International Journal of Mosquito Research

ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2021; 8(3): 51-56
© 2021 IJMR
www.dipterajournal.com
Received: 25-02-2021
Accepted: 27-03-2021

Varun Tyagi
Division of Pharmaceutical Technology, Defence Research Laboratory, Tezpur, Assam, India

Diganta Goswami
Medical Entomology Division, Defence Research Laboratory, Tezpur, Assam, India

Sunil Dhiman
Vector Management Division, Defence Research & Development Establishment, Gwalior, Madhya Pradesh, India

Dipanjan Dey
Cotton University, Guwahati, Assam, India

Santana Saikia
Cotton University, Guwahati, Assam, India

Bipul Rabha
Medical Entomology Division, Defence Research Laboratory, Tezpur, Assam, India

P Chattopadhyay
Division of Pharmaceutical Technology, Defence Research Laboratory, Tezpur, Assam, India

Sanjai K Dwivedi
Division of Pharmaceutical Technology, Defence Research Laboratory, Tezpur, Assam, India

Corresponding Author:
Varun Tyagi
Division of Pharmaceutical Technology, Defence Research Laboratory, Tezpur, Assam, India

Potential malaria vector *Anopheles minimus* (species A) still persisting in North East India

Varun Tyagi, Diganta Goswami, Sunil Dhiman, Dipanjan Dey, Santana Saikia, Bipul Rabha, P Chattopadhyay and Sanjai K Dwivedi

DOI: https://doi.org/10.22271/23487941.2021.v8.i3a.537

Abstract
Vector borne infectious diseases affect two third of the world's human population and cause millions of deaths every year. Among these, malaria remains one of the major killer in the Indian sub-continent and transmitted uninterruptedly by many efficient vectors and their sibling species. In North East India (NE), *Anopheles minimus* has been recognized as an important vector which transmits majority of malaria cases. This study primarily focuses on to recognize the presence and distribution of sibling species of *An. minimus* in certain endemic areas of NE India. *Anopheles* mosquitoes collected were identified using available morphological keys. The genomic DNA was extracted from the mosquitoes and used to perform species specific PCR (ss PCR) for molecular identification of major malaria vector *An. minimus* sibling species. Morphological identification suggested the presence of *An. minimus sl* in low density in the study area. The specimen of *An. minimus* subjected to ss PCR confirmed the prevalence of only one sibling species namely *An. minimus* 'A' in Sialmari and Chandubi areas. Though in low density, but malaria vector *An. minimus* is still present in certain endemic areas of NE India. The ss PCR assay used presently suggested that *An. minimus* sibling species A is prevailing in the region. Presently used ss PCR assay was simpler, faster, cheaper and more readily interpretable than the earlier assays. This information could be useful in understanding of current prevalence and distribution of malaria vector *An. minimus* and its sibling species complex in NE region of India.

Keywords: malaria**,** mosquito vector, *Anopheles minimus*, species specific PCR, North East India

1. Introduction

Malaria is a life threatening disease caused by *Plasmodium* parasites and transmitted among humans by *Anopheles* mosquitoes. Despite comprehensive interventions, malaria cases have shown increase globally during the year 2018 (228 million; 95% confidence interval: 206-258 million) as compared to the year 2017 (219 million; 95% confidence interval: 203-262 million) and year 2016 (216 million; 95% CI: 196-263 million). Malaria attributable deaths showed decline in 2018 (0.41 million), but largely remained unchanged compared to 0.44 million in the year 2017 and 0.45 million in 2016 [1]. The WHO South East Asian region has shown incidence rate fall, however India continue to contribute about 58% of malaria episodes in the region, and reported approximately 0.41 million of confirmed malaria cases during 2018 [1]. *Plasmodium falciparum* dominates the malaria transmission in India and accounts for >60% of malaria cases followed by *P. vivax* (>35% cases) annually. These two malaria parasites are uninterruptedly transmitted by six major vectors namely, *An. culicifacies, An. fluviatilis, An. stephensi, An. minimus, An. dirus and An. annularis* in different parts of the country [1-3].

