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# Morphometric Analysis of Taxonomic Characters of Malaria Vector Mosquito *Anopheles* (*Cellia*) *subpictus* Grassi (Diptera: Culicidae)

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**ABSTRACT**
*Anopheles subpictus* Grassi, a vector of malaria in Oriental region, has gained importance due to its doubtful status as a complex of species. In the present study, twenty-five morphotaxonomic characters from head and wing were studied. The results reveal that the length of antenna, proboscis, palpomere 5, palpomere 4, palpomere 3 and apical pale band have significant positive correlation with the length of palpus while humeral pale spot and preapical dark spot have significant negative correlation and subcostal pale spot has significant positive correlation with the total wing length. It is asserted that some of these morphotaxonomic characters used frequently in species discrimination keys are statistically unreliable and hence result in frequent species discrimination problems being polymorphic. The reliable characters found are length of antenna, proboscis, palpus from the head, and subcostal pale spot from wing. Hence, these characters are recommended for use while preparing reliable morpho-taxonomic keys.

**Keywords:** *Anopheles*, *subpictus*, morphology, mosquito, Pyretophorus.

## 1. Introduction
In the family Culicidae, subfamily Anophelinae, a total of 424 morphologically identifiable species have been described within genus *Anopheles*; out of them 70 have been implicated in transmission of malaria parasite [1]. Some of the members of genus *Anopheles* are also known to form complexes of sibling/cryptic species with ambiguous taxonomic characters and thus the number of species increases to more than 500 when the sibling/cryptic species are also considered. In the bionomics of vector species, correct identification of existing and/or novel species is the first important step for developing vector control strategies [2]. Moreover the taxonomic description of new anopheline species as well as most of the internal classification of subfamily Anophelinae is based primarily on morphology [3, 4].
In this context, *Anopheles subpictus* Grassi 1899, a vector of malaria in Oriental region, has gained considerable importance due to its doubtful status as a complex of species [1, 5, 6]. Initially, two sibling species were identified within its domain based on morphological characters but the number was later raised to four using polytene X chromosome based cytogenetic parameters [7, 8]. As for the vectorial status, it was found to harbour significant level of *Plasmodium* and transmitting it to human populations from some parts of coastal Orissa (India) by Panicker *et al*. [9]. Later, sibling species B, that inhabits brackish waters, has been reported to be an important malaria vector from coastal India, Sri Lanka and South East Asia [1, 10, 11, 12, 13, 14, 15, 16, 17, 18]. Though earlier thought to be zoophagic, some workers have reported it to have an appreciable amount of anthropophagic index (varying from 9% to 41%) readily taking blood meal from human hosts [15, 17]. Recent reports of morphological variations [19] necessitated the present study that was carried out to identify those morphotaxonomic characters which are statistically reliable from the head and wings of this important vector species.

## 2. Materials and methods
Allopatric populations of *A*. (*Cellia*) *subpictus* Grassi 1899, a dominating species in the post-monsoon months of August to November, were collected for the study of adult morphotaxonomic characters from North-west India (Table 1). Unlike most of the anopheline species, it rests indoors during daytime in human dwellings and cattle sheds. The larvae and pupae were collected from clear rainwater pools, irrigation water channels and abandoned flowerbeds etc. They were segregated in separate enamel bowls and fed on protein rich diet of finely powdered dog biscuits & yeast in the ratio of 6:4 [2, 20].

The pupae were allowed to metamorphose into adults to be later used for identification and morphometric study.
Initial identification of the adults was done by following the standard identification keys available for the North western Indian anophelines [21, 22, 23] and a rapid field key developed for this purpose (unpublished). All the populations under study were identified to belong to sibling species A, when confirmed from species-specific salivary polytene X-chromosome banding pattern of the fourth-instar larvae [8, 24].

