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Studies on seasonal abundance & molecular characterization of *Anopheles subpictus* and *Anopheles vagus* based on ITS2 sequence variability

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Abstract
A systematic survey was conducted to know the seasonal abundance as well as morphological variation in *Anopheles* mosquito from Dec2013-Nov2014 in Mogra in Hooghly district West Bengal. Morphological variation and molecular characterization of *An. subpictus* and *An. vagus* have been studied in that above mentioned areas. It has been observed they are closely related on the basis of GC content, repeat sequence and restriction sites but the inter- specific variation in ITS2 length is well marked between *An.subpictus* and *An.vagus.*

Keywords: *An. subpictus*, *An. vagus*, seasonal prevalence, ITS2

1. Introduction
In recent year's rapid urbanization, excessive deforestation and resistance to insecticides cause rapid increase of mosquito population and mosquito borne diseases. *Anopheles* mosquitoes are medically important group of haematophagous vector; serve as an obligate intermediate host for numerous diseases like malaria, filarial, dengue etc [1]. Malarial has become a major health problem in India. It has been reported that in India, around 1.3 million people in 2011 have been affected with malaria [2]. India harbor around 58 Anopheline species among them only 13 are available in Kolkata and sub urban areas [3, 4]. Over the last decades, the global as well as regional climate change has a large effect on adaptive capacity of different species of *Anopheles* mosquitoes. Habitat and climatic conditions determine the dominancy of a species in a particular area. Seasonal prevalence and population dynamics of *Anopheles* mosquitoes of a particular area serve as an indicator point to know the present scenario of malaria. *An.subpictus and An.vagus* are ubiquitous species and appeared abundantly in Kolkata and sub urban areas [4-6]. Morphological variation is a unique feature in the genus *Anopheles* but the rate of mutation is lower magnitude [7, 8]. It has been reported that *An. subpictus* and *An. vagus* manifested their morphological variation in the ornamentation of palpal banding and the structure of wing [9, 10]. Several scientist [11-13] extended their studies on the molecular variation in *Anopheles* mosquitoes through PCR based assay for internal transcribed spacer 2 (non coding region) of r DNA. Various lines of data [14-16] indicated that the repetitive DNA sequences and the variation in their spacer length lead to the genomic diversity. Earlier [4, 17] has been extended their research work to know the DNA sequences and its diversity i.e. the sequence variation in ITS-2 of *An. subpictus* in the Mogra area of Hooghly district of West Bengal. Therefore, an attempt has been made to know the seasonal morphological and molecular diversity of *An. subpictus* and *An. vagus* in Mogra area of Hooghly district in West Bengal.

2. Material & Method
2.1 Source of mosquitoes
Adult *Anopheles* mosquitoes were collected from Mogra. Collection was made in early morning (6-8am) from different biotopes like cattle sheds & human dwellings (near to cattle shed) byusing manual aspirator. Adult female mosquitoes were morphologically identified following classical keys [18, 19] and visualized by using Dewinter Seteriomicroscope and from these collection species man hour density has also been calculated as per the formula given below

$$MHD = N \times 60/T \times P$$

Where, N = No. of mosquitoes collected; T = Time spent in min; P = No. of persons involved in collections.

2.2 DNA Isolation
DNA was isolated from individual adult mosquitoes by phenol chloroform extraction by Standered protocols of [17, 20, 21].

2.3 PCR amplification
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), MgCl2, Taq polymerase (3 units/µl) double distilled water and template DNA. The thermal cycling conditions were: initial denaturation at 950C for 5 min followed by 40 cycles of denaturation at 950C for 30 sec/1 min, annealing at 500-600C for 1 min, extension at 720C for 2-5 min and final extension at 720C for 10 min. The PCR product and standard DNA ladder were electrophoresed in 2% agarose gel and visualized with ethidium bromide.

2.4 DNA Sequencing
The ITS2 regions from some of the collected specimens were amplified according to the condition described above. The DNA of these ITS2 bands was sequenced and aligned between *An.subpictus* and *An. vagus.*

3. Result & observations

Table 1: Seasonal abundance and MHD (Man hour density) of Anophelines in Mogra (Dec 2013-Nov 2014).