North East region of India (latitude- 21°58' N to 29°30'N and longitude- 88°3' E to 97°30' E) shares international border with many endemic countries, such as Bhutan, China, Myanmar and Bangladesh that report considerable malaria cases every year. The region predominantly has humid sub-tropical climate and comprises of hills, wetlands, dense rain forests and forest fringes to support vector mosquito growth and proliferation throughout the year. Although the region inhabits 3.5% of India's population but contributes 17.5% of total malaria deaths reported in the country. Several *Anopheles* species have been incriminated as malaria vector in the region during the recent years [4-6] and associated with the transmission of both *P. falciparum* and *P. vivax* in the endemic pockets, but historically *An. dirus* (monsoon species)

and *An. minimus* (perennial species) have Been Regarded As Important Malaria Vectors Involved in majority of malaria infections. Both these vectors incriminated in different independent investigations unequivocally were major vectors in the region few years ago. However during the recent years the density of *An. minimus* and *An. dirus* has declined considerably. Studies have suggested that both these vectors have been either disappeared [7, 8] or prevailing in very low numbers, which may not be sufficient to maintain perennial malaria transmission [4, 9]. Furthermore, secondary malaria vectors which had insignificant epidemiological importance earlier were incriminated harboring malaria parasites and maintaining uninterrupted malaria transmission in the region [4, 9]. Although many investigations [8, 9] could not collect *An. minimus* during the study, but its prevalence in low density below collectable limit mainly in ecologically suited forest fringed pockets cannot be overruled.

An. minimus s.l. (*Funestus* group) comprises of *An. minimus* s.s. (formerly known as *An. minimus* 'A'), *An. harrisoni* (formerly *An. minimus* 'C') and *An. yaeyamaensis* (formerly *An. minimus* 'E') [10-12]. *An. minimus* 'E' is restricted to Ryukyu Archepelago of Japan and not involved in malaria transmission [13], whereas species 'A' and 'C' are predominantly distributed throughout South East Asia region and responsible for malaria transmission. In North East region *An. minimus* 'A' has been incriminated as malaria vector and found associated in malaria transmission in many states of the region [12]. Although many molecular techniques have been developed to segregate sibling species, Van Bortel *et al.* [14] adopted an alternative method for the molecular identification of sibling species. This included PCR amplification of the rDNA internal transcriber spacer 2 (ITS2) region followed by BsiZI restriction enzyme digestion to distinguish *An. aconitus*, *An. jeyporiensis, An. minimus* 'A' and 'C', *An. pampani, An. varuna* and subsequently *An. Culicifacies* [14, 15]. Proper and accurate identification of any vector involved in malaria transmission, its distribution, behavior, vector competency and relative abundance is essential for successful control management. However, these are not easy to explain owing to difficulties in morphologically distinguishing different species from one another and from the other closely related groups. For instance, in central Vietnam, members of both *An. dirus* and *An. minimus* species complexes were earlier considered primary vectors. However, an earlier study [16] has revealed the fact that those mosquitoes which were identified as *An. minimus* earlier were actually *An. varuna* (a member of the *Funestus* Group that also includes the *Minimus* Complex). *An. varuna,* being an extremely zoophilic species in the study region cannot be regarded as a vector. This incorrect identification led to the misuse of rare and valuable resources and thus creating hindrances in vector management strategies. In order to mitigate the difficulty of morphological identification, varieties of molecular techniques have been developed for distinguishing *An. minimus* s.l. and other closely related species [10, 14, 17, 18].

