CHARACTERIZATION OF ITS2 rDNA OF ANOPHELES PHILIPPINENSIS AND ANOPHELES NIVIPES (DIPTERA: CULICIDAE) FROM NORTH-EAST INDIA

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Abstract. Anopheles (Cellia) philippinensis Ludlow and Anopheles (Cellia) nivipes (Theobald) are two closely related, morphologically very similar, mosquito species in the Annularis group, which play a supportive role in malaria transmission in north-east India. We amplified and performed sequence analysis for the internal transcribed spacer 2 (ITS2) locus of ribosomal DNA (rDNA) gene of morphologically confirmed specimens of these two species from the states of Assam and Nagaland. An. philippinensis and An. nivipes shared 85.2% sequence similarity and no intra-species variation was found in the nucleotide sequences of the two species. Overall, sequence data of the ITS2 marker revealed that both these species from north-east India differed by as much as have been reported from specimens of eastern Thailand.

INTRODUCTION

Anopheles philippinensis and An. nivipes are two closely related mosquito species, which are difficult to separate on morphological features in the adult stage although these species can be reliably identified based on larval and pupal characteristics. The only diagnostic characteristic, the pre-sector dark mark on wing vein 1, used to differentiate the adult females of An. philippinensis from An. nivipes (Reid, 1968) is not unequivocal (Reid, 1967; Prakash et al, 2004), thus posing a significant problem of identification in adult specimens.

An. philippinensis/nivipes is a recognized vector of malaria in countries adjoining the north-eastern region of India namely, Bangladesh (Elias et al, 1982) and Thailand (Rattikhul et al, 1996). In India, An. philippinensis/nivipes is generally restricted to the eastern and north-eastern states (Rao, 1984). While this species is reported to have almost disappeared from the east Indian state of West Bengal where it was once considered an important vector of malaria (Iyenger, 1940), An. philippinensis/nivipes is widely prevalent and possibly playing a supportive role in malaria transmission in the north-eastern states. As far back as the 1930s, this species was incriminated as a vector of human malaria with a 0.1% sporozoite rate in Assam (Anderson and Viswanathan, 1941) and a 0.5% sporozoite rate in an Assam-Meghalaya border area during 1968 (Rajagopal, 1976). Recently, Prakash et al (2005), while investigating the role of prevalent anophelines in malaria transmission in areas of upper Assam and Arunachal Pradesh state, reported 1.7% minimum sporozoite rate in An. philippinensis/nivipes using CSP-ELISA. However, it could not be stated with certainty whether the incriminating species mentioned in all these reports was actually An. philippinensis or An. nivipes or both.
This diagnostic dilemma associated with adult females of An. philippinensis/nivipes emphasizes the urgent need to develop sensitive and reliable method(s) to correctly differentiate the adult philippinensis from nivipes in order to pinpoint the vector species and determine its epidemiological importance. DNA-based molecular identification methods have an advantage over classical morphological and genetic methods because of their reliability, accuracy, precision, ease of handling and their applicability to all mosquito life stages (Collins and Paskewitz, 1996). The second internal transcribed spacer (ITS2) region of ribosomal DNA (rDNA), separating the 5.8S and 28S ribosomal RNA gene, is considered an excellent species diagnostic molecular marker (Walton et al., 1999) as its sequence is likely to vary even between closely related species. The characteristic of generally low level of intra-specific variations at ITS2 locus has been successfully exploited for taxonomic studies of closely related species such as An. nuneztovari (Sierra et al., 2004), for species characterization (Alam et al., 2006), for phylogenetic studies (Sallum et al., 2002) and in developing polymerase chain reaction (PCR)-based assays for the molecular identification of closely related species complexes such as An. gambiae (Paskewitz and Collins, 1990), An. dirus (Walton et al., 1999), and An. minimus (Phuc et al., 2003). An ITS2-based PCR assay has recently been developed for four members of the An. annularis group, including An. philippinensis and An. nivipes using specimens primarily from Thailand and Southeast Asia (Walton et al., unpublished). We have characterized the ITS2 region of An. philippinensis and An. nivipes specimens from Assam and Nagaland states of north-east India with the objective of determining if this method is applicable to these species in north-east India. The ITS2 sequence data for An. philippinensis and An. nivipes from north-east India showed differences similar to those seen in the same species in Thailand.

