

The Taxonomic Signal of the Internal Mosquito Wing Cells.

Somsanith Chonephetsarath

Mahidol University Library and Knowledge Center: Mahidol University

Chadchalem Raksakoon

Kasetsart University Faculty of Science

Suchada Sumruayphol

Mahidol University

Jean-Pierre Dujardin

University of Montpellier

Rutcharin Potiwat (✉ rutcharin.pot@mahidol.edu)

Mahidol University <https://orcid.org/0000-0003-3307-6928>

Research

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Abstract

Background

Previous publications revealed a clear taxonomic signal embedded in the external contour of the wings. Our study explored this signal also for internal cells of the wings, with the following question: do internal cells uniformly provide the same taxonomic information?

Methods

Our study focused on four species, three of them of medical importance: *Ae. aegypti*, *Ae. albopictus*, *Ae. scutellaris* and *Ve. dux*, each of them represented by 30 female wings. Each cell was characterized for size and shape after an elliptic Fourier analysis. For each cell to be tentatively assigned to its respective species, i.e., to measure its amount of taxonomic information, we used the shape of the contour, not its size.

Results

We showed that the taxonomic signal of shape was not uniformly spread among internal cells of the wing, ranging on average from 80% to 94%. Four internal cells out of six performed better than the external contour of the wing. The amount of taxonomic information of internal cells could not be related to their size nor to their shape complexity, but clearly depended on which species were compared. The closest species of our sample, *Ae. albopictus* and *Ae. scutellaris*, two species frequently found in the same water collections in Thailand, were almost perfectly separated (97%) by a single cell.

Conclusion

It has already been said that the wing can be used for mosquito identification even if the whole body is not available, we showed that even partly damaged wings could contain extractable and more accurate taxonomic information.

1. Background

Three species of our sample are known vectors of virus: *Aedes (Stegomyia) aegypti*, *Ae. (St.) albopictus* and *Ae. (St.) scutellaris*. The traditional supraspecific arrangement of these three species, initially belonging to the *Aedes* genus, was tentatively modified two decades ago, restoring *Stegomyia* to genus [1], and suggesting the names *Stegomyia aegypti*, *St. albopicta* and *St. scutellaris* ([1] [2]). In this paper, for these three well known vectors and following the common use among epidemiologists [3], we will use the traditional genus and subgenus names *Aedes (Stegomyia)*.

The fourth species of our sample belongs to the *Verrallina* genus (redefined by Reinert, 1999)[4], subgenus *Verrallina*: *Ve. (Ver.) dux* Dyar & Shannon, 1925 [2, 5].

Aedes aegypti (Linnaeus in Hasselquist, 1762), *Ae. albopictus* (Skuse, 1894) and *Ae. scutellaris* (Walker) are vectors of various viruses [6, 7]. The two first species, *Ae. aegypti* and *Ae. albopictus*, have a wide intercontinental distribution including Thailand, where they recently contributed to an important dengue and chikungunya virus outbreak [8, 9, 10]. In addition to transmitting the chikungunya virus, they are able to spread all of the four dengue serotypes (DENV1-4). *Aedes aegypti* is also a competent vector of Zika virus [11, 12, 13]. *Ae. scutellaris* has a more restricted geographic territory that covers Papua New Guinea, Tonga, Southeast Asia, the South Pacific [14, 15], Australia [16], and central Thailand [17]. It has long been considered as a potential vector of the dengue virus in Papua New Guinea [18]. It was incriminated as a dengue virus vector during a huge endemic of dengue virus serotype 2 in 2005 at the Torres Strait in Australia, where *Ae. aegypti* was absent [16], and as a possible vector of the sylvan dengue fever in Bangkok, Thailand [17].

The fourth mosquito species that has been found in Thailand during our entomological surveillance activities, *Verrallina (Ver.) dux*, is attracted by light and bites humans but has never been reported as a vector of any diseases. It is a predominant species in the mangrove forest of Vietnam [19] and Philippine [20]. In February 2019, *Ve. dux* was collected by us in the mangrove forest that had been reported previously as the breeding place of *Ae. scutellaris* [6]. Actually, both *Ve. dux* and *Ae. scutellaris* may develop in brackish water.

The external morphology has long been the gold standard for taxonomic identification of mosquitoes at different levels of their development [14, 21, 22, 23, 24, 25]. The morphological species determination on adults is generally satisfactory, except in two main situations: (i) some adult morphologies are so similar that they are called “isomorphic” [26], “sibling” [27] or “cryptic” species [28, 29], and (ii) field mosquitoes may be damaged by the capture device or during transportation to the laboratory, losing the few or the only character allowing their reliable identification [30].

Genetic techniques of mosquito identification represent a valuable tool to face these situations [6, 24, 31], but the recently developed modern morphometric approaches, landmark-based and outline-based ones, are more and more suggested as efficient complementary diagnostic tools [32, 33]. The reason for this proposal is that modern morphometrics is a non-traumatic, low-cost and frequently accurate discrimination approach [34]. The method applies after a wing preparation procedure involving slide mounting and imaging preparation: skills in these aspects are common among entomologists and do not represent technical issues [6, 30]

Geometric morphometrics of mosquitoes has already been used to distinguish between genera [35], between species within the same genus [6, 23, 30, 36, 37, 38], between populations of a species [39, 40] and between sexes of a species [38, 41]. Recently, this method has been used by our group to discriminate various organisms as diverse as liver fluke [42], chigger mite [43] or fireflies [30].

The four species of our sample do not contain sibling species, but they are able to generate identification problems when partially damaged, especially for *Ae. albopictus* and *Ae. scutellaris*. These latter have no known clear-cut diagnostic character unless specimens were perfectly preserved. Moreover, larvae and

adults of both species also are very similar and misidentification has widely occurred [2, 14, 22]. Our sample contains also species which are easier to recognize on morphological ground: the *Aedes ssp.* versus *Ve. dux*. We expect their wing metric properties would allow a clear-cut recognition, especially for *Ve. dux*, a species belonging to another genus than *Aedes*.

The three *Aedes (Stegomyia)* species of our sample have recently been examined by both genetic and morphometric techniques [6], including by the outline-based approach used here. Our contribution was to use, one by one, the shape of the various contours offered by the mosquito wing: not only its external border, but also its various internal cells. Our study was designed to answer the question: is the taxonomic signal of the wing spread equally among various internal cells?

2. Materials And Methods

2.1 Study area

The four species of mosquito were collected as larvae in various areas of Thailand between 2009 and 2019, they were reared and maintained in the laboratory under the same environmental conditions, and submitted to morphometric analyses after different generational times (Table 1).

Aedes aegypti was collected from Bangkhae district (Bangkok province) (13°41'43.6"N / 100°23'05.1"E), *Ae. albopictus* was collected from Kanchanaburi Province at 129 km from Bangkok city (14°12'16.2"N / 99°07'58.5"E), *Ae. scutellaris* was collected from Phasi Charoen (Bangkok province) (13°43'19.8"N / 100°26'09.2"E), and *Ve. dux* was collected from the mangrove forest at Bang Pakong (Chachoengsao Province) (13°28'25.0"N / 100°52'19.9"E).

2.2 Mosquito colonization

The *Ae. albopictus* and *Ae. scutellaris* were collected several years ago (Table 1) and maintained in our laboratory (Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University). *Aedes aegypti* and *Ve. dux* were collected more recently from the field and maintained until F3 generation before being used for mounting wings. All four species were identified by external morphology on two days old emerging mosquitoes to avoid losing their scale. We used the taxonomic keys of Huang (1972) and Rattanarithikul et. al. [2, 14].

For all insects, rearing conditions of our laboratory (Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University) were the following: 27 ± 2 °C temperature and 60 ± 10 % of relative humidity (RH) under a natural light cycle until adult emergence. Larvae were reared in plastic trays with filtered water, but *Ve. dux* larvae from mangrove forest were reared in filtered water mixed with their natural breeding water. Larvae were provided 1 ml of fish food solution daily. Pupae were transferred to 30 × 30 × 30 cm cages to facilitate emergence.

2.3 Wings preparation for geometric morphometric analysis

The right and left wings were dissected and mounted using Hoyer's medium (Mixed from Gum arabic 30-40g, Chloral hydrate 200g, Glycerine 20 ml and Distilled water 50 ml) on glass microscope slides. Each slide was photographed by a Nikon DS-Ri1 SIGHT digital camera connected to a Nikon AZ 100 M stereomicroscope (Nikon Corp., Tokyo, Japan) with scale apparent on the photograph. The right wing was used, and in case of damage, the left wing was used instead.

The external contour (cell 0) and the contour of six internal cells (cells 1 to 6) were digitized (Fig. 1) using a computer assisted manual digitization (see Morphometric software).

2.4 Analyses

2.4.1. Size and shape.

An Elliptic Fourier analysis (EFA) [44] was used to describe the shape of the contour and its size. In this approach, the contour is decomposed in terms of sine and cosine curves of successive frequencies called harmonics, each harmonic containing four coefficients. The removal of size effect was obtained by dividing the coefficients by the semi-major axis of the first ellipse. However, for size comparisons we used the perimeter of each contour: the perimeter is highly correlated to the semi-major axis and more understandable as size for a contour. The size variation amongst the four species was illustrated for cell 5 contour (Fig. 2). For both metric properties, i.e., size and shape, statistical comparisons were non-parametric ones, based on random permutations (1000 cycles) between groups.

2.4.2. Validated classification.

The level of taxonomic information likely associated with each contour was measured by the total score of correctly assigned wings after validated classification. The latter was performed using the Mahalanobis distance method: each individual was assigned to the species with which it had the shortest distance. To improve the validity of the method each individual to be identified was previously removed from the total sample, so that its own metric properties could not influence the classification model; this method is called the "validated classification, or "cross checked classification", or also "jackknife classification" [45].

For the external contour and the six internal ones, the following comparisons were performed: (i) A global reclassification of the four species (Table 3), (ii) a global reclassification of the three *Aedes* species (Table 4) and (iii) all possible pairwise reclassifications, i.e., 7 ones (Table 5).

The global reclassification of the three *Aedes* species (Table 4) allowed a direct comparison of our work to the one performed previously on the same species by [6]. For this three species reclassification, the

factor map of the two first discriminant factors were shown (Fig.10).

Each of the 9 comparisons (Tables 3,4,5) was performed separately for each of the seven contours (cell 0 to cell 6) totalizing 63 validated classifications. All of them (Tables 3,4,5) were performed using the Mahalanobis distance as derived from shape variables, thus excluding size variation.

The pairwise comparisons included the one between the two genera, *Aedes (Stegomyia)* and *Verrallina (Verrallina)*, with sample sizes of 90 and 30, respectively (Table 5, second column). All remaining pairwise comparisons were performed with equal sample sizes (30).

For the pairwise comparisons, the superposition of the most discriminant cells only was shown to visualize the shape changes from one species to another (Fig. 3 to 9).