**Table 1:** Collection details of the populations of *A. subpictus* from the States of Haryana (Hr) and Punjab (Pb), India.

| Place of collection | Code | Longitude/Latitude | Life stage of mosquito |
|---|---|---|---|
| Bank Colony, Rohtak Road, Bhiwani, (Haryana) | A | 28.46 N 76.18 E | larvae, pupae, adults |
| Gohana Road, Sonipat, (Haryana) | B | 29.00 N 70.00 E | Larvae |
| Kishan Pura, Patiala Road, Sangrur, (Punjab) | C | 30.12 N 75.53 E | larvae, pupae, adults |
| Garden Colony, Roopnagar, (Punjab) | D | 30.57 N 76.32 E | Larvae |

### 2.1 Morphological analysis
Five adult females from each of the four populations were dissected under binocular dissecting microscope at a suitable magnification. The staining of head and wings was carried out in 70% alcoholic eosin overnight. The parts were dehydrated in alcoholic grades, cleared in clove oil before mounting in Canada balsam. The preparations were studied and photographed under stereo-zoom photomicroscope after which the digital images of the same were transferred to a computer for editing using Adobe Photoshop® Photo editing software version 7.0.

### 2.2 Coding and observation of variable markers
Variations in the morphological details of the selected parts were encoded and documented for different morphotaxonomic characters used in the identification of mosquitoes. Accordingly, as many as 25 morphological characters (9 from head and 16 from wing) were measured using a stage micrometer under a Binocular Research Microscope. The wing venation in anophelines is quite complex where special coding system has been traditionally used for identifying different veins and spots over them. Figures 1 and 2 depict the characters of anopheline head and spotting and venation pattern of the wing used as reference standard for the present study.
The characters from head region included the length of maxillary palpus, palpomeres 2-5, proboscis and antenna. The length of palpomeres 2-5 were measured and then divided by the length of maxillary palpus, while the length of maxillary palpus and length of the antenna was divided by the length of the proboscis (Table 2). For the characters from the wing, different characters of the wing were measured and then divided by the total wing length (excluding the fringe) (Table 6). The standard anopheline morphological terms and abbreviations have been followed [25, 26]. The statistical analysis of the data obtained from all the populations covered under the present study was carried out using SPSS 15.0 programme with n=20.

## 3. Results
### 3.1 Variations in the characters of the head region
The mean values ± standard deviation for various characters for proboscis, antenna and palpi of head and the variations in the ratios of these characters were calculated (Figure 1, Table 2-4) and then statistically analysed by bivariate correlation and multiple regression. The hypothesis tested was that "all the characters of the head ($X_{a1}$, $X_{a2}$…….$X_{a8}$) are significantly and positively related to the length of palpi ($Y_a$)." Accordingly, the results revealed that apart from length of palpomere 2 ($X_{a6}$) and length of subapical dark band ($X_{a7}$), all the other variables had positive and significant correlation with the length of palpus (Table 5). The results of multiple linear regression show that only two variables *viz.* length of proboscis ($X_{a2}$) and length of antenna ($X_{a1}$) are important in differentiating the length of palpus using the regression model $\mathbf{Y_a = 0.617\ X_{a2} + 0.391\ X_{a1}}$ (Table 5).

