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Mini Review

Population genetic structure of malaria vector *Anopheles stephensi* Liston (Diptera: Culicidae)

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Malaria is a complex disease that afflicts human today. Malaria epidemiology is associated with drug resistance in parasite and differential distribution and insecticide resistance in vector. Efforts are being made to eradicate malaria but burden of malaria is still increasing. Vector control is essential for malaria prevention strategies. Knowledge of population genetic structure is pre-requisite for determining prevention strategies, particularly using transgenic mosquitoes. Population genetic study can predict level of gene flow between different populations. *Anopheles stephensi* Liston is urban vector of malaria in Indo-Pakistan subcontinent. About 12% of malaria cases of malaria in India are contributed by *A. stephensi*. Studies conducted on population genetics of *A. stephensi* using various markers in different parts of the world are discussed in this communication.

Keywords: *Anopheles stephensi*, Genetic markers, Malaria, Microsatellite, Population genetics

Introduction

Anopheles stephensi belongs to subgenus Cellia and series Neocellia[1]. *Anopheles stephensi* Liston is an established vector of malaria in India[2], south Iran[3], Pakistan and Afghanistan[4]. It is also distributed in Iran, Iraq, Oman, Saudi Arabia, South China, Myanmar, Thiland and east of Bangladesh. Neither it is reported in Himalaya, Nepal and Sri Lanka[5] nor in other parts of world. A number of studies have been carried out on this species, but till now there is no evidence which indicated that this is a species complex. However, three ecological variants of *A. stephensi* have been reported in all these regions. Earlier these ecological variants were considered as races[6] and as subspecies[7]. But later they were designated variants not subspecies[8].

The three ecological variants of *A. stephensi* 'type', 'intermediate'' and 'mysorensis' can be differentiated on the basis of differences in number of ridges on egg floats[9-11] and on the basis of spiracular indices[12]. The 'type' form is an efficient vector because of its zoophilic nature[11]. Cross mating experiment showed that a definite incompatibility exists between *A. stephensi* 'type' and 'mysorensis'[6]. But fertile offspring on cross mating between both forms were also reported[8,13]. Recently phylogenetic inference of Indian malaria vectors has also been drawn using multilocus approach. They have corroborated the divergence times of radiation of different species of *Anopheles* during the late cretaceous period. Further *A. stephensi* and *A. annularis* were placed in the same monophyletic clad belonging to neocellia series[14].

About 12% of malaria cases in India are contributed by *A. stephensi*[15]. A number of efforts have been made to eradicate malaria in different parts of world. But burden of malaria is still increasing. The variable epidemiology of malaria is associated with high parasitic genetic diversity, rapid evolving insecticide resistance and differential distribution of vector species. Insecticide resistance is causing operational problems for control programmes. Resistance to DDT, dieldrin and malethion is reported in *A. stephensi* in Iran, Iraq, Saudi Arebia and Indian subcontinent[16-18]. In India insecticide resistance has been reported in *A. stephensi* from Delhi, Goa, Haryana, Rajasthan and Karnataka[19-23]. Vector control is foundation of malaria prevention strategies. Genetic control of vector population to make them unable to transmit the parasite is considered a better way to control malaria transmission. A comprehensive knowledge of the population genetic structure of target species and forces that generate and maintain this structure is essentially required to access feasibility of different strategies to control malaria. Distribution pattern of malaria vector, *A. stephensi* and population genetic studies conducted in different parts of world using various markers have been shown in Fig. 1.

