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Research Note

Genetic analyses of Ribosomal loci of *Anopheles minimus* species from North east India

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Abstract. *Anopheles minimus* is one of the major vectors for transmission of malaria disease in north eastern (NE) region of India. The minimus species complex of Minimus subgroup of Myzomyia series of anophelines were studied in malaria affected states- Assam and Arunachal Pradesh (AP) of NE India. Ribosomal DNA markers- second internal transcribed spacer (ITS2) and third domain (D3) of 28S gene were used to characterize *An. minimus* species. Sequence homogeneity was observed in D3 sequences of *An.minimus* specimens throughout both the states. However, a transversion in ITS2 sequence of single specimen collected from Assam-Meghalaya border areas illustrates possibility of intra population polymorphism in ITS2 sequence within the geographical region.

Anopheles minimus sensu lato (s.l.) Theobald 1901, of Minimus subgroup of Myzomyia Series is recognized as an important malaria vector species in Oriental region. *Anopheles minimus* comprises of three sibling species, namely *An. minimus* (formerly species A), *Anopheles harrisoni* Harbach & Manguin (formerly sp. C) and *Anopheles yaeyamaensis* Somboon & Harbach (formerly sp. E). In India (latitude- 22° 00' N and longitude- 77° 00' E), *An. minimus* s.l. is considered as one of the major malaria vectors in North-eastern (NE) region (latitude- 21°58' N to 29°30'N and longitude- 88°3' E to 97°30' E) (Prakash *et al.*, 2004). *Anopheles minimus* had been previously incriminated as vectors in two reportedly malaria endemic states of NE region viz. Assam (latitude- 24° 8'N to 28° 2' N and longitude- 89° 42' E to 96° E) and Arunachal Pradesh (AP) (latitude- 26°30'N to 29° 30'N and longitude- 91° 30' E to 97° 30'E) (Rao, 1984; Dutta & Baruah, 1987; Dutta & Mahanta, 1995; Prakash *et al.*, 2004). The physiography of NE region is very much similar with neighbouring southeastern countries like China, Thailand and Vietnam where *An. minimus* is a major vector (Chen *et al.*, 2011). Thus the objectives of present study were molecular identification and genetic diversity of *An. minimus* s.l. based on ribosomal markers- ITS2 and D3.

Mosquito collections in human dwellings were carried out from 17 sites of Assam and 8 sites of AP during 2008-2011 using Centers for Disease Control (CDC) miniature light traps (Table 1). Mosquitoes were morphologically identified using standard anopheline identification keys (Das *et al.*, 1990). Genomic DNA was extracted by using FTA Classic card nucleic acid extraction technology (Whatman) (Mohanty *et al.*, 2007). Extracted DNA was subjected to Allele Specific PCR (ASPCR) based on Phuc *et al.* (2003). Ribosomal markers- ITS2 and D3 were used to study genetic diversity. ITS2 and

Table 1. Molecular confirmed *An. minimus* species at different geographical localities

