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

Anil Prakash[1], C Walton[2], DR Bhattacharyya[1], PK Mohapatra[1] and J Mahanta[1]

[1]Regional Medical Research Center, NE (Indian Council of Medical Research), Dibrugarh, Assam, India; [2]Faculty of Life Sciences, University of Manchester, Manchester, UK

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 (Rattanarithikul *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. philipinnensis/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.

Correspondence: Dr J Mahanta, Regional Medical Research Centre, NE (Indian Council of Medical Research) Post Box no. 105, Dibrugarh - 786 001 Assam, India.
Tel: +91-373-2381494, 2381506; Fax: +91-373-2381494, 2381548
E-mail: icmrrcdi@hub.nic.in

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 pin point 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 (25º59′N, 91º15′E), Lower Assam; Bokakhat in district Golaghat (26º34′N, 93º14′E), Central Assam; and Jorajan in district Dibrugarh (27º23′N, 95º34′E), Upper Assam; and 1 site in Nagaland state [Sirihima in Dimapur District (25º46′N, 93º54′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 $(NH_4)_2SO_4$, 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).

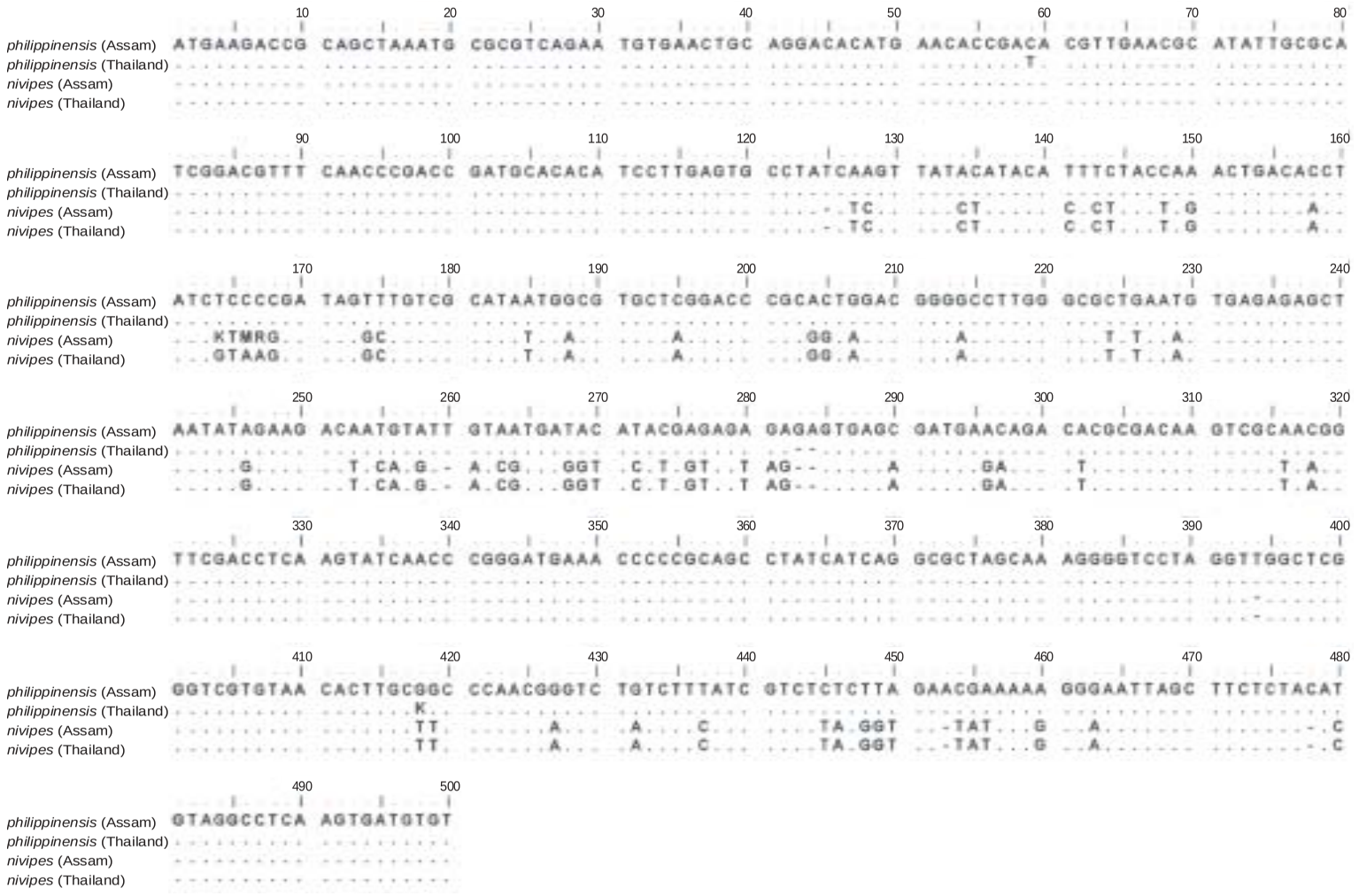

Fig 1-Alignment of the unique ITS2 sequences from the members of the *An. philippinen*sis and *An. nivipes* from Assam and Thailand. Dots indicate identity and a dash denotes a deletion with respect to the reference sequence.

