J Vector Borne Dis 52, June 2015, pp. 122–128

Landmark-based geometric morphometric analysis of wing shape among certain species of *Aedes* mosquitoes in District Dehradun (Uttarakhand), India

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ABSTRACT

Background & objectives: Insect wing morphology has been used in many studies to describe variations among species and populations using traditional morphometrics, and more recently geometric morphometrics. A landmark-based geometric morphometric analysis of the wings of three species of *Aedes* (Diptera: Culicidae), *viz. Ae. aegypti*, *Ae. albopictus* and *Ae. pseudotaeniatus,* at District Dehradun was conducted belling on the fact that it can provide insight into the population structure, ecology and taxonomic identification.

Methods: Adult *Aedes* mosquito specimens were randomly collected using aerial nets and morphologically examined and identified. The landmarks were identified on the basis of landmark based geometric morphometric analysis thin-plate spline (mainly the software tps-Util 1.28; tps-Dig 1.40; tps-Relw 1.53; and tps-Spline 1.20) and integrated morphometrics programme (mainly twogroup win8 and PCA win8) were utilized.

Results: In relative warp (RW) analysis, the first two RW of *Ae. aegypti* accounted for the highest value (95.82%), followed by *Ae. pseudotaeniatus* (90.89%), while the lowest (90.12%) being recorded for *Ae. albopictus.* The bending energies of *Ae. aegypti* and *Ae. pseudotaeniatus* were quite identical being 0.1882 and 0.1858 respectively, while *Ae. albopictus* recorded the highest value of 0.9774. The mean difference values of the distances among *Aedes* species performing Hotelling's T^2 test were significantly high, predicting major differences among the taxa. In PCA analysis, the horizontal and vertical axis summarized 52.41 and 23.30% of variances respectively. The centroid size exhibited significant differences among populations (non-parametric Kruskal-Wallis test, H = 10.56, $p < 0.01$).

Interpretation & conclusion: It has been marked out that the geometric morphometrics utilizes powerful and comprehensive statistical procedures to analyze the shape differences of a morphological feature, assuming that the studied mosquitoes may represent different genotypes and probably come from one diverse gene pool.

Key words *Aedes* mosquito wing; geometric morphometrics; principal component analysis; taxonomic variation; thin-plate spline; Uttarakhand

INTRODUCTION

Since long *Aedes* species (Diptera: Culicidae) are known to spread several diseases like dengue fever, chikungunya, yellow fever, *etc*; originated in Africa[1] and dispersed to tropical and subtropical regions throughout the world[2]. Among the mosquito-borne viral diseases, dengue has become a major international public health concern as it has led to global resurgence of epidemic dengue fever and emergence of dengue haemorrhagic fever (DHF)[3]. Currently, emerging DHF cases have become a leading cause of hospitalization and death among children in the south Asian countries[4]. Although, the production of dengue vaccine is ongoing with satisfactory results[5], vector control and entomological surveillance remain primary issue in controlling the disease due to the challenge of accurately identifying all possible vectors of the disease[6].

In India, *Ae. aegypti* has been identified as a common dengue vector species. With the increasing cases of dengue, mosquito control programmes are faced with problems on vector species diversification and proper identification. As the morphology of insects is under genetic and environmental influences, variation in morphometric traits may provide significant information on many aspects of insect biology[7]. Studies on variations in wing geometry may cater relevant data on proper identification of species and in describing population diversity.

Wings are the excellent structures for studying morphological variations because the intersections of the wing veins provide many well-defined landmarks suitable for morphometrics and that the metric properties of the wing provide precise quantitative information for the identification of insects[8]. Landmarks are the points at which biological structures are sampled. These points produce an exact geometric description of the differences in shape of a structure[9]. A number of entomologists have carried out commendable works on insect wing morphometrics[14-19] as well as in mosquitoes[9, 15-17].

The importance of studying variations in wing geometry in mosquito populations belies on the fact that it can provide insight into the population structure, ecology and even the taxonomic identity of the mosquitoes. Considering the importance of mosquito wings in insect behaviour and physiology, the differences could also provide useful information on vector distribution and disease control.

The present study investigates the variations among the wings of vectors of dengue (*Ae. aegypti* and *Ae. albopictus*) and non-vector species (*Ae. pseudotaeniatus*) through landmark-based geometric morphometric analysis, quantitatively; and more precisely to understand as to where characters could be used later for studies on strains that serve as vectors of the dengue virus.

