

Geometrical Variation of Wing Shape in the Japanese Encephalitis Vector, *Culex tritaeniorhynchus* Giles, 1901 (Diptera: Culicidae), from Different Geographical Locations

Sidiq Setyo NUGROHO^{1*}

Triwibowo Ambar GARJITO²

Mujiyono MUJIYONO³

Rudi NIRWANTONO⁴

¹Research Center for Biosystematics and Evolution, National Research and Innovation Agency, Jakarta-Bogor Highway km 46, Cibinong, Bogor, West Java, 16911, INDONESIA

²Research Center for Public Health and Nutrition, National Research and Innovation Agency, Cibinong Science Center, Jakarta-Bogor Highway, Pakansari, Cibinong, Bogor, West Java, 16911, INDONESIA

³Institute for Vector and Reservoir Control Research and Development, Ministry of Health, Hasanudin Street No. 123 Salatiga City, Central Java, 50721, INDONESIA

⁴Biotechnology Department, Faculty of Engineering, Bina Nusantara University, Kemanggis, Palmerah, Western Jakarta, 11480, INDONESIA

e-mails: ¹sidiqsnugroho148@gmail.com, ²triwibowoa@gmail.com, ³insulaeflorum@gmail.com
⁴rudi.nirwantono@gmail.com

ORCID IDs: ¹0000-0002-7088-3342, ²0000-0002-7697-9759, ³0000-0001-7058-4935
⁴0000-0001-9080-2821

*Corresponding author

ABSTRACT

The wing geometric morphometrics (WGM) is a powerful method for identifying variation within species and differentiating from other species. Therefore, this research aimed to determine the wing geometric variation in *Cx. tritaeniorhynchus* from different locations and also compared it to the wing geometry of the highly resemble species of *Cx. vishnui*. A total of 54 females of *Cx. tritaeniorhynchus* from Ende Regency (Wologai and Nanganesa) and Konawe Regency (Tamesandi and Puuhopa) were collected, while 34 females of *Cx. vishnui* were collected from nearest locations (Mautapaga, Ende Regency, and Bajo Indah, Konawe Regency). In addition, 17 females of *Aedes aegypti* were also used in the analysis representing species outside the Genus *Culex* as an outgroup. After being photographed, the wing was marked to create landmarks (LMs) using the tpsUtil 1.70 and tpsDig2 2.29 software series. The LMs were superimposed using the Procrustes Fit function and the canonical variate analysis (CVA) was performed with MorphoJ 1.06d software, and the Mahalanobis distance matrix and the dendrogram (unweighted squared-change parsimony with 10.000x permutations) were subsequently generated. The

Nugroho, S.S., Garjito, T.A., Mujiyono, M., & Nirwantono R. (2024). Geometrical Variation of Wing Shape in The Japanese Encephalitis Vector, *Culex tritaeniorhynchus* Giles, 1901 (Diptera: Culicidae), from Different Geographical Locations. *Journal of the Entomological Research Society*, 26(2), 239-253.

Received: November 20, 2023

Accepted: July 22, 2024

result demonstrated that the intraspecies geographical variation on the wing geometry occurred in *Cx. tritaeniorhynchus*. Further, WGM also could distinguish *Cx. tritaeniorhynchus* apart from *Cx. vishnui*. The statistical analysis has marked out that the geometrical variation in *Cx. tritaeniorhynchus* wing can be utilized to distinguish different populations from different geographical areas. The feature could also be a tool to differentiate *Cx. tritaeniorhynchus* from *Cx. vishnui*.

Keywords: wing geometric morphometrics, geographical variation, *Cx. tritaeniorhynchus*, *Cx. vishnui*, distinguish species.

INTRODUCTION

Japanese Encephalitis (JE) is considered a health problem in Indonesia and can potentially become an outbreak (Srivastava et al., 2023). This disease is predicted to spread all over the country with a different infection peak season on each island (Hills et al., 2008). In addition, Indonesia is now known as one of the countries that play an important role in JE transmission globally. Previous research revealed that Indonesia is the origin of the JE virus (JEV) which evolved from its ancestor living in the Indomalaya region (Solomon et al., 2003). Japanese encephalitis has spread across Asia and has become the most important cause of epidemic encephalitis worldwide. Four genotypes of Japanese encephalitis virus (JEV. Situated between the Indian and Pacific oceans, Indonesia provides a geographical advantage and allows the virus to spread widely (Weaver & Reisen, 2010) and many cause disease after spillover transmission to humans and agriculturally important domestic animals that are incidental or dead-end hosts. Viruses such as dengue (DENV. Subsequently, JEV then circulated outside the original location to other regions in Asia.

Recently, JEV in Indonesia has been isolated from 10 species of mosquitoes: *Cx. tritaeniorhynchus*, *Cx. gelidus*, *Cx. vishnui*, *Cx. fuscocephala*, *Cx. bitaeniorhynchus*, *Cx. quinquefasciatus*, *An. vagus*, *An. kochi*, *An. annularis*, and *An. subalbatus* (Garjito et al., 2018). However, one of the medically most prominent species as JEV vector is *Cx. tritaeniorhynchus* which is widely widespread and commonly encountered in urban environments near human populations. In Indonesia, the widespread of *Cx. tritaeniorhynchus* is influenced by the presence of many potential breeding sites, especially rice fields (Garjito et al., 2018).

Besides in Indonesia, *Cx. tritaeniorhynchus* has now been proven as the main vector of JEV transmission in other Asian countries (Liu et al., 2013; Longbottom et al., 2017; Seo et al., 2013; Su et al., 2014; Van Peenen et al., 1975). This species is widely distributed in entire South East Asia and surrounding tropical areas, extending to the Middle East, East Asia (Alahmed, 2012; Günay et al., 2015; Navidpour et al., 2012) and Africa (Alves et al., 2014; Fall et al., 2013). It was also reported in Europe recently (Lytra & Emmanouel, 2014). Moreover, *Cx. tritaeniorhynchus* is also known to become a competence vector for several other arboviruses (Hayes et al., 1980; Hubálek & Halouzka, 1999; Jupp et al., 2002).

As mosquito identification is important to study JEV and in mosquito control programs, a proper identification of *Cx. tritaeniorhynchus* becomes a priority. Until now, the field's identification merely relied on morphological features (Longbottom et

al., 2017). However, the identification of *Cx. tritaeniorhynchus* is harder to perform due to highly resemblance morphological features with other species within *Cx. vishnui* Subgroup such as *Cx. vishnui*, *Cx. annulus*, and *Cx. pseudovishnui* (Sirivanakarn, 1977). Further, intraspecific morphological variation also becomes an issue and complicates the identification process.

On the other hand, wing geometric morphometrics (WGM) is a strong, quick, and cheap method to solve problems in taxonomy and morphology, especially in insects (Adams et al., 2013). The wing's shape and venation provide well-defined landmarks for suitable morphometric measurement. The previous study from Mondal et al., (2015) disclosed that the wing shape variation had been observed in several species in genus *Aedes*. Another study by Hounkanrin et al (2023) with *Aedes* species also monitored a significant variation in wing geometry between landscape types in Germany. Considering the biological importance of wings for mosquitoes, the study of variation in mosquito wing shape also could provide useful information on vector taxonomy, distribution, and population diversity (Champakaew et al., 2021; Hounkanrin et al., 2023) particularly in subtropical regions. Different mosquito species display various behaviors, breeding sites, and geographic distribution; however, they can be difficult to distinguish in the field due to morphological similarities between species and damage caused during trapping and transportation. Vector control methods for controlling mosquito-borne disease epidemics require an understanding of which vector species are present in the area as well as the epidemiological patterns of disease transmission. Although molecular techniques can accurately distinguish between mosquito species, they are costly and laborious, making them unsuitable for extensive use in the field. Thus, alternative techniques are required. Geometric morphometrics (GM). Since the information about the wing geometry has not been studied in *Cx. tritaeniorhynchus*, the wing geometry comparison both within species could provide a beneficial information about the population diversity of the species. Hereafter, the comparison of wing geometry of *Cx. tritaeniorhynchus* with *Cx. vishnui* could provide a prospective information to distinguish the species. In the present study, the investigation of wing geometry in *Cx. tritaeniorhynchus* was conducted to learn the variation in wing geometry among different populations collected from different geographic landscapes. Furthermore, we also compared the wing geometry from *Cx. tritaeniorhynchus* with a highly resemble species, *Cx. vishnui* to investigate the wing geometry variation between these species. The natural specimens of *Cx. tritaeniorhynchus* from different landscapes were collected, photographed, digitized, and marked (landmarking). Selected LMs in the wings of *Cx. tritaeniorhynchus* samples were then used to compare the wings geometry from different collection sites. The wing geometry from *Cx. tritaeniorhynchus* was also compared with wing geometries from naturally captured *Cx. vishnui* and laboratory reared *Ae. aegypti* to understand the wing geometry variation between these species.

MATERIAL AND METHODS

Study sites

The study was conducted in two different locations separated by Flores Sea to represent distinct populations separated by natural barrier. Figure 1 displays the map of sampling locations in Flores Island and Celebes Island generated with ArcGIS 10.2 (freely used software for a publication purpose). The first sampling locations were in Ende Regency, East Nusa Tenggara, Flores Island. In this regency, the sampling location were in Nanganesa (Ndona Sub-district), Wologai (Detusoko Sub-district), and Mautapaga (Ende Timur Sub-district). The second sampling locations were in Konawe Regency, South East Sulawesi, Celebes, where the sampling site were in Tamesandi (Uepai Sub-district), Puuhopa (Puriala Sub-district), and Bajo Indah (Soropia Sub-district).

The sampling location in Nanganesa was a low-land area by the coastal area dominated by shrimp farming area and far from the human residence. In contrast, Mautapaga and Bajo Indah were coastal areas surrounded by shrimp farming but near to human settlements. On the other hand, Wologai and Puuhopa were located on a high-land, near to rice farm, far from forest, and near to human settlements. Meanwhile, Tamesandi was a high-land near to a human settlement that residing by the forest.

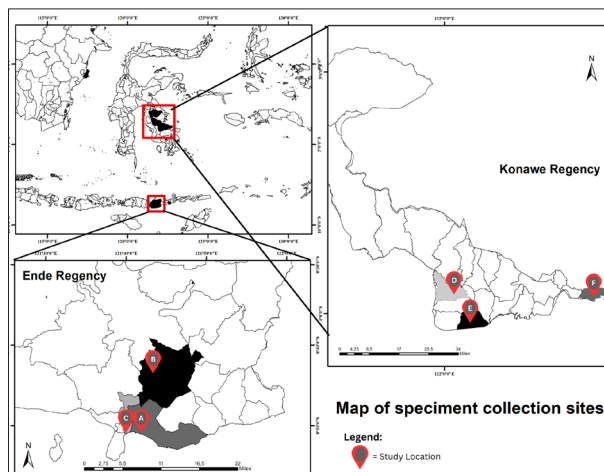


Figure 1. The study was conducted in two different Island, Sulawesi Island and Flores Island, that are separated by Flores Sea. The first location was in Ende Regency, East Nusa Tenggara, Flores Island where the sampling sites were in Nanganesa, Ndona Sub-district (A), Wologai, Detusoko Sub-district (B), and Ende Timur Sub-district, Mautapaga (C). The second location was in Konawe Regency, South East Sulawesi, Celebes, where the sampling site were in Tamesandi, Uepai Sub-district (D), Puuhopa, Puriala Sub-district (E), and Bajo Indah, Soropia Sub-district (F).

Samples collection

A total of 105 female mosquitoes from field collection and laboratory colonies were used in this study (Table 1). *Cx. tritaeniorhynchus* and *Cx. vishnui* samples were collected from fields in April - May 2016. The collection of *Cx. tritaeniorhynchus*

Geometrical Variation of Wing Shape in the Japanese Encephalitis Vector

were performed in Ende Regency (Wologai and Nangesa), and Konawe Regency (Tamesandi and Puuhopa). Due to sample availability, *Cx. vishnui* specimens were captured in same regencies but different location, which were Mautapaga (Ende Regency) and Bajo Indah (Konawe Regency). All mosquitos were collected using the following methods: human landing catch (HLC), animal-baited trap (ABT), collecting near cattle pens, and sweep nets collection. All captured mosquitoes were identified under a stereo dissecting microscope with a built-in camera with 4 – 36 x magnification based on the morphological character keys by (Rattananarithikul et al., 2005). In addition, females of *Ae. aegypti* were obtained from mosquito rearing laboratory at the Institute of Vector & Reservoir Control Research & Development (IVRCRD) Salatiga, Central Java, Indonesia and were used in the study as an outgroup.

Table 1. Mosquito species were used as specimens in this research.

Mosquito species	Collection site			Total number of specimens
	Island	Regency	Village	
<i>Cx. tritaeniorhynchus</i>	Flores	Ende Regency	Nangesa	11
			Wologai	11
	Sulawesi	Konawe Regency	Tamesandi	16
			Puuhopa	16
<i>Cx. vishnui</i>	Flores	Ende Regency	Mautapaga	17
	Sulawesi	Konawe Regency	Bajo Indah	17
<i>Ae. aegypti</i>	Laboratory reared mosquito			17

Wing slide preparation

The left wing of mosquito samples were used in this study since the right wing of most samples were glued on the paper points. The left wing was removed near its base, retaining the basicosta part, using a combination of fine forceps and a fine scalpel blade. The wing was then placed on a glass slide and covered by a coverslip and glued with Euparal mounting medium. The mounting layer was kept as thin as possible to ensure flattening. All mounted wing slides were heated at 56°C overnight to clear air bubbles before imaging. Subsequently, the wing preparation was photographed with a Leica EZ4E dissecting stereo microscope (Leica, Germany) connected to a PC device through tpsUtil 1.70 software (Rohlf, 2019). Twenty identifiable LMs representing the venation pattern were selected, and the coordinates of the LMs were plotted on tpsDig2 2.29 software (Figure 2) according to the method by Rohlf (2015). The description of LMs is presented in Table 2.

Canonical Variate Analysis (CVA) of wing shape

The LMs were superimposed using the Procrustes Fit function in order to remove variation caused by differences in position, scale, and orientation of the coordinates. The averages of Procrustes coordinates obtained from the landmark data were calculated to create a dataset of landmark shift for further statistical analysis (Champakaew et al., 2021; Klingenberg, 2011).

The phenotypic variations based on LMs shift were then decomposed into canonical variates components (CV). The two CVs with the highest variance coverage were

then used to generate a CVA plot to cluster all samples of *Cx. tritaeniorhynchus* within groups representing closeness of the wing geometry. The LMs shift between two groups of mosquitoes were also visualized on a transformation grid to display their different wing shape. In other hand, the phenotypic variations in wings shape between three species of mosquitoes in this study were also determined using similar approach. Further, a cross-validation test based on Mahalanobis distances (discriminant function analysis) was performed to confirm the reliability of classification within each population/species. All the analyses above were conducted in MorphoJ software v.1.07 with 10.000x permutations. Finally, a rooted tree was eventually constructed based on the scores obtained from the Mahalanobis distance matrix using unweighted squared-change parsimony method (with 10.000x permutations) to illustrate the phenetic relationships between the wing data of mosquito within species *Cx. tritaeniorhynchus* and also the phenetic relationships between studied species (Champakaew et al., 2021).

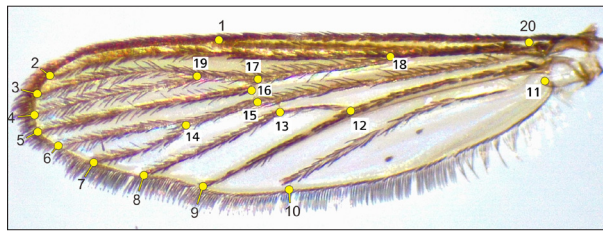


Figure 2. Numbered placement of landmark on the left wing of *Cx. tritaeniorhynchus*. The landmarks description: 1 = intersection of sub-costa and costa; 2 = distal end of the radius (R); 3 = distal end of radial branch 2 (R2); 4 = distal end of radial branch 3 (R3); 5 = distal end of radial 4+5 (R4+5); 6 = distal end of medial branch 1+2 (M1+2); 7 = distal end of medial branch 3+4 (M3+4); 8 = distal end of cubital vein 1 (Cu1); 9 = distal end of cubital vein 2 (Cu2); 10 = distal end of the anal vein (A); 11 = origin of the anal vein (A); 12 = origin of cubital vein 1 (Cu1); 13 = intersection of medio-cubital cross vein (m-cu) and Cu1; 14 = origin of M1+2 and M3+4; 15 = midpoint branch of medial vein (M); 16 = radio-medial cross vein (r-m); 17 = midpoint branch of the radial vein (R); 18 = origin of radial sector vein (Rs); 19 = origin of radial R2 and R3; 20 = humeral cross vein (h).

RESULTS

Wings variation within species *Cx. tritaeniorhynchus*

CVA of wing geometry among population of *Cx. tritaeniorhynchus* displays four different clusters with wing geometry variation indicated by colors, including the population Wologai (red), Nanganesa (light-green), Puuhopa (blue) and Tamesandi (cyan; Figure. 3). The CVA calculation within species *Cx. tritaeniorhynchus* obtained three CVs, among which the two highest covered 84.067% of the total variance (CV1 = 61.730%; CV2 = 22.337%), displaying a geometric difference between populations. The scatter plot of CV1 and CV2 (confidence ellipses = 90%) revealed that the samples from similar sampling locations clump together at a certain location and only overlap with the other location from the similar regency. The four samples from Tamesandi and Puuhopa, in which both locations belong to Konawe Regency, were observed within overlapped zones. Meanwhile, only one sample in overlapping zone was observed

Geometrical Variation of Wing Shape in the Japanese Encephalitis Vector

between population from Wologai and Nanganesa. In contrast, the clusters from different regency did not display overlapping, reflecting a clear distinction.

Mahalanobis distance of wing geometry shown in Table 3 displays the distance values between sample groups of *Cx. tritaeniorhynchus*. The statistical analysis with 10.000x permutations test in MorphoJ revealed significant differences (p-values: < 0.0001). The lowest distance score was obtained from a comparison between Puuhopa and Tamesandi with a score of 3.9741. This record was followed by a comparison between Wologai and Nanganesa with 4.7692. In contrast, the highest distance score was recorded from the comparison of the wing geometry of *Cx. tritaeniorhynchus* from Tamesandi and Nanganesa with 6.8222. This record was followed by the comparison between Tamesandi and Wologai, with a distance value of 6.1674.

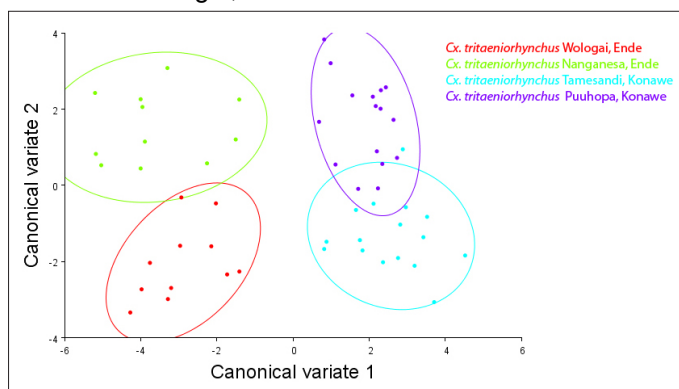


Figure 3. Scatter plot of wings geometry variation among populations. Clusters of all populations were observed in the plot obtaining from the first two canonical variate (CV1 and CV2) with 90% confidence ellipses.

Table 2. Mahalanobis distance of wing shape among sample groups of *Cx. tritaeniorhynchus*.

	Wologai ¹	Nanganesa ¹	Tamesandi ²
Nanganesa ¹	4,7692 ^a		
Tamesandi ²	6,1674 ^b	6,8222 ^c	
Puuhopa ²	6,0569 ^d	6,1907 ^a	3,9741 ^f

Note:

¹ = Ende Regency; ² = Konawe Regency;

a,b,c,d,e,f = indicating the significant difference tested with 10.000x permutations test and with p-values < 0.0001.

Further, the transformation grids in Figure 4 provides information in detail, at which the LMs in the wing of *Cx. tritaeniorhynchus* between populations were different and underwent shifting. The length of the LMs shift within the grid reflects the degree of difference between the two locations. The wing geometry comparison between population collected from different regencies exhibited the most visible LMs shift. In general, the wing geometry alteration in *Cx. tritaeniorhynchus* studied in this study was observed at tree LMs. They were the intersection of the subcosta with the costa (LM1) and in the radius vein branching (LM14 and LM19).

The wing geometry comparison between population from Tamesandi-Nanganesa displayed the most noticeable LMs shift in the grid (LM1, LM 14, LM19, and LM20) that visibly changes its contour (Figure 4A). The LMs shifts with an almost similar

pattern were also apparently monitored between population from Puuhopa-Nangesa and Tamesandi-Wologai, where the most notable LMs shifts were at LM1 and LM19 (Figure 4B and 4C). Further, the wing geometry difference between populations from Puuhopa-Wologai was only observed at LM1, while other LMs were underwent unnoticeable shifts (Figure 4D). Interestingly, a similar shifting pattern of LMs was exhibited from Puuhopa-Tamesandi with the LM shifting at LM1, although both locations are in Konawe regency (Figure 4E). Thus, mosquito samples from Wologai and Nangesa, which both of them are in Ende Regency, were the only populations pair showed small or unnoticeable shifts for all LMs in the transformation grid. Both locations revealed only small shifts at LM19, LM14, LM9, LM10, LM18, and LM11 and displayed unobvious shifts for the rest (Figure 4F).

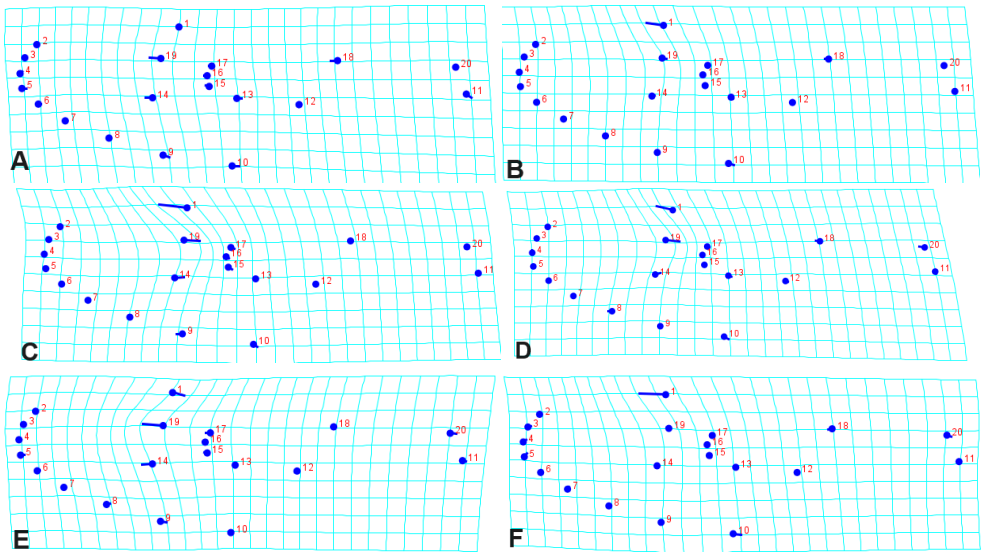


Figure 4. Visualization of LMs shifts on the transformation grid from the wing geometry comparison between population of *Cx. tritaeniorhynchus*. The change of the grids were observed after comparing wings from Tamesandi-Nangesa (A), Puuhopa-Nangesa (B), Tamesandi-Wologai (C), Puuhopa-Wologai (D), Puuhopa-Tamesandi (E), and Wologai-Nangesa (F).

Wing variation between species of mosquito

Figure 5 shows the scatter plot generated from CVA of mosquito wing shape from *Cx. tritaeniorhynchus* (four populations), *Cx. vishnui* (two populations), and *Ae. aegypti* (one population). The CVA analysis based on wing geometry differences across species in this study obtained six CVs, among which the two highest CVs covered 90.751% of the total variance (CV1 = 60.985%; CV2 = 29.766%), displaying the wing geometric difference between clusters. The scatter plot generated from CV1 and CV2 (confidence ellipses = 90%) revealed three different clusters representing three different species. Similar species from different populations, even though collected from different sampling locations, were gathered in a single cluster. All mosquito samples of *Cx. tritaeniorhynchus* from population Wologai (yellow), Nangesa (light-green),

Geometrical Variation of Wing Shape in the Japanese Encephalitis Vector

Tamesandi (cyan), and Puuhopa (light-blue) were clumped together in the first cluster. All samples of *Cx. vishnui* from Mautapaga, Ende Regency (blue) and Bajo Indah, Konawe Regency (purple) were also gathered in a single cluster, while *Ae. aegypti* samples formed the third cluster. The wing topology of mosquitoes from the different species could be distinguished. The apparent LM shifts occurred at LM1, LM12, LM13, LM14, LM18, and LM19, where they affected the length of costa (C), subcostal (SC), cubital vein 1 (Cu1), medio-cubital cross vein, medial branch 1+2 (M1+2), medial branch 3+4 (M3+4), radial sector vein (Rs), radial branch 2 (R2), ad radial branch (R3).

According to the transformation grid, the wing of *Cx. vishnui* has a longer but narrower shape than it *Cx. tritaeniorhynchus*. The grid stretched outward in the base of the wing (LM on the humeral cross vein), but shrank inward in the center (LMs on the intersection of subcosta and costa, and LM on the distal end of the anal vein). R_{2+3} vein on the wing of *Cx. tritaeniorhynchus* was also shorter due to the shift at LM19, so it was farther to the tip of the wing than it was in *Cx. vishnui* wing. Then, the wing shape of *Cx. tritaeniorhynchus* was slightly wider when it was compared with the wing shape of *Ae. aegypti*, and all intersections of its vein (LM12-19) were located closer to the wing base.

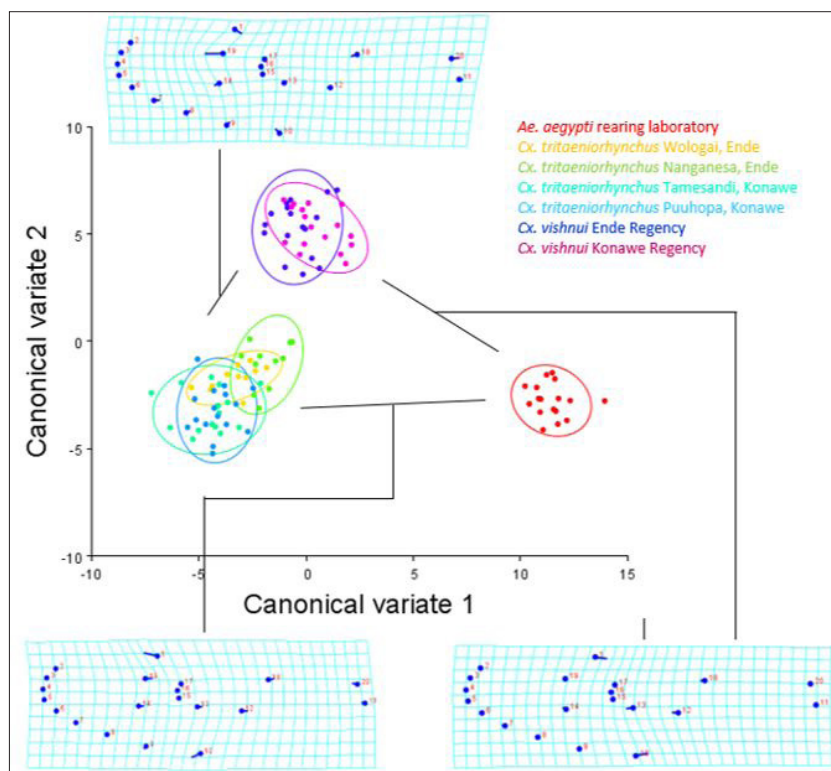


Figure 5. Scatter diagram plot CVA of *Cx. tritaeniorhynchus* wing shape (green, yellow, light blue, Tosca green), *Cx. vishnui* (blue and purple) and *Ae. aegypti* (red).

Dendrogram analysis

The dendrogram derived from the Mahalanobis distance score (Figure 6) also displays the grouping according to the wing shape. Samples of *Cx. tritaeniorhynchus* from each regency formed a distinctive cluster (Wologai clustered with Nanganesa and Tamesandi with Puuhopa). All samples of *Cx. tritaeniorhynchus* from all locations then met in a node and grouped into a bigger cluster. A similar pattern was also exhibited by *Cx. vishnui*. Further, the branch represents all groups of *Cx. tritaeniorhynchus* joined with the branch representing all samples of *Cx. vishnui* to form a cluster representing all samples within the genus *Culex*. In contrast, the outgroup of *Ae. aegypti* was separated into a separate branch, indicating that the species has considerable differences in wing shape from the other two.

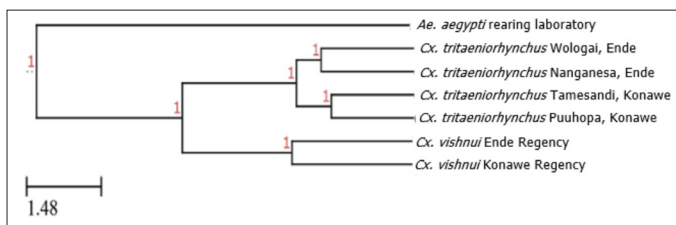


Figure 6. Dendrogram of all studied mosquito species generated based on Mahalanobis distance score. The samples within similar species were positioned in a cluster, although they were from different locations. The outgroup of *Ae. aegypti* was shown to separate at different position. The tree was constructed based on unweighted squared-change parsimony method with 10.000x permutations test (p-value: <.0001).

DISCUSSION

The wing geometric morphometric is a quantitative study of the characteristic like shape in mosquito wings (Rohlf, 2015). Unlike an analytical study, the geometric morphometric is one of the fundamental areas that tightly relates to taxonomy, since it can provide features to discriminate a species with others. Thus, this analysis is widely applied to resolve the problem of the identification of mosquito (Petrarca et al., 1998). Interestingly, this study indicated that *Cx. tritaeniorhynchus* populations from four different locations were able to be distinguished by using wing geometry. The neighbour population within similar regency shared the least difference than populations that were located in different regency.

The trend on the intraspecific wing geometry variation observed in this study was also observed in the studies by Kuclu et al (2011). Four populations of *Ae. vexans* from areas with different location in northeastern Turkey showed variations in wing geometry, where the close locations shared higher degree of similarity in wing geometry. In addition, a study by Wilk-da-Silva et al (2018) in *Ae. aegypti* in urban and conserved areas also indicated that neighbour location shared similar geometry of wing. The wing geometry from mosquitoes captured from conservation areas near to the urban areas was more similar to it from urban area than from other conservation areas that were far away. In contrast, a study Morais et al (2010) on *Ae. aegypti* in Brazil and

Argentina populations found that a close location did not necessary have similar wing geometry, since geographical barrier seemed to affect to the populations.

Both genetic and environmental factors could be were known to influence the morphological features in mosquito. In a study by Kanojia et al (2010) on *Cx. tritaeniorhynchus* in several location India, the genetics and morphological characters variations between populations were significant. They stated that the variations were attributed by selection by the environmental conditions. On the other hand, studies on *Ae. aegypti* by Paupy et al (2010) in Niakhar (Senegal) and Kumar et al (2022) in Tamil Nadu (India) were resulted in an unclear evidence of genetics distinction between population despite the morphological different between populations were observed. A similar result was also obtained by Demirci et al (2012) during a study on *Cx. theileri* populations in the northeast part of Turkey.

However, the driver factors of morphological variation on difference population of *Cx. tritaeniorhynchus* in this study, were still unknown. The variance seems to be affected by distance of locations than local environment characteristics. *Cx. tritaeniorhynchus* from Wologai and Puuhopa, which have similar environment characteristics but located in different islands, shared different wing geometry. On the other hand, Wologai and Nanganesa, which are located at a similar regency (Ende), had a lowest Mahalanobis distance score. A similar case also occurred between Tamesandi and Puuhopa in Konawe Regency. It indicates a high degree of similarity in wing geometry, although they have different environment characteristics. Flores sea that separated Ende Regency and Konawe Regency in different Islands is likely to behave as a geographical barrier.

Perrard et al (2014) said that natural barrier could split the population and drive the genetic isolation that affects morphological variations. It also provides a specific local environment and forcing morphological plasticity. Study by Bai et al (2014) also revealed that a natural barrier including mountainous regions have a role as a barrier that triggers intraspecific fragmentation.

The result from this study also disclosed that the wing shape of the mosquito was able to distinguish species *Cx. tritaeniorhynchus* from the other species. The analysis could significantly differ the *Cx. tritaeniorhynchus* from *Cx. vishnui*, although they share a similar genus. Further, both species also could be separated from *Ae. aegypti* using wing shape. The difference was observed in the width and length of the wing as well as the length of the radius 2 vein, radius 3 vein, and radius 2+3 vein.

A similar finding was also revealed in the study by Mondal et al (2015) that worked on distinguishing *Ae. aegypti* from other species, including *Ae. albopictus* and *Ae. pseudotaeniatatus* using the wing shape (Mondal et al, 2015). The principal component analysis (PCA) analysis of 20 LMs of wings resulted three clusters indicating three separated species. This finding reflects that wing geometry analysis could help to distinguish some resemble species. Vidal et al (2011) also said that the shape and venation of the wing are important features in the identification of different species of *Culex* within the *Cx. vishnui* subgroup. Baylac et al (2003) and Jaramillo-O. et al

(2014) also said that the geometry of wing morphometry could be used to differentiate sibling species and their complex species. In addition, the wing difference between species reflected in the dendrogram tree is also by the taxonomical relationship which also reflects their distance. This supports the fact that the genetics of the species influence the morphological variation.

CONCLUSION

The use of morphometric geometry such as clustering with canonical variate analysis (CVA) and Mahalanobis distance matrix analysis successfully described the variation of the wing geometry of *Cx. tritaeniorhynchus* originating from different locations. The distance of the sampling locations seems to affect to the similarity of the wing geometry between location, since the neighbour locations share lower Mahalanobis distance score. The method was also success to distinguish the wing from *Cx. tritaeniorhynchus* with the wing from *Cx. vishnui* and *Ae. aegypti*. Thus, wing geometric morphometrics in this study can be a reliable alternative to differentiate hard-to-distinguished species in Vishnui Sub-group including between *Cx. tritaeniorhynchus* and *Cx. vishnui*.

ACKNOWLEDGEMENT

Thank you to the Head of the Health Research and Development Agency for permission and funding for this research. Thank you to the Head of B2P2VRP Salatiga for the permission to authors use mosquito specimens. I would also like to thank the Rikhus Vektora 2016 data collection team especially the Ende District vector team in East Nusa Tenggara Province and the Konawe District vector team in Southeast Sulawesi Province, with all efforts to successfully collect data and mosquito specimens from the field in good condition. Thanks to colleagues of the Salatiga B2P2VRP Vector Collection and Reference Laboratory for their assistance in selecting and re-identifying mosquito specimens to be used in this study.

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Geometrical Variation of Wing Shape in the Japanese Encephalitis Vector

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Geometrical Variation of Wing Shape in the Japanese Encephalitis Vector

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