Geometric Morphometric Analysis of Forewings of *Apis mellifera* Linnaeus, 1758 (Hymenoptera: Apidae) Populations in Thailand

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ABSTRACT. – *Apis mellifera* beekeeping in Thailand started in the 1970s and since then has spread throughout the country. There is little information on diversity, especially morphological diversity, of these species in Thailand. The objective of this study was to examine variation among *A. mellifera* populations in Thailand based on geometric morphometrics of forewings. We collected *A. mellifera* worker bees of 160 colonies from 25 apiaries throughout Thailand. Forewing shape variation was examined based on 19 landmark coordinates. The results showed high wing morphological variation. *Apis mellifera* samples from Thailand were found to belong to 5 distinct groups, which were further supported by a hierarchical cluster analysis. In the size analysis, the honey bees in 5 morphotypes differed significantly in wing centroid size ($p < 0.0001$). Canonical variate analysis (CVA) revealed 5 distinct groups with significance in all groups ($P < 0.001$), and a high rate of correct classification based on a cross-validation test (85.63%) was found. Our study provided information on morphological diversity of *A. mellifera* in Thailand and asserts that forewing geometric morphometrics is a simple, reliable, inexpensive method that is sufficient for determination of morphological diversity of this species.

KEYWORDS: *Apis mellifera*, **forewing, geometric morphometrics, landmark, Thailand**

INTRODUCTION

The western honey bee, *Apis mellifera* Linnaeus, 1758 is one of the most beneficial insects. The natural distribution range of the honey bee includes Africa, Europe and the Middle East (Seeley, 1985; Ruttner, 1988). Today, this species is found worldwide due to multiple migrations and importations by humans (Ruttner, 1988; Moritz et al., 2005; Whitfield et al., 2006). This species is a highly variable and there are over 30 recognized subspecies found throughout the world (Engel, 1999; Bouga et al., 2011; Raina et al., 2011). The four widely recognized lineages based on morphometric and molecular data are A (Africa), C (South-Eastern Europe branch), M (Western Europe), and O (Near and Middle Eastern) lineages (Ruttner, 1988; Whitfield et al., 2006; Miguel et al., 2011). Furthermore, two new lineages have been added including the Y lineage in Ethiopia (Franck et al., 2001) and Z lineage in Libya (Alburaki et al., 2013). *Apis mellifera* is an exotic species of great economic importance in Thailand (Suppasat et al., 2007). This species was originally introduced from Taiwan for beekeeping purposes in the 1970s (Wongsiri et al., 2000; Kavinseksan et al., 2004; Suppasat et al., 2007). More than 300,000 colonies were spread throughout the country, with a large number of colonies being distributed in the northern part (Chantawannakul, 2018). At present, this species is distributed across all regions of Thailand by beekeepers. Although beekeeping in Thailand started approximately 50 years ago, there have only been limited studies on *A.*

mellifera in Thailand. Suppasat et al. (2007) demonstrated that there were 4 subspecies from 3 groups of *A. mellifera* in Thailand, based on PCR-RFLP and mtDNA sequences: group ThaiA1 and group ThaiA2 match C lineage (*A. m. carnica* and *A. m. ligustica*) and group ThaiB belongs to O lineage (*A. m. syriaca* and *A. m. lamarckii*). They suggested that the subspecies of group ThaiB was more likely to be *A. m. syriaca* than *A. m. larmarckii* because there was evidence on *A. mellifera* importation from Israel. However, the subspecies of group ThaiB could possibly be *A. m. larmarckii*, since the researchers and beekeepers in Thailand have introduced queens of *A. mellifera* from various countries (Suppasat et al., 2007), this needs to be confirmed with further molecular study. The recent study by Rattanawannee et al. (2019) revealed 3 subspecies (*A. m. caucasica*, *A. m. carnica* and *A. m. ligustica*) using microsatellite analysis and mtDNA sequences. Therefore, 5 subspecies of *A. mellifera* were reported in Thailand (*A. m. caucasica*, *A. m. carnica*, *A. m. larmarckii*, *A. m. ligustica* and *A. m. syriaca*).

Since beekeeping is globally accepted as a highly valued industry (Rizwan et al., 2018) and as a result, many *A. mellifera* populations were freely imported into the country by beekeepers, leading to the admixture of populations. Information on genetic variation and diversity of *A. mellifera* populations in Thailand remain largely unknown.