MATERIAL & METHODS

Study area and sampling

Adult *Aedes* mosquitoes were collected randomly at selected areas of District Dehradun (latitude 30°19' N, 78°04' E; longitude 77°35' E, 78°20' E) from 1 to 20 June 2014, using aerial nets, both early in the morning and late afternoon. The collected specimens were sorted and examined in the laboratory using a stereoscope. Identification of mosquitoes up to species level was performed using standard keys and catalogues[18]. Only 18 female adult species belonging to *Ae. aegypti*, *Ae. albopictus* (potential vectors of dengue) and *Ae. pseudotaeniatus* (non-potential vector of dengue) were utilized in this study. Left wings were detached from the thorax, placed on a glass slide and secured with a coverslip.

Data acquisition

All slides were photographed by using a Kyowa Tokyo No. 204124 stereoscopic zoom dissection microscope (an integrated magnification by 10X–0X; manufactured by Kyowa Optical Co. Ltd, Tokyo, Japan) and a Nikon digital camera system. Photographs were first input to tps-Util 1.28 software[19] and thereafter two dimensional Cartesian coordinates of 20 landmarks identified as the intersections of wing veins with the wing margin, intersection of cross vein with major veins and some vein branch points from left wings (Fig. 1), were digitized by tps-Dig 1.40 software[20]. The description and locations of the identified landmarks are presented in Table 1. All wings were digitized twice in order to reduce the measurement error (ζ)[21]. The second session of the measurement was conducted after having removed the wing and re-placing it under the microscope in order to take the positioning error into account[21]. No analogous systems were used during the whole procedure to keep the digital errors in minimum.

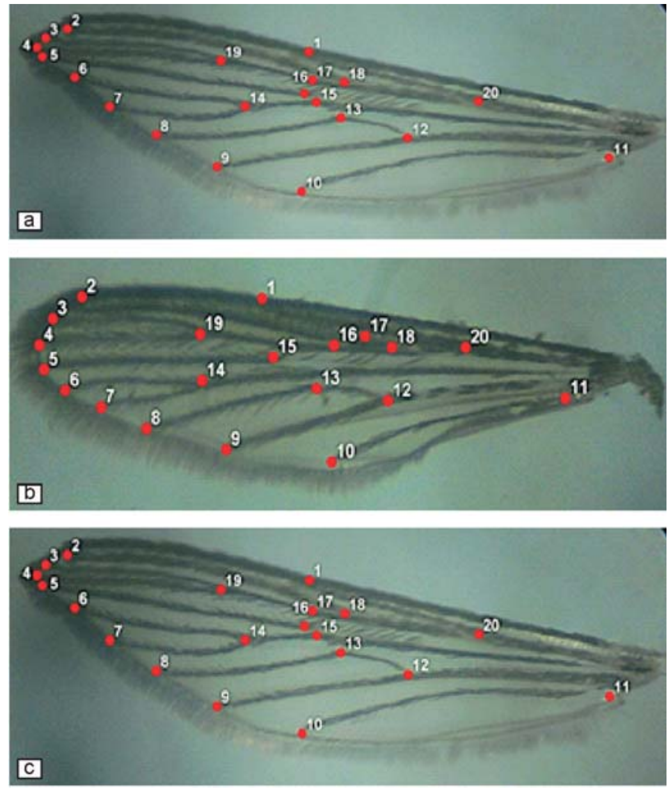

Fig. 1: Digitization of 20 landmarks of left wing of female *Aedes* species using software tps-Dig 1.40. (a) *Ae. aegypti* (b) *Ae. albopictus,* and (c) *Ae. pseudotaeniatus.*

Table 1. Description of assigned landmarks of *Aedes* species

Landmarks	Description of the landmarks
1.	Intersection of costa (C)
2.	Distal end of radius (R)
3.	Radial bnranch 2
4.	Radial branch 3
5.	Distal end of radial branches 4 and 5
6.	Distal end of media 1 and 2
7.	Distal end of media 3 and 4
8.	Distal end of cubital vein 1
9.	Distal end of cubital vein 2
10.	Anal vein
11.	Median vein
12.	Origin of cubital 1
13.	Medio-cubital cross vein
14.	Radio-sectoral vein
15.	Midpoint branch of medial vein
16.	Radio-medial cross vein
17.	Midpoint branch of radial vein
18.	Radial cross vein
19.	Origin of radius branches 2 and 3
20.	Radial sector

Statistical analysis

The coordinates were analyzed using tps-Relw 1.53 software[22] to calculate Eigen values for each principal warp. The landmark configurations were scaled, translated and rotated against the consensus configuration by generalized least squares (GLS) procrustes superimposition method[23]. The consensus configurations per wings were subjected to relative warps analysis, by assessing the variability in the shape space using the scores obtained for each individual landmark which is technically a PCA. The relative warps correspond to the principal components and define a shape space in which individual landmarks are replaced. The bending energies of all taxa were compared using tps-Spline 1.20[24].