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.

ACKNOWLEDGEMENTS

The senior author is thankful to the Commonwealth Scholarship Commission, London, UK, for the award of a Commonwealth Professional Fellowship, 2004, at the University of Manchester, UK, under which this study was undertaken. Field assistance of AC Rabha and D Dutta, Division of Malariology, RMRC, Dibrugarh, is acknowledged. We are also thankful to the anonymous reviewer(s) for improving the manuscript.

REFERENCES

Alam MT, Das MK, Ansari MA, Sharma YD. Molecular identification of *Anopheles (Cellia) sundaicus* from the Andaman and Nicobar islands of India. *Acta Trop* 2006; 97: 10-8.

Anderson LAP, Viswanathan DK.The Assam Medical Research Society, Shillong. A resume of its activities during 1931-1941. Calcutta: Thacker, Spink & Co, 1941.

Collins FH, Paskewitz SM. A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anopheles* species. *Insect Mol Biol* 1996; 5: 1-9.

Elias M, Dewan Z, Ahmed R. Vectors of malaria in Bangladesh. *J Prev Soc Med* 1982; 1: 20-9.

Iyenger MOT. Further observations on vectors of malaria in Bengal and notes on the seasonal infectivity of Anopheles. *J Mal Inst India* 1940; 3: 115-23.

Paskewitz SM, Collins FH. Use of polymerase chain reaction to identify mosquito species of the *Anopheles gambiae* complex. *Med Vet Entomol* 1990; 4: 367-73.

Phuc HK, Ball AJ, Son L, *et al,* Multiplex PCR assay for malaria vector *Anopheles minimus* and four related species in the Myzomia series from Southeast Asia. *Med Vet Entomol* 2003; 17: 423-8.

Prakash A, Bhattacharyya DR, Mohapatra PK, Mahanta J. Taxonomical observations on *Anopheles philippinensis/nivipes* Group mosquitoes in north-east India. *J Commun Dis* 2004; 36: 264-70.

Prakash A, Bhattacharyya DR, Mohapatra PK, Mahanta J. Potential of *Anopheles philippinensis-nivipes* complex mosquitoes as malaria vector in north-east India. *J Environ Biol* 2005; 26: 719-23.

Rao TR. *The Anophelines of India*. Delhi: Malaria Research Centre, ICMR, 1984: 1-518.

Rajagopal R. Studies on persistent transmission of malaria in Burnihat, Meghalaya. *J Commun Dis* 1976; 8: 235-45.

Rattanarithikul R, Konishi E, Linthicum KJ. Detection of *Plasmodium vivax* and *Plasmodium falciparum* circumsporozoite antigen in Anopheline mosquitoes collected in Southern Thailand. *Am J trop Med Hyg* 1996; 54: 114-21.

Reid JA. Two forms of *Anopheles philippinensis* in Malaya. *J Med Entomol* 1967; 4: 175-9.

Reid JA. Anopheles mosquitoes of Malaya and Borneo. *Stud Inst Med Res Malaya* 1968; 31: 1-520.

Sallum MAM, Schultz TR, Foster PG, Aronstein K, Wirtz RA, Wilkerson RC. Phylogeny of Anophelinae (Diptera:Culicidae) based on nuclear ribosomal and mitochondrial DNA sequences. *Syst Entomol* 2002; 27: 361-82.

Sambrook J, Fritsch EF, Meniatis T. Molecular cloning: A laboratory mannual. 2nd ed. New York: Cold Spring Harbour Press, 1989.

Sierra DM, Velez ID, Linton Y-M. The malaria vector *Anopheles (Nysorhynchus) nuneztovari* Gabaldon comprises one genetic species in Columbia based on homogeneity of nucleus ITS2 r-DNA. *J Med Entomol* 2004; 41: 302-07.

Walton C, Handley M, Kuvangkadilok C, *et al,* Identification of five species of the *Anopheles dirus* complex from Thailand using allele-specific polymerase chain reaction. *Med Vet Entomol* 1999; 13: 24-32.