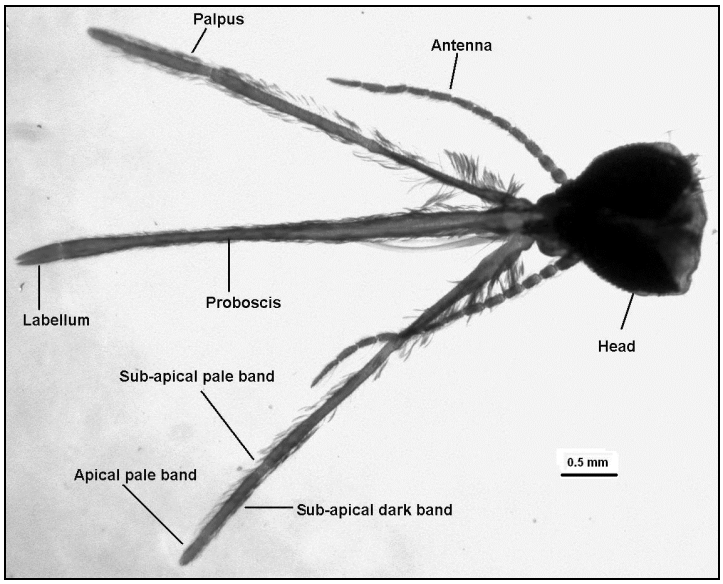


**Fig 1:** Parts of the Head – palpi, proboscis and antennae of *A. subpictus* from Bhiwani (Haryana, India).

**Table 2:** Descriptive values of characters from the head region of *Anopheles subpictus* populations.

| Character/variable (n=20) | Length (mm) |
|---|---|
| Length of palpus | 5.480±1.281 |
| Length of antenna | 3.900±1.383 |
| Length of proboscis | 5.860±1.258 |
| Length of palpomere 5 | 0.680±0.303 |
| Length of palpomere 4 | 1.120±0.258 |
| Length of palpomere 3 | 2.020±0.465 |
| Length of palpomere 2 | 1.940±0.384 |
| Subapical dark band | 0.660±0.114 |
| Apical pale band | 0.840±0.114 |

*Length represented as Mean ± Std. deviation

**Table 3:** Ratios of the variables from head region of *A. subpictus* populations.

| Variable* | Code | Sonipat | Bhiwani | Sangrur | Roop-Nagar |
|---|---|---|---|---|---|
| Length of antenna/length of proboscis | MPlp2/MPlp | 0.63 | 0.53 | 0.55 | 0.8 |
| Length of palpus/length of proboscis | MPlp3/MPlp | 0.91 | 0.91 | 0.88 | 0.97 |
| Length of palpomere 5/length of palpus | MPlp4/MPlp | 0.11 | 0.11 | 0.08 | 0.16 |
| Length of palpomere 4/length of palpus | MPlp5/MPlp | 0.19 | 0.21 | 0.20 | 0.19 |
| Length of palpomere 3/length of palpus | MPlp/P | 0.35 | 0.37 | 0.35 | 0.35 |
| Length of palpomere 2/length of palpus | Ant/P | 0.38 | 0.41 | 0.38 | 0.32 |
| Sub-apical dark band/length of palpus | SADB/MPlp | 0.14 | 0.16 | 0.08 | 0.10 |
| Apical pale band/length of palpus | APB/MPlp | 0.16 | 0.18 | 0.15 | 0.14 |

Note: MPlp, length of maxillary palpus; MPlp2-5, length of palpomeres 2-5; P, length of proboscis; Ant, length of antenna

**Table 4:** Bivariate correlation between length of palpus (dependent variable) and other explanatory variables (* non-significant values) from the head region of *A. subpictus* populations.

| S. No | Explanatory variables | Correlation with Length of Palpus ($Y_a$) | Sig. |
|---|---|---|---|
| 1 | Length of antenna ($X_{a1}$) | 0.956 | 0.006 |
| 2 | Length of proboscis ($X_{a2}$) | 0.978 | 0.002 |
| 3 | Length of palpomere 5 ($X_{a3}$) | 0.848 | 0.035 |
| 4 | Length of palpomere 4 ($X_{a4}$) | 0.944 | 0.008 |
| 5 | Length of palpomere 3 ($X_{a5}$) | 0.968 | 0.003 |
| 6 | Length of palpomere 2 ($X_{a6}$) | 0.768 | *0.065** |
| 7 | Sub-apical dark band ($X_{a7}$) | 0.387 | *0.260** |
| 8 | Apical pale band ($X_{a8}$) | 0.914 | 0.015 |

**Table 5:** Multiple regression model between length of palpus (dependent variable) and other explanatory variables from the head region of *A. subpictus* populations.

| | Un-standardized Coefficients | | Standardized Coefficients | *'t'* test | Sig. |
|---|---|---|---|---|---|
| | B | Std. Error | Beta | | |
| Constant | 3.378 | 2.971 | | 1.137 | *0.373* |
| Length of proboscis ($X_{a2}$) | 0.617 | 0.091 | 0.606 | 6.768 | 0.021 |
| Length of antenna ($X_{a1}$) | 0.391 | 0.083 | 0.423 | 4.720 | 0.042 |

Note: Dependent Variable (Ya): Length of palpus, R square = 0.996, R square adjusted = 0.993, One Way ANOVA '*p*' value = 0.004