For testing significant differences in shape between the two compared species, integrated morphometrics package (IMP) software series[25] were utilized; the data were first superimposed to Bookstein's shape coordinates (BC)[26] by IMP CoordGen8 and then Hotelling's T^2 test was performed by the software IMP twogroup win8. The size morphometric of the examined species was investigated by using the centroid sizes of the wings as estimator with nonparametric Kruskal-Wallis test by SPSS version 17.0[27]. Centroid size is the square root of the sum of squared distances of a set of landmarks from their centroid or in other words it is the square root of the sum of the variances of the landmarks around that centroid in x- and y-directions[27-28].

RESULTS

Relative warps

The relative warps were performed by using an orthogonal alignment projection method (Table 2). Singular values were explained by five relative warps, among which the first two relative warps values for *Ae. aegypti* were 0.17524 and 0.03926 (total percentage of 95.82), whereas for *Ae. albopictus* first two relative warps were 0.22619 and 0.10529 (total percentage of 90.12) and for *Ae. pseudotaeniatus* the warping values were 0.11512 and 0.03843 (total percentage of 90.89). In all the three species, the fifth warping value accounted for the lowest percentage. The values of consensus configuration of the 20 landmarks of three different species of *Aedes* are depicted in scatter plot diagram (Fig. 2). The vector analysis of the magnitude and angle of the consensus Cartesian coordinates values of 20 landmarks reveals that the mean magnitude of the three species are of the same range of 0.197 (*Ae. pseudotaeniatus*) to 0.193 (*Ae. aegypti*), however, the mean angle value recorded was highest in *Ae. albopictus* (25.42°) followed by *Ae. aegypti* (16.87°)

Table 2. Relative warps explaining the variations observed in the venation pattern of *Aedes* sp.

Species	RW	Singular values	%*	% Cumulative†
Ae. aegypti	1	0.17524	91.24	91.24
Ae. albopictus		0.22619	74.07	74.07
Ae. pseudotaeniatus		0.11512	81.77	81.77
Ae. aegypti	2	0.03926	4.58	95.82
Ae. albopictus		0.10529	16.05	90.12
Ae. pseudotaeniatus		0.03843	9.11	90.89
Ae. aegypti	3	0.02637	2.07	97.89
Ae. albopictus		0.06473	6.07	96.18
Ae. pseudotaeniatus		0.03002	5.56	96.45
Ae. aegypti	4	0.02086	1.29	99.18
Ae. albopictus		0.04201	2.56	98.74
Ae. pseudotaeniatus		0.01797	1.99	98.44
Ae. aegypti	5	0.01659	0.82	100
Ae. albopictus		0.02953	1.26	100
Ae. pseudotaeniatus		0.01590	1.56	100

*Calculation of the percentage of warping in each parameter of RW; †Calculating the cumulative percentage on descended manner of RW for each species.

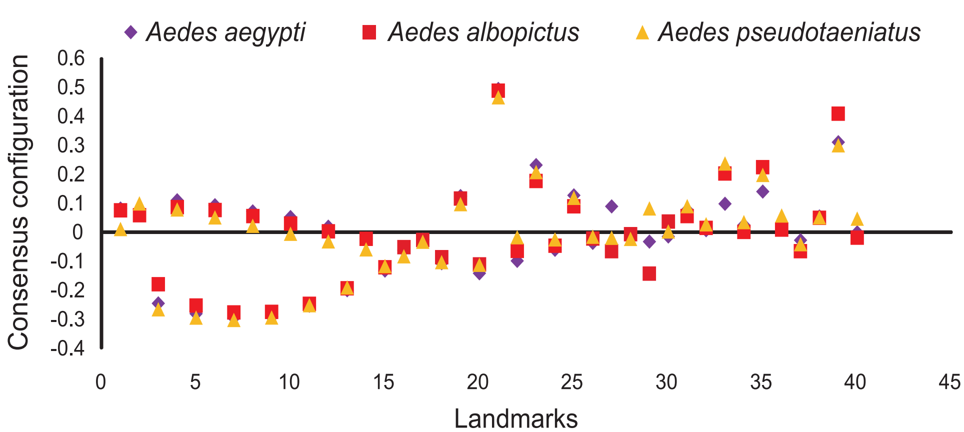

Fig. 2: Scatter plot diagram showing consensus configuration of the 20 landmarks plotted for the three species of *Aedes*.

