Reanalysis of rDNA-ITS2 region sequences of *Anopheles* cf. *culicifacies* 'Bluchistan' revealed conspecificity to *A. dthali*

K. Raghavendra*, B. P. Niranjan Reddy, A. P. Dash and Aparup Das

National Institute of Malaria Research, 22 Shamnath Marg, New Delhi 110 054, India

***Anopheles* cf. *culicifacies* 'Bluchistan' was reported as a new variant of *A. culicifacies* species complex based on internal transcribed spacer 2 (ITS2) region by Djadid and Saifi in 2001 (GenBank accession number AF_402296) and was later stated to be phylogenetically close to *A. culicifacies* sp. A. Though comparison of ITS2 sequences of the members of *A. culicifacies* sp. complex with *A.* cf. *culicifacies* 'Bluchistan' revealed appreciable polymorphism to state the existence of new species, local alignment search and phylogenetic analysis results showed the conspecificity to *A. dthali*. In short, the present study gives a case report of misidentification of a species that highlighted the importance of initial morphotaxonomical identification before conducting the computational molecular phylogenetic studies. Such misidentification may sometimes lead to the suggestion of wrong vector control strategies for disease management.**

Keywords: *Anopheles culicifacies*, *Anopheles dthali*, misidentification, phylogeny, rDNA-ITS2.

ANOPHELES culicifacies sensu lato, is recognized as a complex of five sibling species provisionally designated as species A, B (ref. 1), C (ref. 2), D (ref. 3) on the basis of diagnostic inversion genotypes on polythene chromosome, and species E based on Y-chromosome mitotic karyotype variations and sporozoite positivity[4]. Recently, different DNA markers, viz. cytochrome oxidase I (COI)[5], cytochrome oxidase II (COII)[6], internal transcribed spacer 2 (ITS2)[7,8] and 28S D3 region[9] are reported to distinguish different members of the *A. culicifacies* species complex.

In 2001, Djadid and Saifi (http://www.ncbi.nlm.nih.gov/nuccore/16903461) reported the presence of an entirely new species of *A. culicifacies* identified from Zeineddini, Baluchistan province and named it as *A.* cf. *culicifacies* 'Bluchistan' based on variance in ITS2 region of ribosomal DNA with the available sequences of members of the *A. culicifacies* complex.

Thereafter, using ribosomal DNA (rDNA)-ITS2, randomly amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) primers, this new species was reported to be distributed in wide geographic regions of Iran; in Koutiji, near Kerman Province and in Baluchistan of southeastern province. This species was earlier designated as sp. X (refs 10 and 11). Moreover, the author also reported a close identity of the new species *A.* cf. *culicifacies* 'Bluchistan' to *A. culicifacies* sibling species A at the ITS2 DNA sequence region (GenBank accession number AF_402297)[10].

The *ITS2* marker gene was extensively used to differentiate the members of various anophelines and their sibling species[12–14]. Though molecular techniques hold some merits over morphotaxonomical methods for species identification, the importance of the latter cannot be overlooked. In Vietnam, misidentification of *A. minimus* as *A. varuna* led to the suggestion of wrong vector control methods[15]. Here, we present a case of morphological misidentification, which has led to the report of existence of a new variant of *A. culicifacies* species that was actually *A. dthali*. *A. dthali* is reported as malaria vector of secondary importance with wide distribution in southern parts of Iran including Baluchisthan[16] and it is worth mentioning here that *A.* cf. *culicifacies* 'Bluchistan' was reported to have been collected from this area[10].