### 3.2 Variation in the characters of the wing

Wings were examined for recording the variations in wing spots, veins and cross veins from which the mean values ± standard deviation and ratios were calculated (Figure 2, Table 6-8). Accordingly, some of those characters which showed considerable variation were the apical dark spot, length b/w r-m and m-cu cross veins and length of vein r2+3 beyond r-rs cross vein (Table 6, Figure 2). The analysis involved bivariate correlation with '*t*' test and multiple linear regressions using SPSS 15.0 to test the hypothesis that all the characters of the wing ($X_{b1}$, $X_{b2}$…….$X_{b15}$) are significantly and positively related to the total wing length ($Y_b$)." Accordingly, the results revealed that humeral pale spot ($X_{b3}$) and preapical dark spot ($X_{b5}$) had a significant negative correlation while subcostal pale spot ($X_{b6}$) had a significant positive correlation with the total wing length ($Y_b$) (Table 8). The multiple linear regression results reveal that the length of subcostal pale spot ($X_{b6}$) was significantly important in differentiating the total wing length using model $\mathbf{Y_b = 100.399 + 1.885\ X_{b6}}$ (Table 9).

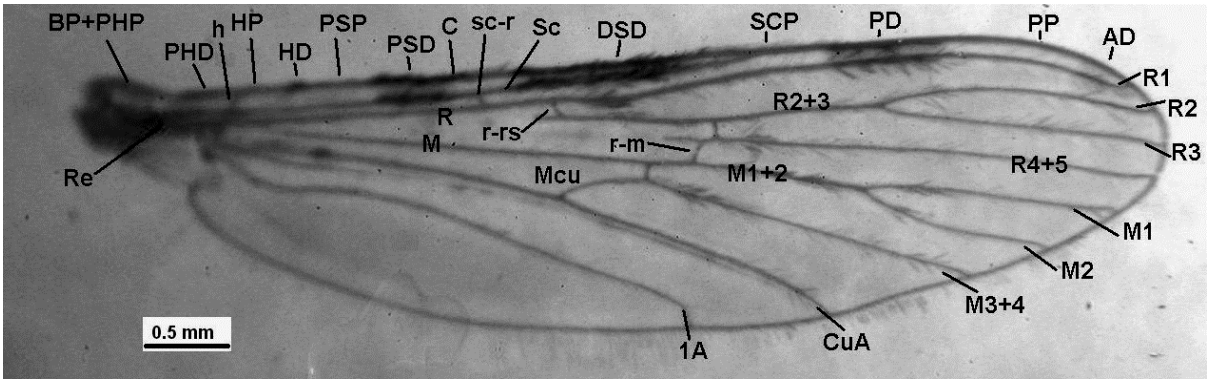


**Fig 2:** Wing spotting and venation characters of *A. subpictus* from Bhiwani (Haryana, India). C: Costa, CuA: cubitus anterior, h: humeral crossvein, r-rs: radial crossvein, $R_1$: radius one, $R_2$: radius two, $R_{2+3}$: radius two plus three, $R_3$: radius three, $R_{4+5}$: radius four plus five, R: radial, Re: remigium, Sc: subcosta, sc-r: subcostal crossvein, M: media, $M_1$: media one, $M_2$: media two, M2+3: media two plus three, r-m: radial media crossvein, 1A: anal vein, Mcu: media cubitus crossvein, Spotting - BP+PHP: basal pale + pre humeral pale, PHD: pre humeral dark, HP: humeral pale, HD: humeral dark, PSP: proximal sector pale, PSD: proximal sector dark, SP: sector pale, ASP: accessory sector pale, DSD: distal sector dark, SCP: subcostal pale, PD: preapical dark, PP: preapical pale, AD: apical dark).

**Table 6:** Descriptive values of characters from the wing of *A. subpictus* populations.

| Character/variable (n=20) | Length (mm) |
|---|---|
| Total wing length | 5.328±0.324 |
| Length of apical dark spot | 0.296±0.021 |
| Prehumeral dark | 0.104±0.035 |
| Humeral pale | 0.288±0.395 |
| Preapical pale | 0.52±0.116 |
| Preapical dark | 0.648±0.076 |
| Subcostal pale | 0.696±0.153 |
| Distal sector dark | 0.856±0.163 |
| Proximal sector dark | 0.624±0.128 |
| Humeral dark | 0.16±0.176 |
| Basal pale and prehumeral pale | 0.32±0.109 |
| Length b/w r-m and m-cu crossveins | 0.156±0.060 |
| Length of vein r2+3 beyond r-rs crossvein | 0.152±0.065 |
| Length of r4+5 beyond r-m crossvein | 0.192±0.017 |
| Length of r2 vein | 1.184±0.199 |
| Length of r3 vein | 1.256±0.186 |

