On the conspecificity of *Anopheles fluviatilis* species S with *Anopheles minimus* species C

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Anopheles fluviatilis and *An. minimus* complexes, each comprising of at least three sibling species, are closely related and important malaria vectors in Oriental Region. Recently *An. fluviatilis* species S, which is a highly efficient malaria vector in India, has been made conspecific with *An. minimus* species C (senior synonym) on the basis of homology in 335 base pair nucleotide sequence of D3 domain of 28S ribosomal DNA (rDNA). We examined the conspecificity of these two nominal species by obtaining and analysing the DNA sequences of nuclear ribosomal loci internal transcribed spacer 2 (ITS2) and D2-D3 domain of 28S rDNA (28S-D2/D3) from those of *An. fluviatilis* S and *An. minimus* C. We found that the sequences of *An. fluviatilis* S are appreciably different from those of *An. minimus* C with pair-wise distance (Kimura-2-parametre model) of 3.6 and 0.7% for loci ITS2 and 28S-D2/D3, respectively. Pair-wise distance and phylogenetic analyses using ITS2 sequences of members of Minimus and Fluviatilis Complexes revealed that *An. fluviatilis* S is distantly related to *An. minimus* C as compared to any other members of the Fluviatilis Complex. These findings suggest that the two nominal species, *An. fluviatilis* S and *An. minimus* C, do not merit synonymy. The study also confirms that the reported species *An. fluviatilis* X is synonym with species S.

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1. Introduction

Anopheles fluviatilis s.l. and *An. minimus s.l*., two important malaria vector species in Oriental Region, are closely related and belong to the Minimus Subgroup of Anopheles (Harbach 2004). The *An. fluviatilis s.l.* has been described to be extended from Yemen to Taiwan (Knight and Stone 1977) but their presence beyond east of northeastern states of India is doubtful (Harrison 1980). The distribution of *An. minimus s.l.* extends from India across the Indochina-Malay peninsular countries down to the Thai-Malay border and north across the People's Republic of China (up to 30"N latitude) to Taiwan and the Ryukyu islands (Harrison 1980). *An. fluviatilis* and *An. minimus* are now recognized as species complexes, each comprising of at least three sibling species—species S, T and U in the Fluviatilis Complex (Subbarao *et al* 1994) and species A, C and E in the Minimus Complex (Harbach 2004). Significant differences in their biological characteristics have been reported that are important from vector-control or epidemiological point of views. Among Fluviatilis Complex species S has been recognized as highly efficient malaria

Keywords. *Anopheles fluviatilis*, *Anopheles minimus*, malaria, ribosomal DNA, molecular taxonomy, sibling species

Abbreviations used: ITS2, Internal transcribed spacer 2; ML, Maximum Likelihood; MP, Maximum Parsimony; NJ, Neighbour-Joining; rDNA, ribosomal DNA.

vector due to high anthropophagy whereas species T and U are regarded as non-vectors being almost exclusively zoophagic (Nanda *et al* 1996; Subbarao 1998). Among *An. minimus* complex, species C has been reported to be likely more exophagic and zoophilic as compared to species A (Van Bortel *et al* 1999). Correct identification of members of these closely related species-complexes is therefore crucial for planning vector-specific malaria control strategies.

In recent years *An. fluviatilis* S, which is among the highly efficient malaria vectors from India, has been considered a synonym of *An. minimus* species C (Harbach 2004; Garros *et al* 2005; Chen *et al* 2006). These reports are based on a comparison of the 335 base pair nucleotide sequence of the D3 domain of 28S (28S-D3) ribosomal DNA (rDNA) of *An. fluviatilis* S (GenBank accession No. AF437880, Singh *et al* 2004a) with that of *An. minimus* species C (Garros *et al* 2005). However the internal transcribed spacer 2 (ITS2) of a putative species *An. fluviatilis* X (Manonmani *et al* 2001), which was later recognized as species S by Manonmani *et al* (2003), was not homologous to that of *An. minimus* C. Disagreeing with Manonmani *et al* (2003), Chen *et al* (2006) however believe that *An. fluviatilis* X may represent another species within the *An. fluviatilis* complex. In the present study we determined the nucleotide sequences of the ITS2 and D2-D3 domain of 28S rDNA (28S-D2/D3) from *An. fluviatilis* S and *An. minimus* C. and analysed the data to resolve these taxonomic problems. The rDNA multigene family was specifically selected for this purpose due to its high intraspecific homogeneity resulting from concerted evolution, which however does not affect interspecific divergence.