Available 20 DNA sequences of ITS2 region of the members of *A. culicifacies* complex (of which, 8 sequences were of sp. B, 7-sp. A, 2-sp. E, 1-sp. C and 1-sp. D) were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/). To substantiate the data further, more specimens of species B, C and D were sequenced. Field collected specimens of *A. culicifacies* from villages of Alwar District (76°38′E long. and 27°34′N lat.; Rajasthan, north-west India), Surat District (72°54′ long. and 21°10′ lat.; Gujarat, West India), were identified to sibling species B, C and D, following COII-PCR assay[6], and used for sequencing. DNA was isolated following the procedure described in Collins *et al.*[17]. The rDNA-ITS2 region was amplified using primers ITS2a (TGTGAACTGCAGGACACAT) and ITS2b (TATGCTTAAATTCAGGGGGT), and by following the PCR protocol described in Goswami *et al.*[7]. The PCR product was cleaned using QIAquick PCR purification kit (QIAGEN) according to manufacturer's instructions. Direct sequencing was performed, transposing the cloning intermediate step, using the Big Dye® terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in volume of 10 µl containing 10 ηg of purified DNA and 2 µl of Big Dye terminator master mix, and 1.6 p moles of primer, according to manufacturer's instructions. Sequencing reactions were performed 25 cycles of 30 S at 96°C, 30 S at 50°C, 4 min at 60°C. Excess of dye terminators were removed by ethanol/sodium acetate precipitation method. Sequencing reactions were electrophoresed using the POP-4™ polymer on ABI PRISM® 3730 Genetic Analyser (Applied Biosystems).

The accession numbers of the new sequences were highlighted with asterisk mark (*) in Figure 1. Pairwise and multiple sequence alignments were performed using the program clustalX, version 1.83 (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/)[18]. Inter- and intraspecific

*For correspondence. (e-mail: kamarajur2000@yahoo.com)