*Length represented as Mean ± Std. deviation

**Table 7:** Ratios of different character variables from wings of *A. subpictus* populations. (TWL - Total wing length)

| Variable* | Sonipat | Bhiwani | Sangrur | Roop-Nagar |
|---|---|---|---|---|
| Length of apical dark spot/ TWL | 0.056 | 0.063 | 0.05 | 0.048 |
| Prehumeral dark/ TWL | 0.016 | 0.015 | 0.021 | 0.013 |
| Humeral pale/ TWL | 0.136 | 0.150 | - | - |
| Preapical pale/ TWL | 0.104 | 0.087 | 0.078 | 0.125 |
| Preapical dark/ TWL | 0.136 | 0.150 | 0.1 | 0.104 |
| Subcostal pale/ TWL | 0.096 | 0.126 | 0.157 | 0.139 |
| Distal sector dark/ TWL | 0.144 | 0.142 | 0.15 | 0.153 |
| Proximal sector dark/TWL | 0.08 | 0.134 | 0.121 | 0.125 |
| Humeral dark/TWL | - | - | 0.05 | 0.020 |
| Basal pale and prehumeral pale/ TWL | 0.08 | 0.039 | 0.035 | 0.069 |
| Length b/w r-m and m-cu crossveins/ TWL | 0.04 | 0.023 | 0.017 | 0.041 |
| Length of vein r2+3 beyond r-rs crossvein/ TWL | 0.024 | 0.015 | 0.035 | 0.020 |
| Length of r4+5 beyond r-m crossvein/ TWL | 0.032 | 0.039 | 0.035 | 0.034 |
| Length of r2 vein/ TWL | 0.24 | 0.246 | 0.178 | 0.258 |
| Length of r3 vein/ TWL | 0.256 | 0.261 | 0.185 | 0.265 |

**Table 8:** Bivariate correlation between total wing length (dependent variable) and other explanatory variables (* non-significant values) from the wing of *A. subpictus* populations.

| S. No | Explanatory variables | Correlation with Total wing length ($Y_b$) | Significance value |
|---|---|---|---|
| 1 | Length of apical dark spot ($X_{b1}$) | -0.473 | *0.211** |
| 2 | Prehumeral dark ($X_{b2}$) | 0.152 | *0.404** |
| 3 | Humeral pale ($X_{b3}$) | -0.862 | 0.030 |
| 4 | Preapical pale ($X_{b4}$) | 0.540 | *0.174** |
| 5 | Preapical dark ($X_{b5}$) | -0.853 | 0.033 |
| 6 | Subcostal pale ($X_{b6}$) | 0.895 | 0.020 |
| 7 | Distal sector dark ($X_{b7}$) | 0.358 | *0.277** |
| 8 | Proximal sector dark ($X_{b8}$) | 0.657 | *0.114** |
| 9 | Humeral dark ($X_{b9}$) | 0.454 | *0.221** |
| 10 | Basal pale and prehumeral pale ($X_{b10}$) | 0.023 | *0.486** |
| 11 | Length b/w r-m and m-cu crossveins ($X_{b11}$) | 0.195 | *0.377** |
| 12 | Length of vein r2+3 beyond r-rs crossvein ($X_{b12}$) | 0.323 | *0.298** |
| 13 | Length of r4+5 beyond r-m crossvein ($X_{b13}$) | 0.566 | *0.160** |
| 14 | Length of r2 vein ($X_{b14}$) | 0.207 | *0.369** |
| 15 | Length of r3 vein ($X_{b15}$) | 0.103 | *0.435** |

**Table 9:** Multiple regression model between total wing length (dependent variable) and other explanatory variables from the wing of *A. subpictus* populations.