2.5 Morphometric Software

We used two packages, the CLIC package version 97 [32] which is available at (<https://xyom-clic.eu>) and the current online morphometric package, XYOM (<https://xyom.io>, Dujardin & Dujardin, 2019). The manual digitization as performed by the XYOM software is a computer assisted digitization. It allows to increase the number of pseudolandmarks by automatically adding more points between the ones digitized by the user, provided they fall exactly on the contour. This process is under visual control and allows to multiply the number of pseudo landmarks, thus it allows to increase the capture of shape.

3. Results

This study used 120 wings belonging to four species (30 wings per species): *Ae. aegypti*, *Ae. albopictus*, *Ae. scutellaris* and *Ve. dux*. The repeatability score was computed as an indirect estimate of measurement error. The repeatability score for size was always above 99%. It ranged from 80% to 89% for shape.

3.1. Wing size analysis

The size was described by the perimeter (Fig. 2). *Aedes albopictus* presented the largest average sizes, while *Ve. dux* presented the smallest ones (Table 2). This pattern was observed for each contour.

3.2. Wing shape analysis

The Mahalanobis distances computed from the external outline, cell 2 and cell 5 were statistically different between the four species after non-parametric tests (1,000 cycles) ($p < 0.05$). No significant difference could be detected between *Ae. aegypti* and *Ae. albopictus* for cells 1, 4 and 6 ($p > 0.05$).

3.2.1 Comparing the taxonomic information of different cells

Cells had consistently different discriminating power, but the external contour (cell 0) never provided the best total score. The less informative contour was cell 6 (see Fig.1), which was obvious in most comparisons (Table 3, Table 4 and Table 5 second column). According to the groups included into the comparisons, the scores of cells 6 ranged from 58% between genera (Table 5, second column) and to 92% between *Ae. albopictus* and *Ve. dux* (Table 5). On the total of 9 comparisons (Table 3 to Table 5), its average taxonomic information reached 69%, while the most informative cell (cell 5) could reach an average of 91% of correct species attribution.

This was obviously dependent also on two factors, (i) first, the number of groups included in the validated reclassification, and (ii) second the sample sizes of each group involved. For instance, when comparing the four species in one global analysis (Table 3), or the three species together (Table 4), the average score of correct attribution for cell 6 was 33.5%; this average was 80% when two groups only were considered (Table 5). The sample sizes of these two groups was also influencing the final score: cell 6 was reliable at only 58% to recognize two groups with strongly unequal samples (90 and 30, see second column of Table 5), while the same cell could produce 84% of average correct attribution score when sample sizes were equal (30 and 30) (Table 5, columns 3 to 8).

3.2.2 Reclassifying four species (Table 3)

The total scores of correct group assignment were low for cell 6 (31%) and cell 1 (61%), and acceptable but not excellent for the remaining cells (from 77% to 84%).

3.2.3 Reclassifying three species (Table 4)

The same pattern of performances as observed for the four-species comparison was disclosed: low scores for cell 6 (36%) and cell 1 (59%), and acceptable (from 72% to 83%), or even very good (88% from cell2) scores for the remaining cells. The factor map (Fig10) obtained from cell2, which was the most informative cell between these three taxa, showed a clear tendency of species separation. *Aedes albopictus* and *Ae. scutellaris* were clustered together on one side of the first discriminant factor, and *Ae. aegypti* lied on the other side. This configuration conformed to the phylogenetic tree of Sumruayphol et al., 2016 [6].

3.2.4 Pairwise reclassifications (Table 5)

The Table 5 presents all pairwise reclassifications, including the one between two genera (Table 5, second column). In this between genera reclassification, the *Aedes* genus is represented by the totality of the three species sample, with 90 individuals, and the *Verralina* genus contains only one species, the *Ve. dux*, with 30 individuals. Results of this comparison between genera were shown in the second column of

Table 5. Except for cell 6, each contour could provide satisfactory scores of genus recognition ranging from 82 to 96%. Cell 4 (94%) and cell 5 (96%, Fig. 3) were the most informative cells about genera.

It was between two groups having the same sample size (n=30) that the highest scores of correct attributions were obtained (Table 5). The lowest scores were found for cell 1. Each interspecific comparison had its best discriminating cell, as for instance cell 5 to discriminate *Ae. albopictus* from *Ae. scutellaris* (97%, Fig. 4), cell 2 between *Ae. aegypti* and *Ae. scutellaris* (98%, Fig. 5) or cell 3 between *Ae. aegypti* and *Ae. albopictus* (95%, Fig. 6). Excellent scores were reached when the two species compared belonged to different genera 98% (cell 5, Fig. 7), 92% (cell 6, Fig. 8), 98% (cell 4, Fig. 9).

4. Discussion

In this study, we used two *Aedini* genera, *Aedes* (three species) and *Verrallina* (one species). The *Verrallina* species, *Ve. dux*, was examined here by modern morphometrics for the first time. Its morphology is clearly distinct from the *Aedes* genus, and as a different genus, it was expected to give us a clearly different, maybe non-overlapping, geometry of the wing. The other species have been examined previously for the external contour of the wing [6], but not for the internal cells.

Internal cells were considered here to check whether the taxonomic signal of wing contour was spread equally among various internal structures of the same wing. We did not use a landmark-based approach for internal cells because it would be based on too few landmarks (3 to 5 according to the cell).

Various amounts of size and shape differences were disclosed at each outline. We showed that some of the shape differences were strong enough to recognize species with high accuracy. We attributed these shape differences to evolutionary divergence, even if there were probably also environmentally induced variation. In our sample, the main sources of possible environmental influence on metric properties could be the following: the number of generations spent in the (same) laboratory, the water used for larval development, the number of generations spent in the laboratory. The number of generations before morphometric analyses differed between the four species, therefore some of the metric differences we found here could be due also to laboratory effects, especially for *Ae. albopictus* and *Ae. scutellaris* which spent many generations in the laboratory. Previous studies about the influence of the number of generations in the laboratory showed clear changes in the size of the insects, but confirmed the stability of shape [46, 47] and of its inheritance [48]. We tried to maintain the laboratory conditions similar for each species: temperature, humidity, food, nutrition, water and container were the same. However, the water solution of *Ve. dux* was different regarding salt concentration and nutrition. To reduce possible laboratory mortality, the origin of water from the collected area (mangrove forest) was used in these experiments. Thus, we could not exclude some contribution of the microenvironment to the observed interspecific differences, but these external factors have been already shown to affect size much more than shape [48].

4.1. Wing size variation

Even within the same species, size may be consistently affected by the number of laboratory generations [46, 47], by changes in temperature [49] or humidity [50].

Among the three *Aedes* species there was considerable overlapping of global size, with *Ae. albopictus* tending to be the largest species. Statistical comparisons showed significant differences, except for the comparison between *Ae. aegypti* and *Ae. scutellaris* (Table 2). In previous studies, *Ae. aegypti* was statistically larger than *Ae. scutellaris* [6]. This apparent contradiction confirms the lability of size across geographic areas and seasons [30, 40].

In our sample, there was a striking difference in size between the two genera, *Aedes* and *Verrallina*. Whatever the contour considered, *Verrallina* was the smallest species with no overlapping of size. Such difference is likely to be a generic trait, and could be *per se* a simple generic character. However, since size is sample-dependent much more than shape [32], it was excluded from our validated reclassifications.

4.2. Wing shape variation

Shape, as a metric character, is much less dependent than size on environmental factors, especially regarding interspecific differences [32]. Our working hypothesis is that the morphometric variation of shape distinguishing species in our sample was mainly due to evolutionary differences [28, 32, 51].

4.2.1. Shape divergence between species

As expected, *Verrallina*(*Ver.*) *dux* was generally the most discriminated species. It was recognized at 100% in the four groups comparisons (Table 3). Although some species were nicely recognized when considering the detailed scores in the global comparisons involving four or three groups, the total scores were relatively low: from 31% to 84% in the four species comparisons (Table 3), and from 36% to 88% in the three species comparison (Table 4). These total scores were much lower than the ones obtained in pairwise comparisons (from 80% to 94%, see Table 5). In pairwise comparisons, *Ve. (Ver.) dux* was recognized with an accuracy ranging from 92% to 98%, which was comparable to most other pairwise classifications (Table 5).

Considering the external contour of the wing, this study supported the previous results highlighting the interest of the outline-based approach to discriminate between the wings of *Ae. aegypti*, *Ae. albopictus* and *Ae. scutellaris* [6]. Our comparison of the three *Aedes* species together (Table 4) provided scores of total correct recognitions (83%) higher than the ones observed in females by Sumruayphol et al. (2016) (76%) [6]. This could have various causes, among which the digitization method or the sampling bias. We used the improved manual digitization technique of XYOM (<https://xyom.io>), which allows us to increase the number of valid pseudolandmarks by 10 times or more. More pseudolandmarks means a better capture of shape. In addition, there also could be a sampling effect: Mahalanobis distances are more reliable indeed when group sizes are not too different (30,30,30 in our study versus 93,51,45 in the previous one) [52]. Other reasons concurring to different results could be a different geographic or laboratory origin of the specimens.

The sampling configuration of groups included in a validated reclassification appeared as an important factor to be considered. In our study, the best scores were obtained when considering two groups having the same number of specimens (Table 5). Grouping the three species of *Aedes* into one group (n=90) versus *Ve. dux* (Table 5, second column), the scores did not reach the ones obtained comparing the same genera using only one species by genus (Table 5, columns 4, 6 and 7). This result is likely to be due to the unequal sample sizes when mixing three *Aedes* (n=90) versus one *Verrallina* species (n=30) (Table 5, second column): strongly unequal sample sizes may bias the Mahalanobis distances [52].

4.2.2. Taxonomic signal among cells

The global analysis including four species highlighted the different taxonomic information associated with each cell, going from 31% for cell 6 to 84% for cell 5, with intermediate results as 61% for cell 1 or 77% for cells 3, 4, or 82% for the external contour (Table 3). This divergence of taxonomic information between cells may be observed also examining Tables 4 and Table 5. When considering a three groups comparison (Table 4), cells 1 and 6 were obviously uninformative cells (59% and 36%, respectively) relative to the other ones ranging from 72% to 88%. The pairwise comparisons (Table 5) showed many other examples. For instance, between *Ae. aegypti* and *Ae. albopictus*, cell 3 could recognize species with a 95% accuracy, while the contiguous cell 4 of the same wing reached 63% only (see Table 5).

The external contour generally produced slightly lower identification scores than internal cells (see Tables from 3 to 5). This weaker taxonomic signal could have a simple technical explanation. Indeed, the contour used here was not a completely anatomic one: the starting point and the ending point, both at the area of junction with the thorax, did not coincide and were artificially joined by a straight line. This line was obviously not an anatomic part (see arrow on Fig.1). It was however not possible to avoid this way of digitizing because each dissected wing was more or less damaged at its articulation with the thorax. Thus, the capture of shape was not complete, even if the loss was very small relative to the remaining part of the external contour. Another explanation could be that the external contour of the wing suffers more biomechanical forces related to fly conditions, constraining its shape to adapt to aerodynamic necessities.