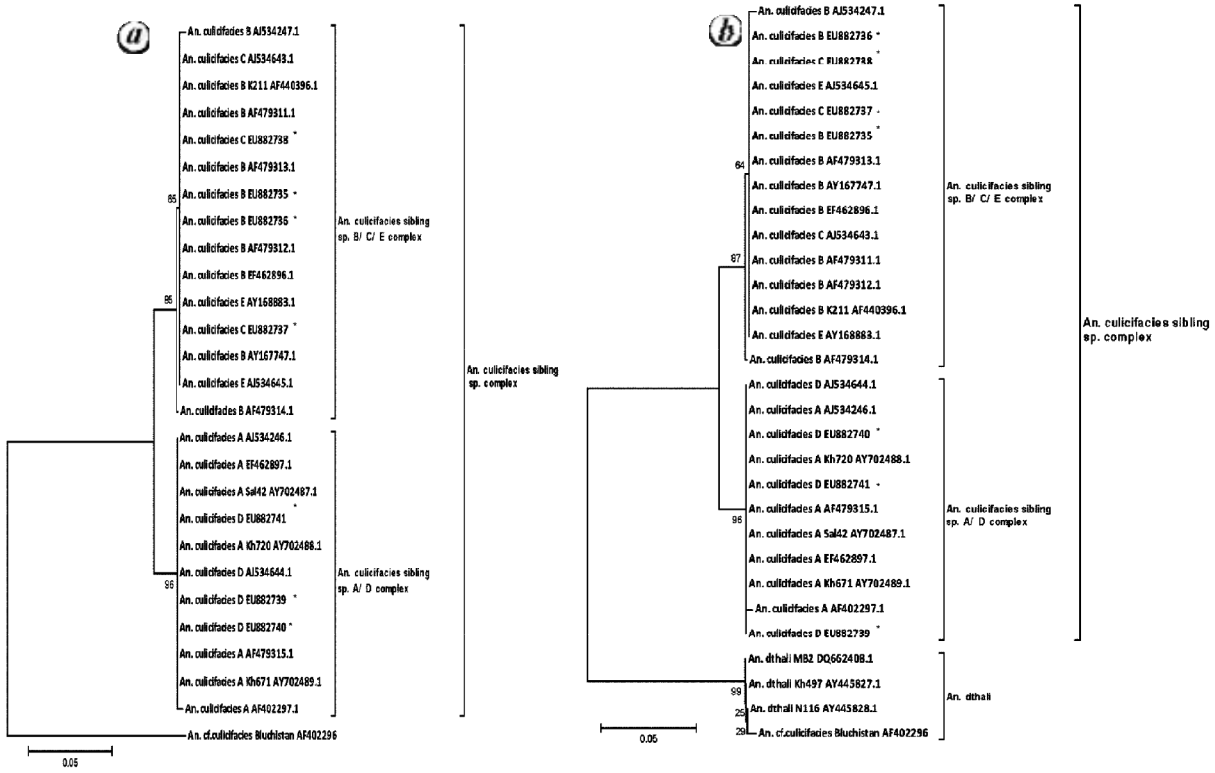

Figure 1. ***a***, Phylogenetic analysis based on rDNA-ITS2 sequences revealed the formation of two separate clades by members of *A. culicifacies* complex and *A.* cf. *culicifacies* 'Bluchistan'. ***b***, The phylogenetic analysis shows the formation of separate clades by *A. culicifacies* complex and together by *A.* cf. *culicifacies* 'Bluchistan' with *A. dthali*. The accession numbers of new sequences are indicated with asterisk mark (*).

genetic divergences were calculated following Kimura's 2 parameter method (K2P)[19]. Neighbour Joining (NJ)[20] method was used to construct the phylogenetic tree. All analyses were performed using MEGA 4 software[21].

The available sequences of the ITS2 region in GenBank were utilized to ascertain the phylogenetic position of *A.* cf. *culicifacies* 'Bluchistan' species with regard to other members of the *A. culicifacies* species complex. Contrary to the earlier report of Djadid[10], not much sequence similarity between *A.* cf. *culicifacies* 'Bluchistan' and *A. culicifacies* sibling species A was observed (Figure 1 *a*). In order to determine the phylogenetic position of *A.* cf. *culicifacies* 'Bluchistan' with other members of the species complex, NJ phylogenetic tree was constructed with K2P using MEGA 4 software. It was clear from the tree (Figure 1 *a*), that *A. culicifacies* forms single clades of species A and D (96% bootstrapped value) and of species B, C and E (85% bootstrapped value). However, the *A.* cf. *culicifacies* 'Bluchistan' formed a clear single but separate clade. Intraspecific sequence diversity in sibling species A, D, B, C and E was 0.001, 0.0, 0.001, 0.0 and 0.0 respectively. Since only one sequence of *A.* cf. *culicifacies* 'Bluchistan' was available, no intraspecific sequence diversity could be estimated for this species. Notably, within-cluster, namely A/D and B/C/E, sequence divergence was <1%. Whereas, interspecific sequence divergences between *A. culicifacies* sp. complex (spp. A, B, C, D and E) and *A.* cf. *culicifacies* 'Bluchistan' was 0.342; *A. culicifacies* sp. complex and *A. dthali* was 0.336; *A.* cf. *culicifacies* 'Bluchistan' and *A. dthali* was 0.003 respectively. The inter-species divergence between *A.* cf. *culicifacies* 'Bluchistan' and *A. dthali* is comparable to intraspecies divergence in *A. culicifacies* sp. complex. Moreover, the maximum number of nucleotide differences within the members of *A. culicifacies* species complex ranged from 0 to 20 bp, whereas the nucleotide differences between the members of this complex and *A.* cf. *culicifacies* 'Bluchistan' was in the range of 96–106 bp. The above results suggest that the *A.* cf. *culicifacies* 'Bluchistan' is clearly different from members of *A. culicifacies* species complex in contrast to the earlier findings of Djadid[10].

