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Identification and molecular characterization of *Anopheles* mosquitoes in some rural areas of West Bengal, India

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ABSTRACT: During the year 2015-2016 a systemic survey has been undertaken to know the temporal and spatial distribution of *Anopheles* mosquitoes in Southern and Northern parts of West Bengal. *Anopheles vagus* and *An. subpictus* predominate in southern Bengal while *An. barbirostris* is more abundant in northern Bengal. *Anopheles* species were identified morphologically as well as by the sequencing of ITS 2 region of rDNA. © 2018 Association for Advancement of Entomology

KEY WORDS: *Anopheles*, distribution, molecular identification

INTRODUCTION

Thirty-six percent of the global population becomes the victim of the disease malaria and almost two thousand twenty million of people are at the risk of the same in ~90 countries. In the South-East Asian region India itself estimates for approximately two third of the confirmed malarial cases. In accordance with Singh and Sharma (2002) the central and eastern parts of India are the most vulnerable areas of the disease malaria. According to World malaria report, 2009 five states including West Bengal account for sixty percent of cases of malaria. The *Anopheles* is the only mosquito taxon that is root cause for the transmission of malaria. *Anopheles* also transmits dirofilarial nematodes and arboviruses of veterinary importance (Ramachandra, 1984). 444 formally named species and 40 unnamed species complexes are identified as distinct species of *Anopheles* (Harbach, 2004). India possesses about 58 morphologically identified species of *Anopheles*. It has been narrated that about 13 species are available in Kolkata and sub urban areas of West Bengal. Out of 58 species of *Anopheles* found in our country, six taxa are considered as major malaria vectors with different regional specificities. As for example, *Anopheles culicifacies* is a vector of rural areas in our country and generates about sixty five percent of malaria per year. *An. fluviatilis* is found in the plains as well as in foothills and is responsible for almost 15% of malarial cases, *An. minimus* is an important vector in northeast, *An. dirus* is found in the forest areas of northeastern states where as *An. sundaicus* is mainly present in Andaman and Nicobar islands, and *An. stephensi* is a dominant vector mosquito in urban areas like Kolkata of West Bengal. Population load of this genus is mainly generated by the *An. subpictus* and *An. vagus* in different areas of West Bengal (Paul *et al.*, 2015). Each of the 40 species of anophelines transmitting human malaria differ in their transmission potential (WHO, 2005). All these mosquito species except *An. stephensi* have been characterized as species

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complexes with number of sibling species and have different roles in the transmission of malaria. The density of these vector mosquitoes varies with the seasons as well as available habitats. Therefore studies of temporal and spatial abundance of different types of *Anopheles* mosquitoes are essential for formulating the controlling strategies. The Ribosomal DNA has been used extensively and very successfully for phylogenetic analysis of both closely and distantly related organisms. Due to overlapping morphological characteristics of malaria vectors and difficulties in their identification based on morphological features, it becomes essential to distinguish closely related groups of *Anopheles* using alternative methods other than morphological taxonomy. Among the available alternative methods, the cytological method of polytene chromosome based identification has been utilized to distinguish of the cryptic species. However there are few problems namely, it defines only female mosquitoes, further it cannot be utilized in females that are unfed or fully gravid; moreover the method requires high level of technical expertise. Biochemical assays are also developed for species identification in *Anopheles* in some cases. Therefore the Ribosomal DNA probes are used for species identification in *Anopheles* (Collins *et al.,* 1996). The Polymerase chain reaction (PCR) based diagnostic assay reflects its own advantage in taxonomy as it reliable and sensitive. The PCR methods have been effectively used to distinguish Anopheles species.The internal transcribed spacer 2 (ITS2) sequence, which like the internal transcribed spacer 1 (ITS1), evolves faster than coding sequence. So, for isolation and molecular characterization of closely related *Anopheles* mosquitoes the internal transcribed spacer 2(ITS-2) region of ribosomal DNA (r DNA) has widely been used (Walton *et al.,* 1999). ITS2 regions alone have been successfully utilized in distinguishing closely related mosquito species that belongs to various genera such as *Anopheles* (Marrelli *et al.,* 2006), *Culex* (Toma *et al.,* 2000) and *Aedes* (Beebe *et al.,* 2007). Recent developments in the field of DNA-based tools, such as allele-specific PCR, PCR restriction fragment length polymorphism and single-strand conformational polymorphism assay (Wilkerson, 2005) have proven to be potential techniques for the differentiation of numerous *Anopheles* species. The present investigations were concentrated for the proper identification of the *Anopheles* species and the comparative study of sequence variations in ITS2 of the different species of *Anopheles* found in the studied areas of both south and north Bengal.