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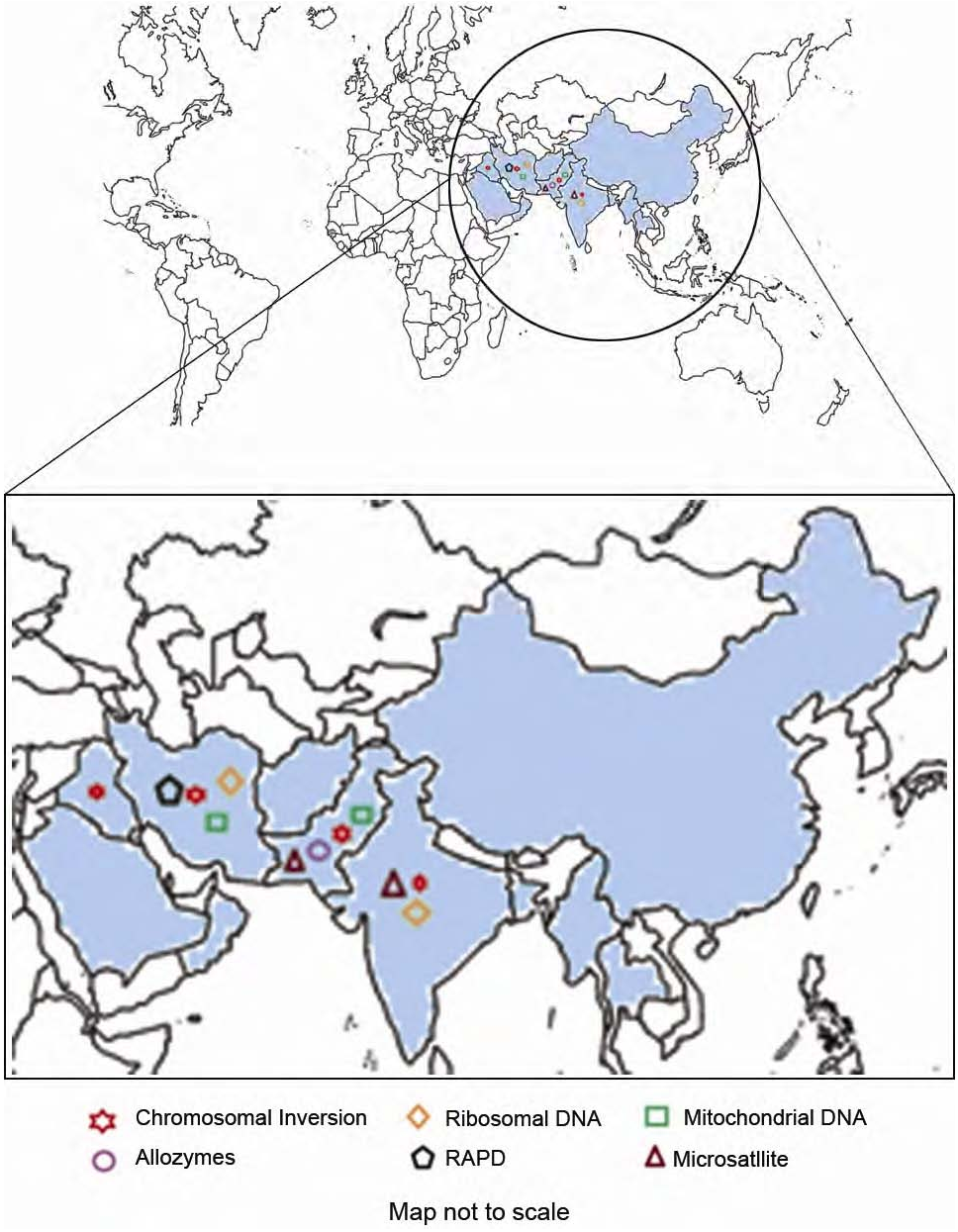

Fig. 1—Distribution pattern and population genetic studies of *A. stephensi* conducted in different parts of world using various markers.

A wide variety of genetic markers are available for population genetic study of malaria vector[24]. These include traditional tools and classical genetic markers to highly polymorphic markers. The present review summaries population genetic study of *A. stephensi* conducted in different parts of world using different genetic markers.

Traditional markers

Chromosomal polymorphism—Cytogenetic analysis has remained a reliable tool for species identification and population genetic study. Chromosomal number in *Anopheles* is 6[25]. Inversion polymorphism is widespread in *Anopheles.* Studies of chromosomal inversion are helpful in understanding the population genetic structure of any species. Chromosomal polymorphisms so far in *Anopheles* involve paracentric inversion. Single pericentric inversion was observed in *A. pulcherrimus*[26]. There is no random distribution of chromosomal inversions on chromosomal arms[27]. Maximum rearrangements are observed on arm R of chromosome 2 in subgenus

Cellia[28]. Cytogenetic maps of *A. stephensi* have been developed that are useful in cytotaxonomic and population genetic study[29]. Similarly cytogenetic photomaps of *A. stephensi* were developed from salivary gland[30] and ovarian nurse cells[29,31]. Cytogenetic photomap is a genetic term referring to the visual appearance of a chromosome when stained and examined under a microscope, visually distinct regions, called light and dark bands give each chromosomes a unique appearance.