Internal cells are close anatomical contours with no artificial joining of two points like in the external contour of the wing (Fig. 1, see arrow). The unequal taxonomic information of the shape of the various cells examined could not be put in relation with their size. Actually, each cell could be very informative or not according to the taxa under comparison. For instance, cell 1 correctly assigned 63% of individuals to their respective species when comparing *Ae. aegypti* and *Ae. albopictus*, whereas it could recognize 95% of individuals when comparing *Ae. scutellaris* and *Ve. dux* (see Table 5). Because the taxonomic information of each cell changed unpredictably with the taxa under comparisons, there may be some biological, unknown explanation. For another group of insects (bees), some variation of the amount of taxonomic information was also observed and remained unexplained [53].

The taxonomic signal of the contours was dependent on the taxa under comparison. but within the same comparison, the taxonomic information could vary between them. Intuitively, one possible reason for

having different recognition power for the same taxa could be related to the complexity of the contour: the more complex the contour, the more substantial the capture of shape. For instance, the most discriminant cell (cell 5) presented indeed a slightly more complex contour than the others. However, cells as simple as cell 4 produced better scores than cell 5 in some pairwise comparisons (see Table 5 between *Ae. aegypti* and *Ae. scutellaris*, also between *Ve. dux* and *Ae. scutellaris*), it could even recognize 100% of *Ve. dux* in the four groups comparisons (Table 3, detailed score).

5. Conclusions

Our main results could be summarized by two main observations: (i) the taxonomic information was not spread equally from one cell to the other, and (ii) the taxonomic signal of one or more of the internal cells was generally better than the one associated with the external contour of the wing. An information of practical interest obtained from these observations is that some internal cells may be used instead of the complete contour. This is useful information for epidemiologists and entomological surveillance programs because wings of field collected specimens may be damaged while still presenting perfect internal cells. It has already been said that outlines can be used for mosquito identification even if the whole body is not available, we showed that even partly damaged wings would contain extractable and more accurate taxonomic information.

Declarations

Ethics approval and consent to participate

This study was approved by the Faculty of Tropical Medicine-Animal care and Use Committee of Faculty of Tropical Medicine, Mahidol University (Approval No: FTM-ACUC 013/2019).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SC prepared sample for GM, analyzed data and wrote the manuscript. CR designed experiment, collected sample and wrote the manuscript. SS conceptualized the study idea and analyzed data. JP analyzed

data and wrote the manuscript. RP designed experiments, collected sample and wrote the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and analyzed for this study are available from the corresponding author upon reasonable request.

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Tables

Table 1. Materials

Species	Locality	Province	Latitude	Longitude	Year	F
<i>Ae. aegypti</i>	Bangkhuae	Bangkok	13°41'43.6"N	100°23'05.1"E	2019	F3
<i>Ae. albopictus</i>	Lum Sum	Kanchanaburi	14°12'16.2"N	99°07'58.5"E	2009	F49
<i>Ae. scutellaris</i>	Phasi charoen	Bangkok	13°43'19.8"N	100°26'09.2"E	2011	F33
<i>Ve. dux</i>	Bang Pakong	Chachoengsao	13°28'25.0"N	100°52'19.9"E	2019	F3

Legend of Table 1. "Materials". Geographic localization and years of capture of the mosquitoes, collected as larvae. From these collections, 30 females were used for morphometric analyses after a different number of generations (F) in the same laboratory.

Table 2. Outline-based statistical comparisons of the perimeters between four mosquitoes in seven outlines

Species	N	Mean (mm)	S.D.	S.E.
Cell 0				
<i>Ae. aegypti</i>	30	5.60 (4.78-6.45) ^a	0.45	0.08
<i>Ae. albopictus</i>	30	6.10 (5.60-6.54) ^b	0.27	0.04
<i>Ae. scutellaris</i>	30	5.43 (4.87-5.72) ^a	0.16	0.03
<i>Ve. dux</i>	30	3.59 (3.09-3.83) ^c	0.19	0.03
Cell 1				
<i>Ae. aegypti</i>	30	1.55 (1.30-1.90) ^a	0.14	0.02
<i>Ae. albopictus</i>	30	1.58 (1.37-1.80) ^{a,b}	0.11	0.02
<i>Ae. scutellaris</i>	30	1.48 (1.24-1.59) ^{a,c}	0.07	0.01
<i>Ve. dux</i>	30	0.99 (0.80-1.12) ^d	0.07	0.01
Cell 2				
<i>Ae. aegypti</i>	30	2.19 (1.86-2.52) ^a	0.16	0.02
<i>Ae. albopictus</i>	30	2.46 (2.23-2.65) ^b	0.12	0.02
<i>Ae. scutellaris</i>	30	2.20 (1.95-2.30) ^a	0.07	0.01
<i>Ve. dux</i>	30	1.46 (1.24-1.61) ^c	0.08	0.01
Cell 3				
<i>Ae. aegypti</i>	30	2.10 (1.81-2.36) ^a	0.14	0.02
<i>Ae. albopictus</i>	30	2.37 (2.12-2.57) ^b	0.11	0.02
<i>Ae. scutellaris</i>	30	2.08 (1.88-2.20) ^a	0.07	0.01
<i>Ve. dux</i>	30	1.41 (1.21-1.55) ^c	0.08	0.01
Cell 4				
<i>Ae. aegypti</i>	30	1.22 (1.04-1.46) ^a	0.10	0.01
<i>Ae. albopictus</i>	30	1.27 (1.09-1.45) ^a	0.09	0.01
<i>Ae. scutellaris</i>	30	1.25 (1.06-1.40) ^a	0.06	0.01
<i>Ve. dux</i>	30	0.83 (0.67-0.96) ^b	0.06	0.01

Cell 5				
<i>Ae. aegypti</i>	30	1.84 (1.54-2.14) ^a	0.14	0.02
<i>Ae. albopictus</i>	30	2.12 (1.92-2.34) ^b	0.10	0.01
<i>Ae. scutellaris</i>	30	1.89 (1.69-1.98) ^a	0.06	0.01
<i>Ve. dux</i>	30	1.25 (1.06-1.37) ^c	0.07	0.01
Cell 6				
<i>Ae. aegypti</i>	30	2.13 (1.75-2.54) ^a	0.17	0.03
<i>Ae. albopictus</i>	30	2.37 (2.13-2.62) ^b	0.14	0.02
<i>Ae. scutellaris</i>	30	2.14 (1.97-2.34) ^a	0.09	0.01
<i>Ve. dux</i>	30	1.42 (1.22-1.58) ^c	0.09	0.01

Legend of Table 2 Different superscript letters (a, b, c and d) indicate significant differences between species at P<0.05. Mean: average perimeter length calculated using the outline-based method; min: minimum; max: maximum; S.D.: standard deviation and S.E.: standard error.

Table 3. Validated classification of four *Aedes* spp. based on shape-derived Mahalanobis distances.

	<i>Ae. aegypti</i>	<i>Ae. albopictus</i>	<i>Ae. scutellaris</i>	<i>Ve. dux</i>	Total
Cell 0	80% (24/30)	80% (24/30)	93% (28/30)	77% (23/30)	82% (99/120)
Cell 1	43% (16/30)	40% (12/30)	70% (21/30)	80% (24/30)	61% (73/120)
Cell 2	83% (25/30)	77% (23/30)	90% (27/30)	83% (25/30)	83% (100/120)
Cell 3	77% (23/30)	83% (25/30)	70% (21/30)	80% (24/30)	77% (93/120)
Cell 4	70% (21/30)	60% (18/30)	80% (24/30)	100% (30/30)	77% (93/100)
Cell 5	73% (22/30)	83% (25/30)	87% (26/30)	93% (28/30)	84% (101/120)
Cell 6	3% (1/30)	53% (16/30)	7% (2/30)	60% (18/30)	31% (37/120)

Legend of Table 3 For each contour (from cell 0 to cell 6, see Fig. 1), **detailed and total** scores of correct species assignment after validated classification based on Mahalanobis distances among four species.

Table 4. Percentage of correct assignment scores (assigned/observed)

	<i>Ae. aegypti</i>	<i>Ae. albopictus</i>	<i>Ae. scutellaris</i>	Total
Cell 0	80% (24/30)	77% (23/30)	93% (28/30)	83% (75/90)
Cell 1	57% (17/30)	53% (16/30)	67% (20/30)	59% (53/90)
Cell 2	90% (27/30)	83% (25/30)	90% (27/30)	88% (79/90)
Cell 3	77% (23/30)	87% (26/30)	77% (23/30)	80% (72/90)
Cell 4	70% (21/30)	63% (19/30)	83% (25/30)	72% (65/90)
Cell 5	80% (24/30)	83% (25/30)	83% (25/30)	82% (74/90)
Cell 6	43% (13/30)	57% (17/30)	7% (2/30)	36% (32/90)

Legend of Table 4 For each contour (from cell 0 to cell 6 (see Fig. 1), **detailed and total** scores of correct species assignment after validated classification based on Mahalanobis distances between two groups.

Table 5. Total scores of validated pairwise classifications.

	<i>Aedes spp.</i>	aeg	aeg	aeg	alb	alb	scu	Average
Contours	<i>Ve. dux</i>	alb	scu	dux	scu	dux	dux	
cell0	90%	77%	77%	85%	93%	83%	90%	85%
cell1	82%	67%	82%	75%	77%	87%	95%	81%
cell2	84%	92%	98%	85%	87%	85%	85%	88%
cell3	86%	95%	83%	83%	92%	90%	97%	89%
cell4	94%	63%	95%	92%	78%	90%	98%	87%
cell5	96%	92%	88%	98%	97%	92%	93%	94%
cell6	58%	83%	80%	87%	77%	92%	82%	80%

Legend of Table 5 For each contour (from cell 0 to cell 6, see Fig. 1), **total** scores of validated classifications based on Mahalanobis distances between two groups (**detailed scores not shown**). The second column shows the classification of *Aedes spp.* (n=90) versus *Ve. dux* (n=30). The average score of each cell is presented in the last column.