2. Experimental procedures

2.1 *Mosquitoes: collection, identification and DNA isolation*

The adult females of *An. fluviatilis* used in this study were collected from Sundergarh (22.1^0 N, 84.0^0 E) and Koraput (18.8^0 N, 82.7^0 E) districts of Orissa state (India)—the localities from which *An. fluviatilis* S (Subbarao *et al* 1994) and X (Manonmani *et al* 2001), respectively, were originally described. All collections were made from human dwellings. The mosquitoes were morphologically identified following Christophers (1933) and cytologically characterized for species-specific inversions on chromosome arm 2 following Subbarao *et al* (1994). Since q1 inversion (used for differentiation of species S and T) is polymorphic in some areas (Subbarao 1998), making it difficult to distinguish species S from species T, the specific status of *An. fluviatilis* was also confirmed on the basis of 28S-D3 rDNA sequences of members of Fluviatilis Complex (Singh *et al* 2004a).

The adult females of *An. minimus s.l.* were collected from Pyin Oo Lwin Township (Mandalay division; 22.2^0 N, 96.27^0 E) located in the central part of Myanmar, identified morphologically by field staff of Medical Entomology Research Division, Yangon, Myanmar following Delphin and Rao (1957), and placed individually in micro-centrifuge tubes containing silica gel plugged with a small piece of cotton wool. Mosquitoes were re-identified at National Institute of Malaria Research following Christophers (1933). The DNA of individual mosquitoes was isolated following Coen *et al* (1982). The assignment of sibling species was done on the basis of homology of ITS2 sequences of individual mosquitoes obtained during this study with the published sequences of members of Minimus group (Sharpe *et al* 2000; Van Bortel *et al* 2000; Garros *et al* 2005; Chen *et al* 2006).

2.2 *PCR amplification and DNA sequencing*

The ITS2 region of rDNA was amplified using primers ITSA (5'-TGT GAA CTG CAG GAC ACA T-3') and ITSB (5'-TAT GCT TAA ATT CAG GGG GT-3') (Beebe and Saul 1995). The D2-D3 domain of 28S rDNA was amplified using primers D2A (5'-AGT CGT GTT GCT TGA TAG TGC AG-3') (Campbell *et al* 1993) and D3B (5-TCG GAA GGA ACC AGT TAC TA-3') (Litvaitis *et al* 1994). For each PCR assay, we used 50 µl PCR reaction mixture which contained 0.50 µM of each primer, 200 µM of each dNTP, 1.5 mM of $MgCl_2$ and 1.25 unit of Taq polymerase. The PCR conditions for both PCRs were an initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min followed by a final extension at 72°C for 7 min. The PCR products were purified using Quiaquick PCR Purification Kit (Qiagen, California, USA) to remove unincorporated primers and dNTPs prior to sequencing. The sequencing was done on both strands of amplified DNA using the BigDye v3.1 Terminator Cycle Sequencing Ready Kit (Applied Biosystem, Foster City, USA) following the manufacturer's protocol. The primers used for sequencing were the same as those used in the original PCR amplification. Two additional internal primers i.e D3A (5'- GAC CCG TCT TGA AAC ACG GA-3') (Litvaitis *et al* 1994) and D2B (5'-TTG GTC CGT GTT TCA AGA CGG G-3') (Campbell *et al* 1993) were also used for sequencing of 28S-D2/D3 to remove any ambiguity in sequence reading. The sequences have been deposited in GenBank and their accession numbers are given in table 1. Fifteen specimens of *An. minimus* C and 22 specimens of *An. fluviatilis* S were sequenced for both ITS2 and 28S-D2/D3.

