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Anopheles (Cellia) maculatus group: Its spatial distribution and molecular characterization of member species in north-east India

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ABSTRACT

Anopheles (Cellia) maculatus is considered a group of at least nine formally named species. Faced with the difficulty of correct morphological identification due to overlapping characters, several member species of the *An. maculatus* group are known to play important role in malaria transmission in the Oriental region. Current assemblage, distribution and vectorial importance of the member species within the Maculatus group is far from clear in the north-eastern region of India. Our study encompassing 410 individuals, collected from 67 geo-referenced spots across the eight north-east Indian states, identified the presence of 6 member species of the Maculatus group using the molecular tools. *Anopheles dravidicus* and *Anopheles rampae* were documented for the first time in this part of India with latter forming the new country record. While *Anopheles pseudowillmori* (59.5%) and *An. maculatus* (32%) were widely available species in most of the north-eastern states, restricted distribution of *Anopheles willmori* to Nagaland and that of *Anopheles sawadwongporni* and *An. rampae* to Mizoram state was noted. None of the species was found positive for human malaria parasite. While no intraspecific differences existed in the sequences of second internal transcribed spacer (ITS2) region of ribosomal DNA (r-DNA) of the member species of the Maculatus group within north-east India, few differences were detected in the sequences of *An. dravidicus*, *An. maculatus* and *An. pseudowillmori* from north-east India with species from the neighbouring countries.

1. Introduction

North-east India (20–30°N, 87–98°E), regarded as one of the hot spots for global bio-diversity (Myers et al., 2000), comprises of eight administrative states, namely Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim and Tripura sharing over 2000 km of international border with four neighbouring countries, *i.e.*, Bhutan, China, Myanmar and Bangladesh. Malaria is a major public health problem in this part of the country contributing 10–15% of reported cases and 8–10% recorded deaths due to malaria (Mohapatra et al., 1998). Malaria in the north-east India is transmitted by three primary vectors, namely *Anopheles minimus*, *Anopheles baimaii* and *Anopheles fluviatilis* (Mohapatra et al., 1998) aided by few secondary vectors such as *Anopheles nivipes* (Bhattacharyya et al., 2010) and possibly *Anopheles maculatus* (Rao, 1984). However, a recent study (Singh et al., 2010) found that *An. fluviatilis s.l.* reported from Assam was actually a seasonal variant of *An. minimus*. Earlier studies considered *An. maculatus* as a single species with two varietal forms (Christophers, 1933). While the type form *An. maculatus* was widely prevalent throughout India, the variety *willmori* was abundant all along the Himalayan region (Christophers, 1933). Application of morphology, cytogenetics, cross-mating and molecular methods now confirms *An. maculatus* to be a group of nine formally named species *viz. Anopheles dispar*, *Anopheles dravidicus*, *Anopheles greeni*, *An. maculatus*, *Anopheles notanandai*, *Anopheles pseudowillmori*, *Anopheles rampae*, *Anopheles sawadwongporni* and *Anopheles willmori* (Harbach, 2011; Somboon et al., 2011). Although member species of the Maculatus group are known to be variously involved in malaria transmission in the Oriental region (Reid, 1968; Rao, 1984; Upatham et al., 1988) but exact vectorial role of each species within this group remains unclear due to the error prone morphological species identification owing to overlapping characters (Manguin et al., 2008) or data based mainly on undifferentiated Maculatus group. Natural infection of malaria was detected in *An. maculatus* as well as variety *willmori* from parts of Assam and Meghalaya several decades ago (Anderson and Viswanathan, 1941; Viswanathan et al., 1941) implicating their importance in malaria transmission in the north-eastern region of India. Moreover, *An.*

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pseudowillmori is considered to be the main vector of malaria in countries bordering north-east India like Bhutan (Sharma, 2002; Yangzom et al., 2012) and Tibet autonomous region of China (Song et al., 2009). Recently, ELISA positivity for *Plasmodium vivax* was reported in *An. maculatus* from Habiganj district of Bangladesh just across the Indo-Bangladesh border (Alam et al., 2010). Moreover, members of the Maculatus group of mosquitoes were found infected with human malaria parasite in different parts of Oriental region (Green et al., 1991; Rattanarithikul et al., 1996; Somboon et al., 1998; Coleman et al., 2002; Manh et al., 2010). Therefore, epidemiological importance of members of the Maculatus group in the north-east India has increased but there is a paucity of information on composition, geographical distribution and current vectorial role of available member species of Maculatus group leading to the inadequate understanding of fast changing malaria epidemiology in this part of India. Therefore, in order to fill this void we undertook a study, using DNA based methods, aiming at identifying, characterizing and investigating current vector status/vector potentiality of the available member species of the Maculatus group in the north-eastern states of India.