Figures

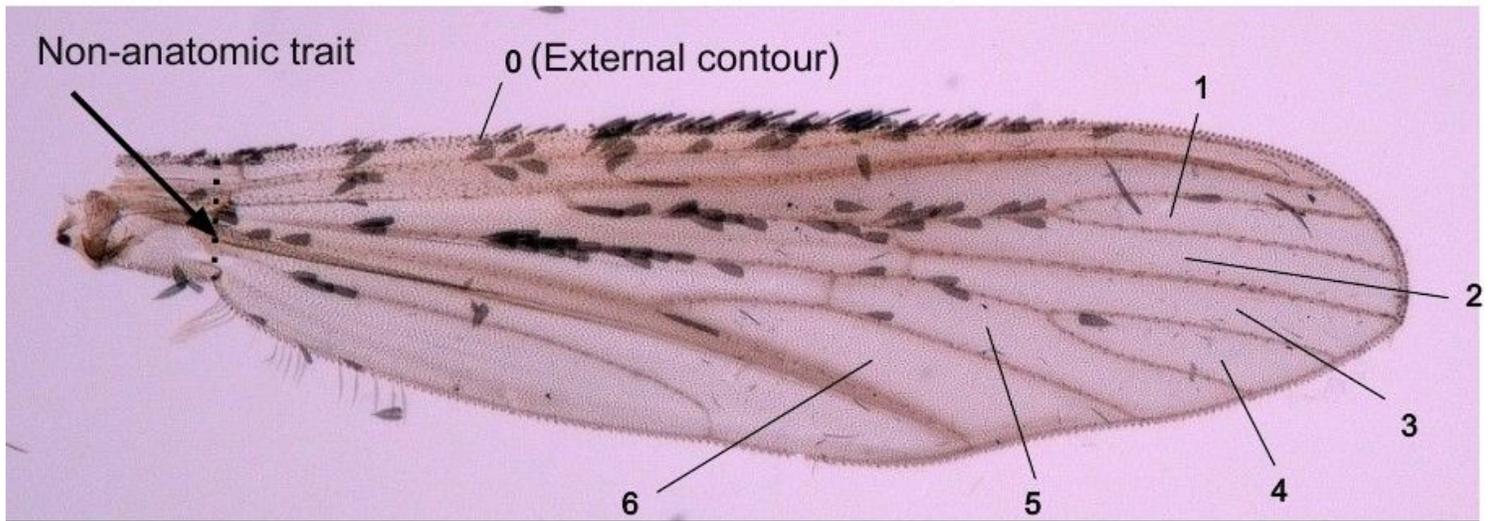


Figure 1

Seven contours digitized on the wing for outline-based geometric morphometric analysis. Cell 0, the external contour of the wing; Cell 1, between veins R2 and R3; Cell 2, delimited by veins R2+3, R3 and R4+5 and rm; Cell 3: delimited by veins R4+5, M1, M1+2 and rm; Cell 4, between veins M1 and M2; Cell 5, delimited by veins M1+2, M2 and M3+4; cell 6 between M3+4 CuA and mCu. The nomenclature of veins follows Rattaranaritikul et al. 2010 (p. 71). The arrow shows the small part of the external contour which was artificially joined to obtain a completely close outline.

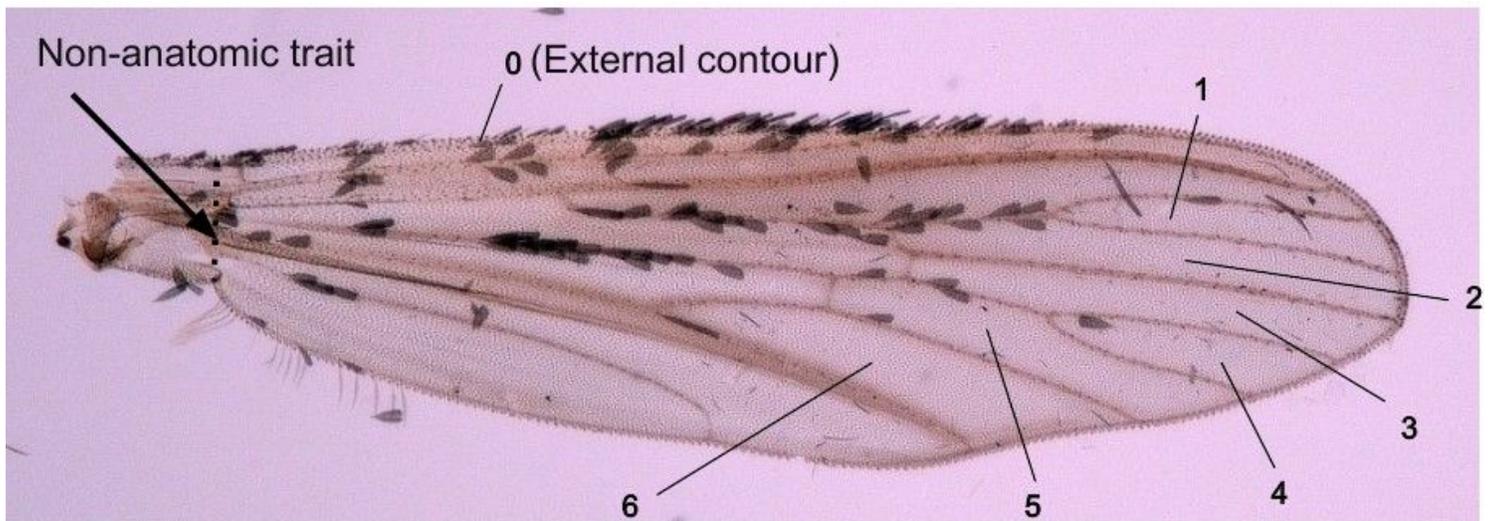


Figure 1

Seven contours digitized on the wing for outline-based geometric morphometric analysis. Cell 0, the external contour of the wing; Cell 1, between veins R2 and R3; Cell 2, delimited by veins R2+3, R3 and R4+5 and rm; Cell 3: delimited by veins R4+5, M1, M1+2 and rm; Cell 4, between veins M1 and M2; Cell 5, delimited by veins M1+2, M2 and M3+4; cell 6 between M3+4 CuA and mCu. The nomenclature of veins follows Rattaranaritikul et al. 2010 (p. 71). The arrow shows the small part of the external contour which was artificially joined to obtain a completely close outline.

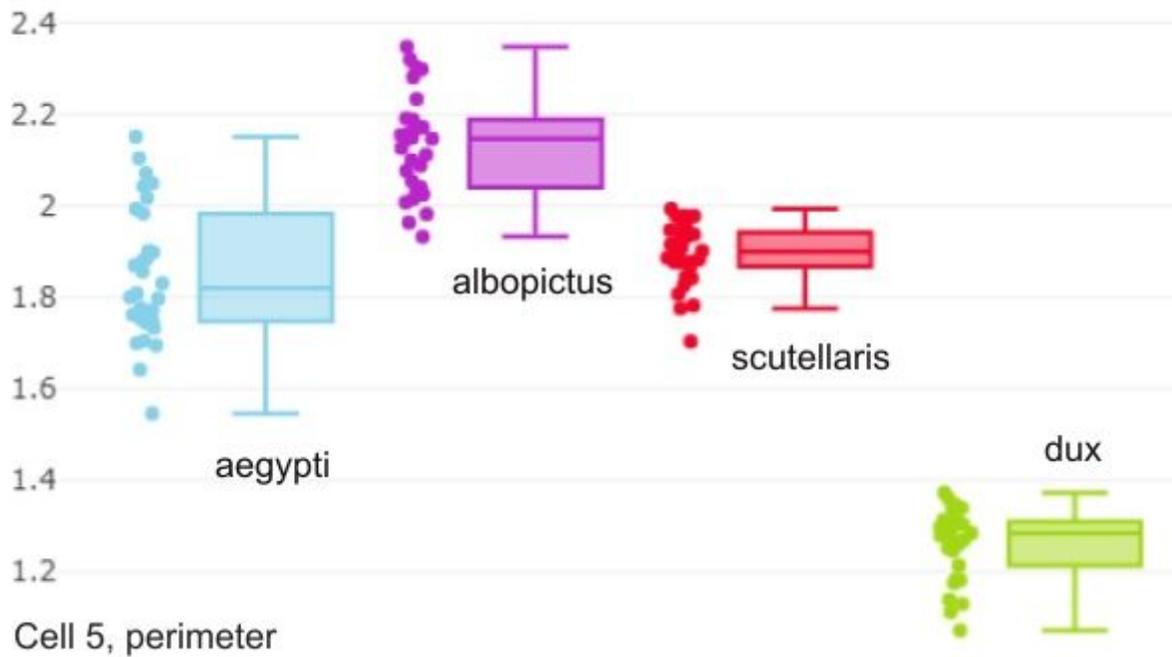


Figure 2

Quantile boxes showing the wing perimeter (mm) derived from outline-based geometric morphometrics analysis. Each box shows the group median that separates the 25th and 75th quantiles. From left to right: *Ae. aegypti*; *Ae. albopictus*; *Ae. scutellaris* and *Ve. dux*. The figure shows the perimeter of the fifth cell. The other contours (not shown) showed almost exactly the same interspecific variation: the non-overlapping smaller size of *Ve. dux* versus the other species, and the trend for larger sizes in *Ae. albopictus*.

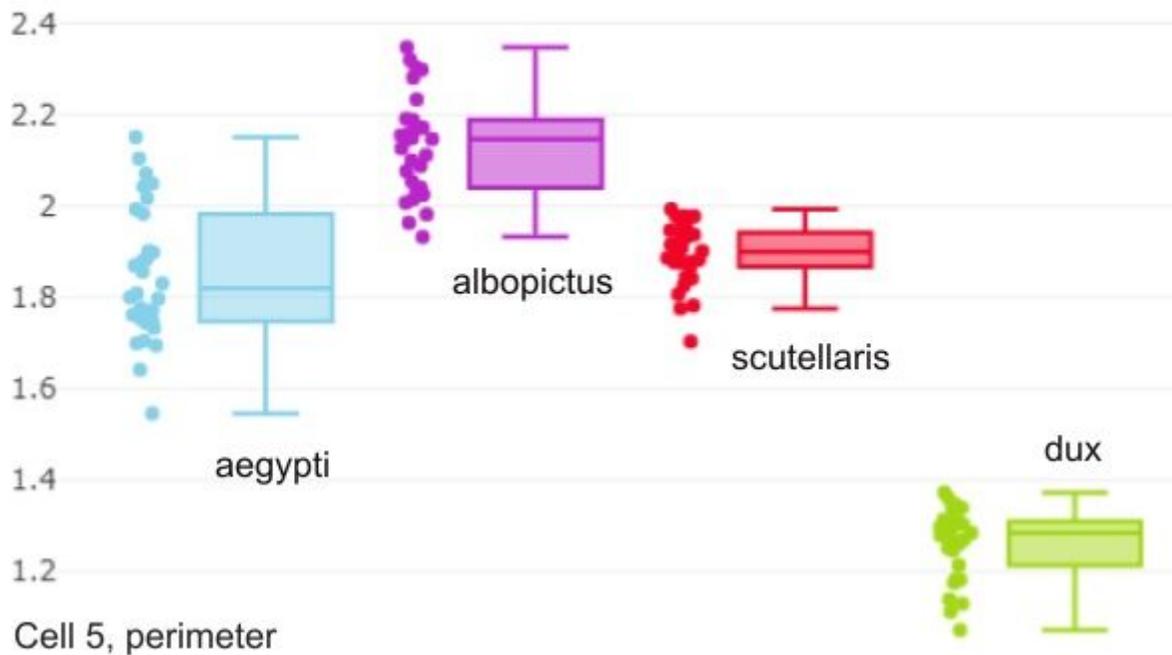


Figure 2

Quantile boxes showing the wing perimeter (mm) derived from outline-based geometric morphometrics analysis. Each box shows the group median that separates the 25th and 75th quantiles. From left to right: *Ae. aegypti*; *Ae. albopictus*; *Ae. scutellaris* and *Ve. dux*. The figure shows the perimeter of the fifth cell. The other contours (not shown) showed almost exactly the same interspecific variation: the non-overlapping smaller size of *Ve. dux* versus the other species, and the trend for larger sizes in *Ae. albopictus*.