Table 1. DNA sequences used in the study.

Species	Locality	Locus	GenBank accession number	Reference
An. fluviatilis S	Sundergarh, Orissa (India) Koraput, Orissa (India)	ITS2	DQ345964	This study
An. fluviatilis S	Sundergarh, Orissa (India) Koraput, Orissa (India)	28S-D2/D3	DQ665846	This study
An. fluviatilis X	Koraput and Malkangiri, Orissa (India)	ITS2	AF167298†	Manonmani *et al* 2001
An. fluviatilis T	Hardwar, Uttaranchal (India)	ITS2	DQ238490	Chen *et al* 2006
An. fluviatilis U	Hardwar, Uttaranchal (India)	ITS2	DQ238492	Chen *et al* 2006
An. minimus C	Pyin Oo Lwin, Mandalay (Myanmar)	28S-D2/D3	DQ665848	This study
An. minimus C	Pyin Oo Lwin, Mandalay (Myanmar)	ITS2	DQ665847	This study
An. minimus C	Kanchanburi Province (Thailand)	ITS2	AF194506	Sharpe *et al* 2000
An. minimus C	Guangxi; Guizhou; Sichuan; Yunnan (China) Chiang Mai; Kanchanaburi (Thailand); Hoa Binh (Vietnam)	ITS2	DQ238493	Chen *et al* 2006
An. minimus C	Hanoi suburbs, Hoa Binh (Vietnam)	ITS2	AF230462	Van Bortel *et al* 2000
An. minimus C	Hoa Binh Pr. (Vietnam)	ITS2	AY255109	Garros *et al* 2005
An. minimus C	Not known	ITS2	AF416784	Shuisen and Linhua, unpublished
An. minimus A	Guangdong; Guangxi; Hainan; Taiwan; Yunnan (China); Chiang Mai; Kanchanaburi; Krabi; Loei; Nakhon; Si Thammarat; Prachuap Khiri Khan; Ranong; Songkhla (Thailand); Hoa Binh (Vietnam)	ITS2	DQ238494	Chen *et al* 2006
An. leesoni	Northern Province, South Africa	ITS2	AY255107	Garros *et al* 2004
An. flavirostris	Philippines	ITS2	AY943666	Torres *et al* 2006

†The GenBank sequence differes from corresponding published sequence (Acta Trop. 78:3, 2001) by one base pair. We used published sequence in this study.

2.3 *Sequence analysis*

The sources of DNA sequence data that were used in the analyses are shown in table 1. For the phylogenetic analysis, ITS2 sequences of some closely related members were downloaded from GenBank.

DNA sequences were aligned using the ClustalW method implemented in software Molecular Evolutionary Genetics Analysis version 3.1 (MEGA 3.1) (Kumar *et al* 2004). Genetic distances were estimated by the Kimura 2–parameter model using software MEGA 3.1. Sites containing alignment gaps were not used in the distance analyses and were treated as missing information. The analysis of phylogenetic relationship between members of the Fluviatilis and Minimus Complexes was carried out using ITS2 sequences. Maximum Parsimony (MP), Maximum Likelihood (ML) and Neighbour-Joining (NJ) methods were used for construction of phylogenetic trees with the software PAUP* version 4.0 beta 10 (Swofford 2001). The MP and ML analyses were carried out using the heuristic searches and TBR branch-swapping algorithm with ten random taxon addition sequences. For ML-tree we obtained the best evolutionary model of nucleotide substitution that fits the data using software Modeltest3.7 (Posada and Crandall 1998). Results from these analyses showed that the best fit for our data was JC+G model as selected by the Hierarchical Likelihood Ratio Test (hLRTs). The robustness of the resulting phylogenetic topologies was examined by bootstrapping (500 pseudo-replicates for MP and ML, and 2000 replicates for NJ). The sequences of *An. flavirostris* and *An. leesoni* were taken as outgroup during construction of the tree.