Sl. No.	Area of collection	Ecological terrain	Type of collection	Latitude Longitude	Anopheles Collected	*An minimus* identified
1.	Torajan, Dibrugarh	Foothills	Cattleshed & Human dwellings	27.3 N, 95.4E	Cattleshed-71 Human dwelling-59	–
2.	Lezai, Dibrugarh	Plains	Human dwellings	27.4N, 94.8E	60	–
3.	Margherita, Dibrugarh	Plains	Human dwellings	27.2N, 95.6E	95	–
4.	Boko, Kamrup	Foothills	Cattleshed & Human dwellings	25.9N, 91.2E	Cattleshed-75 Human dwellings-68	4
5.	Sonapur, Kamrup	Foothills	Cattleshed & Human dwellings	26.1N, 91.9E	Cattleshed-24 Human dwellings-12	2
6.	Manash, Barpeta	Forest	Cattleshed & Human dwellings	26.7N, 91.0E	Cattleshed-63 Human-33	2
7.	Tamulpur Nalbari	Foothills	Cattleshed & Human dwellings	26.6N, 91.5E	Cattleshed-26 Human-14	1
8.	Koilamari, Lakhimpur	Forest	Cattleshed & Human dwellings	27.3N, 94E	Cattleshed-6 Human-3	–
9.	Bhitoripam, Lakhimpur	Plains	Cattleshed & Human dwellings	27.2N, 93.9E	Cattleshed-9 Human-7	–
10.	Kohora, Golaghat	Foothills	Human dwellings	26.5N, 93.4E	340	–
11.	Bokakhat, Golaghat	Plains	Cattleshed & Human dwellings	26.6N, 93.6E	Cattleshed-33 Human-26	2
12.	Titabor, Jorhat	Plains	Cattleshed & Human dwellings	26.5N, 94.1E	Cattleshed-289 Human-178	–
13.	Soru Amsoi, Nagaon	Foothills	Human dwellings	26N, 92.1E	4	1
14.	Goalpara, Kamrup	Plains	Cattleshed & Human dwellings	26.1N, 90.6E	Cattleshed-19 Human-9	–
15.	Hamren, Karbi Anglong	Foothills	Cattleshed & Human dwellings	25.8N, 92.5E	Cattleshed-34 Human-23	2
16.	Hafflong,	Foothills	Cattleshed & Human dwellings	25.1N, 93.0E	Cattleshed-23 Human-12	
17.	Chirang,	Foothills	Cattleshed & Human dwellings	26.6N, 90.3E	Cattleshed-30 Human-19	
18.	Bandardewa, AP	Forest	Cattleshed & Human dwellings	27.1N, 93.8E	Cattleshed-7 Human-4	3
19.	Piong, AP	Foothills	Cattleshed & Human dwellings	27.5N, 95.9E	Cattleshed-201 Human-184	1
20.	Kimin, AP	Foothills	Cattleshed & Human dwellings	27.3N, 93.9E	Cattleshed-93 Human-62	15
21.	Namsai, AP	Foothills	Cattleshed & Human dwellings	27.6N, 95.8E	Catteshed-65 Human-28	
22.	Khunsa, AP	Foothills	Cattleshed & Human dwellings	26.9N, 95.5E	Cattleshed-31 Human-23	
23.	Ruksin, AP	Foothills	Cattleshed & Human dwellings	28.0N, 95.3E	Cattleshed-14 Human-6	
24.	Bhalukpong, AP	Foothills	Cattleshed & Human dwellings	27N, 92.5E	Cattleshed-15 Human-7	6
25.	Ampen, Miao, AP	Foothills	Human dwellings	27.4N, 96.2E	164	37

D3 region were amplified based on Walton *et al.* (1999) and Singh *et al.* (2004) respectively with modifications. Amplified PCR products were purified by Invitrogen PureLink™ PCR purification kits. Purified products were then sequenced in both directions. Sequencing was outsourced to Anshul Biotech, India. Both forward and reverse direction of each sequence was checked and edited manually using BioEdit Sequence Alignment Editor Software. ITS2 and D3 sequences were aligned using default parameters in Clustal W software. Edited sequences were submitted to NCBI GENBANK and respective accession numbers were obtained. Sequences inferences were done by using Maximum Likelihood method based on Tamura- Nei model. Evolutionary analyses were conducted in Molecular Evolutionary Genetics Analysis (MEGA) version 5.0.

Out of 2582 anophelines collected from 17 sites in Assam and 8 sites in AP, only 76 were confirmed by AS-PCR to be *An. minimus* from 12 sites (Assam- 6 and AP-6). Molecular characterization based on ITS2 and D3 region of *An.minimus* was carried out. Two specimens each representing all 12 sites was further processed for sequence analysis. Interestingly, in ITS2 amplification, an AS-PCR confirmed *minimus* species from Sonapur area of Assam-Meghalaya border showed amplification at 585 bp, whereas other ITS2 sequences showed amplification product at 562 bp. ITS2 amplicons were sequenced, edited with 5.8S and 28S borders defined region, actual length of ITS2 sequences was determined to be 370 bp. The tree generated with nucleotide sequences with highest log likelihood (-485.7631) is shown (Figure 1). 100% homogeneity with no intraspecific variations among the sequences was observed. However, the single specimen of *An. minimus* collected from Sonapur area of Assam- Meghalaya border (GenBank accession no. JQ046375) showed variations from rest of *An. minimus* species. This particular specimen had a transversion (T-A) at nucleotide position 4 (Figure 2). The sequence showed 100% similarity with a sequence from China (Gen Bank accession no: AF 416783.1). D3 region of confirmed species were amplified showing amplification at 375bp. Phylogeny analysis based on D3 region for *An. minimus* from all sequences revealed no differences. Our submitted sequences showed 100% homology with sequences previously reported from the region (GenBank accession no. EF221771.1).