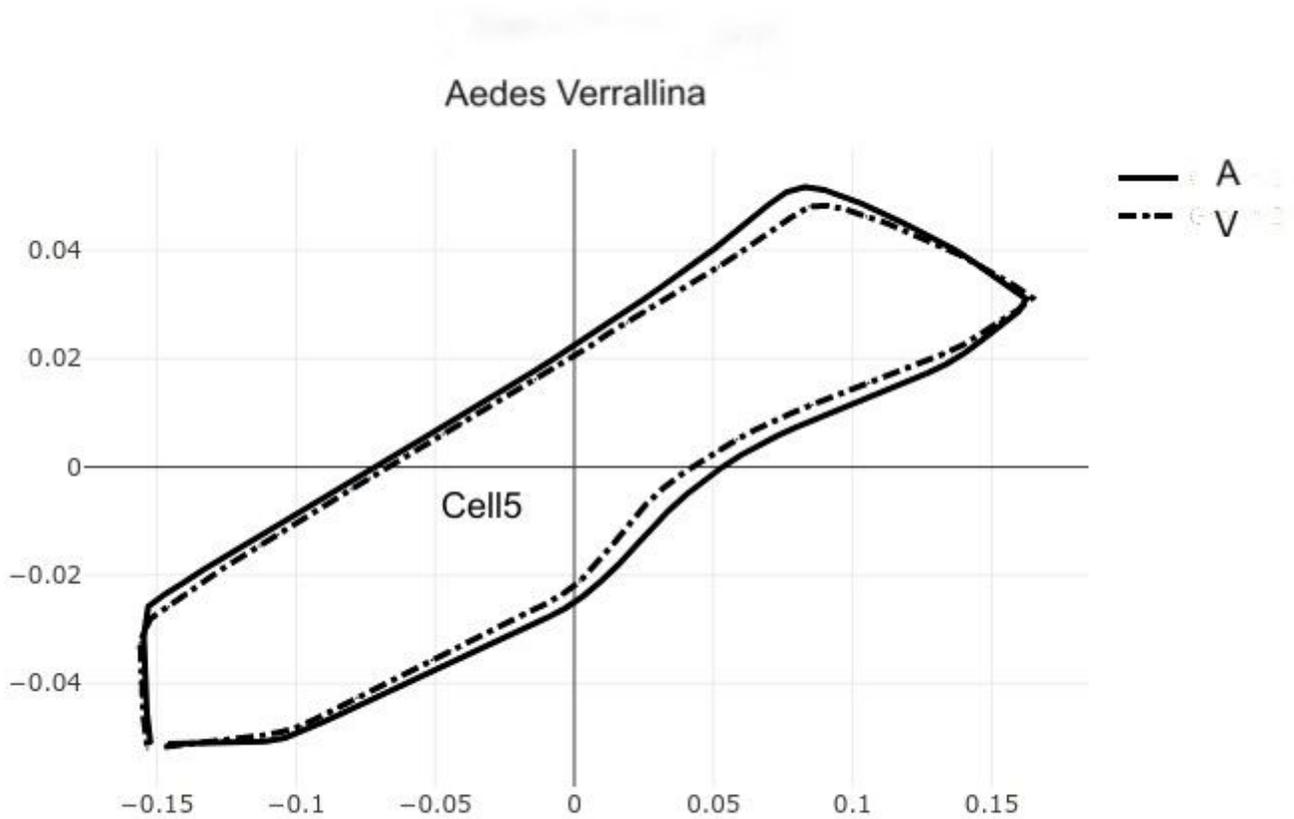


Figure 3

Superposition of size-free contours of cell 5 (see Fig. 1) showing shape differences between two genera: *Aedes* ssp. (averaging *Ae. aegypti*, *Ae. albopictus* and *Ae. scutellaris*) and *Ve. dux* (dashed traits). See also Figs. 7, 8 and 9.

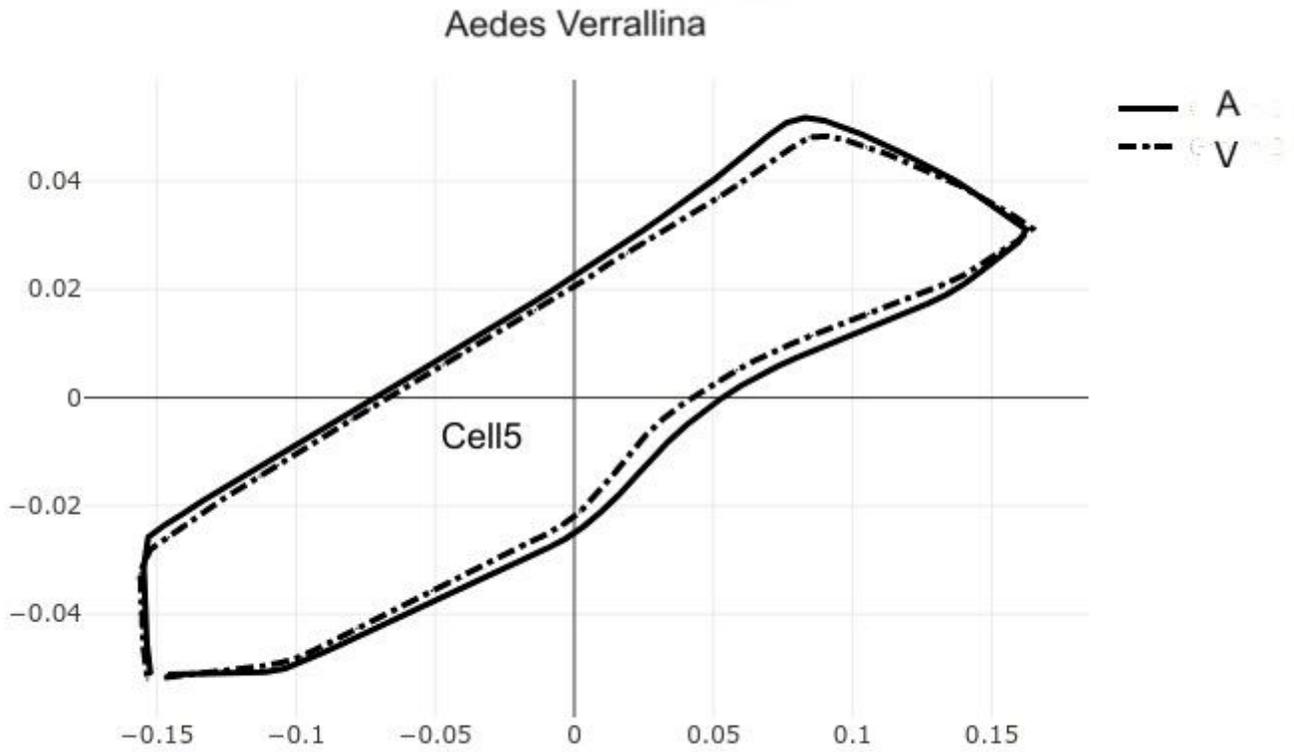


Figure 3

Superposition of size-free contours of cell 5 (see Fig. 1) showing shape differences between two genera: *Aedes* ssp. (averaging *Ae. aegypti*, *Ae. albopictus* and *Ae. scutellaris*) and *Ve. dux* (dashed traits). See also Figs. 7, 8 and 9.

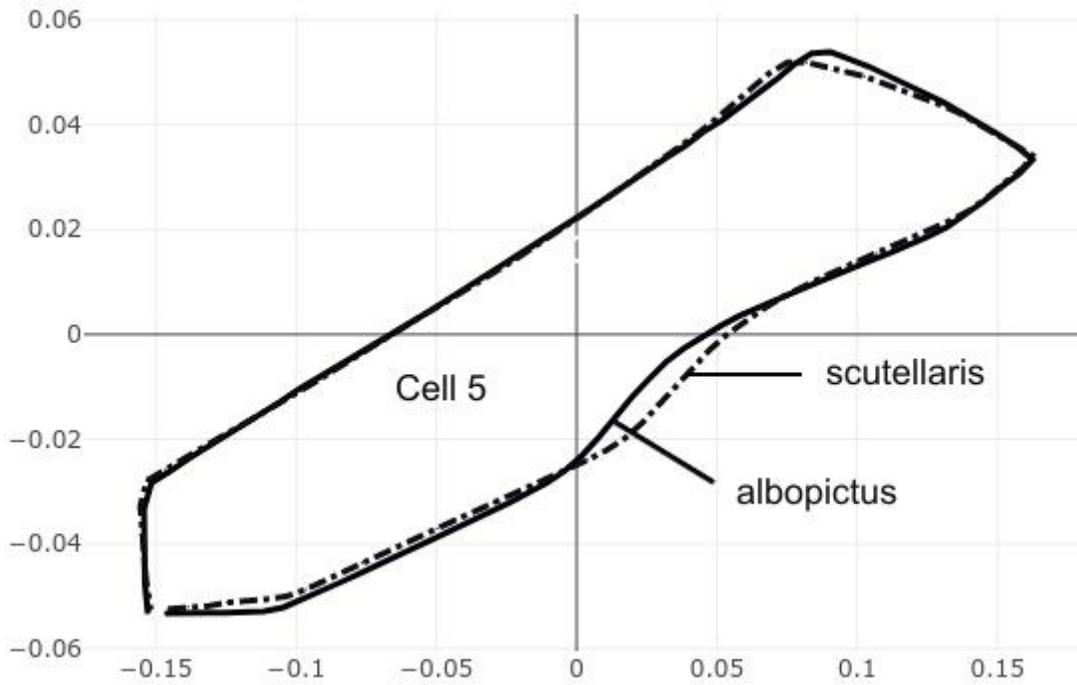


Figure 4

Superposition of size-free contours of cell 5 (see Fig. 1) showing shape differences between two species: *Aedes albopictus* (solid line) and *Ae. scutellaris* (dashed traits).