The present study was aimed at investigating the presence of potential malaria vector *An. minimus* in the region and to identify its sibling species in northeastern region of India

Methods

Study area and collection of *Anopheles* mosquitoes: Field collection of mosquitoes was performed during March 2016 to September 2016 (pre-monsoon and monsoon season). The adult *Anopheles* mosquitoes were collected from certain ecologically suited sentinel locations in Nameri (26.93° N - 92.88° E), Sialmari (26.71° N – 93.09° E), Jongakholi (25.98° N - 91.24° E), Chandubi (25.88° N - 91.43° E) areas of Assam (Fig. 1). These areas have vast forest land, scattered tea meadows and forest fringed inhabited areas with many streams, rivers and small irrigation canals with grassy margins, thereby providing conducive breeding habitat for *An. minimus* as well as for other *Anopheles* mosquitoes.

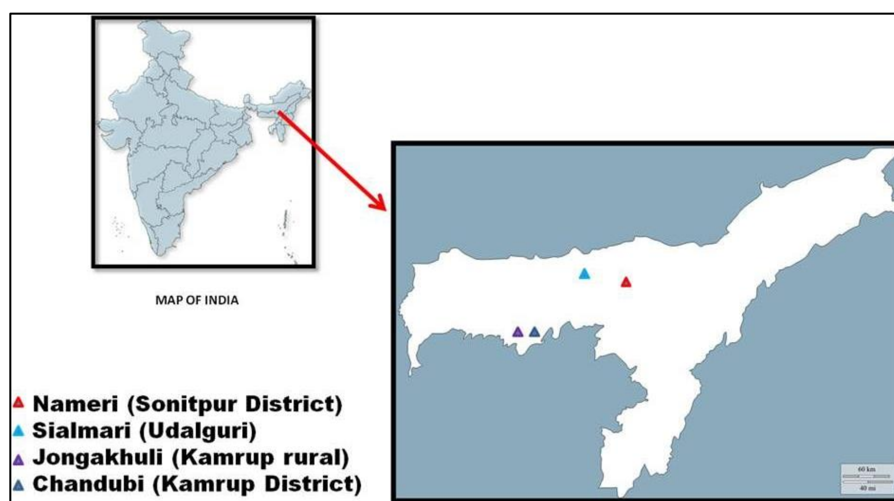

Fig 1: Collection sites: *Anopheles minimus* collection locations in Assam, North East India (map base taken from Google map; not to be scaled)

The indoor resting mosquitoes were located by torch light and collected by using mouth aspirators during early morning time (0500 – 0700 hr); whereas 6V battery operated CDC developed light traps were installed between 1800 hr to 0800 hr inside the human houses (total 4 trap nights/4 man hours at each site). The mosquitoes were morphologically identified using standard keys [19-21]. Since the study was primarily aimed at *An. minimus* mosquitoes, we separated mosquitoes that broadly look similar to *An. minimus*. These included *An. jeyporiensis, An. minimus, An. aconitus* and *An. varuna* and broadly categorized into *An. minimus* group in the present study. These mosquitoes were first used to identify *An. minimus* s.l. and subsequently the identified *An. minimus* s.l. specimens were processed for sibling species identification

using species specific PCR assays. In the present study

DNA Isolation: The DNA extraction was performed as described previously [22]. Briefly, each adult female mosquito was homogenized by using polypropylene micro pestle (Tarson, India) in 2 ml micro centrifuge tube (Tarson, India) having filled with 100 µl lysis buffer containing 0.1 M Tris–HCl, 0.05 M EDTA, 0.2 M Sucrose, 0.05 % SDS, 0.1 M NaCl. The homogenate was immediately kept on ice for 10 min followed by heat treatment at 65 ºC for 30 min. Subsequently, 30 µl 5M potassium acetate was added and immediately transferred to ice for one hour followed by centrifugation at 13,000 rpm for 15 minutes at 10 ºC. A double volume of absolute chilled ethanol was added to the supernatant. The tube was left undisturbed for precipitation of DNA and stored at -20 ºC for overnight. After centrifugation at 13,000 rpm for 15 min at 10 ºC, the precipitated DNA was washed in 70% ethanol twice. The DNA pallet was allowed to air dry and finally dissolved in 50 µl TE buffer for use as DNA template in PCR assays.