| | Un-standardized Coefficients | | Standardized Coefficients | *t* test | Sig. |
|---|---|---|---|---|---|
| | B | Std. Error | Beta | | |
| Constant | 100.399 | 9.636 | | 10.419 | 0.002 |
| Subcostal pale ($X_{b6}$) | 1.885 | 0.543 | 0.895 | 3.470 | 0.040 |

Note: Dependent Variable (Yb): total wing length, R square = 0.801, R square adjusted = 0.734, One Way ANOVA '*p*' value = 0.040

## 4. Discussion

Morphology has been traditionally used for description of the species and for discrimination of problematic species. With the advancement of technology some of the characters used traditionally in taxonomy and systematics have been found to be polymorphic resulting in identification of sibling/cryptic species complexes. Hence the cytogenetic and molecular description of such problematic and traditional species have resulted in revision of the phylogeny and systematics of anophelines [3, 4, 27, 28, 29, 30, 31].

*A.* (*Cellia*) *subpictus* Grassi 1899 which is a member of Pyretophorus series was found to have sibling species within its domain based on the morphological and cytogenetic parameters [7, 32]. Suguna *et al.* [8] provided some more cytogenetic and morphological evidences to raise the total to four sibling species, which were provisionally named as A, B, C and D. However, these were never formally described with the relevant illustrations to support the differences in important characters like in adult palpi, egg chorion architecture, polytene chromosome polymorphism and larval chaetotaxy. As per, Suguna *et al.* [8], apical white band of adult females can be used to segregate species A, B and C, while X-chromosome inversion can be used to segregate all the four species. It was contended that the palpal character is not polymorphic and X-chromosome hybrids have not been reported in nature. In conflict, Kirti and Kaur [21] have reported large number of morphological variations in palpi while Chhilar and Chaudhry [33] have already reported natural X-chromosome hybrids. A review of the entire data accumulated so far from Suguna [32], Reuben and Suguna [7], Suguna *et al.* [8], Kirti and Kaur [19], Chhilar and Chaudhry [33] and other salivary polytene chromosome polymorphism data available with the author (unpublished) has revealed that the characters of the palpi, wing venation and spotting, egg chorion architecture (egg float number), and salivary polytene X-chromosome banding pattern are polymorphic in nature. Further, identification of characters from various stages of the same individual has practical problems for field application and designing suitable vector control strategies.

In context with the present study several *Anopheles* species have been reported to be polymorphic for characters such as wing spotting pattern, venation and length. These include *A. nuneztovari* [34], *A. darlingi* [35, 36], *A. sundaicus* [37], *A. galvaoi*, *strodei*, *aquasalis* [38], *A. marajoara* [39], and members of funestus and minimus group [30, 40]. Some workers have even reported the wing spot intensity and pattern to vary depending on environmental conditions [41].

Historically, it was Reid [42] who started the debate by raising the then subspecies *indefinitus* to the full status of species separating it from *subpictus*, the brackish water breeding species (Probably the sibling species B). He also reviewed the status of *A. vagus*, *A. indefinitus*, *A. subpictus* and *A. sundaicus* and contended that most of these species are hard to discriminiate based on adult morphology and only can be separated using other characters from eggs, larva, pupa and polytene chromosomes. These species and recently described *A. pseudosundaicus* [43] have overlapping highly variable characters with the *A. subpictus* sensu lato. Even the characters like apical white band and subapical dark band which are highly relied upon for discrimination between these species have considerable polymorphism [6, 8, 19, 43, 44, 45, 46]. Very recently Surenderan *et al.* [46] have used molecular tools to provide genetic support of *A. sundaicus* species misidentified to be *A. subpictus* sibling species B, thus justifying the need of testing the morphological characters for their reliability.

Based on the statistical analysis of present morphotaxonomic

characters, it can be concluded that most of these characters though used for species discrimination using dichotomous keys are statistically unreliable and hence result in frequent problems in species discrimination due to their polymorphic nature. The reliable characters found during the present study are the length of the antenna, the proboscis and the palpus from head region, and the subcostal pale spot from the wing. Subcostal pale spot has been successfully used earlier in discrimination of *A. sinensis* and *A. lesteri* [47]. Hence, these characters are recommended for use while preparing reliable morpho-taxonomic keys of this and related species.

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