3. Results

Of 28 mosquitoes collected in Myanmar and identified as *An. minimus s.l.* by the field staff of Department of Medical Research, Yangan (Myanmar), 24 were reconfirmed to be *An. minimus* on the basis of morphological characters following Christophers (1933). The others could not be confirmed due to loss of essential characters required for

Table 2. Pair-wise distances (below diagonal) and number of nucleotide substitutions (above diagonal) in ITS2 among members of the Fluviatilis and Minimus Complexes.

	An. fluviatilis S	*An. fluviatilis* T	*An. fluviatilis* U	*An. fluviatilis X*	*An. minimus C*	*An. minimus* A
An. fluviatilis S	—	10	9	0	13	19
An. fluviatilis T	0.028	—	1	10	15	21
An. fluviatilis U	0.025	0.003	—	9	14	20
An. fluviatilis X	0	0.028	0.025	—	13	19
An. minimus C	0.036	0.042	0.039	0.036	—	19
An. minimus A	0.054	0.060	0.057	0.054	0.054	—

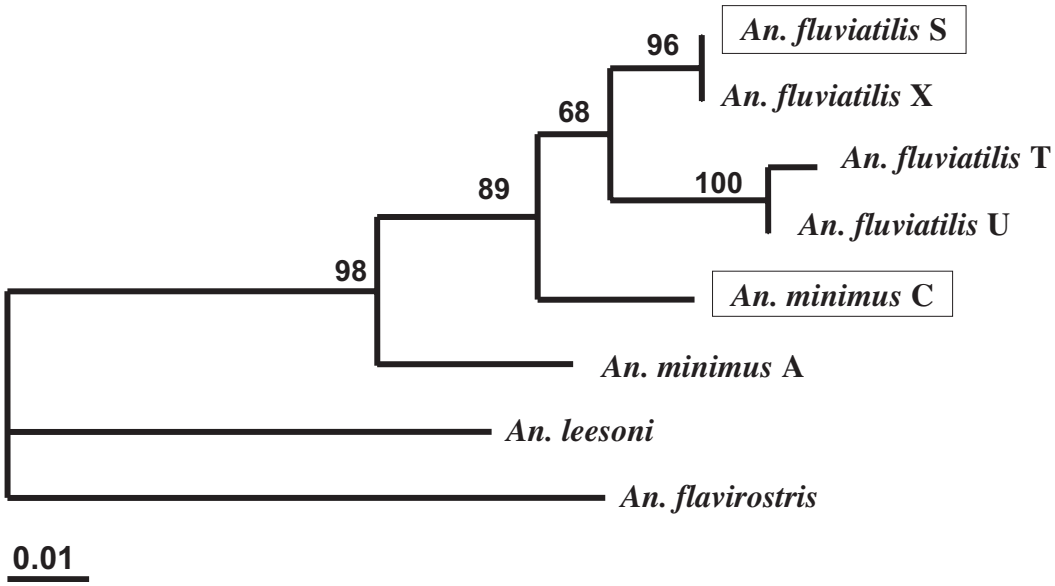

Figure 1. Maximum likelihood (ML) tree inferred from ITS2 sequences from members of Fluviatilis and Minimus Complexes. Numbers above the branches are bootstrap values. Scale bar represents 0.01 nucleotide substitution per site. *Anopheles leesoni* and *An. flavirostris* are taken as outgroups.

morphological identification. Fifteen samples, which were randomly selected for sequencing, were identified as *An. minimus* C based on homology with published sequences of ITS2 of *An. minimus* C (Sharpe *et al* 2000; Van Bortel *et al* 2000; Garros *et al* 2005; Chen *et al* 2006).