***Anopheles* sp**	**Seasons**							
	Summer (Mar-May)		**Monsoon (Jun-Aug)**		**Post Monsoon (Sept-Nov)**		**Winter (Dec-Feb)**	
	Total	**MHD**	**Total**	**MHD**	**Total**	**MHD**	**Total**	**MHD**
Anopheles subpictus	51	17	37	12.33	19	6.33	8	2.66
Anopheles vagus	45	15	112	37.33	4	1.33	0	0

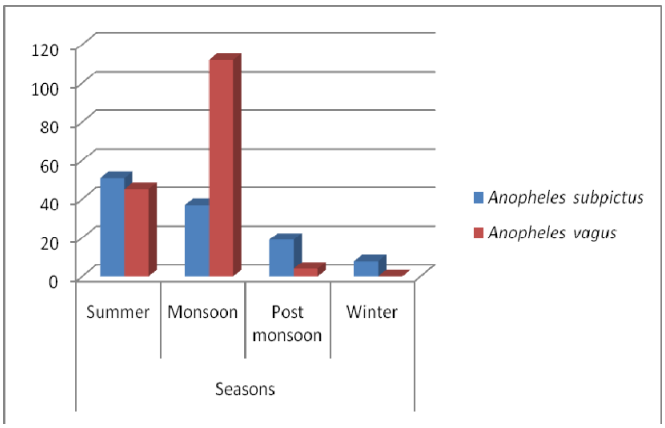

Fig 1: Graphical representation of Sesonal abundance of *An .subpictus* and *An .vagus* (Dec 2013-Nov 2014).

Table 2: Morphological variation of *An .vagus* in different seasons.

Name of Species	**Type of variation**	**Number**	**Season**
An. vagus	*An. vagus* with unequal palp (1)	1	Summer
	Palp with black tip(1)	1	Summer
	Palp with black tip (3)	5	Monsoon

Fig 2: Palp with black tip (2a) and unequal length of palp (2b) in *An. vagus.*

An.subpictus

GATTATTGACGCATATGGCGCATCGGACGTTTCAACCCGACC
GATGCACACATCCTTGAGTGCCTACTAGGTACTGAGAGATTC
CTATAACTTGACTACAGACGGCGCCACAAACGGGCTGACGGG
CCATCCGTCGTCCGGCGTGCGACTGTGCAGCATGGCGTGCTC
GGGTCTCGGCGTGGACCCTTGGGCGCTGAAAGTGGACACTGT
TTGGCGGCACCTGTGCGTGTGCTTTCAGTGTTGATGTATGGT
GAGGGTAGTGTCAAATCGCACGGTTAGACAACAAGTGTACCG
TCGAGTTTGGTGCAATGGGATGCCTACTACCATGGGCGGAGC
CGGGGTGCATTCAACACTGGATGTCCTGTATCAACCGGATGC
CAACTTGGTTGGTGGTGCGGGCGCAGACAGGACAGTGAATAG
ATCTTGGTGGTACAACCCACATGTGGGTTAGTAGGTAGGTGG
TCGATGTGTGCAGGTGACAACCGGATGCCAGCGATGGCGGTG
CCGGCGCACACCAGCACACTCGCCCCTAGGTCGCTTGTTGCG
TGTAACGCGTGTGATCCATACACATACCTGTTTGAGCTGTGC
GTTGAACACAAGAGGATGAGAGTTGCCAAACACACAGACCAC
ACTCCAGAGCCTTAAAAA.