Coluzzi *et al.*[32] reported six autosomal paracentric inversions in 10 strain of *A. stephensi* from India, Pakistan, Iran and Iraq. Three overlapping inversion (b, c and d) were observed on arm R of chromosome 2, one inversion on arm L of chromosome 2 and two inversions were observed on arm L of chromosome 3. No chromosomal inversion was observed on arm R of chromosome 3 and sex chromosome. *A. stephensi* of Delhi (India) was found to be more polymorphic than from Karachi (Pakistan). Inversion b was found to more prevalent in all populations and inversion d was reported only in Delhi strain.

Sixteen polymorphic inversions were reported in Pakistani population of *A. stephensi*[33]. Previously described inversion 'b'[31] and two new inversions 'e' and f' were observed on arm R of chromosome 2. 'c' and 'd' inversions were not observed in these populations. On arm L of chromosome 2, two paracentric inversions 'c and d' were observed. But in population of Karachi inversion'd' was not detected. Coluzzi *et al.*[31] has not reported any inversion on 3R but in population of Karachi 'b and c' inversions were observed on this chromosome and inversion 'a' was observed on the same chromosomal arm of *A. stephensi* donated by National Institute of Malaria Research, New Delhi. Out of nine inversions observed on 3L, seven inversions were new and remaining two similar to described by Coluzzi *et al*[32]. Therefore, 12 new and 4 old inversions were observed in field populations. The inversions that were observed in rural population were not observed in urban populations. Similarly, Subbarao[34] observed 10 inversions in urban population of *A. stephensi* in India while in rural population only b inversions on chromosome 2 and inversions h1 in heterozygous form in only one specimen was observed. Inversion h1 was not detected in urban population.

Allozyme—Allozymes are allelic variants of enzymes encoded by structural genes. Allozyme are cheap, easy to use, quicker to isolate and develop. Alozyme analysis has been used in population genetic study of *A. minimus*[35], differentiating species groups and sympatric taxa of *A. albitarsis*, *A. culicifacies* complex and *A. flavirostrisl / filipinae*[36-38].

Van Dreil *et al.*[39] analyzed allozyme variability at 16 loci in 11 lines of *A. stephensi* from Pakistan. Six lines were field collected populations and remaining was laboratory reared. Only six loci were found to be polymorphic. Number of alleles ranged between 1.31-1.63 and degree of polymorphism varied between 0.188-0.375. Population specific alleles were not observed.

Modern markers

mt DNA—mt DNA has been the most popular marker for population genetic study over the last three decades. The reason for adoption of mt DNA as a marker is that it is easy to amplify because it appears in multiple copies in cell. Mitochondrial genes are strongly conserved in animals, no intron and very short intergenic regions. mt DNA has been widely used in population genetic study due to its rapid evolution, maternal inheritance and no recombination. The complete sequence of mt DNA of *Anopheles* is near about 15500 bp long. mtDNA genome of *A. quadrimaculatus*, *A. gambaie*, *A. funestus* and *A. darlingi* are available till date[40-43]. mt DNA contain genes for 2 ribosomal RNA, tRNA, ND1-6 (NADH dehydrogenase), COI-III (cytochrome oxydase) and ATP synthase. mt DNA has been utilized in investigations of a wide variety of *Anophelines* using COI-II region. But no such study has been conducted using COIII sequence.

Genetic structure of different populations and biological forms of *A. stephensi* of South Iran was studied using mt DNA by Oshangi *et al.*[44] This was the first DNA based study on *Anopheles stphensi*. Mosquitoes collected from different regions of South Iran and an Indian strain (BEECH) donated from Liverpool School of Tropical Medicine, UK were analyzed. All three biological forms did not showed any inter or intrapopulation polymorphism in COI and COII region. Digestion of PCR products with different restriction enzymes also not showed any polymorphism except for DraI. Restriction enzyme DraI produced two haplotype A and B. Both haplotypes were observed in 'type' and 'mysorensis' form but 'intermediate' form consisted of haplotype B. Indian strain (BEECH) was 'type' form and found to be identical in sequence of COII of mysorensis

form haplotype A of Baluchistan, Iran. They have demonstrated that lack of geographical barriers was responsible for similarity and gene flow between different ecological forms.