MATERIALS AND METHODS

***Collection of mosquito*:** Adult *Anopheles* mosquitoes have been collected from different areas of West Bengal. The mosquitoes have been captured in early morning (6-8 am) from different biotopes like cattle sheds and human dwellings, near to cattle shed by using manual aspirator, when the mosquitoes take rest after feeding at night.

Location	Lattitude/ Longitude
1. Mogra (Hooghly District)	$22^0$59N/ $88^0$22E
2. Singur (Hooghly District)	22.82N/ 88.23E
3. Bhotpatti (Jalpaiguri District)	26.54N/ 88.72E
4. Berubari (Jalpaigur***i*** District)	26.42N/88.70E

It is established that Sibling species A or fresh water form of *An. subpictus* is a potential vector of malaria in some regions of Hooghly district of West Bengal (Chatterjee and Chandra, 2000). Again the sub Himalayan Dooars area of the Jalpaiguri district in West Bengal is an endemic area for malaria. Rudra *et al*. (2010) reported that *Anopheles minimus, An. varuna, An. vagus, An. maculatus, An. fluviatilis, An. hyrcanus, An. barbirostris, An. culicifacies* etc. have been recorded from the tea garden dwellings of the Jalpaiguri district. Therefore the abovementioned areas have been selected for our present survey.

Identification of species based on the external morphology:

Stereozoom of Dewinter Technologies were used for the identification and for the classification of the physiological stages. All larvae were reared up to adult stage in the water collected from the water bodies from where the larvae were collected. Dried

specimens were used in morphological and molecular identifications. The collected specimens were morphologically identified according to the identification key of Christophers (1933) and Nagpal *et al*. (2005).

DNA isolation and PCR amplification:

DNA was isolated from individual adult mosquito by phenol chloroform extraction method following the protocols of Ausubel *et al*. (1999), Neetu and Choudhury (2005), Choudhury and Sharma (2006) and standardized in our laboratory. The ITS2 region of r DNA was amplified using the specific forward and reverse primer (FP, RP) consisting of 20 - 21 base oligomers having the sequence 5' TGTGAACTGCAGGACACACAT-3' (CODE 46JB) and 5'- TGTGCTTAAATTCAGGGGGT-3' (code 47JB) respectively. A PCR master mix was prepared by mixing 10X PCR buffer, dNTP mix (100mM each), $MgCl_2$, Taq polymerase, double distilled water and the template DNA. The thermal cycling condition was: initial denaturation at 95^0C for 5 min followed by 40 cycles of denaturation at 95^0C for 30 sec/1 min, annealing at 50^0-60^0C for 1 min, extension at 72^0C for 2-5 min and again final extension at 72^0C for 10 min. The PCR product and standard DNA ladder was electrophoresed in 2% agarose gel and visualized by ethidium bromide.

Statistical analysis: Mean, Standard Deviation, Standard Error were calculated using Graphpad software and chi square test was performed to make it clear whether there were any significant variation of population density of different species of *Anopheles* in different seasons.