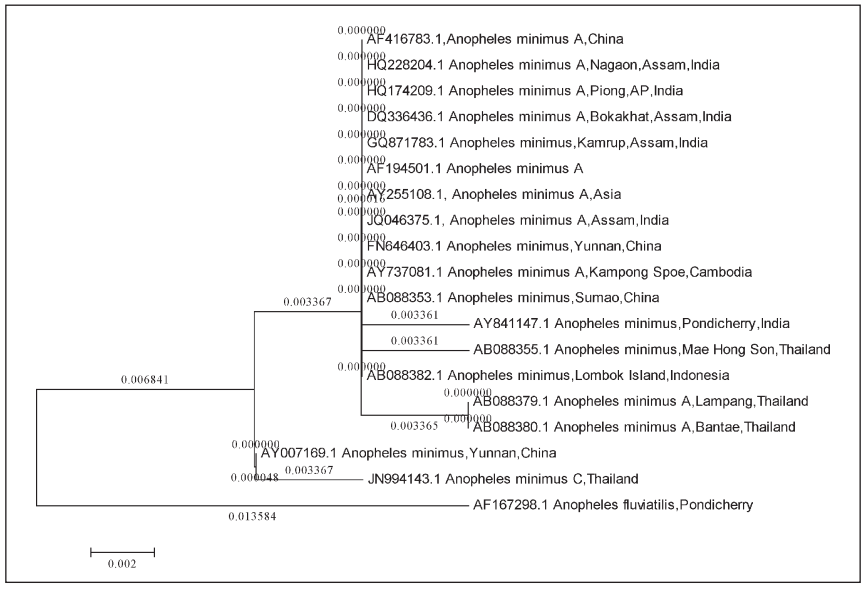


Figure 1. Phylogeny of *An. minimus* based on ITS2. Phylogenetic analysis is based on ITS2 region of rDNA. Maximum Likelihood phylogeny was constructed based on Tamura- Nei model