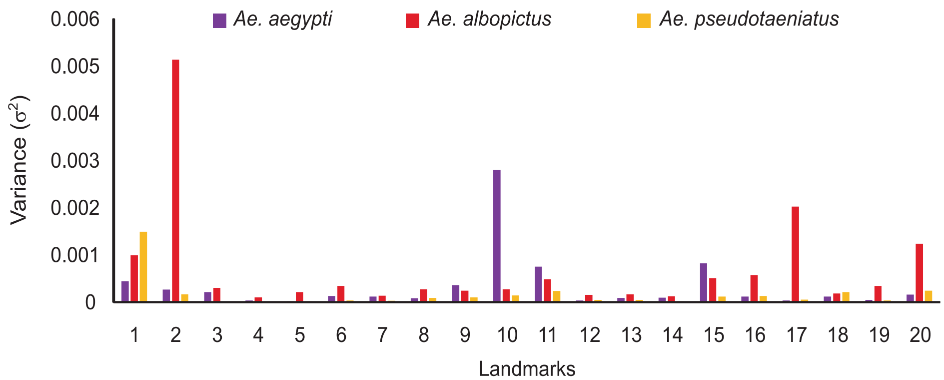

Fig. 3: Column diagram depicting variances (σ^2) at each landmark for aligned *Aedes* species.

and lowest being recorded for *Ae. pseudotaeniatus* (–9.38°).

In case of variance (σ^2), the mean value recorded highest for *Ae. albopictus* (0.000691), followed by *Ae. aegypti* (0.000336), whereas lowest depicted in *Ae. pseudotaeniatus* (0.000162). The landmarks 10, 2 and 1 account for the highest values for the aligned species *Ae. aegypti*, *Ae. albopictus* and *Ae. pseudotaeniatus* with the values of 0.002799, 0.005136 and 0.001493 respectively. Similarly, landmarks 5, 4 and 3 depict the lowest values of variance (s^2), *i.e*, 0.000019, 0.000102 and 0.000007 for *Ae. aegypti*, *Ae. albopictus*, and *Ae. pseudotaeniatus* respectively (Fig. 3).

The relative positions of the average configurations of the subgenera are clustered together in the shape space defined by the first two relative warps, as the first two relative warps explained highest percentage of the singular values (Fig. 4). The relative positions of the landmarks 11, 17, 18 and 20 in *Ae. pseudotaeniatus* give a different basal shape to the wings than those of *Ae. aegypti* and *Ae. albopictus.*

The bending energies, procrustes distances and angles calculated from the wing consensus data are depicted in Table 3. Similarity in the means of energies of the wing shapes between *Ae. aegypti* and *Ae. pseudotaeniatus* is quite remarkable, *i.e.*, 0.1882 and 0.1858 respectively, while, *Ae. albopictus* recorded the highest value (0.9774).

Table 3. Bending energies, procrustes distance (d) and angles (radians) among the examined species

Species	Energy	Angle	d
Ae. aegypti	0.1882	0.0749	0.0749
Ae. albopictus	0.9774	0.1860	0.9983
Ae. pseudotaeniatus	0.1858	0.0516	0.0516