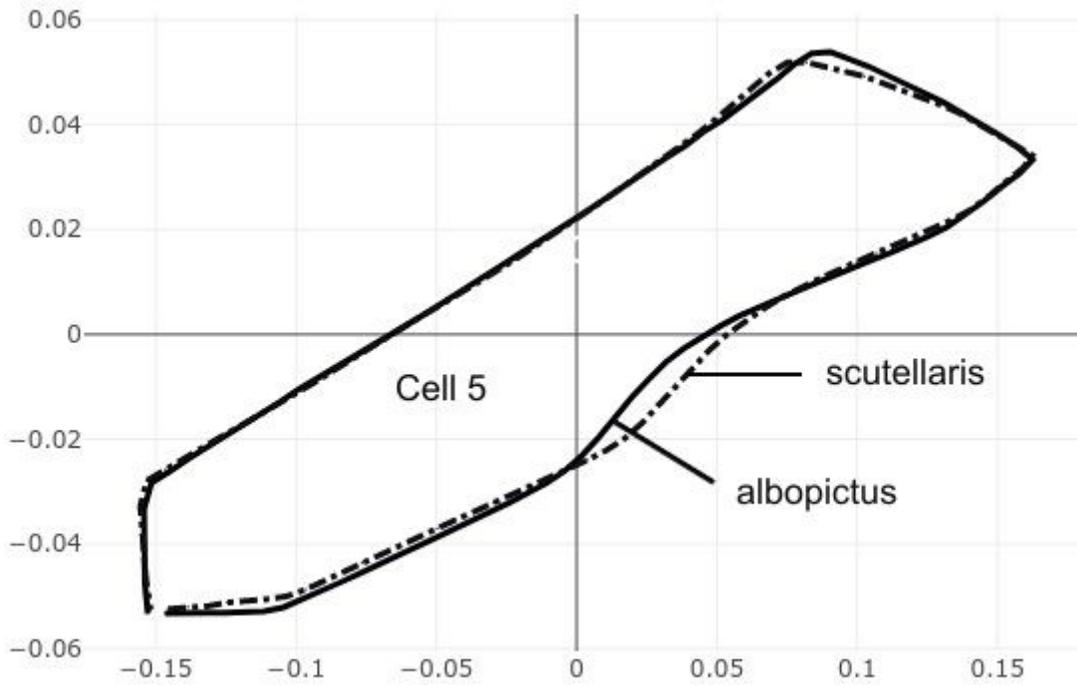


Figure 4

Superposition of size-free contours of cell 5 (see Fig. 1) showing shape differences between two species: *Aedes albopictus* (solid line) and *Ae. scutellaris* (dashed traits).

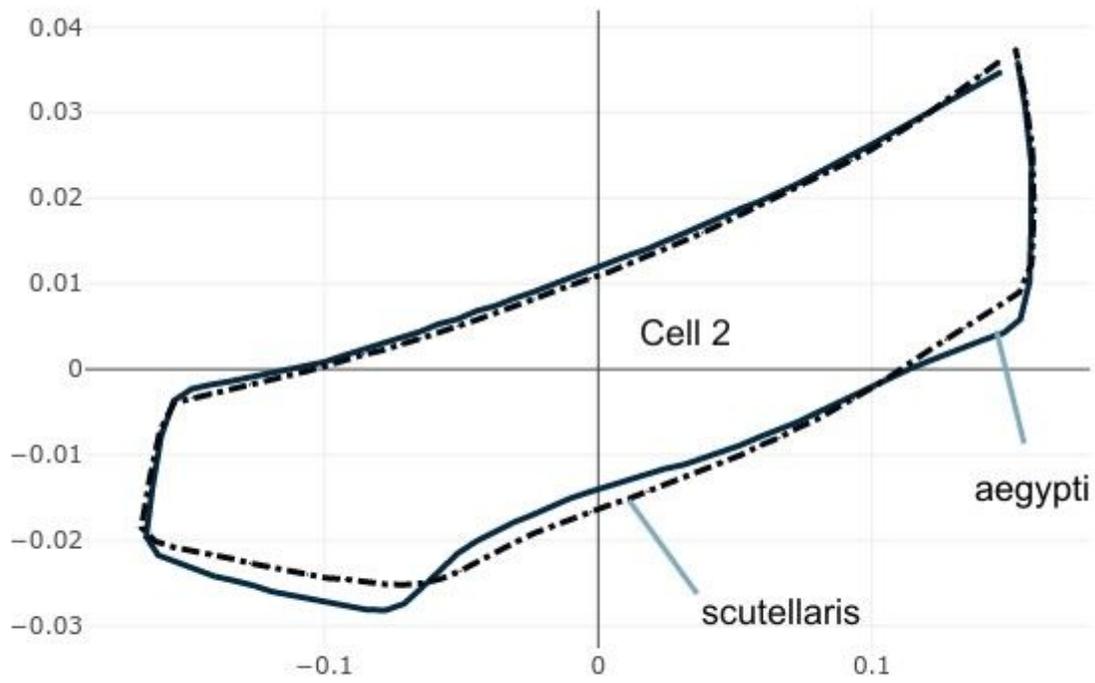


Figure 5

Superposition of size-free contours of cell 2 (see Fig. 1) showing shape differences between two species: *Aedes aegypti* (solid line) and *Ae. scutellaris* (dashed traits).

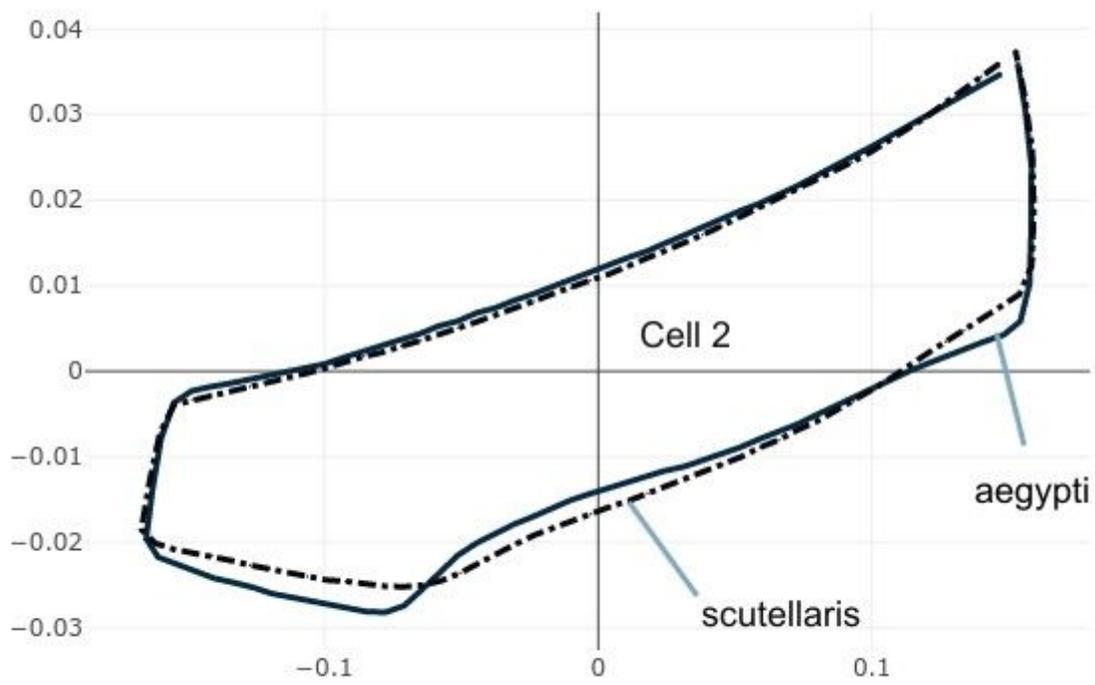


Figure 5

Superposition of size-free contours of cell 2 (see Fig. 1) showing shape differences between two species: *Aedes aegypti* (solid line) and *Ae. scutellaris* (dashed traits).

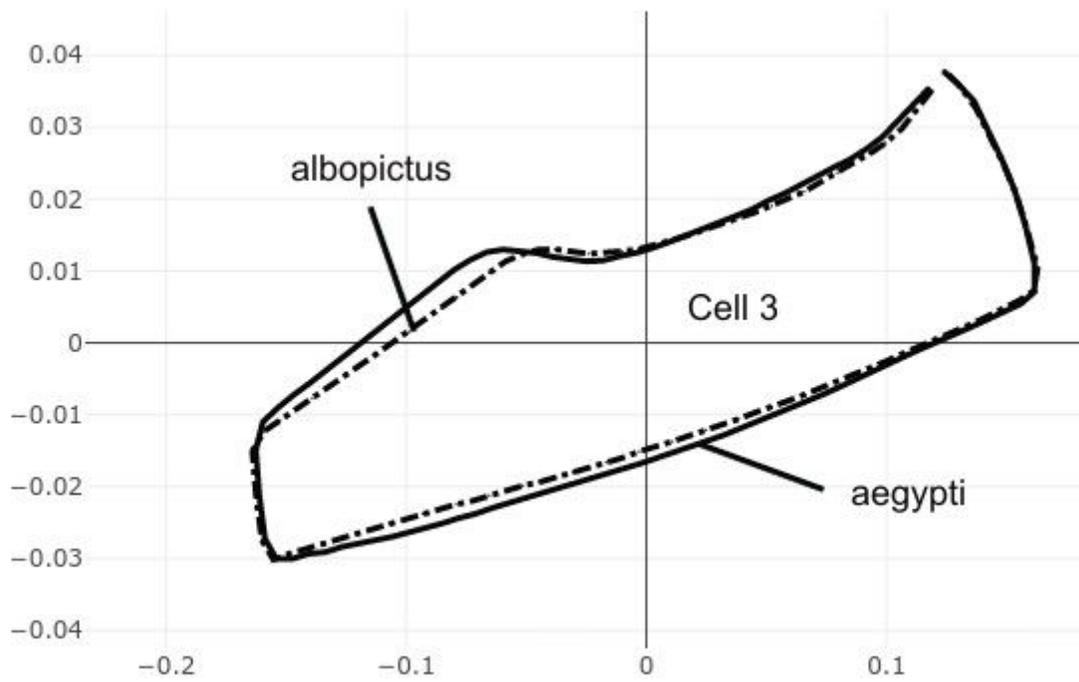


Figure 6

Superposition of size-free contours of cell 3 (see Fig. 1) showing shape differences between two species: *Aedes aegypti* (solid line) and *Ae. albopictus* (dashed traits).

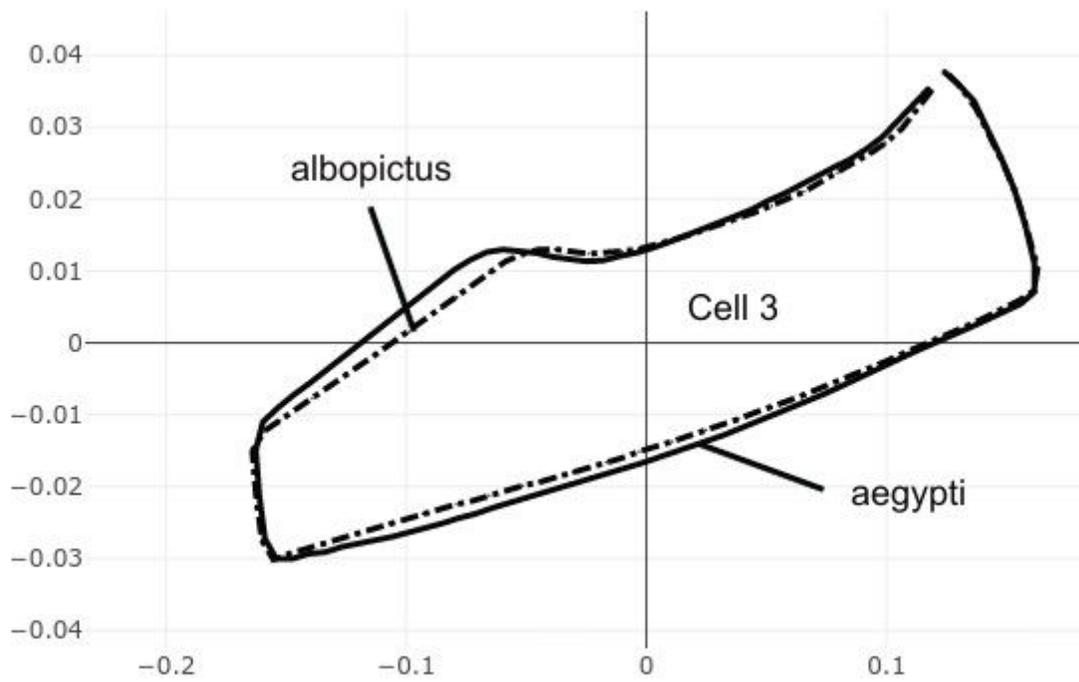


Figure 6

Superposition of size-free contours of cell 3 (see Fig. 1) showing shape differences between two species: *Aedes aegypti* (solid line) and *Ae. albopictus* (dashed traits).