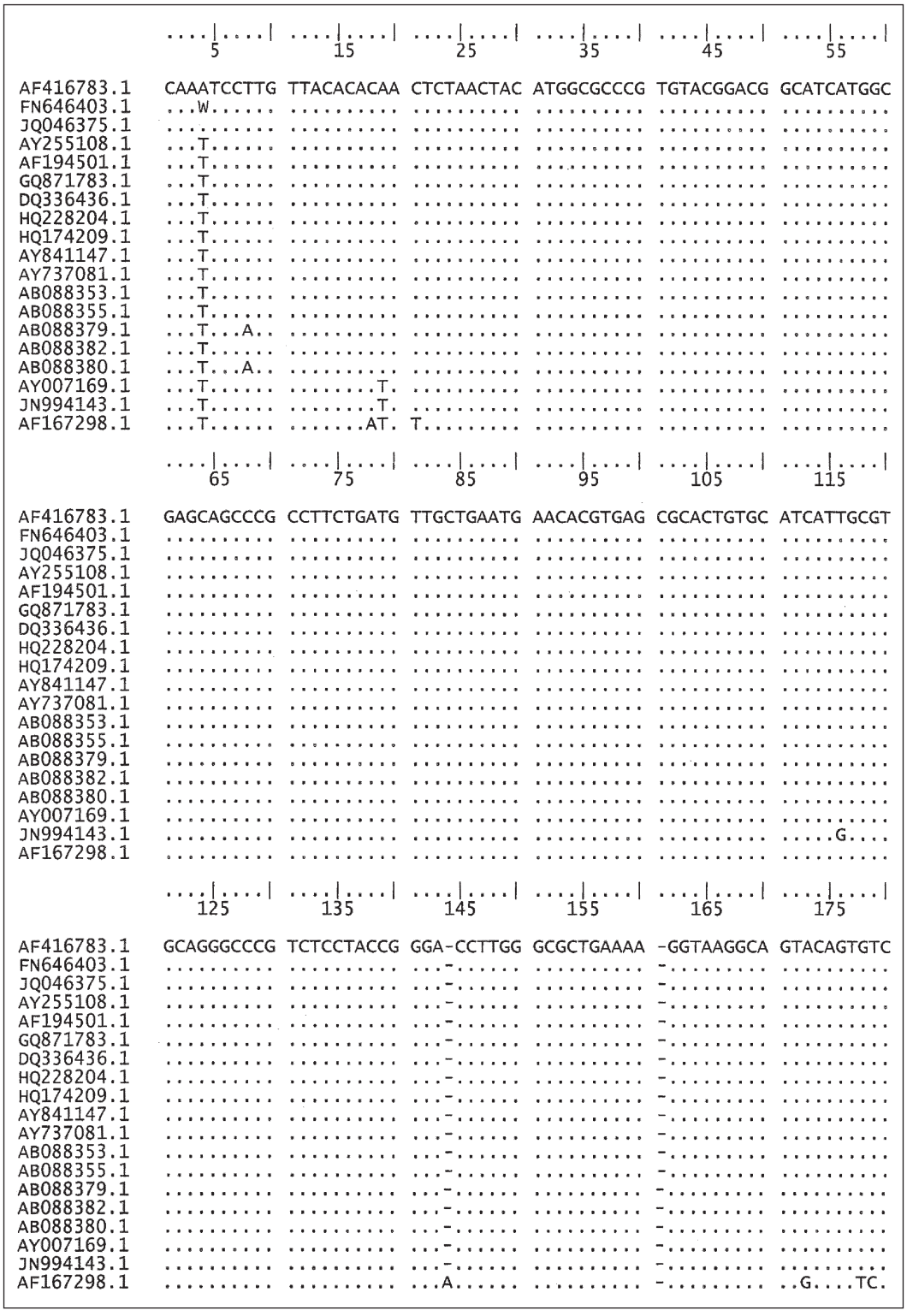
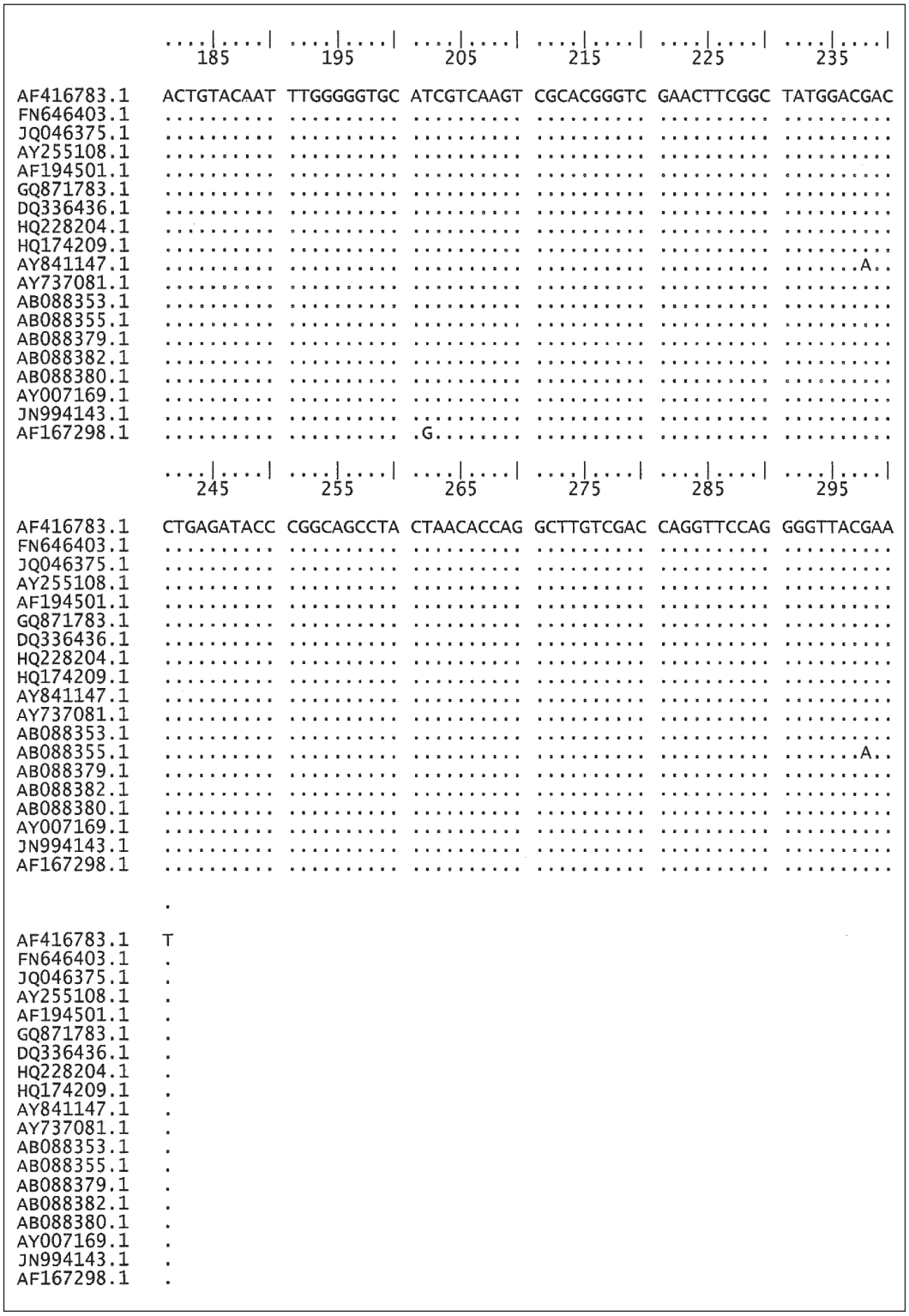