RESULTS AND DISCUSSION

Adult *Anopheles* mosquitoes were identified morphologically. In *An. subpictus* maxillary palp possesses three pale bands. Maxillary palp is with sub-apical pale band 0.33or less in length of pre-apical dark band which is 0.5 or more in length of apical pale band. In *An. vagus* the apical pale band is larger than sub-apical pale band that is equal to pre-apical dark band (Fig. 1). Maxillary palp of *An. barbirostris* is totally black and bushy (Fig. 4). In south Bengal the mean abundance of *An. subpictus* is maximum during the period of March-May (Fig. 2a) season while *An. vagus* predominates during June to August (Fig. 2b). In case of north Bengal *An. barbirostris* could be collected throughout the year but it is most abundant during September to November and *An. pseudowillmori* is the least abundant among the collected species and it has been mostly collected during the period of September to November (Fig. 2c and 2d).

Length of ITS 2 sequences and % of GC content of the collected specimens, *An. subpictus* and *An. vagus* collected from the South Bengal, respectively were 686 bp and 831 bp and both are rich in GC 55.9 and 56.8 % respectively. Length of ITS 2 sequences and % of GC content of the collected specimens from North Bengal, indicated that *An. barbirostris* collected from North Bengal richness in the ITS 2 sequence (872), but the *An. pseudowillmori* showed 452; whereas percentage of GC content was 54.7% in *An. barbirostris* and 52.1 % in *An. pseudowillmori*.

The Gene Accession Numbers of the collected species are given in Table 1.The sequences have also been subjected to Spectral Repeat Finder (SRF) and Tandem Repeat Occurrence Locator (TROLL) programs (Sharma *et al.*, 2004; Benson, 1999) for identifying the occurrence of interspersed and tandem repeats respectively. The SRF represents various repeats which are further categorized as dimers, trimers, tetramers, pentamers and polymers. In the present sequence AC repeat shows the highest copy in *An. subpictus* where as CA and TG repeats are mostly found in *An. vagus* (Table 2). Tetramers, pentamers as well as polymers are present in both of the species populations. In Northern Bengal *An. barbirostris* predominates but *An. pseudowilmori* has also been found. TG and CA dimers show highest copies in *An. barbirostris* where as in *An. pseudowilmori* AC dimers are highest in numbers and in this species no polymers are found (Table 3). It is known that *An. pseudowillmori* is one of the predominant malarial vectors in Tibet (Song, 2009) but record of its occurrence in West Bengal is very poor. But this study reveals the presence of this

Table 1. Collected species with the collection sites and their gene accession numbers

Site of collection	Name of species	Accession Number
Hooghly district, South Bengal	*An. subpictus*	KC191825
Hooghly district, South Bengal	*An. vagus*	KT 716079
Jalpaiguri district, North Bengal	*An. barbirostris*	KU378200.1
Jalpaiguri district, North Bengal	*An. pseudowillmori*	KU378201.1

Table 2. Spectral repeat finder (SRF) based ITS2 sequence characteristics of *An. subpictus* and *An.vagus*

Sequence	*An. vagus*	*An. subpictus*
Dimers		
AC	130	113
TG	138	116
CA	138	100
Trimers		
GTG	48	42
Tetramers		
CCTA	4	5
GCAT	13	8
CGTG	15	10
GTGC	23	13
TGCA	16	8
GCGT	16	10
Pentamers		
GGTGC	6	3
Polymers		
GACGTG	0	1
CTCGGCGTG	1	1

Table 3. Spectral repeat finder (SRF) based ITS2 sequence characteristics of *An. barbirostris* and *An. pseudowilmori*

Sequence	*An. barbirostris*	*An. pesudowillmori*
Dimers		
AC	109	51
TG	129	50
CA	129	50
Trimers		
GTG	28	13
Tetramers		
CCTA	2	2
GCAT	7	2
CGTG	6	3
GTGC	8	3
TGCA	8	0
GCGT	3	4
Petamers		
GGTGC	1	1
Polymers		
GACGTG	3	0
CTCGGCGTG	0	0

