WING GEOMETRY ANALYSIS AS A POTENTIAL TOOL FOR SPECIES IDENTIFICATION FOR *Anopheles* MOSQUITOES (DIPTERA: CULICIDAE) IN INDONESIA

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ABSTRACT

In the last decade, wing geometry has been investigated intensively as an alternative powerful method for solving taxonomic problems in insects. The objectives of this research were to describe wing geometry variation among seven *Anopheles* species and to confirm the sensitivity of wing geometry analysis for identifying single specimen of *Anopheles* mosquito. Thus, the potential of wing geometry analysis as an alternative tool for species identification for *Anopheles* mosquitoes can be recognized. Left wing of seven *Anopheles* species were detached and photographed. Wing geometry was represented by 18 landmarks (LMs). Wing geometry analysis was conducted by MorphoJ and tps software series. Comparison among species and identification simulation were done using canonical variate analysis (CVA). Wing geometry was successfully discriminated and grouped seven *Anopheles* species into correct subgenera and series. This method also gave good results in identifying single specimen. Nine out of 11 specimens (81, 8%) obtained identification results that match their phylogenetic relationships. Weakness using wing geometry in species identification can be overcome by adding template species. In conclusion, wing geometry analysis has good potential to be used as an alternative tool for species identification for *Anopheles* mosquito in Indonesia.

Keywords: Wing geometry, mosquitoes, *Anopheles*, species identification.

ABSTRAK

Dalam beberapa dekad ini geometri sayap telah diteliti secara mendalam sebagai kaedah alternatif berpotensi dalam menyelesaikan masalah taksonomi serangga. Tujuan kajian ini adalah untuk memperihalkan variasi geometri sayap di antara tujuh spesies *Anopheles* dan mengesahkan analisis geometri sayap dalam mengecamkan spesimen tunggal nyamuk *Anopheles*. Maka dengan itu, potensi analisis geometri sayap sebagai kaedah alternatif untuk pengecaman spesies nyamuk *Anopheles* dapat dikenalpasti. Sayap kiri dari tujuh spesies *Anopheles* dipisahkan dan diambil gambar fotonya. Geometri sayap nyamuk diwakili oleh 18 tanda. Analisis geometri sayap dilakukan menggunakan rangkaian perangkat MorphoJ dan tps. Perbandingan di antara spesies dan simulasi identifikasi dilakukan menggunakan analisis kanonikal variat (CVA). Geometri sayap berhasil membezakan dan mengelompokkan tujuh spesies *Anopheles* ke dalam subgenus dan seriesnya. Kaedah ini juga memberikan hasil yang baik untuk pengecaman spesimen tunggal. Sembilan dari 11 spesimen (81, 8%) mendapatkan hasil pengecaman yang sesuai dengan hubungan filogenetiknya yang hampir. Kelemahan kaedah geometri sayap dalam pengecaman spesies dapat di atasi dengan menambahkan spesies templat. Kesimpulannya, analisis geometri sayap memiliki potensi yang baik untuk digunakan sebagai kaedah alternatif dalam pengecaman spesies nyamuk *Anopheles* di Indonesia.

Kata kunci: Geometri sayap, nyamuk, *Anopheles*, pengecaman spesies.

INTRODUCTION

In the period of 2007-2017, the malaria control in Indonesia showed encouraging results, and annual parasite incidence showed decline significantly by three times, from 2.89 per 1000 to 0.9 per 1000 population. More than 50% of districts officially declared as malaria free areas (Sitohang et al. 2018). Comprehensive malaria control efforts with local specific approaches have conducted with coordination between the Ministry of Health with support from UNICEF, WHO, Global Fund, local government, community organization, and the private sector has led to major achievement in malaria control in Indonesia. However, malaria is still one of the major infectious diseases, especially in the eastern part of Indonesia (Hakim 2011; Pusdatin 2016). For that reason, strengthening surveillance systems with early and prompt diagnostic and treatment alongside vector control in high-transmission areas and coupled with tailored approaches in low-transmission areas are still needed.

Indonesia is home to 71 identified *Anopheles* mosquito species, which has been divided into two subgenera, namely *Anopheles* and *Cellia* (O'Connor & Sopa 1981). The subgenera are further segregated into section, series and groups. Twenty *Anopheles* species have been confirmed as malaria vector in Indonesia (Elyazar et al. 2013). Knowledge of species composition is vital to provide database of mosquito diversity, which are medically important, so that limited resources could be applied effectively in malaria control (Ng et al. 2016). Precise identification of mosquito species is crucial to understand the epidemiological patterns of disease transmission, which are related to the abundance of the vector, the infectivity and the capacity and competence of vectors (Laurito et al. 2015; Vidal et al. 2011). The most common method for mosquito identification is using identification keys based on morphological characters (Harbach & Kitching, 1998). Nowadays a lot of studies had been conducted to identify mosquito species by molecular analysis. However, molecular method requires expensive equipment and reagents as well as in depth training for the operators. Not all regions in Indonesia have adequate facilities and human resources to carry out molecular analysis. Therefore, an alternative method is needed to solve taxonomic problems in limited conditions.

Although wing geometry analysis not really a taxonomic tool, it is a powerful method to explore variation in wing shape. It also proven to be very useful to help in identification, as it complements the traditional qualitative description of diagnostic features (Börstler et al. 2014). Previous studies had been used the wing geometry to distinguished 11 species of *Anopheles* subgenus *Nyssorhynchus* in Colombia (Calle et al. 2008; Jaramillo-O et al. 2014). Wing geometry is also used to describe variation in *Anopheles flavirostris* detected positive and negative filarial (Sendaydiego & Demayo 2015). It can also be an aid for the taxonomic identification of poorly conserved specimens that are difficult to classify. Based on shape, one of the strengths of the wing geometry is the ability to assign individuals of initial unknown species to the correct species with relative accuracy. But before using wing geometry, first species identification using conventional morphological keys is needed. The purposes of this study were to described wing geometry variation among seven *Anopheles* species and confirm the sensitivity of wing geometry analysis for identifying single specimen of *Anopheles* mosquito.

MATERIALS AND METHODS

The study was conducted at the Institute for Vector and Reservoir Control Research and Development (IVRCRD), Indonesia in November 2017. This study examined 105 female *Anopheles* mosquitoes belonging to seven species, called as template species, representing two subgenera, i.e. *Anopheles* and *Cellia*. Species included in the *Anopheles* subgenus are *An. barbirostris* and *An. sinensis*, while *Cellia* subgenus consists of the remaining species: *An. aconitus, An. farauti, An. maculatus, An. tessellatus*, and *An. vagus.* The accuracy of identification was tested using 11 species which are represented by single specimen each, called as test species. A validated classification procedure was used, also called jack-knife classification, where each individual is allocated to its closest group without being used to help determine a group center (Kaba et al. 2016). Mosquitoes were identified with morphological key identification provided by O'Connor & Soepanto (1999).

Mosquitoes' left wing was detached using fine forceps and mounted dry between slide microscopy glass and cover slip. The photographs of mosquito wings were taken using a Leica EZ4E dissecting microscope camera. Eighteen LMs placement (Figure 1) was done using tpsDig2 software developed by Rohlf (2015).Wing geometry analysis was conducted using MorphoJ software developed by Klingenberg (2011). Comparison of wing shapes between species studied was analyzed using canonical variate analysis (CVA) (Mondal et al. 2015). Each individual was represented by a point depicted in the CVA graph. The location of the point coordinates in the CVA illustrates the relative resemblance of an individual to other individuals studied. The relative similarity is quantitatively expressed in the Mahalanobis distance. To determine the relationship among the species studied, the Mahalanobis distance was used to compile a dendrogram using the unweighted pair group method with arithmetic mean (UPGMA) method. Furthermore, CVA analysis was carried out on 11 test specimens toward template specimens that had been analyzed before. The Mahalanobis distance among template and test species was presented in Table 2, then identification simulation results have summarized in Table 3. The smallest Mahalanobis distance shows the most similar wing geometry.

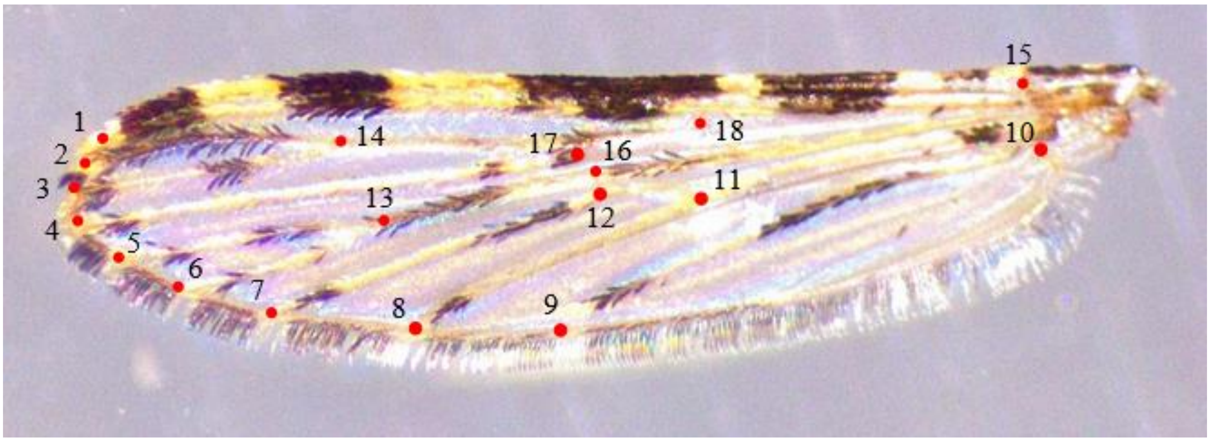


Figure 1. Landmarks placement on the *Anopheles* left wing. 1, distal end of radius; 2, distal end of radial branch 2; 3, distal end of radial branch 3; 4, distal branch of radial 4+5; 5, distal end of medial branch 1; 6, distal end of medial branch 2; 7, distal end of medial branch 3+4; 8, distal end of cubital anterior vein; 9, distal end of anal vein; 10, origin of anal vein; 11, medio-cubital cross-vein; 12, intersection of medio-cubital cross-vein and medial 3+4; 13, origin of medial branch 1 and 2;14, origin of radial branch 2 and 3; 15, humeral cross-vein; 16, origin of medial branch 3+4; 17, origin of radial branch 4+5; 18, origin of radial sector vein.

RESULTS

The wing geometry analysis was able to correctly distinguish seven template *Anopheles* species (Figure 2). Canonical variate analysis as visualized in a graph with different symbols for each species. Each cluster of species is given a confidence ellipse to help distinguish one cluster from another. Species with the closest cluster distance are between *An. maculatus* and *An. vagus*. Three other species of the *Cellia* subgenus are located some distance from the two species above. Cluster *An. aconitus* seems closer to *An. tessellatus* than to *An. farauti*. Meanwhile, the Mahalanobis distance of *An. tessellatus* is closer to *An. farauti* than to *An. aconitus* (Table 1). Likewise, *An. barbirostris* cluster seemed to be closer to *An. aconitus* rather than to *An. sinensis* which belongs to the same subgenus. Actually, the Mahalobis distance of *An. barbirostris* closer to *An. sinensis* than to *An. aconitus*. Dendrogram arranged from Mahalanobis distance shows the relationship among template species (Figure 3). Wing geometry analysis was able to grouped species in the appropriate subgenera and series. A mismatch occurs in constructing dendrogram that does not accordance with phylogenetic relationship, the explanation is visualized in Figure 4.

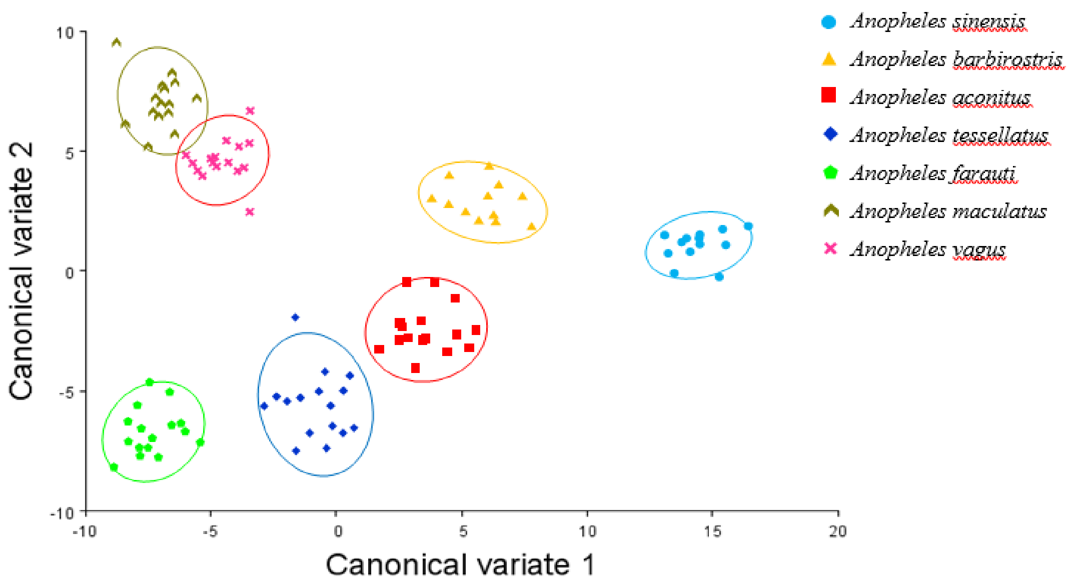


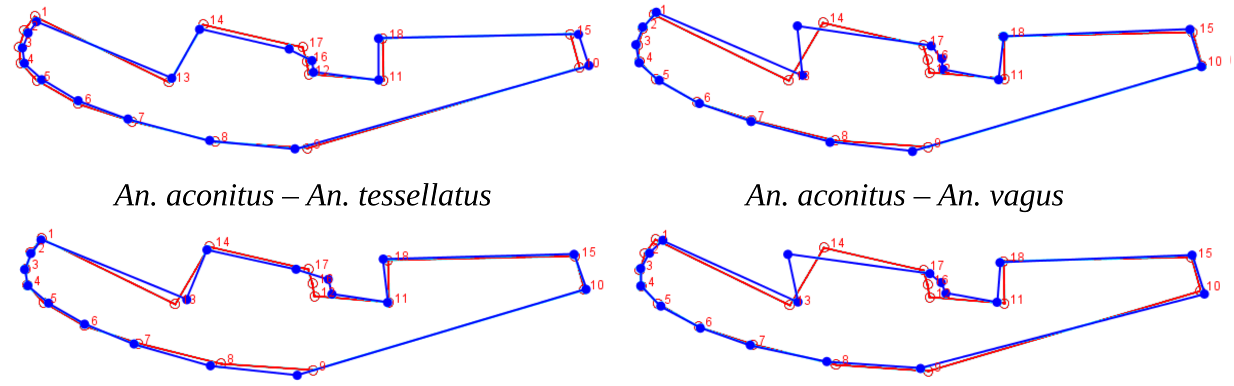
Figure 2. Graphic of canonical variate analysis result on *Anopheles* wings.

Table1. Mahalanobis and Procrustes distances among seven *Anopheles* species.

		Procrustes distance					
Anopheles	*aconitus*	*barbirostris*	*farauti*	*maculatus*	*sinensis*	*tessellatus*	*vagus*
aconitus		0,0508	0,0488	0,0629	0,0685	0,0377	00543
barbirostris	15,1517		0,0649	0,0661	0,0522	0,0434	0,0673
farauti	16,2328	18,2057		0,0573	0,0915	0,0347	0,0445
maculatus	16,2227	16,0489	14,9526		0,0885	0,0590	0,0282
sinensis	15,8275	14,2387	23,3634	23,0613		0,0749	0,0866
tessellatus	10,6096	13,2529	9,1331	14,6940	17,9230		0,0544
vagus	14,3051	15,9995	13,4236	9,2316	20,0577	12,9927	

Mahalanobis distance

Figure 3. Dendrogram constructed from Mahalanobis distance using UPGMA method.



An. aconitus – An. tessellatus *An. aconitus – An. vagus*

An. aconitus – An. farauti *An. aconitus – An. maculatus*

Figure 4. Comparison of wireframe graphs of *An. aconitus* (red) versus another species in the *Cellia* subgenus.

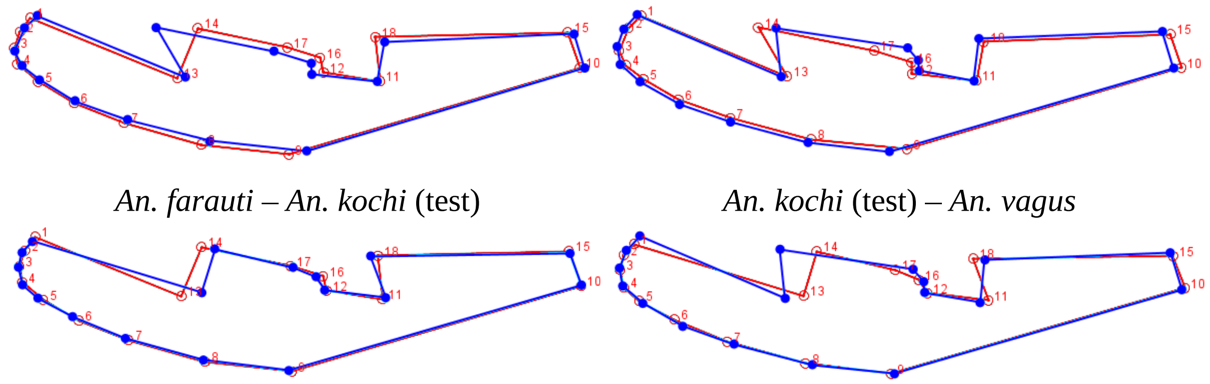
The second step was to simulate species identification using CVA by placing test specimens on template specimens that have been tested previously. Nine out of 11 (81, 8%) species tested could be correctly identified according to the closest relationship in this simulation. Two species that not correctly identified were *An. kochi* that identified closest to *An. vagus* and An. *ludlowae* that identified closest to *An. farauti* (Table 2). Specimen *An. kochi* according to the previous phylogenetic study should be closest to *An. farauti* that both included in the *Neomyzomyia* Series, while *An. ludlowae* should be closest to *An. vagus* that both are members of the *Pyretophorus* Series (Table 3). Wireframe graphs of species with incorrect identification are visualized in Figure 5.

Table 2. Mahalanobis distance resulted from CVA among template and test specimens.

		Template specimens						
	Anopheles	*barbirostris*	*sinensis*	*aconitus*	*farauti*	*maculatus*	*tessellatus*	*vagus*
Test specimens	*flavirostris*	14,4025	17,8207	10,5284	15,1306	17,5780	10,4971	17,9610
	indefinitus	17,6416	19,8427	13,6728	12,0098	12,1185	11,3591	6,9974
	kochi	15,5536	20,4050	18,2112	18,9334	16,5013	16,8851	14,8346
	leucosphyrus	18,1848	24,6619	19,1568	11,3663	15,7164	12,4150	15,9456
	ludlowae	19,7779	22,2488	18,7359	12,2313	18,8825	13,6718	16,4360
	maculatus	15,9056	22,9867	16,2225	13,8170	7,4070	12,9088	11,4639
	peditaeniatus	11,4556	11,0126	13,6293	20,0897	18,8069	14,6587	16,7280
	sinensis	17,1351	6,0399	17,9112	25,5066	25,5597	20,1231	22,9479
	subpictus	16,5297	17,0304	15,7236	13,2778	15,7489	12,2226	11,3007
	sundaicus	18,0292	19,9985	16,1750	16,1728	13,7135	15,0737	8,4298
	vagus	14,1610	21,0988	14,8641	14,1019	10,4394	12,6663	9,0607

Table 3. Summary on identification simulation results on *Anopheles* species using wing geometry analysis.

No.	Species	Result	Closest species	Closest relationship
1	*An. flavirostris*	Correct	*An. aconitus*	*Funestus* Group
2	*An. indefinitus*	Correct	*An. vagus*	*Pyretophorus* Series
3	*An. kochi*	Incorrect	*An. farauti*	*Neomyzomyia* Series
4	*An. leucosphyrus*	Correct	*An. farauti*	*Neomyzomyia* Series
5	*An. ludlowae*	Incorrect	*An. vagus*	*Pyretophorus* Series
6	*An. maculatus*	Correct	*An. maculatus*	Same species
7	*An. peditaeniatus*	Correct	*An. barbirostris*	*Myzorhynchus* Series
8	*An. sinensis*	Correct	*An. sinensis*	Same species
9	*An. subpictus*	Correct	*An. vagus*	*Pyretophorus* Series
10	*An. sundaicus*	Correct	*An. vagus*	*Pyretophorus* Series
11	*An. vagus*	Correct	*An. vagus*	Same species



An. farauti – *An. kochi* (test) *An. kochi* (test) – *An. vagus*

An. farauti – *An. ludlowae* (test) *An. ludlowae* (test) – *An. vagus*

Figure 5. Comparison of wireframe graphs of *An. farauti* (left-red) and *An. vagus* (right-blue) versus *An. kochi* and *An. ludlowae* as test specimens.

DISCUSSION

Our results indicate that seven medically important *Anopheles* species were correctly distinguished by wing geometry analysis as clearly shown in the CVA results graph. Geometric differences among species are indicated by Mahalanobis distance, i.e. the greater value, the greater the difference. Therefore, wing geometry analysis can be considered as a valid alternative method for identification of the seven mosquito species studied here identification by taxonomic method is not possible. Some *Anopheles* species in Indonesia have similar morphological characteristic, making it difficult to identify using dichotomous keys. Wing geometry analysis is important to be developed in Indonesia as an alternative tool to assist in the identification of *Anopheles* species.

Wing shape was chosen over wing size because wing size is known to be easily affected by environmental factors (Gómez et al. 2014). Size variation did not interfere with species delimitation and size comparison cannot ascertained to be holds in nature because size is commonly subject to environment and genetic plasticity (Dujardin 2008; Henry et al. 2010). Wing shape has proven to be a stable character that is less labile than size and is very informative on the genetics and evolution of organisms (Klingenberg 2010). For *Anopheles* taxonomy, wing shape has been species-discriminative in at least two studies (Calle et al. 2008; Lorenz et al. 2012), but it was yield no significant correlation in two other studies in which molecular taxonomy worked (Gómez et al. 2013; Vicente et al. 2011).

Wing geometry analysis was previously used by Lorenz et al. (2012), who were able to identify the species *An. cruzii*, *An. homunculus* and *An. bellator* included in *Kerteszia* subgenus. There was partial overlapping in the morphospace of canonical variables between *An. cruzii* and *An. homunculus*. They suggested that slight divergence between species might be a result of recent diversification of the subgenera (Collucci & Sallum 2003) or due to evolutionary constraints. The close evolutionary relationship among *Kerteszia* representatives might be reflected in the wing shape because of heritability of this structure (Lorenz et al. 2012). A study on identifying *Anopheles* mosquitoes using wing geometry has also been carried on subgenus *Nyssorhynchus* (Jaramillo-O et al. 2014). They succeeded in using wing geometry to identify several sibling species that lived sympatrically, namely *An. bennarrochi* B and *An. oswaldoi s.l.* in Colombia. Morphological identification of sibling species usually utilizes male terminalia, hence wing geometry analysis can help in identification if only female mosquitoes are found (Wilke et al. 2016).

The dendrogram compiled in this study encountered a mismatch with the results of phylogenetic research on *Anopheles* reported before. At the subgenus level it can be separated properly, but at the series level discrepancies are found. The dendrogram shows that *An. aconitus* is closer to the branch of *Neomyzomyia* series occupied by *An. tessellatus* and *An. farauti*. According to the phylogenetic, the relationship of *An. aconitus* should be closer to *An. vagus* (*Pyretophorus* series) (Harbach 2013). Wireframe graphs show the difference between wing geometry of *An. aconitus* compared with *An. vagus* and *An. maculatus* (Figure 4). It showed that the intersection of radial vein (LM 14) in *An. vagus* and *An. maculatus* was more distal to wing base compared with the intersection of medial vein (LM 13). While in *An. aconitus* as well as in *An. tessellatus* and *An. farauti* LM 14 was more proximal to wing base compared with LM 13.

Identification mismatch also occurs between *An. kochi* and *An. ludlowae*. According to the genus *Anopheles* phylogenetic analyzed by Harbach (2013), *An. kochi* (*Koch*i group) is closely related with *An. farauti* (*Punctulatus* group), both species are included in *Neomyzomyia* series, while *An. ludlowae* together with *An. vagus* are included in *Pyretophorus* series. When observed in wireframe graph, *An. kochi* have LM 14 that more distal to wing base than LM 13, so it's more similar to *An. vagus*. In *An. ludlowae*, LM 14 conspicuously more proximal to wing base than LM 13, so the geometry is closer to *An. farauti*. The wing venation has a role in wing rigidity which is important for flight (Mountcastle & Combes 2013). Furthermore, wing geometry is likely involved in the production of the aerodynamic forces during the flight (Young et al. 2009). However, it was said that the results of phylogenetic studies based on morphological and molecular data sets of *Anopheles* mosquitoes were conspicuously ambiguous (Harbach & Kitching 2015). Therefore, wing geometry is not possible to stand alone for use in compiling phylogenetic of *Anopheles* mosquitoes. Wing geometry analysis is reliable only when used as an alternative tool for the identification of *Anopheles* species. Accuracy of wing geometry analysis for species identification can be improved by adding template species according to the species found in an area that conducts *Anopheles* surveys.

CONCLUSIONS

Wing geometry analysis can be used to distinguish seven *Anopheles* species in the *Anopheles* and *Cellia* subgenera and grouped them according to their phylogenetic relationship. With the right approach, the accuracy of wing geometry analysis as an alternative method for species identification could be improved.

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