***An. minimus* species specific PCR assay:** The assay employed *An. minimus* species-specific reverse primers along with a universal forward primer [18] derived from highly conserved 5.8S coding region (Table 1). The PCR reaction was performed in 25 µl reaction volume containing - 1x PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl), 0.2 mM NTPs, 1.5 mM $MgCl_2$, 25 ng each of six primers, 0.625 U Taq, and 20 ng of DNA template[18]. The thermal cycle profile was optimized to the following conditions - 94 ºC for 5 min; then 32 cycles of 94 ºC for 1 min, 60 ºC for 2 min 72 ºC for 2 min and a final extension at 72 ºC for 7 min. Ten µl of PCR product mixed with 2 µl of ethidium bromide (EtBr) was run on 1.0 % agarose gel and the results were visualized under UV–VIS gel documentation system (Syngene, G-Box, UK). The method reported previously [18] is able to identify *An. minimus* s.s. (species 'A') and species 'C' of *An. minimus* complex and two other members of the *An. minimus* group (*An. aconitus* and *An. varuna*) along with *An. jeyporiensis*. However we have used primers that were specific to *An. minimus* species 'A' and 'C' only to identify species 'A', species 'C' or hybrid of 'A' and 'C' from the collected samples.

Table 1: Detail of primers used for differentiating *An. minimus* 'A' and 'C' within *An. minimus* sibling species complex

S. No.	Primer	Sequence	PCR Product
1	Universal forward (anneals to 5.8s coding region)	ATCACTCGGCTCATGGATCG	-
2	*An. minimus* 'A' specific	GGGCGCCATGTAGTTAGAGTTG	184 bp
3	*An. minimus* 'C' specific	GGTTGCCCACTCAATACGGGTG	509 bp

Results

Mosquito collection

In the present study different *Anopheles* species were collected from the sentinel study sites using mouth aspirator and CDC light traps (Table 2). Among the collected mosquitoes, two mosquito species namely, *An. annularis* and *An. vagus* were predominant and recorded in high number in all the collection sites. In Nameri, *An. vagus* was recorded in highest number both in trap collection (35.8%) ($\chi^2 = 51.6$; $p < 0.0001$) and indoor resting collection ($\chi^2 = 16.7$; $p = 0.01$) followed by *An. annularis* (26.9%). However in Jongakhuli, *An. annularis* was predominant in both CDC trap collection (72.7%; ($\chi^2 = 96.3$; $p < 0.0001$) and indoor resting collections ($\chi^2 = 43.6$; $p < 0.0001$).

During the study, *An. minimus* mosquitoes were collected in very low number. In Sialmari collection area, we found 4 *Anopheles* mosquitoes in bad condition and the morphological features of these specimens were deteriorated. Therefore we could not identify them correctly; however these were identified to *An. minimus* species 'A' using species specific PCR assay.

Table 2: Morphological identified *Anopheles* species, collected in CDC Trap and hand catch during the study

S. No	Location	*Anopheles* species	CDC trap collection (%)	χ^2 (p)	Indoor resting collection (%)	χ^2 (p)
1	Nameri	*An. jeyporiensis*	3 (4.5)	51.6 (<0.0001); df=6	1 (2.6)	16.7 (0.01); df=6
		An. aconitus	2 (3.0)		2 (5.1)	
		An. varuna	4 (6.0)		5 (12.8)	
		An. vagus	24 (35.8)		12 (30.9)	
		An. annularis	18 (26.9)		7 (17.9)	
		An. philippinensis	10 (14.9)		7 (17.9)	
		An. crawfordi	6 (9.0)		5 (12.8)	
2	Sialmari	*An. jeyporiensis*	7 (13.2)	25.3 (0.0001); df=5	2 (5.4)	28.2 (<0.0001); df=5
		An. minimus/ An. varuna	4 (7.5)		0 (0.0)	
		An. aconitus	4 (7.5)		4 (10.8)	
		An. varuna	6 (11.3)		6 (16.2)	
		An. vagus	18 (34.0)		11 (29.7)	
		An. annularis	14 (26.4)		14 (37.8)	
3	Jongakholi	*An. aconitus*	1 (1.8)	96.3 (<0.0001); df-3	2 (5.4)	43.6 (<0.0001); df=3
		An. vagus	12 (21.8)		7 (18.9)	
		An. annularis	40 (72.7)		24 (64.9)	
		An. crawfordi	2 (3.6)		4 (10.8)	
4	Chandubi	*An. varuna*	6 (14.3)	10.5 (0.01); df=3	5 (13.2)	9.1 9 (0.03); df=3
		An. vagus	18 (42.9)		16 (42.1)	
		An. annularis	10 (23.8)		9 (23.7)	