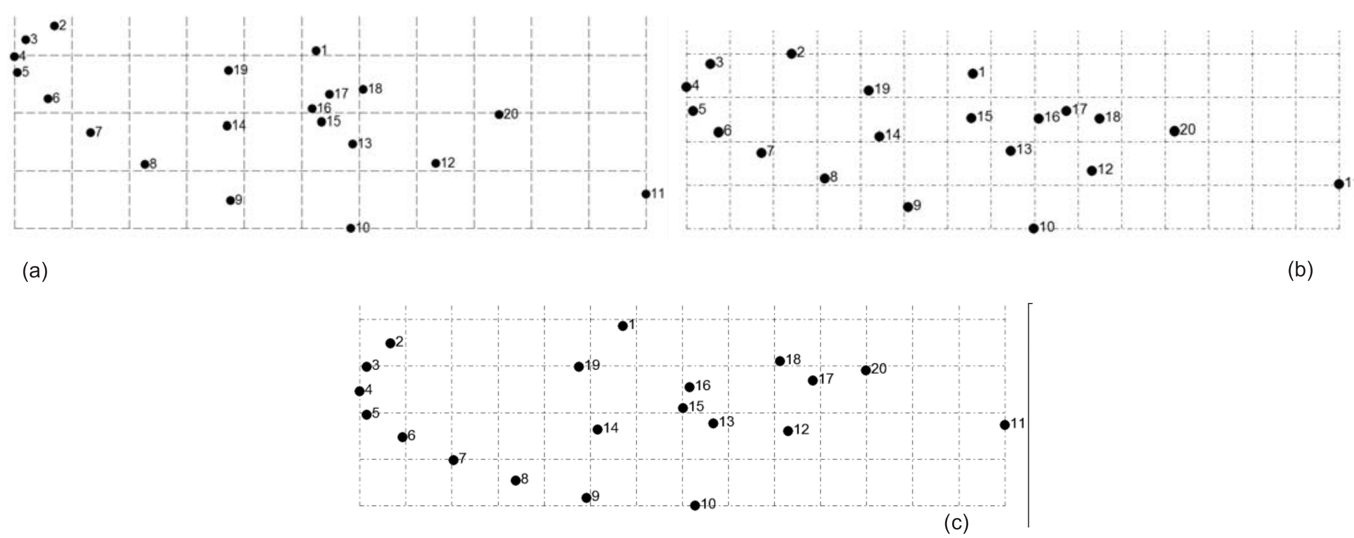

Fig. 4: Relative positions of the mean configurations of the species for *Aedes* in the shape space defined by the first two relative warps ($x = 1$, $y = 2$ and $d = 0$). Circles with numbers indicate the landmarks—(a) *Ae. aegypti*, (b) *Ae. albopictus,* and (c) *Ae. pseudotaeniatus*.

Bookstein's shape coordinates (BC)

Distance between mean values of the Hotelling's T^2 test based on the data in Bookstein two point registration upon use of software IMP twogroup win8 yielded quite significant results ($p > 0.05$), which predict significant differences among the taxa (Table 4).

Table 4. Comparison of the mean shapes using twogroup win8 software

Studied *Aedes* species	Distance between mean values (Hotelling's T^2 test)	$p > 0.05$
Ae. aegypti vs Ae. albopictus	0.9519	Significant
Ae. aegypti vs Ae. pseudotaeniatus	0.1441	Significant
Ae.albopictus vs Ae. pseudotaeniatus	1.2169	Significant

Principal component analysis (PCA)

When a PCA was conducted on the 20 wing landmarks, the first two PC's summarized 52.41 and 23.30% of the total variance respectively. The first PC value suggests relative differences in the relative positions of the landmarks regarding the base of the wing. Main deformations centred on the medial of the wings of the landmarks 15–16 and 17–18 (Fig. 5). The distribution of individuals along the first two PCs is shown in Fig. 6. *Ae. aegypti* and *Ae. pseudotaeniatus* tended to cluster along positive axis of PC1 and PC2 respectively, however, specimens of *Ae. albopictus* were found to gather along negative of both the axes suggesting higher phenotypic distances among themselves.

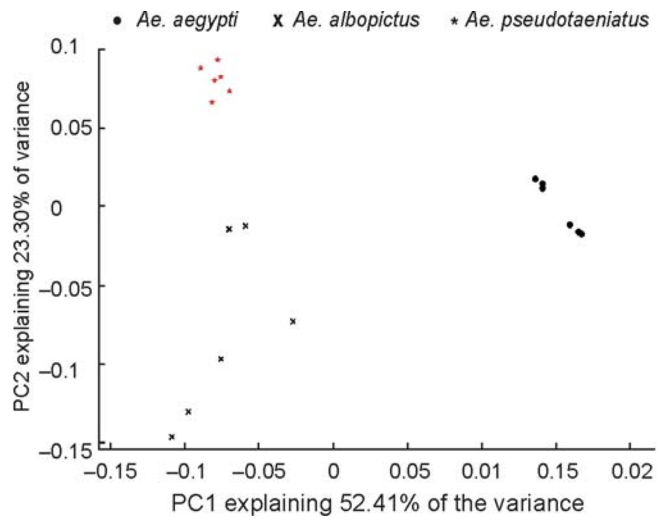

Fig. 5: Distribution of the three different species of *Aedes* along the first two PCs.