The BLASTN (nucleotide BLAST) search in GenBank resulted in very high sequence homology (99.2–100%) of *A.* cf. *culicifacies* 'Bluchistan' with three sequences of *A. dthali* (strains MB2, N116A and Kh497 with accession numbers DQ_662408, AY_445828 and AY_445827 respectively). Using the same alignment, a NJ phylogenetic tree was constructed (Figure 1 *b*), and also tested for homogeneity of substitution pattern employing available sequences of *A. dthali*, *A.* cf. *culicifacies* 'Bluchistan', and other members of the *A. culicifacies* sp. complex (A, B, C, D and E). Three non-overlapping, unambiguously distinguishable species clusters were formed with A/D and B/C/E and *A.* cf. *culicifacies* 'Bluchistan' – *A. dthali*, with the bootstrap values 96%, 87% and 99% respec-

tively (Figure 1 *b*). Further, *A.* cf. *culicifacies* 'Bluchistan' and *A. dthali* were found to have shared substitution homogeneity that was different from the members of *A. culicifacies* species complex, while all the five members of the sibling species complex shared similar substitution pattern. The results, thus, confirmed that *A.* cf. *culicifacies* 'Bluchistan' is close to *A. dthali*, than to any member of the *A. culicifacies* species complex.

In conclusion, the DNA sequence analyses of the ribosomal ITS2 region clearly revealed that *A.* cf. *culicifacies* 'Bluchistan' and *A. dthali* are the same species. Our conclusion is further substantiated by the fact that *A. dthali* is widely distributed in southern parts of Iran including Baluchisthan. Current developments in genome analysis and barcode genes have further facilitated the identification of new species in addition to existing methods such as morphological, cytological, isozyme based methods. However, misidentification of disease vectors sometimes leads to the suggestion of wrong vector control strategies. This study, thus, highlights the importance of accurate morphological identification of field collected specimens before applying modern molecular and computational phylogenetic techniques to establish the taxonomic relationships.