		An. philippinensis	8 (19.0)		8 (21.1)	

Identification of *Anopheles minimus* sibling species

Species specific PCR for *Anopheles minimus* sibling species diagnosis used genomic DNA of field collected *An. minimus* s.l. and amplified separately with primer specific for species 'A', species 'C' and both 'A' and 'C' for hybrid detection along with 5.8S forward primer for each reaction. First reaction with primers set 'A' yielded 184 base pair band that are specific to *An. minimus* species 'A' while PCR reactions for species 'C' and 'A/C' hybrid did not produce any band (Fig. 2).

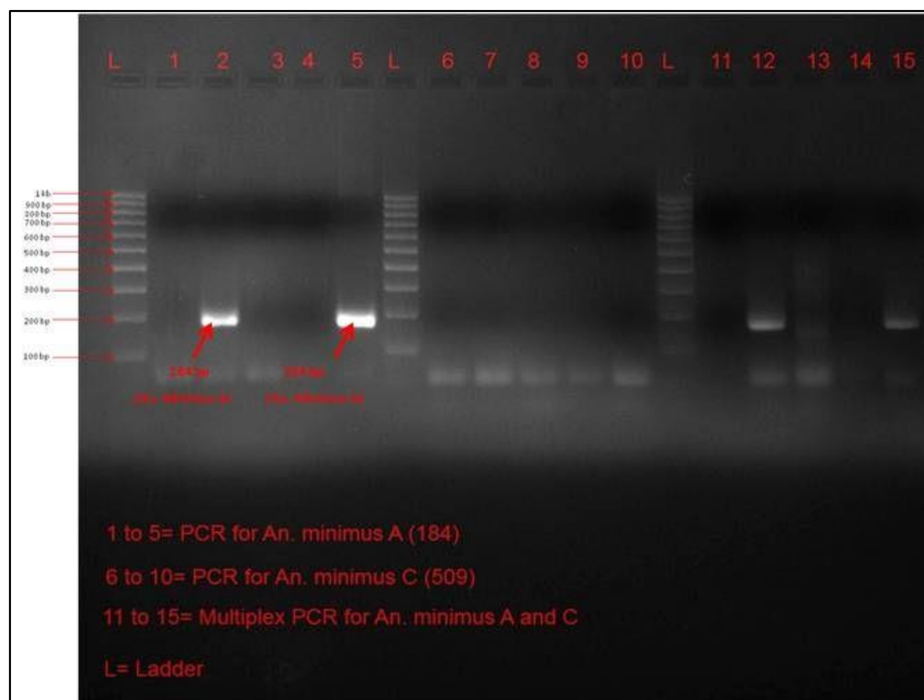

Fig 2: *An. minimus* sibling species identification using species specific PCR assay

