial variation. Additionally, they can be analyzed via Polymerase Chain Reaction (PCR).

Madhya Pradesh is located in the Central part of India with geographical area of about 308 thousand km$^2$. In Madhya Pradesh, 76,429 km$^2$ of area constituting 31% of the total geographical area of the state is covered with thick forest. The forests cover of Madhya Pradesh constitutes 12.44% of total forest cover in India. Despite 50 years of rigorous efforts to manage this disease, malaria is still a major health problem in Central India. Madhya Pradesh is one of the worst malaria affected states in India (Breman, 2001). 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) contributes for more than 60% of reported annual malaria cases in India (Sharma et al., 2014). Madhya Pradesh alone contributes roughly 27% of the total malaria cases among the other Indian states (Diamond-Smith et al., 2009).
Area wise Madhya Pradesh is second largest state of India with the total area of Madhya Pradesh state divided into 50 districts. It is sixth largest state in India by population, with 75% of the total population residing in the rural areas. Tribes constitute about 20% of total population of Madhya Pradesh.

Malaria is complex in this state due to vast tract of forest with tribal settlement (Singh et al., 2004a; Mishra et al., 2012). The nomadism practiced by tribal people and various development activities has posed a sizeable problem with increase in malaria cases (Singh et al., 2003; Singh et al., 2004a, b). Malaria control remains difficult in forested areas, owing to the complexities of human behavior and of scaling up malaria-control measures (Singh et al., 2014). The treatment and control of malaria has become more difficult with the spread of insecticide resistance in vectors (Mishra et al., 2012).

Malaria control in Central India relies mainly upon two powerful tools, i.e. vector control by indoor residual spraying (IRS) using DDT (1 gm/m2) and Parasite control using chloroquine (CQ). Their use is becoming challenging day by day due to increased resistance in vector against DDT and against chloroquine (CQ) in malaria parasites.

Control of malaria in tribal areas needs specific approaches and management strategies. Keeping this in mind Enhanced Malaria Control Project (EMCP), also known as Tribal Malaria Action Plan was launched by National Anti-Malaria Programme (NAMP) in collaboration with World Bank in September 1997, to control malaria specially focusing on tribal areas. Amount of 165 million USD was credited to Indian government for the implementation of this project in 100 areas in 8 north Indian states having high malaria risk factor (Barat, 2006; Sarbib et al., 2006). The program mainly focused on inaccessible area with tribal settlement having high malaria burden with clear predefined eradication strategies enabling NAMP to make shift from unyielding conventional methods to modern malaria control strategies. Though, the EMCP project was designed specifically to benefit mainly the tribal populations in Northeastern states, it has the flexibility of diverting resources in the case of malaria outbreak in any other area (Singh et al., 2009). Out of existing 50 districts in Madhya Pradesh only 20 districts contributing 60% of malaria cases were included under EMCP
project. Initially, only 18 districts were included but later on extended to 20 due to increasing malaria incidences in these areas. The project after its completion in December 2005 is under sustenance phase.

Due to various novel control strategies applied during EMCP project, a gradual decline in malaria cases was observed initially, but later on from 2000 onwards malaria cases stated increasing endangering the whole project. In the year 2000, maximum numbers of malaria cases were reported estimating an overall increase of 29% in malaria cases compared to the year 1996, before introduction of EMCP project. Thereafter, number of malaria cases and *P. falciparum* decreased moderately in 2002 and 2003. This decline in the malaria cases could not be continued especially in the case of *P. falciparum* infections and an increase of about 40% in malaria cases due to *P. falciparum* was observed in the year 2004, with only a marginal reduction in the overall malaria cases. In the following years *i.e.* 2005, 2006 and 2007, a remarkable decline in overall malaria cases was observed. In spite of declining malaria cases, the share of malaria cases due to *P. falciparum* increased from 33% in 1996 to 62% in 2007. The similar situation was observed in number of deaths due to malaria as many explosive outbreaks of malaria were recorded in Madhya Pradesh from year 1996 to 2006 resulting in increased death toll from 6 to 35 (Singh *et al*., 2009; Singh *et al*., 2013). Even in districts where EMCP project was not applied, several focal malaria outbreaks occurred between the years 2003 to 2006 resulting in a rapid increase of malaria incidence from 25% to 50% (Singh *et al*., 2009).

There seems to be no information available about the population genetic structure and gene flow of *An. stephensi* in India, except in Rajasthan (Vipin *et al*., 2010a) where gene flow in *An. stephensi* was studied across the Aravalli mountain range and in Haryana (Vipin *et al*., 2010b) where genetic differentiation between three ecological variants i.e. ‘type’, ‘intermediate’ and ‘mysorensis’ was studied using microsatellite markers.

No such study appears to have been carried out in Central India. Therefore, the present investigation was undertaken considering complex topography in Central India. Madhya Pradesh was selected for current study owing to its complex topography with
vast forest tract, huge river basins and several different mountain ranges. Two of most famous mountain ranges in Madhya Pradesh are Satpura and Vindhya mountain range. These mountain ranges give rise to main river systems: Narmada and Tapti running from east to west and Sone, Chambal, Mahanadi, Betwa west to east which provides ample breeding areas for \textit{Anopheles} mosquitoes. It was hypothesized that “There should be significant genetic structuring in \textit{An. stephensi}, owing to its complex topography”.