Figure 2. Alignment of ITS2 sequences. Dots (.) indicate identity of nucleotides within the alignment

Figure 2. *Cont'd*



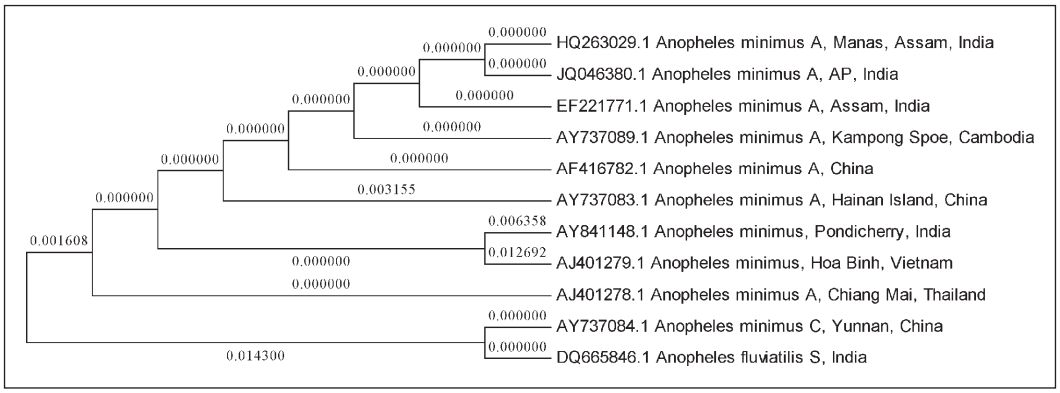


Figure 3. Phylogeny of *An. minimus* based on D3. Phylogenetic analysis is based on D3 region of rDNA. Maximum Likelihood phylogeny was constructed based on Tamura- Nei model

We studied genetic diversity of *An. minimus* species of mosquitoes from two states- Assam and AP of NE India covering almost all malaria endemic areas. Since ITS2 sequences are likely to be fixed within the species and vary between closely related species due to high intraspecific homogeneity and interspecific variability, ITS2 region of rDNA of ASPCR confirmed *An. minimus* was amplified. ITS2 sequences of specimens in our collected sites were completely identical with sequences of that previously recorded from China, Thailand and other parts of India (Prakash *et al.*, 2004) (Figure 1). However, a single specimen showed a single base substitution (Transversion at site 4) (Figure 2) in ITS2 sequence from Sonapur area of Assam-Meghalaya border. This variation showed a distinct population structure. Larger number of samples from the area is needed to sequence to determine extent and nature of any distinct population structure since this bit of information has potential implications for malaria control in malaria endemic PHC of Sonapur (NIMR, 2012). The sequence analysis of *An. minimus* species showed 100% homology in 28S- D3 rDNA (Figure 3). Thus, it can be stated that no intra- specific variations were found among the specimens. It has been observed in the present study that our submitted sequences showed similarity with previously submitted sequences from the region and also with the sequences from China, Thailand and Cambodia. However, the sequences showed variations with sequence of Vietnam and placed in different clades. Distant geographical locations may be the possible reason for diversity of sequences. D3 sequence homogeneity remains over a local geographical scale. The molecular phylogenetic analysis of *An. minimus* species from these two states showed homogeneity in D3 regions. These similarities showed that there is lack of intra specific variations at the loci throughout the geographical range of Assam and AP states.

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Conflict of Interest:
None to declare.

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