Ali *et al.*[45] has also analyzed COI region of mtDNA of *Anopheles stephensi* from Pakistan. Four haplotypes were observed but genetic differentiation was not observed despite of geographical distance. No such study has been carried out from any other part of the world.

Ribosomal DNA—Nuclear ribosomal genes are the largest and most ancient multigene family, occurring in tandem repeats, hundred or thousand of copies long. Nuclear ribosomal genes are a mosaic of variability and are therefore useful for broad range of comparative studies. rDNA is composed of tandemly repeated transcriptional units 18s, 5.8s and 28s separated by two Internal Transcribed Sequences, ITS-1 and ITS-2, respectively. The ITS regions show high degree of variation within species as well as difference between them, therefore it has great potential as a genetic marker in a wide variety of studies. ITS-2 sequence has been used to study phylogenetic relationships and to distinguish closely related species of mosquitoes[46,47]. These regions are therefore also used as a molecular marker for phylogenetic and population genetic analysis of various species of *Anopheles*. Population genetic structure of *A. darling* was studied by Mirabello[48] using rDNA ITS-1 and ITS-2 as marker. But, no study appears to be conducted in *A. stephensi* using rDNA ITS-1 region.

rDNA-ITS2 region of *A. stephensi* collected from Iran was analysed by Djadid *et al.*[49] to examine the hypothesis that *A. stephensi* is a single species despite of its geographical range. The length of ITS2 region ranged from 466 to 468 base pairs. Based on this sequence *Anopheles stephensi* in Iran divided into three groups and these three types of ITS2 sequence are due to presence of 'type', 'intermediate' and 'mysorensis' forms in Iran. Transition, transversion and single base pair insertion are responsible for polymorphism. It was concluded that these three types of sequence are due to presence of three ecological variants of *Anopheles stephensi* in Iran. Two microsatellite regions (CA repeats) were also reported in the ITS2 sequence. Presence of CA repeat in ITS2 region resulted in longer region in *Anopheles stephensi* as compared to other species of *Anopheles* found in Iran. Phylogenetic analysis based on ITS2 sequence unveiled that different populations of *Anopheles stephensi* are clustered in a single main branch and main branch further divided into three sub-branches.

Alam *et al.*[50] studied rDNA ITS2 and D_3 sequence of field collected (mosquitoes collected from Mysore, Bhuvneshwar and Pondicherry) as well as laboratory colonized Indian *Anopheles stephensi* (type and mysorensis). It was for the first time that Indian population of *Anopheles stephensi* sequenced for ITS2 locus. Size and sequence of ITS2 and D_3 loci were found to be same in 'type' and 'mysorensis' forms. Only one ITS2 haplotype was observed. Indian species of *Anopheles stephensi* showed 100% sequence similarity with one haplotype out of eight haplotype of Iranian population. On the basis of this study it was concluded that 'type' and 'mysorensis' forms cannot be distinguished using ITS-2 and D_3 loci and Indian and Iranian strains of *Anopheles stephensi* are single species.

RAPD—RAPD markers are DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence. Like other PCR based methods, RAPD is also an efficient tool to differentiate geographically and genetically isolated populations. RAPD proved to be an efficient and quick method to distinguish two morphologically indistinguishable malaria vectors, *Anopheles gambaie* and *Anopheles arabiensis*[51]. Posso *et al.*[52] used RAPD markers to study genetic variations of *Anopheles nuneztovari* populations from Western and Northeastern Colombia. Diversity of *Anopheles darling* was analyzed by Pinedo-Cincina *et al.*[53] using RAPD.

Djadid *et al.*[49] analyzed RAPD loci along with rDNA-ITS2 in field population of *Anopheles stephensi* from Iran. Three primers AB-1, AB-4 and UBC-353 were selected for analysis. AB-1 amplified region was found to be highly conserved. Two microsatellite regions (GT repeat) were found in this segment and this region showed 100% similarity with fme 1 gene of *Drosophila*, *Mus musculus* and *Homo sapiens*. AB-4 and UBC-353 amplified regions showed polymorphism in rural and urban population but it was unclear whether these samples belonged to 'type' or 'mysorensis' form. Two microsatellite regions (GT repates) were observed in RAPD sequence. No other study seems to have been carried out from other parts of the world.