**MATERIAL AND METHODS**

Immatures of An. philippinensis/nivipes were collected during August 2004 from breeding habitats in 3 geographically separated sites in Assam state: Boko in district Kamrup (25°59′ N, 91°15′ E), Lower Assam; Bokakhat in district Golaghat (26°34′ N, 93°14′ E), Central Assam; and Jorajan in district Dibrugarh (27°23′ N, 95°34′ E), Upper Assam; and 1 site in Nagaland state [Sirihima in Dimapur District (25°46′ N, 93°54′ E)]. The mosquito collection sites of Sirihima, Bokakhat and Boko are situated at a distance of 275 kms (south-west), 220 kms (west) and 550 kms (west) from the Dibrugarh District of Assam, respectively. These mosquito immatures were reared individually in plastic vials to obtain adults with associated larval and pupal exuviae. Emerged adults were identified positively either as An. philippinensis or An. nivipes based on larval and pupal characteristics using the keys of Reid (1968). Only confirmed specimens of the two species were used to determine ITS2 sequences. Genomic DNA from the individual adult whole mosquito was extracted using a phenol-chloroform method (Sambrook et al., 1989). Amplification of ITS2 was achieved using primers modified from Paskewitz and Collins (1990), namely, 5′-ATC ACT CGG CTC GTG GAT CG-3′ and 5′-ATG CTT AAA TTT AGG GGG TAG T-3′. Reaction conditions and thermocycler parameters were as described by Walton et al. (1999) with following modifications: (i) Taq Red DNA Polymerase (Continental Laboratory Products, Northampton, UK) and its 10X buffer [160 mM (NH₄)₂SO₄, 670 mM Tris-HCl (pH 8.8), 0.1% (v/v) Tween 20] were used, (ii) dimethylsulphoxide was not added, and (iii) 39 amplification cycles were used. The amplified PCR products were purified using a commercially available DNA purification kit (Millipore, Billerica, USA) and sequenced in both directions (by MWG-Biotech AG Germany). The forward and reverse sequences from each specimen were checked
and edited using SeqEd ver 1.03 multiple-sequence editor program (ABI, 1992) and aligned using the default parameters within the CLUSTAL W multiple sequence alignment program in Bio Edit version 5.0.9 software (http://www.mbio.nesu.edu/BioEdit).

RESULTS

ITS2 sequences from two morphologically confirmed specimens of An. philippinensis (one each from Kamrup and Dibrugarh Districts, Assam) and two morphologically confirmed specimens of An. nivipes (1 each from Golaghat District, Assam and Dimapur District, Nagaland) were obtained. Sequences generated in this study were deposited in GenBank (http://www.ncbi.nlm.nih.gov) under the following accession numbers: An. philippinensis from Boko, district Kamrup, Assam (DQ 319187) and An. nivipes from Bokakhat, district Golaghat, Assam (DQ 336434). Amplified fragment containing ITS2 and flanking regions of 5.8S and 28S subunits of rDNA was 500 bp and 493 bp in An. philippinensis and An. nivipes, respectively. No intra-species differences were found in the ITS2 sequences of either species. An. philippinensis and An. nivipes shared 85.2% sequence similarity. The inter-species variations were mostly located in the ITS2 region and not in the flanking sequences of the 5.8S and 28S genes (Fig 1).

A homology search in the GenBank database for ITS2 sequences of An. philippinensis and An. nivipes did not find any entry. However, the ITS2 sequences of An. philippinensis and An. nivipes obtained in this study were compared with the sequences of these species from eastern Thailand (Walton C et al, unpublished). The ITS2 sequence of An. philippinensis from Thailand shared 99.2% similarity with that of the same species from Assam. The Assam sequence differed from the Thai sequence by a transition at position 59 (C in place of T), and an insertion of an AG repeat unit at positions 283-284 (Fig 1). Furthermore, position 418 had a base G in the Assam sequence but was heterozygous for G and T in specimens collected from various geographical regions of Thailand. The An. philippinensis sequences from specimens from Kamrup and Dibrugarh Districts, Assam, were the same as that obtained from Nameri Park of Sonitpur District, Assam (Walton C et al, unpublished). In the case of An. nivipes, the sequence similarity was 99.3% between the Thailand and Assam specimens. The sequence of An. nivipes from eastern Thailand had base G, A and A at position 164, 166 and 167, respectively, whereas the Assam and Nagaland sequences had G/T, A/C and A/G at these positions. Such polymorphic sites were also found in ITS2 sequences of An. philippinensis and An. nivipes collected from different areas of Thailand (Walton C et al, unpublished). Though both the sequences of An. nivipes in our study were identical as regards to polymorphic sites at positions 164, 166 and 167, clearly there is a need to sequence more specimens from diverse areas of north-east India to determine if there are any other sequence variants present and whether this will affect the application of the An. annularis group molecular identification method (Walton et al, unpublished).

DISCUSSION

This is the first study reporting the ITS2 sequences of An. philippinensis and An. nivipes, two closely related mosquito species, from several sites in north-east India. Overall, sequence data of the ITS2 marker revealed that both these species from north-east India differed as much as have been observed from the same species in Thailand. In contrast, no variations in ITS2 sequences were found in the case of An. baimaii (=dirus D) and An. minimus s.s (=minimus A) specimens from north-east India and Thailand (Prakash et al, unpublished).
Fig 1-Alignment of the unique ITS2 sequences from the members of the An. philippinensis and An. nivipes from Assam and Thailand. Dots indicate identity and a dash denotes a deletion with respect to the reference sequence.
Moreover, studies on the mitochondrial marker, cytochrome oxidase 2 (COII), have revealed a unique bio-diversity in north-east India and have hinted that there may be considerable population genetic structures within the geographical region of NE India (O’ Loughlin S, unpublished). It is, therefore, important to gain further knowledge on the genetic diversity and divergence of vector mosquitoes in north-east India in order to devise effective species-specific vector control measures.

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