Discussion

An. minimus has been recognized as an efficient malaria vector in North East region of India since long and held responsible for perennial transmission of *Plasmodium* parasite, as evidenced by high sporozoite rates recorded in different seasons [23]. In the region, *An. minimus* s.l. is endophilic and prefers to rest hiding on darker areas of walls, ceilings, cloths folding and temporary structures inside the human dwellings after taking blood meal. Studies have shown that *An. minimus* has very high affinity for human blood and reported anthropophilic index of > 90% in North East region of India [4, 6, 24]. Considering this, the mosquito collections in the present study were made inside the human houses with an objective to collect *An. minimus* mosquitoes. This species is still sensitive to various insecticidal formulations used in intervention programmes; hence it is argued that *An. minimus* may be exhibiting behavioral changes by preferring to bite and rest outdoors than indoors in order to avoid direct contact with the indoor sprayed insecticides. Although investigations have reported significant declining trend in *An. minimus* density[4] to virtually nil level in different states of North East region of India, however none of the study so far has evidenced that density has not declined but behavioral plasticity in *An. minimus* has led it to feed human blood and rest outdoor.

In the recent years there has been considerable change in the ecology in the region involving deforestation, increasing spread of cultivated lands and reduction in the breeding habitat which has influenced the vector composition drastically [25, 26]. *An. minimus* females are highly selective for egg laying places and lay eggs in grassy margins of unpolluted rivers and streams. Therefore water bodies lacking such supported ecology for *An. minimus* breeding may sometime account for absence of larvae in water and adults in the nearby places.

In the present study, we could not collect *An. minimus* mosquitoes in high number but still showed the *An. minimus* are still present in the region. Species specific PCR assay used was able to identify species 'A' of *An. minimus* complex. Different PCR based methods have been used successfully in identification of mosquito complexes accurately [18, 22, 27-31]. These methods are simple, precise and more accurately interpreted, hence could be used in segregating the closely related sibling species from each other. Furthermore, the multiplex PCR assay used presently was also able to identify sibling species 'A' of *An. minimus* among all the mosquito species used for the assay in the study. The PCR assay used produced unambiguous variation in PCR products of various *An. minimus* group mosquitoes in South East Asian countries, thus making this assay suitable and reliable for accurate identification of this mosquito complex [18].

Studies have reported that *An. minimus* was once widely prevalent and involved in disease transmission in Himalayan foothills of northern to eastern region of India [8, 23, 24]. However, later thought to have been disappeared as studies did not report this species from Sub-Himalayan foothill regions [9]. However, this species appeared in eastern region after more than four decades and involved in malaria transmission [32, 33]. Similarly, malaria vectors that were disappeared completely and believed to be eliminated under

the influence of control interventions or ecological changes were found to have been re-emerged later and incriminated as vector involved in malaria outbreaks [33].

Many studies conducted during past few years have reported malaria cases in North East region of India in the absence of *An. minimus*, while establishing that other anopheline mosquitoes that played insignificant role in malaria transmission previously, have taken over as important vectors and involved in continuous transmission of *Plasmodium* species throughout the year [8, 9]. The present study conducted in forest fringed foothills areas that provide suitable breeding and proliferation ecology for *An. minimus* s.l. confirms that this important vector has not disappeared completely but limited its prevalence into certain favorable areas. Present study has identified *An. minimus* species ‘A’ which is a well known vector in the region. The findings further suggest that *An. minimus* may be thriving under selection pressure as numbers of insecticidal products are in place to target this important malaria vector. However it may re-surge in high density once these large scale interventions targeting this mosquito are withdrawn considering that it is disappeared completely.

Conclusion

Present study has attempted to establish that *An. minimus* ‘A’ is existing in the North Eastern region of India but limited to certain ecologically suitable pockets in forest fringed areas. The environmental conditions still favour the prevalence of this potential vector which is probably striving under selection by changing ecology as well as increasing insecticidal pressure. Therefore complete disappearance of this mosquito in the study region can be overruled. Study also emphasizes that it is not appropriate to conclude *An. minimus* group identification without using PCR like sensitive methods, as it may give confusing results uninvited for vector control programmes.