Microsatellite—Microsatellites are simple tandem repetitive DNA. These are densely distributed throughout eukaryotic genome, making them preferred

marker for genetic analysis[54,55]. Microsatellite loci are selectively neutral found in large number and evenly spaced throughout genome. Microsatellite markers can be used in genome mapping, paternity analysis and population genetic studies. Use of microsatellites in population genetic study is two decade old. Since then they are the first choice for most of population genetic and molecular ecology study.

The field of population genetics and ecology has been revolutionized by the discovery of microsatellite sequences[56]. Microsatellite DNA has become a popular tool for genetic studies of *Anopheles* mosquitoes. Up to 150 polymorphic loci have been characterized for seven species in *A. gambaie* complex[57]. Similarly, investigators have also analyzed population genetic structure of *A. stephensi* using microsatellite polymorphism. Veradi *et al*.[58] developed a set of 16 microsatellite markers of *A. stephensi* and also analyzed 24 individual collected from Northwest Frontier Province of Pakistan at these 16 loci. Out of 16 microsatellite loci 13 loci were found to be highly polymorphic.

There are two studies on the population genetics of *A. stephensi* using microsatellite loci developed by Veradi *et al*.[58] The first is from two villages in irrigation zone of Indus Basin Irrigation System (IBIS) and one village outside the irrigation scheme in Northwest Frontier Province (NWFP), Pakistan by Ali *et al*.[45] They used seven microsatellite loci for analysis. The microsatellite loci were found to be moderate to highly polymorphic. They have reported that there is no significant variation between populations. The study indicates that negligible genetic differentiation occurs within the population analyzed irrespective of geographical distance between sample collection sites.

However, the second study pertains to be from North-West India. Vipin *et al*.[59] studied gene flow in *A. stephensi* across Aravalli Hills. They concluded that Aravalli hills are not working as effective barrier to gene flow. Vipin *et al*.[60] also examined the genetic diversity and gene flow within three ecological variants of *A. stephensi* in India. Low level of gene flow and high genetic differentiation was observed between ecological variants. A significant negative correlation between Fst and geographical distance was observed among three variants which indicate that distance played no role in genetic differentiation.

In population of Pakistan locus E12 and A7 were found to be highly polymorphic but in Indian population of *A. stephensi* locus A7 was not analyzed due to unscorable band and locus E12 was moderately polymorphic. Locus G11 was highly and E7T least polymorphic in Indian population. While in population of Pakistan both loci were not studied. That's why comparison of Indian and Pakistani population of *A. stephensi* is not possible.

Population genetics can be better understood by knowing the chromosomal location of microsatellite marker with respect to polymorphic inversions. Recently, Kamali *et al*.[61] mapped 12 microsatellite loci on ovarian polytene chromosomes of *A. stephensi* using FISH. Ten microsatellites were mapped inside previously described polymorphic chromosomal inversions. Out of these 10 microsatellites four were found inside the 2Rb (largest) inversion. Remaining 2 microsatellites were cytogenetically indistinguishable because they hybridized to same band.

Single Nucleotide Polymorphism—Single nucleotide polymorphism (SNP), a variation at a single site in DNA, is the most frequent type of variation in the genome. SNP is widely used in analysis of population and species. It is also used in population genetic study of *A. gambiae*[62-64] and *A. funestus*[65]. But no such study has been conducted on *A. stephensi* using SNP markers.

Conclusion

A. stephensi urban malaria vector has been the focus of genetic studies for several years. Researchers are using a wide combination of genetic markers to examine population structure, genetic differentiation and gene flow of this species. However, the present information is insufficient to explain population genetic structure of *A. stephensi*. An important limitation of present studies is that these are confined to small areas. It is extremely important to estimate gene flow by extending research to other sites. Further studies should use extensive sampling of field populations in large geographical areas. It could provide more knowledge of gene flow and factors that affect gene flow. One more drawback of these studies is that investigators used different markers in different regions of world due to which comparison of data is not possible. Study using combination of traditional and modern molecular markers will contribute to more detailed understanding of population genetic structure of *A. stephensi*. This study may serve as a basis of malaria control programme in those parts of world where *A. stephensi* is main malaria vector.

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