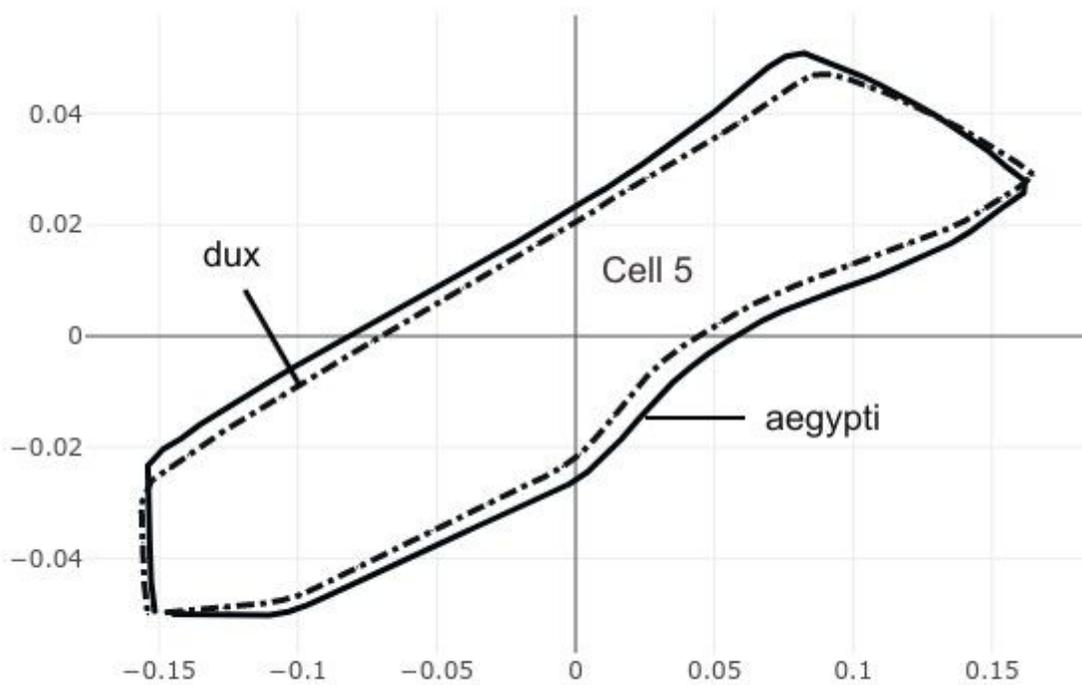


Figure 7

Superposition of size-free contours of cell 5 (see Fig. 1) showing shape differences between two species, also two genera: *Aedes aegypti* (solid line) and *Ve. dux* (dashed traits).

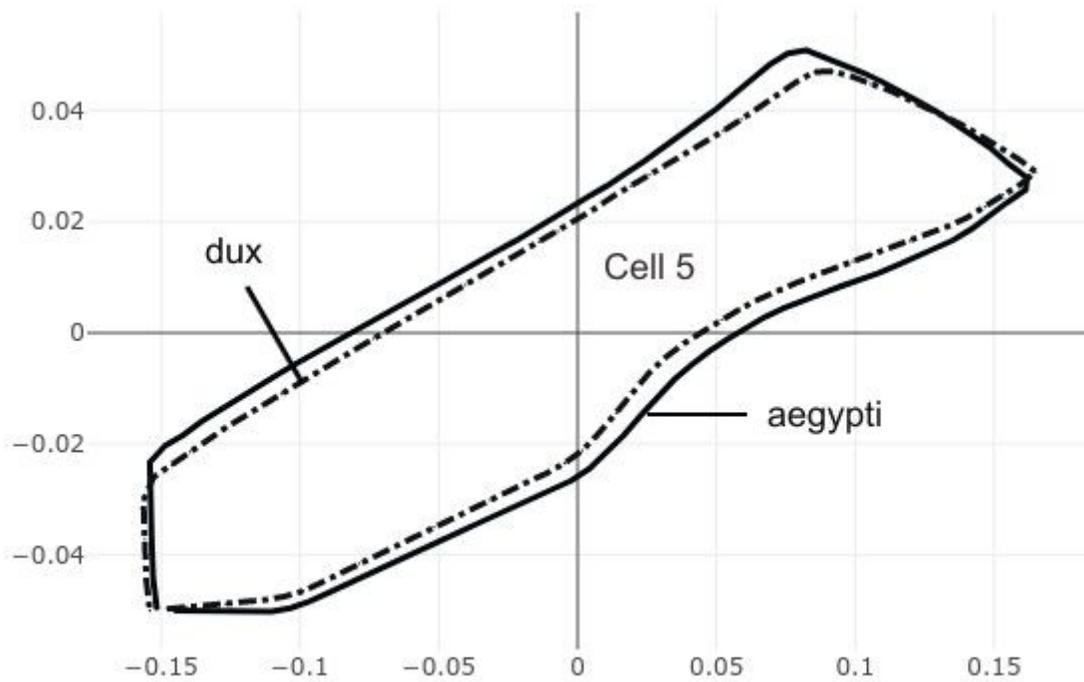


Figure 7

Superposition of size-free contours of cell 5 (see Fig. 1) showing shape differences between two species, also two genera: *Aedes aegypti* (solid line) and *Ve. dux* (dashed traits).

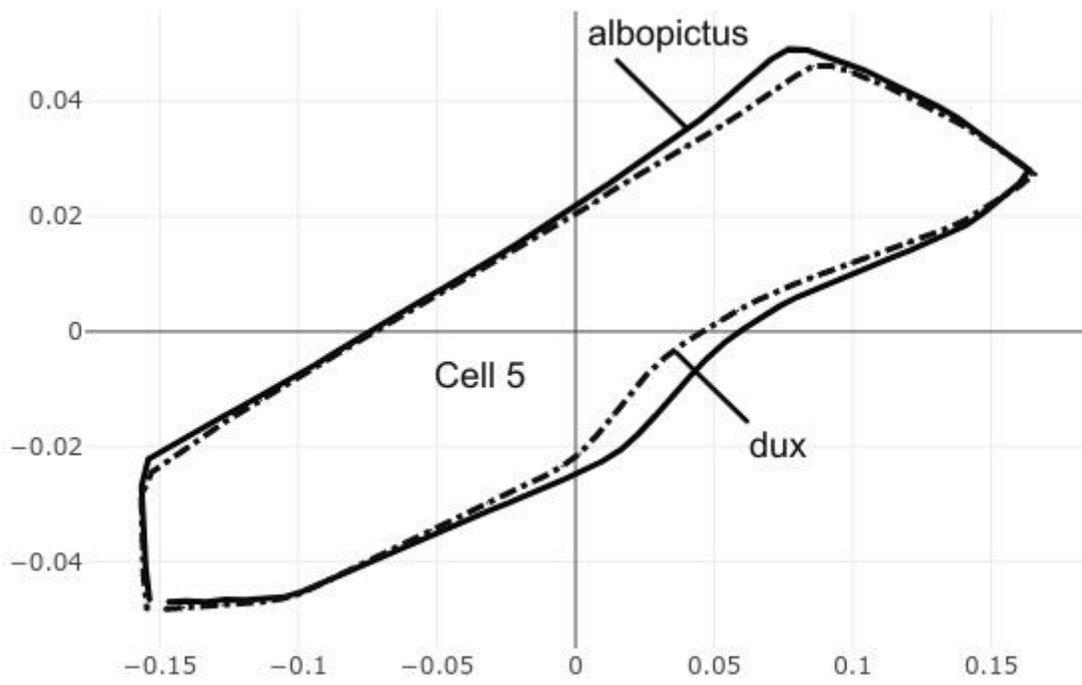


Figure 8

Superposition of size-free contours of cell 5 (see Fig. 1) showing shape differences between two species, also two genera: *Aedes albopictus* (solid line) and *Ve. dux* (dashed traits).

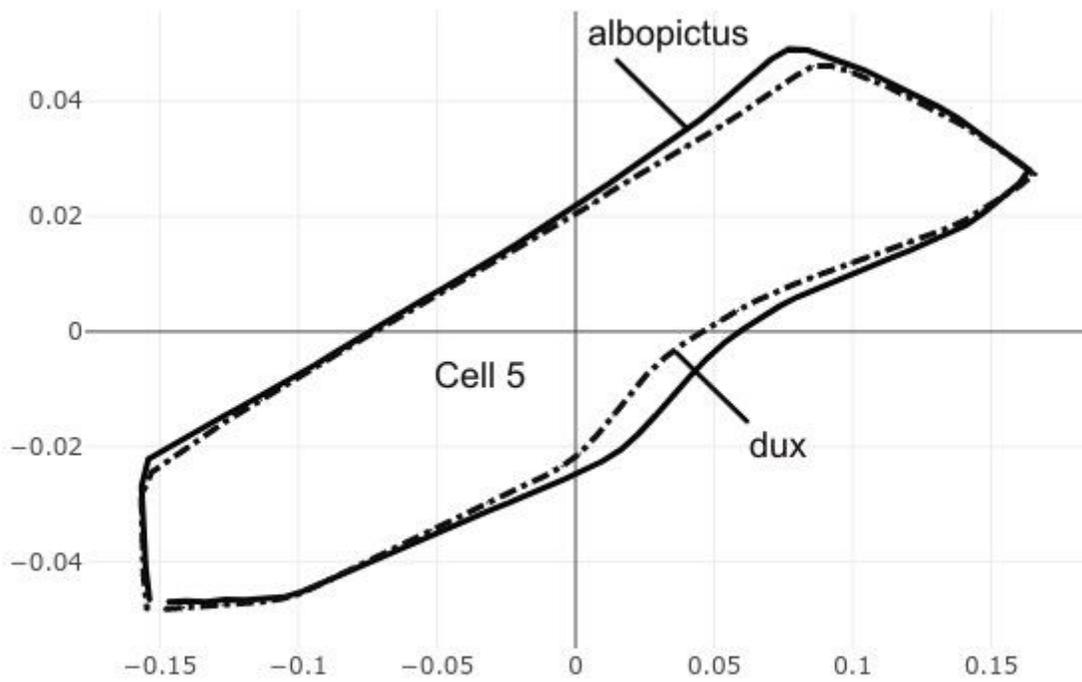


Figure 8

Superposition of size-free contours of cell 5 (see Fig. 1) showing shape differences between two species, also two genera: *Aedes albopictus* (solid line) and *Ve. dux* (dashed traits).

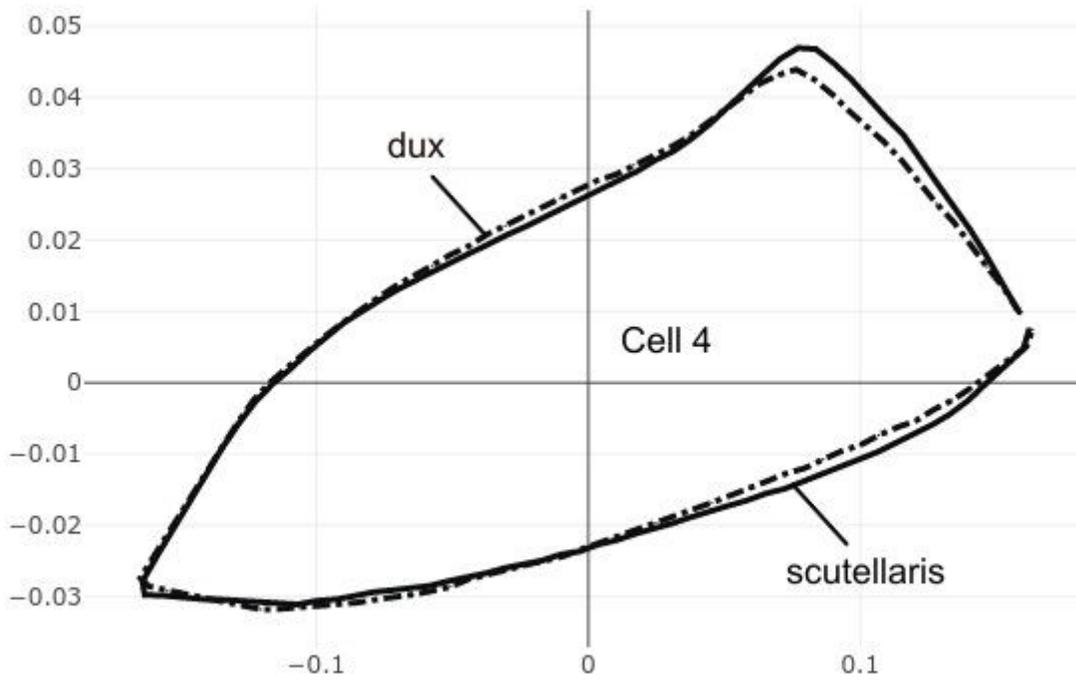


Figure 9

Superposition of size-free contours of cell 4 (see Fig. 1) showing shape differences between two species, also two genera: *Aedes scutellaris* (solid line) and *Ve. dux* (dashed traits).

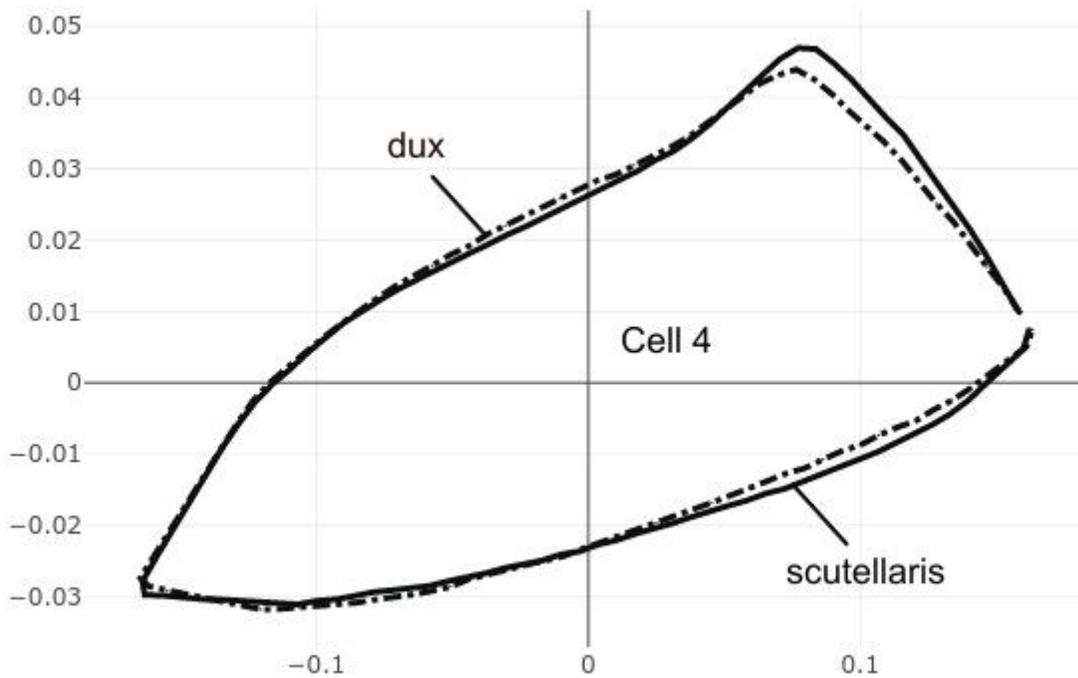


Figure 9

Superposition of size-free contours of cell 4 (see Fig. 1) showing shape differences between two species, also two genera: *Aedes scutellaris* (solid line) and *Ve. dux* (dashed traits).

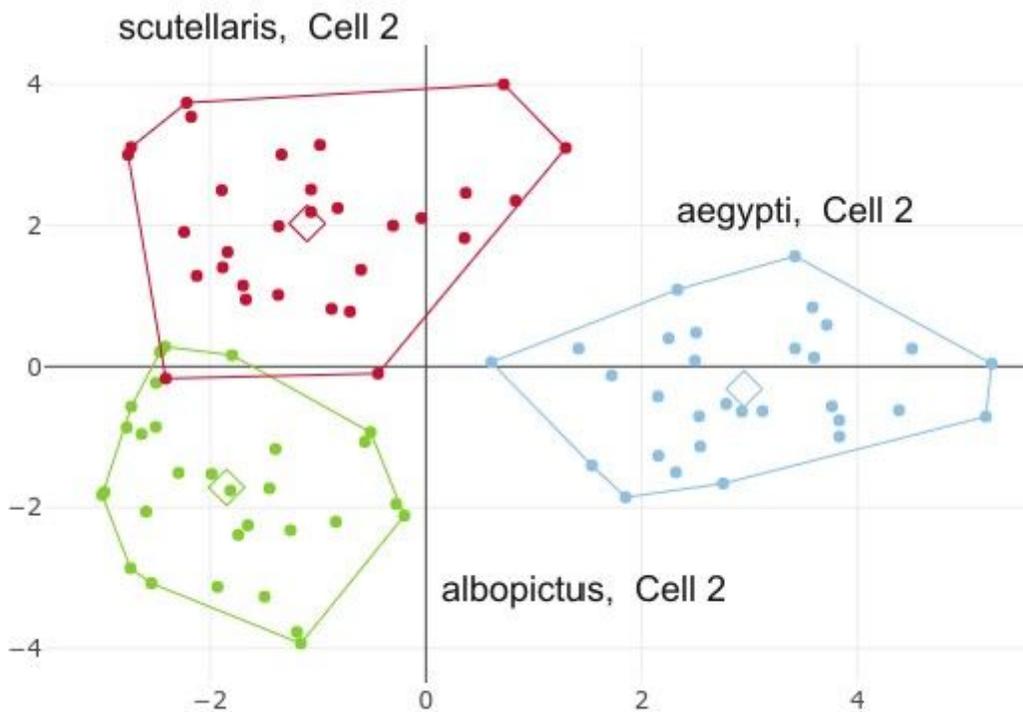


Figure 10

Factor map of the two discriminant factors of shape variables derived from cell 2, the most discriminant cell in the three groups comparisons (see Table 4). *Ae. aegypti* (blue); *Ae. albopictus* (green); *Ae. scutellaris* (red). The first discriminant factor is the horizontal axis.

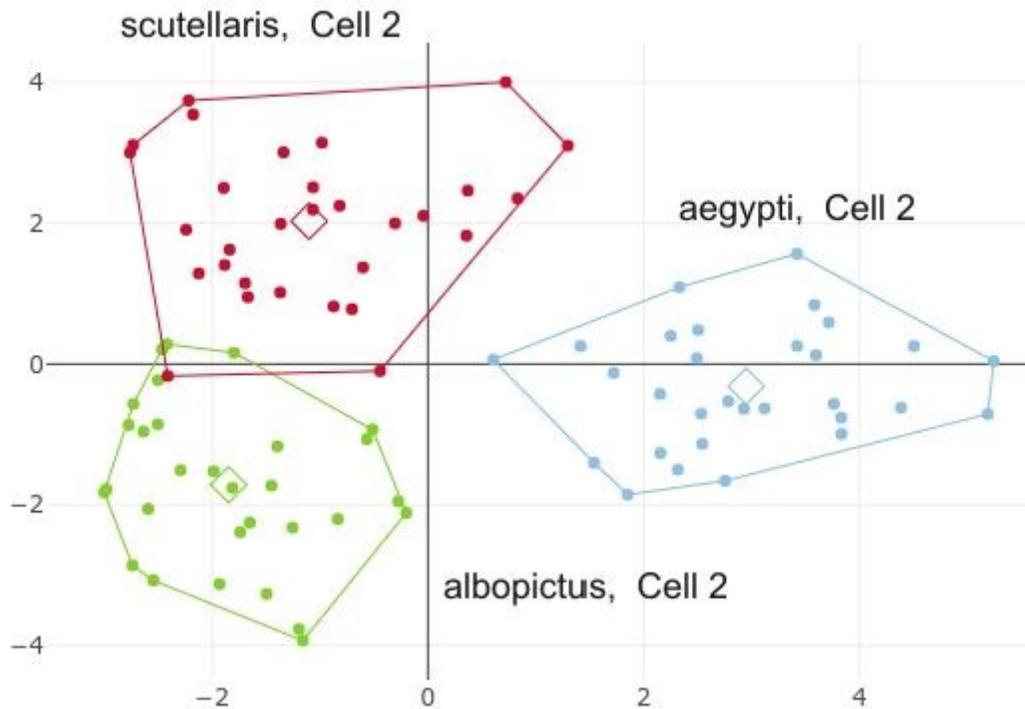


Figure 10

Factor map of the two discriminant factors of shape variables derived from cell 2, the most discriminant cell in the three groups comparisons (see Table 4). *Ae. aegypti* (blue); *Ae. albopictus* (green); *Ae. scutellaris* (red). The first discriminant factor is the horizontal axis.

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