2. Materials and methods

2.1. Mosquito collections

Host seeking female mosquitoes of *An. maculatus* group were collected from the human dwellings in the main malaria transmission season (April–October) during 2008–2011 from 67 spots out of 124 surveyed across all the eight north-eastern states of India using CDC miniature light traps (Bio Quip Products, USA) operated throughout the night. As this study was designed to collect malaria vector mosquitoes attracted to humans, CDC light traps were placed in a room inside human dwellings where at least one person slept. On an average 4–6 trap night collections were made at each spot. Next morning trapped adults belonging to the Maculatus group were separated using the key of Das et al. (1990b) and kept individually in beam capsules with silica gel, numbered and stored at 4 °C till further processing.

2.2. DNA extraction

The individual mosquito was bisected to head-thorax and abdomen under aseptic conditions and genomic DNA extracted separately from the two body parts using FTA Classic card (Whatman) protocol (Mohanty et al., 2007). Head-thorax DNA was used for species identification and detection of human *Plasmodium* sporozoites. DNA from the abdominal region of the blood fed individuals was used for the presence of human blood.

2.3. Allele specific PCR for species identification

Species identification of the Maculatus group individuals was carried out using the methods of Ma et al. (2006) and Walton et al. (2007) in conjunction, with minor adjustments. Both are allele-specific polymerase chain reaction (ASPCR) methods based on the fixed differences in the second internal transcribed spacer (ITS2) separating the 5.8 s and 28 s subunits of ribosomal DNA (r-DNA) giving species-specific bands of diagnostic lengths. The protocol of Walton et al. (2007) distinguished five species of the Maculatus group *viz. An. maculatus*, *An. dravidicus*, *An. pseudowillmori*, *An. sawadwongporni* and *An. rampae* and that of Ma et al. (2006) identified *An. willmori*.

2.4. Detection of human Plasmodium infection

Sporozoite detection of human *Plasmodium* in the salivary glands of mosquitoes was done using the head-thorax DNA by nested PCR method (Snounou et al., 1993). This method uses genus specific primers for the first round of amplification and in the second round each parasite species is detected separately by using species specific primers.

2.5. Detection of human blood

Presence of human blood in the abdominal DNA of blood fed individuals was detected using the primers and protocol described by Mohanty et al. (2007) with minor adjustments.

2.6. Characterization of r-DNA ITS2

r-DNA ITS2 region of 28 randomly chosen individuals belonging to 6 species (*An. maculatus*-11, *An. pseudowillmori*-7, *An. sawadwongporni*-4, *An. dravidicus*-3, *An. willmori*-2 and *An. rampae*-1) representing all the eight states of the north-east India were amplified following Walton et al. (2007). Amplicons were purified using high pure PCR product purification kits (Roche Diagnostics GmbH, Germany) and sequenced in both directions using Big Dye Terminator V3.1 kit in a 16 capillary automated DNA sequencer (Applied Biosystems, USA3130XL Genetic Analyzer). The sequences were edited manually using BioEdit v 7.0.9 (Hall, 1999) and aligned in Clustal W2 (Larkin et al., 2007) using default parameters to study the intraspecific variations.

Available sequences of the Maculatus group were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/) and a multiple sequence alignment file was prepared to compare our sequences and sequences from the neighbouring countries. Neighbour-Joining (NJ) phylogeny was constructed in MEGA version 5.05 (Tamura et al., 2011) using Kimura 2 parameters distance matrix with 1000 bootstrap replicates (Kimura, 1980). All positions containing gaps and missing data were eliminated.

3. Results

Sixty seven of the 124 surveyed spots spread over 34 districts across the eight north-eastern states, yielded 410 females of the Maculatus group with lesser abundance in the states of Sikkim and Manipur (Fig. 1). Molecular identification revealed the presence of 6 member species of the Maculatus group *viz. An. pseudowillmori*, *An. maculatus*, *An. willmori*, *An. dravidicus*, *An. sawadwongporni* and *An. rampae* (Table 1) with the predominance of *An. pseudowillmori* (59.5% of the Maculatus group) followed by *An. maculatus* (31.9%). *An. pseudowillmori* was detected throughout the region with the highest abundance in Arunachal Pradesh state (97.5% of the Maculatus group collection in the state). *An. maculatus* was the predominant species of the group in Meghalaya (83.3%), Assam (79.2%) and Tripura (70.8%) states. *An. dravidicus* (1.5% of overall collections) was detected in 3 states *viz.* Assam, Manipur and Sikkim. Distribution of *An. willmori* (2.4%) was restricted only to Nagaland state, and that of *An. sawadwongporni* (4.1%) and *An. rampae* (0.5%) to the state of Mizoram.

Altogether 351 individuals of the Maculatus group were processed for the presence of human malaria infection but none was found positive (Table 2). Blood meal analysis of 34 fed females of *An. pseudowillmori* and 20 of *An. maculatus* revealed the presence of human blood in 50% and 65% individuals of the two species, respectively.

The length of ITS2 region of different species of the Maculatus group ranged between 330 and 338 bp with 57.1–59.1% GC content. It was the shortest and of same length (330 bp) in *An. pseudowillmori*