An.vagus

TAAGGAGTGCAAACGCTGAGAGACGCATGCGCAGTGCCGCAA
GACTTGTCACATTCAGGCCCCAAGTGTACGCGAGAGCACCGA
GTCACGCCTGCTGCACAGTGCATCGCGGACAAAGATGCCCGT
CAGCCCCTTAGAGCGCCGTGTGCGTACAAGTCTATAGATAAA
TACTCAGTACACGTAGGCACTCACGATGTGTGCATCTGTCTG
GTGAAACGTCCGTTGCCAAAGGCAATAAAAGGGAATTTTTTG
AAATATTCCGCACACGAACACCGATAAGTTGAACGCATATGG
CGCATCGGACGTTTCAACCCGACCGATGCACACATCCTTGAG
TGCCTACTAGGTACTGAGATTTAACTATGACTTGACTACAGA
CGGCGCCACTAAAGGGCTGACGGGCCATCCGTCGTCCGGCGT
GCGACTGTGCAGCATGGCGTGCTCGGGTCTCGGCGTGGACCC
TTGGGCGCTGAAAGTGGACACTGTTTGGCGGCACCTGCGCGT
GTGCTCTCAGTGTTGACGTATGGTGAGGGTAGTGTCAAGTCG
CACGGTTCGACAACAAGCGTACCGTCGAGTTTGGTGCAATCG
GATGCCTACTACCATGGGCGGTGCCGGCGTGCATTCAACACA
CTCGACGTCCCGTACCAACCGGATGCCTGTGAAGGCGGTGCC
GGCGCAGACGGGACACTGAATTGATCTTGGTGATATTGGGGG
ATGATGGATGATGTGTGTCGCGAGTGACAACCGGATGCCAGC
GATGGCGGTGCCGGCGCACACGAGCGCTCACACACGCCTCTC
CCCTCGGTCGCTTGTGGCGTGTAACGCGTGTGA.

Fig 3: DNA sequence in ITS2 region of *An. subpictus* and *An. vagus* in Mogra, Hoooghly district West Bengal.

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EMBOSS_001 ------------------------------------------------------------
EMBOSS_002 TAAGGAGTGCAAACGCTGAGAGACGCATGCGCAGTGCCGCAAGACTTGTCACATTCAGGC


EMBOSS_001 ------------------------------------------------------------
EMBOSS_002 CCCAAGTGTACGCGAGAGCACCGAGTCACGCCTGCTGCACAGTGCATCGCGGACAAAGAT


EMBOSS_001 ------------------------------------------------------------
EMBOSS_002 GCCCGTCAGCCCCTTAGAGCGCCGTGTGCGTACAAGTCTATAGATAAATACTCAGTACAC


EMBOSS_001 ------------------------------------------------------------
EMBOSS_002 GTAGGCACTCACGATGTGTGCATCTGTCTGGTGAAACGTCCGTTGCCAAAGGCAATAAAA


EMBOSS_001 -----------------------------------GATTATTGACGCATATGGCGCATC
EMBOSS_002 GGGAATTTTTTGAAATATTCCGCACACGAACACCGATAAGTTGAACGCATATGGCGCATC
 * * ****************

EMBOSS_001 GGACGTTTCAACCCGACCGATGCACACATCCTTGAGTGCCTACTAGGTACTGAGAGATTC
EMBOSS_002 GGACGTTTCAACCCGACCGATGCACACATCCTTGAGTGCCTACTAGGTACTGAGATTTAA
 ************************************************** *

EMBOSS_001 CTATAACTTGACTACAGACGGCGCCACAAACGGGCTGACGGGCCATCCGTCGTCCGGCGT
EMBOSS_002 CTATGACTTGACTACAGACGGCGCCACTAAAGGGCTGACGGGCCATCCGTCGTCCGGCGT
 **** ********************** ** ******************************

EMBOSS_001 GCGACTGTGCAGCATGGCGTGCTCGGGTCTCGGCGTGGACCCTTGGGCGCTGAAAGTGGA
EMBOSS_002 GCGACTGTGCAGCATGGCGTGCTCGGGTCTCGGCGTGGACCCTTGGGCGCTGAAAGTGGA
 ************************************************************

EMBOSS_001 CACTGTTTGGCGGCACCTGTGCGTGTGCTTTCAGTGTTGATGTATGGTGAGGGTAGTGTC
EMBOSS_002 CACTGTTTGGCGGCACCTGCGCGTGTGCTCTCAGTGTTGACGTATGGTGAGGGTAGTGTC
 ******************* ********* ********** *******************

EMBOSS_001 AAATCGCACGGTTAGACAACAAGTGTACCGTCGAGTTTGGTGCAATGGGATGCCTACTAC
EMBOSS_002 AAGTCGCACGGTTCGACAACAAGCGTACCGTCGAGTTTGGTGCAATCGGATGCCTACTAC
 ** ********** ********* ********************** *************

EMBOSS_001 CATGGGCGGAGCCGGGGTGCATTCAACACTGGATG--TCCTGTATCAACCGGATGCCAAC
EMBOSS_002 CATGGGCGGTGCCGGCGTGCATTCAACACACTCGACGTCCCGTACCAACCGGATGCC---
 ********* ***** ************* *** *** ************

EMBOSS_001 TTGGTTGGTGGTGCGGGCGCAGACAGGACAGTGAATAGATCTTGGTGGTACAACCCACAT
EMBOSS_002 TGTGAAGGCGGTGCCGGCGCAGACGGGACACTGAATTGATCTTGGTGATATTGGGGGATG
 * * ** ***** ********* ***** ***** ********** **

EMBOSS_001 GTGGGTTAGTAGGTAGGTGGTCGATGTGTGCAGGTGACAACCGGATGCCAGCGATGGCGG
EMBOSS_002 ATGGAT-------------GATGTGTGTCGCGAGTGACAACCGGATGCCAGCGATGGCGG
 *** * * * ** ***************************

EMBOSS_001 TGCCGGCGCACACCAGCACACTC---------GCCCCTAGGTCGCTTGTTGCGTGTAACG
EMBOSS_002 TGCCGGCGCACACGAGCGCTCACACACGCCTCTCCCCTCGGTCGCTTGTGGCGTGTAACG
 ************* *** * * * ***** ********** **********

EMBOSS_001 CGTGTGATCCATACACATACCTGTTTGAGCTGTGCGTTGAACACAAGAGGATGAGAGTTG
EMBOSS_002 CGTGTGA-------------------------------------------------------
 *******

EMBOSS_001 CCAAACACACAGACCACACTCCAGAGCCTTAAAAA
EMBOSS_002 ---------------------------------
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Fig 4: Alignment of ITS2 region of *An. subpictus* and *An. vagus*.

Table 3: Base pair length and GC content of ITS2 region of *An. subpictus* and *An. vagus*:

Species	Bp	GC content
An. subpictus	648	55.4%
An. vagus	831	56.8%

Table 4: Repeat sequence of ITS 2 region:

Name of repeat sequence	*An. vagus*	*An. subpictus*
Dimers		
AC	130	110
TG	138	121
CA	138	121
Trimers		
GTG	48	39
Tetramers		
CCTA	4	8
GCAT	13	8
CGTG	15	7
GTGC	23	17
TGCA	16	10
GCGT	16	9
Petamers		
GGTGC	6	5
Polymers		
GACGTG	0	0
CTCGGCGTG	1	1

4. Discussion

The present investigation indicated that the Anopheline population load is mainly maintained by the population volume of *An. subpictus* and *An .vagus* in Mogra area of Hooghly district of West Bengal during 2013-2014. Seasonal variation in the Anopheline population is well marked in the studied area. Our result (Table-1) indicates that in *An. subpictus* MHD was highest in summer (March to May) and in *An. vagus* it was in Monsoon (June to August).Our data (Table: 1) reveal that *An.subpictus* is more or less present throughout the year. On the other hand *An .vagus* population is rapidly decreased in winter season (Fig: 1). Present investigation shows that *An .subpictus* is prevalent in summer and monsoon along with *An.vagus* population. (Fig: 1). In monsoon the population of *An.vagus* is more than that of *An. subpictus* while the *An. subpictus* population is gradually decreased with the onset of post monsoon. Morphological variation is a unique feature in the genus *Anopheles* which is manifested through palp proboscis and wing. Our present study indicated that the variation in palp is well marked in *An. vagus* (Fig- 2a, b). Our investigation has also been extended to find out the molecular variation in ITS2 region of *An. subpictus* and *An. vagus*. Our present observation reveals that *An. subpictus* A is more prevalent in our investigated area and it shows 98% similarity with *An. subpictus* A (KC191825) of Sri Lanka [22]. Furthermore our investigation also reveals that *An. subpictus* A and *An. vagus* (KT716079) is closely related on the basis of molecular characteristics viz the GC content, the number of repeat sequence and restriction sites (Table 3, 4). Earlier it has also been reported [23] that *An. vagus* is genetically similar to *An. subpictus* A. No tandem repeat has been observed in both these species. Besides that, in our present observation interspecific variation through ITS2 length (Table: 3) is well marked between *An. subpictus and An. vagus.*

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