1. Green, C. A. and Miles, S. J., Chromosomal evidence for sibling species of the malaria vector *Anopheles* (Cellia) *culicifacies* Giles. *J. Trop. Med. Hyg.*, 1980, **83**, 75–78.
2. Subbarao, S. K., Vasantha, K., Adak, T. and Sharma, V. P., *Anopheles culicifacies* complex: evidence for a new sibling species, species C. *Ann. Entomol. Soc. Am.*, 1983, **76**, 985–986.
3. Vasantha, K., Subbarao, S. K. and Sharma, V. P., *Anopheles culicifacies* complex: population cytogenetic evidence for species D (Diptera: Culicidae). *Ann. Entomol. Soc. Am.*, 1991, **84**, 531–536.
4. Kar, I. *et al.*, Evidence for a new malaria vector species, species E, within the *Anopheles culicifacies* complex (Diptera: Culicidae). *J. Med. Entomol.*, 1999, **36**, 595–600.
5. Kumar, P. N., Rajavel, A. R., Natarajan, R. and Jambulingam, P., DNA barcodes can distinguish species of Indian mosquitoes (Diptera: Culicidae). *J. Med. Entomol.*, 2007, **44**, 1–7.
6. Goswami, G., Singh, O. P., Nanda, N., Raghavendra, K., Gakhar, S. K. and Subbarao, S. K., Identification of all members of the *Anopheles culicifacies* complex using allele-specific polymerase chain reaction assays. *Am. J. Trop. Med. Hyg.*, 2006, **75**, 454–460.
7. Goswami, G., Raghavendra, K., Nanda, N., Gakhar, S. K. and Subbarao, S. K., PCR-RFLP of mitochondrial cytochrome oxidase subunit II and ITS2 of ribosomal DNA: markers for the identification of members of the *Anopheles culicifacies* complex (Diptera: Culicidae). *Acta Trop.*, 2005, **95**, 92–99.
8. Manonmani, A. M., Sadanandane, C., Sahua, S. S., Mathivanana, A. and Jambulingam, P., rDNA-ITS2-PCR assay for grouping the cryptic species of *Anopheles culicifacies* complex (Diptera: Culicidae). *Acta Trop.*, 2007, **104**, 72–77.
9. Singh, O. P., Goswami, G., Nanda, N., Raghavendra, K., Chandra, D. and Subbarao, S. K., An allele-specific polymerase chain reaction assay for the differentiation of members of *Anopheles culicifacies* complex. *J. Biosci.*, 2004, **29**, 275–280.
10. Djadid, N. D., Operational research in tropical and other communicable diseases. Final report summaries 2001–02, 2001, pp. 52–53; (www.emro.who.int/TDR/PDF/FinalReportSeries01-02.pdf)
11. Djadid, N. D., Sanati, M. H., Zare, M. and Hassanzehi, A., rDNA-ITS2 identification of *Anopheles pulcherrimus* (Diptera: Culicidae): Genetic differences and phylogenetic relation with other Iranian vectors and its implications for malaria control. *Iran. Biomed. J.*, 2003, **7**, 1–6.
12. Porter, C. H. and Collins, F. H., Species-diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* (Diptera: Culicidae). *Am. J. Trop. Med. Hyg.*, 1991, **45**, 271–279.
13. Wilkerson, R. C., Reinert, J. F. and Li, C., Ribosomal DNA ITS2 sequences differentiate six species in the *Anopheles crucians* complex (Diptera: Culicidae). *J. Med. Entomol.*, 2004, **41**, 392–401.
14. Sedaghat, M. M., Linton, Y. M., Oshaghi, M. A., Vatandoost, H. and Harbach, R. E., The *Anopheles maculipennis* complex (Diptera: Culicidae) in Iran: molecular characterization and recognition of a new species. *Bull. Entomol. Res.*, 2003, **93**, 527–535.
15. Van Bortel, W., Harbach, R. E., Trung, H. D., Roelants, P., Backeljau, T. and 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**, 729–732.
16. Vatandoost, H., Shahi, M., Hanafi-Bojd, A. A., Abai, M. R., Oshaghi, M. A. and Rafii, Ecology of *Anopheles dthali* patton in Bandar Abbas district, Hormozgan province, Southern Iran. *Iran. J. Arthopod-Borne Dis.*, 2007, **1**, 21–27.
17. Collins, F. H., Mendez, M. E., Rasmussen, M. O., Mehafy, P. C., Besansky, N. J. and Finnerty, V., A ribosomal RNA gene probe differentiates a member species of the *Anopheles gambiae* complex. *Am. J. Trop. Med. Hyg.*, 1987, **37**, 37–41.
18. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G., The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, 1997, **25**, 4876–4882.
19. Kimura, M., A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.*, 1980, **16**, 111–120.
20. Saitou, N. and Nei, M., The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, 1987, **4**, 406–425.
21. Tamura, K., Dudley, J., Nei, M. and Kumar, S., MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.*, 2007, **24**, 1596–1599.

Received 3 December 2008; revised accepted 4 August 2009

Increasing prolificacy and ewe efficiency in sheep through *FecB* gene introgression

A. K. Mishra[1,*] A. L. Arora[1] and Satish Kumar[2]

[1]Division of Animal Genetics and Breeding, and
[2]Animal Biotechnology Section, Central Sheep and Wool Research Institute, Avikanagar 304 501, India

The *FecB* gene of Garole (G) was introgressed into non-prolific Malpura (M) sheep and the performance of the GM half-breds is being monitored. DNA samples of Garole, Malpura and Garole × Malpura (GM) crossbreds were screened by PCR–RFLP to determine the presence of *FecB* mutation. The majority of Garole (96%) and GM crossbred (72%) were carriers (BB and B+) for the *FecB* mutation. The $FecB^{BB}$ and $FecB^{B+}$ carrier ewes resulted in 81.19 and 69.31% higher prolificacy respectively, as compared to non-carrier Malpura ewes. The viable benefits accrued by

*For correspondence. (e-mail: anilmishra65@yahoo.co.in)