species in North Bengal and it shows 93% similarity with the existing sequence in gene bank. According to Zhang (1998) repetitive sequences are important in a number of regulatory functions and are principle causes of genomic instability. Tandem repeats were lacking in all the collected species which may reveal the genomic stability of the collected specimens. The tetramer TGCA has restriction site for a restriction enzyme HpyCH4IV. The present studies suggest that the distribution of sequence polymorphism throughout the populations of a species is a type of intra-genomic mechanism that can promote genotypic variations by constantly changing sequences. Chi square tests revealed that there exists significant difference in seasonal abundance of different *Anopheles* mosquitoes in South-Bengal within the groups *An. subpictus* and *An. vagus* ($\chi^2 = 45.797^{**}$ as against the table value 16.919). But there is no significant difference among seasonal abundance of different *Anopheles* mosquitoes in North-Bengal within the groups of *An. barbirostris* and *An. psudowillmori* ($\chi^2 = 3.928^{NS}$ as against the table value 16.919).

ITS2 rDNA is a non-coding DNA sequence that is reliable and dependable for differentiation of closely related species and restriction fragment length

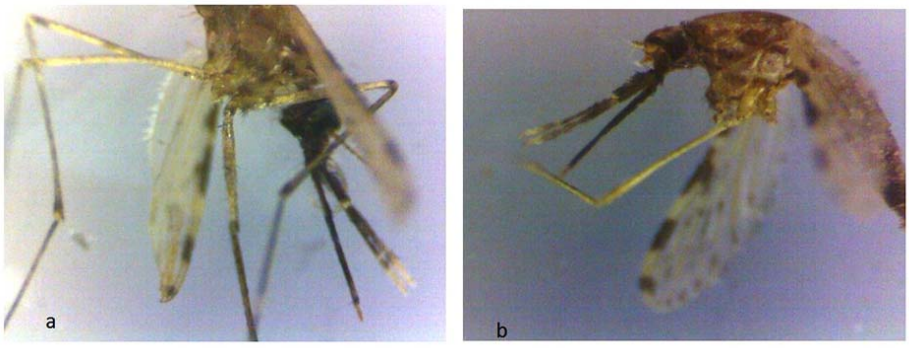
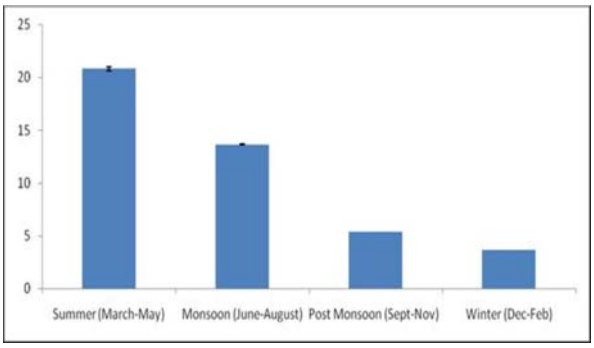
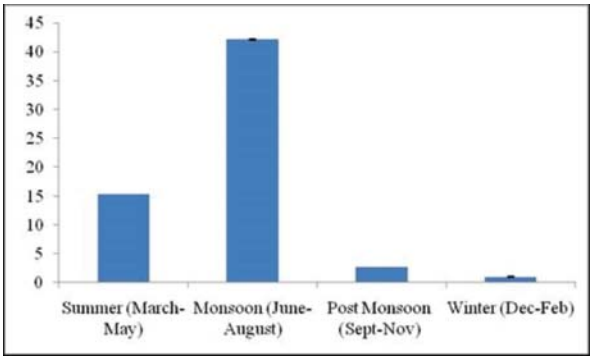


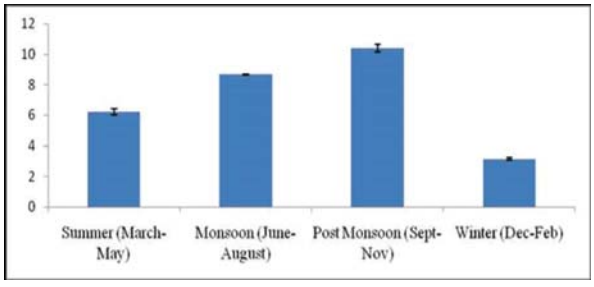
Fig. 1 Morphological identification of *Anopheles subpictus* and *An. vagus* collected from South Bengal.