A total of 22 samples of *An. fluviatilis* S, seven from Koraput and 15 from Sundergarh, with chromosomal inversion genotype $2+^{q1}+^{r1}$ were sequenced for ITS2 and 28S-D2/D3. Based on chromosomal inversion genotype and 28S-D3 sequence homology with AF437880 (Singh *et al* 2004a) all were assigned to be classical *An. fluviatilis* S.

Analysis of ITS2 sequences revealed that there is no intraspecific sequence variation among the *An. minimus* C and *An. fluviatilis* S specimens sequenced in this study. It was noted that the ITS2 sequences of all *An. minimus* samples (this study) were homologous to all *An. minimus* C sequences available through GenBank (AF194506, DQ238493, AF416784, AF230462, AY953517, AY255109) originating from Vietnam, Thailand and China. Thus ITS2 sequences of *An. minimus* species C across different geographical regions are absolutely conserved making it an ideal candidate region of species-diagnostic and phylogenetic importance.

The pair-wise distance analysis of ITS2 sequences between members of the Fluviatilis and Minimus (species A and C only, no authentic data available for species E) Complexes and their phylogenetic relationship (ML tree) inferred from ITS2 sequences are shown in table 2 and figure 1, respectively. The tree-topologies inferred from different methods employed in this study (MP, ML and NJ) were same. In all phylogenetic analysis all nodes were supported with >50% bootstrap value.

Comparison of ITS2 sequences from *An. minimus* C and *An. fluviatilis* S revealed that they differ by 13 base

substitutions (pair wise distance=3.6%, Kimura-2-parametre model) and two indels. Such difference in a locus which is highly conserved within a species across all geographical regions of known distribution is clearly suggestive of non-synonymous status of these two species. It was also noted that the *An. fluviatilis* S is more distant to *An. minimus* C as compared to any other members of the Fluviatilis Complex. However, *An. minimus* C is closer to members of the Fluviatilis Complex compared to species A (table 2 and figure 1).

The ITS2 sequence of *An. fluviatilis* S was also compared with that of putative species X reported by Manonmani *et al* (2001). We found that the ITS2 sequences of all 22 *An. fluviatilis* S are homologous to the published ITS2 sequence of species X. This further confirms that species X is synonymous with species S. It may be noted that seven specimens of species S used in this study were from the same area (Koraput, Orissa) where Manonmani *et al* (2001) described species X, which were later found to be correlated with species S while comparing their PCR assay with cytotaxonomy (Manonmani *et al* 2003).

The analysis of the 853 base pair partial 28S rDNA (D2 and D3 domain) of *An. fluviatilis* S and *An. minimus* C revealed six substitutions (pair-wise distance = 0.7%, Kimura-2-parametre model). All the substitutions are restricted to the D2 domains and there is no difference in the D3 domain. These differences are conserved and species-specific as no intra-specific variations were found among samples sequenced. Thus ITS2 and 28S-D2 sequences of all *An. fluviatilis* S are appreciably different from those of *An. minimus* C (19 substitutions+2 indels). However all *An. fluviatilis* S had ITS2 sequence similar to published sequence of species X. Therefore *An. fluviatilis* S and *An. minimus* C are different species, while former is synonymous to *An. fluviatilis* X.

4. Discussion

An. fluviatilis S was made conspecific with *An. minimus* species C on the basis of 335 base pairs sequence similarity over relatively conserved 28S rDNA (D3 domain). However when we sequenced the upstream D2 domain of 28S rDNA, we found a difference of six base pairs between these two species. Similarity in small regions of conserved 28S rDNA is not uncommon in closely related species. For example, there is 100% homology in 28S-D3 rDNA between species B, C and E and also between species A and D of the Culicifacies Complex (Singh *et al* 2004b). Similarly, there is a difference of only one base pair in 28S-D3 rDNA between species T and U of the Fluviatilis Complex (Singh *et al* 2004a), which are sympatric in northern India and exhibit total reproductive isolation as evidenced by the absence of inversion heterozygotes (Subbarao *et al* 1994, Shukla *et al* 2001; Sharma *et al* 1995). Therefore homology in a small region of rDNA should not be considered as a sufficient evidence for synonymy. The present study involving ITS2 and a larger region of 28S rDNA clearly indicates that *An. fluviatilis* S and *An. minimus* C are, in fact, independent species.