Centroid size (CS)

Centroid sizes were used as measures of overall wing size differences among populations. *Ae. aegypti* accounted for the highest centroid size value (3914.74 ± 270.079), followed by *Ae. pseudotaeniatus* (3543.50 ± 118.655), while lowest being accounted for *Ae. albopictus* (3398.72 ± 189.485). The size differences among the populations were significant (Kruskal-Wallis test: H = 10.56; $p < 0.01$) (Fig. 6).

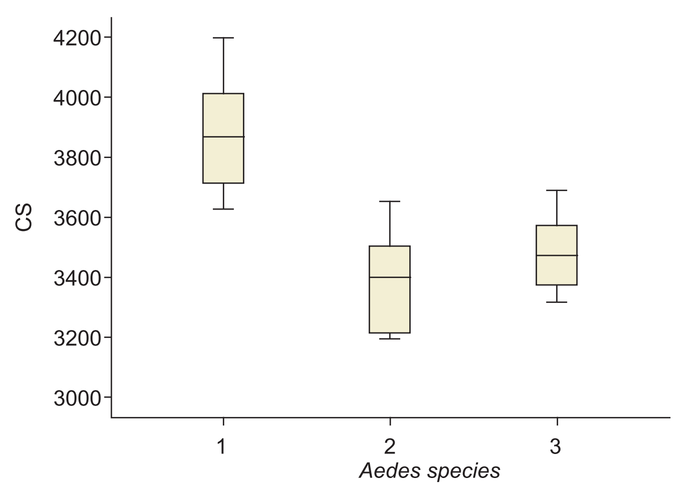

Fig. 6: Kruskal-Wallis test based on centroid size (CS) in SPSS. 1 = *Ae. aegypti* (n = 6); 2 = *Ae. albopictus* (n = 6); and 3 = *Ae. pseudotaeniatus* (n = 6).

DISCUSSION

Morphometrics is defined as the quantitative description, analysis and interpretation of shape and variation of structures in biology[29]. In a fundamental area of research, unlike the analytical approaches, the geometric one is aimed at comparison of the shapes[28]. Moreover, morphometric studies have contributed main role in resolving taxonomic problem in mosquito identification[30].

Our results show that the wing shapes exhibited significant difference among the three *Aedes* species, based upon the results of RW, BC, PCA and CS as reported for Hemipterans[11-12]. In mosquitoes[14], the species of *Culex viz. Cx. quinquefasciatus vs Cx. nigripalpus* and *Cx. pipiens vs Cx. torrentium* were differentiated based on the wing shape and wing veins, thus supporting our study. The observations of Dhivya and Manimegalai[16] also support our findings as their studies were based on geometric morphometric methods, but the difference was that they showed variations among the male and female individuals of a species. Though, in the past there had been studies on wing geometry of *Aedes* species including *Ae. aegypti*[9] and *Ae. albopictus*[15], but on intraspecific variations between the species.

The reasons for observed variations in wing morphology of mosquitoes need more exploration. As of now, we can only hypothesize that the mosquitoes represent different genotypes and probably come from diverse gene pool. Henceforth, further studies should be done to determine whether variations in the wings of mosquitoes have genetic basis or are mere reflections of the existence of high phenotypic plasticity brought about by varied environmental conditions during growth and development of the larvae[10].

In general, geometric morphometrics utilizes powerful and comprehensive statistical procedures to analyse shape differences of a morphological feature, using either homologous landmarks or outlines of the structure[7]. Unlike the wing shape, the wing size did not vary significantly in the present study. These uncorrelated size and shape variation patterns appear to be due to the existence of distinct determinants for those biological variables, as discussed in the light of earlier studies[10].

ACKNOWLEDGEMENTS

Authors are thankful to the Council for Scientific and Industrial Research, New Delhi for financial support and to Mr. J.P. Bahuguna, District Vector Borne Disease Control Officer, Office of CMO, Dehradun for cooperation.

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Received: 12 February 2015 *Accepted in revised form:* 26 March 2015