Acknowledgements

We are thankful to the Director, Defence Research Laboratory Tezpur to support the work. The help rendered by the villagers during the study is deeply acknowledged.

Reference

1. World Health Organisation. World malaria report 2019. Geneva: WHO Press, World Health Organization 2019.
2. Dhiman S, Veer V, Dev V. Declining transmission of malaria in India: accelerating towards elimination. In: Manguin, S.; Dev, V. (editors). Towards malaria elimination - a leap forward. Intech Open, 2018. London, UK. https://doi.org/10.5772/intechopen.77046.
3. Dhiman S. Are malaria elimination efforts on right track? An analysis of gains achieved and challenges ahead? Infect. Dis. Poverty 2019;8(14).
4. Dhiman S, Bhola RK, Goswami D, Rabha B, Kumar D, Baruah I *et al*. Polymerase chain reaction detection of human host preference and *Plasmodium* parasite infections in field collected potential malaria vectors. Pathogen Global Hlth 2012;106(3):177-80.
5. Dhiman S, Yadav K, Rabha B, Goswami D, Hazarika S, Tyagi V. Evaluation of insecticides susceptibility and malaria vector potential of *Anopheles annularis* s.l. and *Anopheles vagus* in Assam, India. PLoS ONE 2016;11(3):e0151786. oi:10.1371/journal.pone.0151786.
6. Dev V, Sangma BM, Das A. Persistent transmission of malaria in Garo Hills of Meghalaya bordering Bangladesh, north-east India. Malaria J 2010;9(1):263.
7. Saxena R, Das MK, Nagpal BN, Srivastava A, Gupta SK, Kumar A *et al*. Identification of risk factors for malaria control by focused interventions in Ranchi district, Jharkhand, India. J. Vector Borne Dis 2014;51:276-81.
8. Dev V, Sharma VP, Barman K. Mosquito-borne diseases in Assam, north-east India: current status and key challenges. WHO South-East Asian J Public Hlth 2015;4:20-9.
9. Yadav K, Dhiman S, Rabha B, Goswami D, Saikia PK, Veer V. Disappearance of *An. minimus* and *An. dirus* from certain malaria endemic areas of Assam, India. J Arthropod-Borne Dis 2017;11(1):27-35.
10. Green CA, Gass RF, Munstermann LE. Population genetic evidence for two species in *Anopheles minimus* in Thailand. Med. Vet. Entomol 1990;4:25-34.
11. Harbach RE. The classification of genus *Anopheles* (Diptera: Culicidae): a working hypothesis of phylogenetic relationships. Bull. Entomol. Res 2004;94:537-53.
12. Dutta P, Khan SA, Topno R, Chowdhury P, Baishya M, Chowdhury P *et al*. Genetic diversity and gene structure of mitochondrial region of *Anopheles minimus* (Diptera: Culicidae) - major malaria vector of North East India. Asian Pacific J. Trop. Med 2014, 952-5.
13. Somboon P, Walton C, Sharpe RG, Higa Y, Tuno N, Tsuda Y *et al*. Evidence for a new sibling species of *Anopheles minimus* from the Ryukyu Archipilago, Japan. J Am. Mos. Control Assoc 2001;17:98-113.
14. Van Bortel W, Trung HD, Roelants P, Harbach RE, Backeljau T, Cooseman M. Molecular identification of *Anopheles minimus* s.l. beyond distinguishing the members of the species complex. Insect Mol. Biol 2000;9:335-40.
15. Van Bortel W, Sochanta T, Harbach RE, Socheat D, Roelants P, Backeljau T *et al*. Presence of *Anopheles culicifacies* B in Cambodia established by the PCRRFLP assay developed for the identification of *Anopheles minimus* species A and C and four related species. Med. Vet. Entomol 2002;16:329-34.
16. Van Bortel W, Harbach RE, Trung HD, Roelants P, Backeljau T, Coosemans M. Confirmation of *Anopheles varuna* in Vietnam, previously misidentified and mistargeted as the malaria vector *Anopheles minimus*. Am. J. Trop. Med. Hyg 2001;65:729-32.
17. Van Bortel W, Trung HD, Manh ND, Roelants P, Verle P, Cooseman M. Identification of two species within the *Anopheles minimus* complex in northern Vietnam and their behavioural divergences. Trop. Med. Int. Health 1999;4:257-65.
18. Phuc HK, Ball AJ, Son L. Multiplex PCR assay for the malaria vector *Anopheles minimus* and four related species in the Myzomyia Series from Southeast Asia. Med. Vet. Entomol 2003;17:423-8.
19. Das BP, Rajagopal R, Akiyama J. Pictorial key to the species of Indian anopheline mosquitoes. Zoology 1990;2:131-62.
20. Harrison BA. Medical entomology studies. XIII. The Myzomyia series of *Anopheles (*Cellia) in Thailand, with emphasis on intra-inter specific variations (Diptera: Culicidae). Contr. Am. Entomol. Inst 1980;17:1-195.

21. Nagpal BN, Sharma VP. Indian Anophelines. Oxford & IBH Publishing Co. Pvt. Ltd., Mohan Primlani, New Delhi 1995, 1-416.
22. Tyagi V, Sharma AK, Dhiman S, Srivastava AR, Yadav R, Sukumaran D *et al*. Malaria vector *Anopheles culicifacies* sibling species differentiation using egg morphometry and morphology. Parasit. Vect 2016;9:202.
23. Dev V, Manguin S. Biology, distribution and control of *Anopheles (Cellia) minimus* in the context of malaria transmission in northeastern India. Parasit. Vect 2016;9:585.
24. Dev V. *Anopheles minimus*: its bionomics and role in the transmission of malaria in Assam India. Bull. World Health Org. 1996;74(1):61-6.
25. Nath MJ, Bora A, Talukdar PK, Das NG, Dhiman S, Baruah I *et al*. A longitudinal study of malaria associated with deforestation in Sonitpur district of Assam, India. Geocarto Int 2012;27(1):79-88.
26. Yadav K, Nath MJ, Talukdar PK, Saikia PK, Baruah I, Singh L. Malaria risk areas of Udalguri district of Assam, India: a GIS-based study. International J. Geograp. Info. Sci 2012;26(1): 123-31.
27. Walton C, Handley M, Kuvangkadilok C, Collins FH, Harbach RE, Raimai V *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.
28. Chen B, Harbach RE, Butlin RK. Molecular and morphological studies on the *An. minimus* group of mosquitoes in southern China: taxonomic review, distribution and malaria vector status. Med. Vet. Entomol 2002;16:253-65.
29. Sharpe RG, Hims MM, Harbach RE, Butlin RK. PCR-based methods for identification of species of the *Anopheles minimus* group: allele-specific amplification and single-strand conformation polymorphism. Med. Vet. Entomol 1999;13:265-73.
30. Kengue P, Trung HD, Baimai V, Coosemans M, Manguin S. A multiplex PCR-based method derived from random amplified polymorphic DNA (RAPD) markers for the identification of species of the *Anopheles minimus* group in Southeast Asia. Insect Mol. Biol 2001;10:427-35.
31. Singh OP, Goswami G, Nanda MN, Raghavendra K, Chandra D, Subbarao SK. An allele-specific polymerase chain reaction assay for the differentiation of members of the *Anopheles culicifacies* complex. J Biosci 2004;29:275-280.
32. Gunasekaran K, Sahu SS, Jambulingam P. Estimation of vectorial capacity of *Anopheles minimus* Theobald & *An. fluviatilis James* (Diptera: Culicidae) in a malaria endemic area of Odisha State, India. Indian J Med. Res 2014;140(5): 653-9.
33. Jambulingam P, Sahu SS, Manonmani A. Reappearance of *Anopheles minimus* in Singhbum hills of East-Central India. Acta Trop 2005;96:31-5.