Recently Chen *et al* (2006) have contemplated the similarity in bionomics and malaria transmission data as further evidence to support synonymy of *An. fluviatilis* S with *An. minimus* C. The similarity in habitat (i.e. hilly and forested), high anthropophilic index (i.e. > 90%) and high sporozoite infection rates in *An. fluviatilis* S and *An. minimus* from India (Assam), led them to conclude that *An. fluviatilis* S and *An. minimus* C are the same species (Chen *et al* 2006). However, molecular characterization of *An. minimus* from Assam, India, using ITS2 and 28S-D3 (O P Singh *et al*, unpublished data) revealed the presence of species A only, and species C has not been recorded from Assam so far. Thus the data on bionomics and malaria transmission of *An. minimus* from Assam relates to species A.

The identification of *An. fluviatilis* S in India, according to Chen *et al* (2006), is the case of misidentification of *An. minimus* as *An. fluviatilis* due to variation in the pale bands on the maxillary palpi of females as has been reported in Southeast Asian countries (Harrison 1980; Chen *et al* 2002). The molecular characterization of *An. fluviatilis* S from India presented in this study reveals that they are not misidentified *An. minimus.* In a study carried out in Assam, India, it was found that such variations are found in *An. minimus* (species A) but are limited to a small proportion of the population, one that is found in the winter months only (O P Singh *et al,* unpublished data). So far there is not any report of misidentification of *An. fluviatilis* as *An. minimus.* However this issue needs to be extensively investigated using molecular tools.

The ITS2 and 28S-D2/D3 sequences are conserved in *An. fluviatilis* S and *An. minimus* C with no intraspecific divergence noted in the present study. Interestingly, the ITS2 of *An. minimus* C, which is most extensively studied DNA from different parts of China, Vietnam, Thailand and central Myanmar (see table 1) are homologous. The appreciable degree of sequence difference between *An. fluviatilis* S and *An. minimus* C in such extremely conserved rDNA leaves little space for doubt about their independent species status. This is further substantiated by the fact that cytochrome oxidase subunit II-sequences of *An. fluviatilis* S (Singh *et al*, unpublished data, GenBank accession Nos DQ383278 and DQ383279) differ from those of *An. minimus* C (AJ512737) by 22 base pairs (d=3.33%, Kimura 2–parameter model). These molecular studies in conjunction with the morphological difference between these two species bring the synonymy of *An. fluviatilis* S with *An. minimus* C into question and support an independent specific status of *An. fluviatilis* S.

According to our current knowledge about the distribution of *An. fluviatilis* S and *An. minimus* C, these are allopatric species making it impossible to perform population genetic analyses to prove their specificity. The former is restricted to some parts of India and the latter has not been identified from India so far. Recent molecular studies carried out by Jambulingam *et al* (2005) in Orissa (India), and by A Prakash *et al* (personal communication) and O P Singh *et al* (unpublished data) in northeastern states of India (where *An. minimus* is most abundantly found), revealed the existence of *An. minimus* A only. In the absence of sympatric populations of *An. fluviatilis* S and *An. minimus* C, DNA sequence data are sufficient to prove their independent specific status. A polytene chromosome map of *An. minimus* C, which is unavailable so far, may add further evidence of genetic differentiation between these two species.

As a consequence of recognition of *An. fluviatilis* S as synonym of *An. minimus* C, the distribution of the latter has been shown in various parts of India by Chen *et al* (2006). With reinstatement of specific status of *An. fluviatilis* S, as a result of this study, there is no evidence that *An. minimus* C occurs in India. The present study however extends the distribution of *An. minimus* C, which has been reported from China, Laos, Thailand and Vietnam (Garros *et al* 2006), to include Myanmar.

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