Knowledge on diversity of *A. mellifera* populations is important for effective management strategies and conservation program. Therefore, determination of honey bee diversity is essential and will provide a

valuable resource for breeding and conservation purposes. Generally, the determination of honey bee diversity is mainly based on traditional morphological characteristics (Raina and Kimbu, 2005; Shaibi et al., 2009; Nedic et al., 2011) and advanced molecular approaches (Garnery et al., 1993; Jensen et al., 2005; Whitfield et al., 2006; Bodur et al., 2007; Ilyasov et al., 2011; Gruber et al., 2013). However, the use of classical morphology is based on multiple measurements from several body parts and required highly time-consuming specimen preparation and numerous measurements (Kandemir et al., 2011). Molecular methods are not only expensive methodology, but they require advanced and sophisticated equipment, and often are more time consuming than morphological methods (Francoy et al., 2008; Nawrocka et al., 2018). An alternative method, called geometric morphometrics (GM), has been developed based on the coordinates of landmarks on wing venations (Bookstein, 1991; Slice, 2007), this method is informative, easy to perform, less time-consuming, less costly and more accessible to beekeepers to use as a tool for identifying *A. mellifera* populations (Tofilski, 2008). In addition, geometric morphometrics on wings of honey bee has been proven to be highly effective in its ability to describe inter- and intra-specific variation of honey bees (Bouga and Hatjina, 2005; Francoy et al., 2006, 2008, 2009; Tofilski, 2008; Rattanawannee et al., 2010, 2012; Kandemir et al., 2011; Miguel et al., 2011; Oleksa and Tofilski, 2015; Henriques et al., 2020). In *A. mellifera*, wing geometric morphometrics has been widely used in discriminating subspecies (Tofilski, 2008; Francoy et al., 2009; Kandemir et al., 2011; Miguel et al., 2011; Abou-Shaara and Al-Ghamdi, 2012; Charistos et al., 2014; Oleksa and Tofilski, 2015; Barour and Baylac, 2016; Porini et al., 2019), variation within subspecies (Barour et al., 2011; Dolati et al., 2013; Henriques et al., 2020) and hybrids (Oleksa and Tofilski, 2015). For example, three subspecies of honey bee (*A. m. carnica*, *A. m. cauasica* and *A. m. mellifera*) can be distinguished based on geometric morphometric analysis (Tofilski, 2008). Furthermore, Miguel et al. (2011) reported that geometric morphometrics is appropriate for classification of honey bees within A, M or C branches. Moreover, geometric morphometrics is a powerful tool to classify A branch of African honey bee subspecies: *A. m. intermissa, A. m. sahariensis* and *A. m. capensis* (Barour and Baylac, 2016). Besides honey bees, this method is acknowledged as useful for identification of stingless bees (Francisco et al., 2008; Francoy et al., 2009, 2011; Bonatti et al., 2014), bumble bees (Aytekin et al., 2007), *Megachile* bees (Falamarzi et al., 2016),

Euglossa bees (Francoy et al., 2012) and also for medical entomology (Dujardin, 2008; de Souza et al., 2020). Moreover, several studies proposed that geometric morphometrics provide higher power in discrimination and may have advantages over traditional morphometrics, and exhibited relatively high agreement with molecular methods (Kekecoglu et al., 2007; Kandemir et al., 2011; Miguel et al., 2011; Oleksa and Tofilski, 2015; Zhou et al., 2016). In addition to the genetic variation and genetic structure, morphological variation of these species is also important information regarding diversity and species recognition. To date, morphological variation is an understudied research topic in commercial honey bees in Thailand. Therefore, we aim to test a landmark-based geometric morphometric analysis of forewings for determination of morphological diversity in *A. mellifera* populations that are economically important insect of Thailand.

MATERIALS AND METHODS

Sample collection

Worker bees of 160 *Apis mellifera* colonies were collected from 25 apiaries throughout Thailand during October 2015 to May 2017 (Table 1, Fig. 1). Approximately, twenty adult worker bees per colony were sampled from the brood area. All specimens were preserved in 80% (*v/v*) ethanol and then kept in a freezer (-20 ºC) for wing morphological investigation. The research project had been reviewed and approved by the Institutional Animal Care and Use Committee, Mahasarakham University (IACUC-MSU). The approval number is 025/2018.

Wing processing and data acquisition

Ten adult worker bees per colony were randomly sampled from 160 *A. mellifera* colonies, so a total of 1,600 bees were used for the analysis. The right forewing of each individual was removed and mounted flat on a microscope slide with Hoyer's medium solution. The mounted wings were photographed with a digital camera enhanced through a stereomicroscope (OLYMPUS SZX7). Each wing was captured with the same accessories and to the same scale. A TPS file was generated from all the captured images through the tpsUtil version 1.63 software (Rohlf, 2015). Nineteen coordinates of homologous landmarks were manually plotted at the wing vein intersections (Fig. 2) using tpsDig2 version 2.16 software (Rohlf, 2015). The landmark coordinates obtained from tpsDig (Raw coordinates) were used as input in the MorphoJ 1.07a package (Klingenberg, 2011) and PAST version 3.15 software (Hammer et al., 2001) for wing analysis. The raw coordinates of the landmarks on wings were

TABLE 1. Sampling locations of *Apis mellifera* in Thailand.

Location	Sample code	Coordinates	No. of colonies (No. of individuals)	Collection date
1. Maerim, