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NOTES

Variations in palpal ornamentation of *Anopheles fluviatilis* species T and U (Diptera: Culicidae) and their taxonomic consequence

Gunjan Sharma, Manila Lather & Om P Singh*

National Institute of Malaria Research, Sector 8, Dwarka, New Delhi-110 077, India

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Anopheles fluviatilis sensu lato James is a highly efficient malaria vector in Indian subcontinent and Iran which is comprised of at least four sibling species provisionally designated as species S, T, U and V. An important morphological characteristic for differentiation of this species complex from other closely related anopheline species complex, the Minimus Complex, is the ratio of length of subapical pale band to dark band intervening apical and subapical pale bands on the maxillary palps of female mosquito. Here, we report variation in the subapical pale band in *An. fluviatilis*, especially in species U, to the extent that palps of some specimens resemble members of Minimus Complex, inferring that palpal ornamentation may not be reliable characteristics for identification of *An. fluviatilis*. Taxonomic consequence of such variation is discussed.

Keywords: *Anopheles minimus*, Fluviatilis Complex, hypomelanism, malaria vectors, Minimus Complex, mosquitoes, Vector control

Vector control is a crucial component of malaria prevention, control and elimination strategies[1], the success of which relies on correct identification of vector species[2]. In India, there are six malaria vectors recognized as primary malaria vectors, viz., *An. culicifacies*, *An. fluviatilis*, *An. minimus*, *An. stephensi*, *An. sundaicus* and *An. dirus*[3]. All of them, except *An. stephensi*, are comprised of several cryptic species[3]. Traditionally, identification of vector species is performed on the basis of morphological characteristics that is used to differentiate mosquito species[2]. However, there are some reports where vectors have been misidentified owing to overlapping morphological characteristics in closely related species[4-7]. Identification of vector species is further challenged by the presence of sibling species. Although considerable progress has been made toward the development of advanced tools for the identification of sibling species of Indian malaria vectors using chromosomal, biochemical and molecular markers[3,8-12], but use of such techniques is limited to differentiation of members of a specific species complex only provided they are correctly identified morphologically prior to application of such techniques. It has been observed that incorrect morphological identification of mosquitoes prior to cytotaxonomy[5] or PCR assay[2] can lead to misleading result. Therefore, correct morphological identification of mosquitoes prior to application of such techniques is essential[2].

Morphological differentiation of two closely related vector species complexes, *An. fluviatilis s.l.* and *An. minimus s.l.*, which belongs to Minimus subgroup of *Anopheles*[13], is often challenging due to overlapping morphological characteristics. As on date, there are four reported sibling species in the Fluviatilis Complex, i.e., species S, T, U and V[9,12] and three sibling species in the Minimus Complex, i.e., *Anopheles minimus*[14], *An. harrisoni*[15] and *An. yaeyamaensis*[16]. Instances exist in literature where *An. minimus s.l.* have been misidentified as *An. fluviatilis s.l.* due to overlapping morphological characteristics[4-6], especially the ratio of length of the subapical pale band to dark band intervening apical and subapical pale bands which is an important morphological characteristic for differentiation of these two species. Such misidentification was attributed to hyper-melanisation of palpi of *An. minimus s.l.* mainly in the cooler season[5], where the length of the subapical pale band is reduced substantially resembling *An. fluviatilis s.l.* However, there is no published report of misidentification of *An. fluviatilis s.l.* as *An. minimus s.l.* Here, we examined a scenario where palps of *An. fluviatilis* may resemble *An. minimus s.l.* which questions the validity of such a morphological characteristic in morphological discrimination of these two species.

Materials and Methods

Mosquito collection and processing

Adult *An. fluviatilis s.l.* were collected from villages Ismailpur, Dargahpur and Oaspur under Laksar CHC (Community Health Centre) of district

*Correspondence:
E-mail: singh@nimr.org.in

Hardwar, India (latitude 29°N, longitude 78°E, Fig. 1), in the month of March 2014 from cattle sheds and human dwellings using a hand aspirator and flashlight torch in the morning (06:00 to 08:00 h). Presence of three sibling species of *An. fluviatilis* i.e., species T, U and V has been recorded in the past in these villages[12]. The outdoor daytime temperature during the collection period was 28-32°C and 17-18°C during night. Mosquitoes were carefully transferred to a polystyrene foam box with one side opening mounted with nylon netting and transported to laboratory at Delhi. Mosquitoes were provided access to 10% glucose-soaked cotton pad during transport. Besides, some of the field mosquitoes with palpi resembling *An. minimus s.l.* (having subapical pale band equal to intervening dark band between apical and subapical pale band) were selectively pinned and preserved. Live mosquitoes were allowed to lay eggs in laboratory and reared till emergence into adult (F_1). Rearing was done at constant room temperature maintained at 25±1°C, close to the prevailing average temperature in collection sites and relative humidity of 70±5%. During rearing, larvae were fed on grinded mixture of dog biscuit and yeast in a ratio of 3:2. Upon pupation, pupae were transferred in bowl containing water and placed into mosquito cage. Adults were provided access to 10% glucose-soaked cotton pad. Four to five day's old adult female mosquitoes were anesthetized with ether and palpal characteristics were examined under Leica M165-C stereoscopic microscope. Pinned mosquitoes were also examined.

DNA isolation

DNA was isolated from individual mosquitoes following examination of morphological characteristics. Briefly, whole mosquito was initially grinded in 1.5 mL microfuge tube with 50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and final volume was made up to 200 µL. This was incubated for 10 min on a heat block maintained at 96°C. Finally, the content was centrifuged at 10000 RPM for 5 min, and the supernatant transferred in another microcentrifuge tube and stored at −20°C.

Sibling species identification and DNA sequencing

For identification of sibling species of Fluviatilis Complex, allele-specific PCR (ASPCR) was carried out as described by Singh *et al.*[10]. The PCR product was visualized on 2% agarose gel (Fig. 2). For confirmation of ASPCR results, some samples were sequenced for domain D3 of 28S rDNA. For sequencing, samples were amplified using primers

Fig. 1 — Map of Hardwar district, Uttarakhand, showing study villages

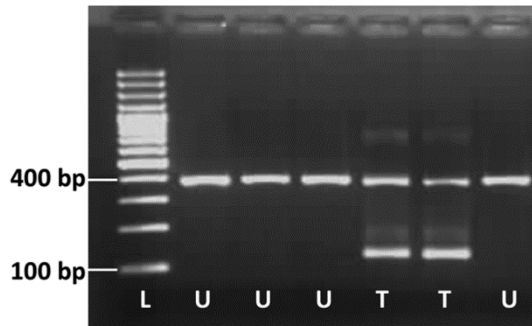

Fig. 2 — Gel photograph showing allele-specific PCR product. L=100 bp DNA ladder, T= *An. fluviatilis* species T, U= species U

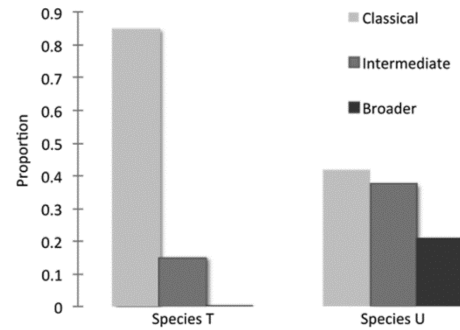

Fig. 3 — Proportion of mosquitoes with three different classified categories of subapical pale band in *An. fluviatilis* species T and U

D3A and D3B as described by Singh *et al*.[4]. The PCR products were sequenced from both strands of DNA using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific).

Results and Discussion

Palpal ornamentation is important taxonomic characteristics for the identification of *An. fluviatilis*, *An. minimus* and *An. varuna.* Christophers (1933)[17] in his monograph "The Fauna of British India, Including Ceylon and Burma" has mentioned that "Distinction (of *An. fluviatilis*) from *An. minimus*, and especially from *An. varuna*, may be difficult if the palpal ornamentation is ambiguous" and that "Specimens with typically marked palpi, which form the great majority, should offer no difficulty". These statements underline the importance of palpal characteristics in the differentiation of the *An. fluviatilis s.l.* from *An. An. minimus s.l.* The main distinguishing characteristics of *An. fluviatilis* according to Christophers is "black band between apical and the subapical pale band usually four to five times length of the subapical pale band and at least half length of dark area between subapical and more basal pale band"[10]. There has been no change on this criterion since then. In our own experience, we often receive misidentified *An. fluviatilis* by field entomologist as *An. minimus*. We realized that such mistakes are due to the presence of a broad subapical band resembling *An. minimus.* We, therefore, examined the presence of such variation in field population of *An. fluviatilis* and their significance in the formal taxonomy.

A total of 53 samples were identified for sibling species by ASPCR assay following Singh *et al.*[10], of which 20 samples were subjected to DNA sequencing for D3 domain of 28S-rDNA for confirmation of ASPCR-based results. DNA sequencing results of six specimens of species T and 14 specimens of species U (having broader subapical pale band on maxillary palpi), were in agreement of ASPCR. Of the 53 samples identified for sibling species, 44 samples were randomly sampled from the F_1 generation of field collected mosquitoes and nine pinned specimens which were suspected to be *An. minimus* based on palpal characteristics. Mosquitoes were classified in three categories based on the ratio of width of dark band intervening apical and subapical pale band on the palpi to the width of subapical pale band, i.e., (i) "classical" with ratio <1/3; (ii) "intermediate" with ratio between 1/3 and 3/4; and (iii) "broader" with ratio >3/4 to 1. The distribution of these three categories of palpi in different sibling species has been shown in Table 1. Species V, which was recorded in an earlier study[3], was absent in this collection. It was observed that classical subapical pale band was present in the majority of species T (85%) and "broader" category was absent. In contrast, species U showed wide variation in subapical pale band with "broader" category present in 21% of individuals (Fig. 3). All the nine pinned specimens (field collected) with subapical pale band similar to *An. minimus,* i.e., equal to the intervening dark band between apical and subapical pale band

Table 1 — Distribution of ratio of subapical pale band to dark band intervening apical and subapical pale bands in members of *An. fluviatilis* species

	Species T	Species U	Total
Randomly sampled (F_1)			
Classical	17 (85%)	10 (42%)	27
Intermediate	3 (15%)	9 (37%)	12
Broader	0	5 (21%)	5
Total	20	24	44
Selectively sampled (wild caught)			
Broader	0	9 (100%)	9
Grand total			53

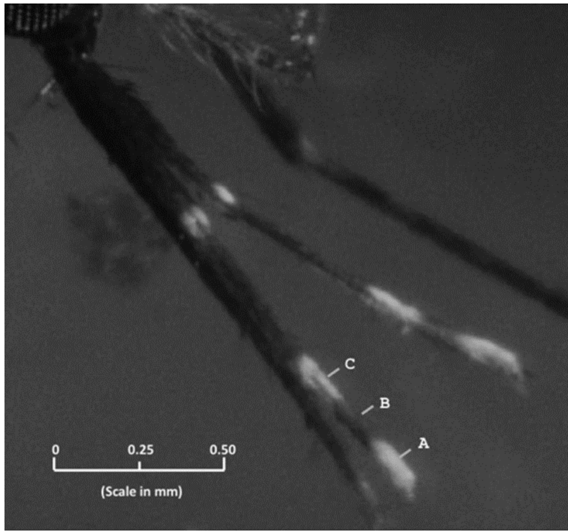

Fig. 4 — Palpal ornamentation of an *An. fluviatilis* species U resembling *An. minimus s.l.* (A) apical pale band; (B) intervening dark area between apical pale band and subapical pale band; (C) subapical pale band

(category "broader"), were found to be species U. Thus, species U tended to have a broader subapical pale band as compared to species T. A photograph of palpal ornamentation in species U with "broader" subapical pale band has been displayed as Fig. 4.

Identification of *An. fluviatilis s.l.* has always been complicated due to overlapping morphological characteristics with *An. minimus* complex and *An. varuna*[4-6]. Earlier record showed the widespread distribution of *An. fluviatilis* from Yemen to Taiwan[18]. However, later work by Harrison[5], Chen *et al.*[6] and Singh *et al.*[4] have reported misidentification of *An. minimus s.l.* as *An. fluviatilis s.l.* resulting from overlapping maxillary palpal characteristics in China, Thailand and a northeast state of India (Assam). Therefore, their presence in countries eastward to India and in north-eastern states of India was very much doubted[5]. As a consequence, Harrison[5] and Chen *et al.*[6] have recommended removal of *An. fluviatilis* from fauna-list of Thailand and China, respectively. Interestingly, the chromosomal complements of *An. minimus s.s.* and *An. fluviatilis* species U were found to be homosequential, which was another reason for misidentification of *An. minimus* precisely as *An. fluviatilis* species U[4]. These instances related to misidentification of *An. minimus* as *An. fluviatilis* have been attributed to hyper-melanism in mosquitoes in cooler months[5]. All these reports indicate misidentification of *An. minimus* as *An. fluviatilis* but there is no any report where *An. fluviatilis* have been misidentified as *An. minimus* due to hypomelanism. This study reports that such hypomelanism in palpi of *An. Fluviatilis*, especially in the species U, can lead to misidentification of *An. fluviatilis* as *An. minimus*.

Correct identification of vector species is crucial for the success of vector control programme. Due to challenge in the identification of sibling species (which may differ in epidemiologically important biological attributes, such as vectorial competence, insecticide resistance, etc), there has been significant advancement in the development of diagnostic tools for the identification of cryptic species present in various malaria vectors. Application of such tool on incorrectly-identified mosquitoes based on morphological characteristics may be seriously misleading. A recent study[2] carried out in South Africa reports that when 11 morphological species were subjected to standard PCR used for discrimination of *An. gambiae* complex as well as *An. funestus* group, three morphological species were incorrectly identified belonging to *An. funestus* group and four morphological species were incorrectly identified as member of *An. gambiae* complex. This report signifies importance of morphological identification of vector species in the malaria control programme, especially before applying molecular tool. Similar precaution should be taken before carrying out cytotaxonomic identification of sibling species. It has been reported that the chromosome complements of *An. fluviatilis* species U is identical to *An. minimus s.s.* and this has resulted in misidentification of *An. minimus s.s* as *An. fluviatilis* species U[4]. It is thus desired that careful studies should be carried out on formal taxonomy involving morphological, chromosomal and molecular tools together.

Conclusion

The present study reports variation in the subapical pale band in *An. fluviatilis s.l.*, especially in species U, to the extent that palps of some specimens resemble *An. minimus s.l.*, inferring that palpal ornamentation may not be a reliable characteristic for identification of *An. fluviatilis s.l.*

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Conflict of interest

The authors declare no conflict of interest.

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