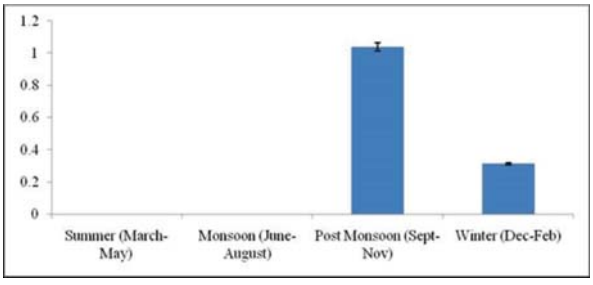
2a: An. subpictus



2b: An. vagus



2c: An. barbirostris



2d: An. pseudowillmori

Fig. 2 Graphical representation of the seasonal abundance of different types of *Anopheles* in *rural areas* during 2015-16 (Mean + Standard Error)

polymorphism of the ITS2 is a sensitive, specific and rapid method for molecular confirmation (Loaiza, 2010). ITS2 is a prudent choice to study phylogenetic relationship of closely related *Anopheles* species, as well as biodiversity and geographic races of a particular species of mosquitoes. The present investigation reveals that *An. subpictus* and *An.vagus* are predominant *Anopheles* mosquitoes in some rural and sub-urban areas of South Bengal. In rural areas *An.vagus* is most teeming in number during monsoon while *An.subpictus* predominates in sub-urban areas throughout the year. Analysis of ITS 2 indicates that both the species are GC rich and dimers are mostly found in SRF based ITS2 study. *An.subpictus* is one of the most abundant species in most parts of India. *An. subpictus* has been incriminated as a potent vector of malaria in Maldive Islands, Lakshadweep Islands etc. It is assumed (Panicker *et al.*, 1981) that this species might be responsible for the transmission of malaria in the coastal villages of Pondicherry and Tamil

(I) ITS 2 sequence of *An. subpictus*:

GTGGTATTGCTGACGCATATGGCGCATCGGACGTTTCAACCCGACCGATGCACACATCCT
TGAGTGCCTACTAGGTACTGAGAGATTCCTATAACTTGACTACAGACGGCGCCACAAACG
GGCTGACGGGCCATCCGTCGTCCGGCGTGCGACTGTGCAGCATGGCGTGCTCGGGTCTCG
GCGTGGACCCTTGGGCGCTGAAAGTGGACACTGTTTGGCGGCACCTGCGCGTGTGCTCTC
AGTGTTGACGTATGGTGAGGGTAGTGTCAAATCGCACGGTTCGACAACAAGCGTACCGTC
GAGTTTGGTGCAATCGGATGCCTACTACCATGGGCGGAGCCGGCGTGCATTCAACACTCG
ACGTCCTGTATCAACCGGATGCCAACTTGGTTGGTGGTGCCGGCGCAGACAGGACACTGA
ATCGATCTTGGTGGTACAACCCACATGTGGGTGATCAAGGAGGGGGTGTAAGTGTGGGAG
GGACACGAGGGTGGCGCCGACGCACACGCCGGCACTCACCCCCACGTCCCTCGTTGCGTG
TATTGCGGGTGATCCATAGAGTGATCTGTTTGAATAGTGGGTTGAACTGGGTATGAAAAA
AGTTACCAAAAAAGCTGACCACACTCCCAGTAGGCCTTCCATGATGTGTGACTAGATGTG
GGACTACACCCAGAATTTAAGCATAA

(II) ITS 2 sequence of *An. vagus*:

TAAGGAGTGCAAACGCTGAGAGACGCATGCGCAGTGCCGCAAGACTTGTCACATTCAGGCCCCAAGT
GTACGCGAGAGCACCGAGTCACGCCTGCTGCACAGTGCATCGCGGACAAAGATGCCCGTCAGCCCCTT
AGAGCGCCGTGTGCGTACAAGTCTATAGATAAATACTCAGTACACGTAGGCACTCACGATGTGTGCAT
CTGTCTGGTGAAACGTCCGTTGCCAAAGGCAATAAAAGGGAATTTTTTGAAATATTCCGCACACGAAC
ACCGATAAGTTGAACGCATATGGCGCATCGGACGTTTCAACCCGACCGATGCACACATCCTTGAGTGC
CTACTAGGTACTGAGATTTAACTATGACTTGACTACAGACGGCGCCACTAAAGGGCTGACGGGCCATC
CGTCGTCCGGCGTGCGACTGTGCAGCATGGCGTGCTCGGGTCTCGGCGTGGACCCTTGGGCGCTGAAA
GTGGACACTGTTTGGCGGCACCTGCGCGTGTGCTCTCAGTGTTGACGTATGGTGAGGGTAGTGTCAAG
TCGCACGGTTCGACAACAAGCGTACCGTCGAGTTTGGTGCAATCGGATGCCTACTACCATGGGCGGTG
CCGGCGTGCATTCAACACACTCGACGTCCCGTACCAACCGGATGCCTGTGAAGGCGGTGCCGGCGCAG
ACGGGACACTGAATTGATCTTGGTGATATTGGGGGATGATGGATGATGTGTGTCGCGAGTGACAACCG
GATGCCAGCGATGGCGGTGCCGGCGCACACGAGCGCTCACACACGCCTCTCCCCTCGGTCGCTTGTGG
CGTGTAACGCGTGTGA

Fig. 3 ITS2 sequences of the collected samples from South Bengal

Fig. 4 Mouth parts and tip of the hind limb of *An. barbirostris.*

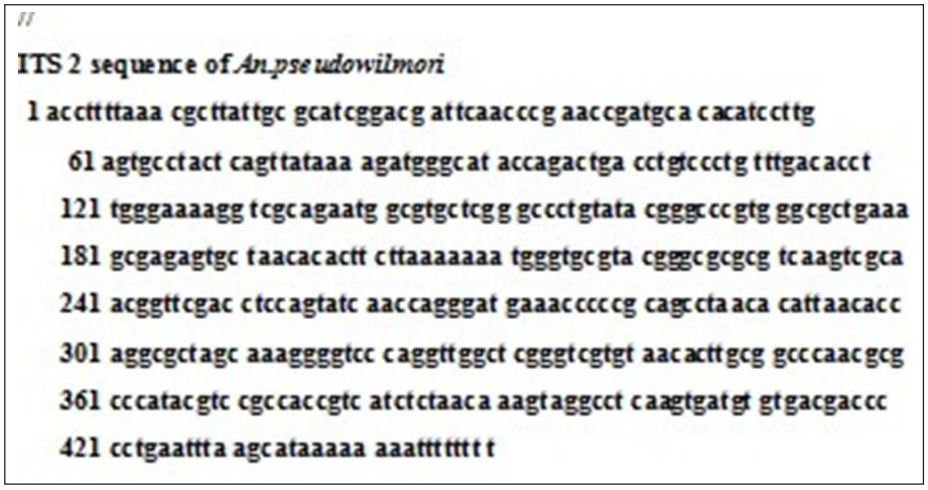


Fig. 5 ITS2 sequences of collected samples from North Bengal

Nadu. *An. subpictus* is also considered as a secondary vector in certain parts of India (Singh *et al.,* 2014) *Anopheles vagus*, is widely distributed in Asia. Evidence shows that it can function as a secondary vector. *Anopheles barbirostris* is a vector of malaria in Sri Lanka (*Amerasinghe et al., 1999),* India and Southeast Asia, and also a vector of Brugian filariasis in Southeast Asia (Lien *et al.*, 1977). In Tibet, *A. pseudowilmori* , both an indoor and outdoor species, is recognized as the principal malarial vector. The ability to efficiently and unequivocally identify the species is a priority for obtaining a clear understanding of malarial transmission in any region. These are therefore key areas for the application of this diagnostic AS-PCR assay that is relevant to vector control. Since vector incrimination is dependent upon accurate species identification, so, proper identification and study of biological characteristics are part and parcel.

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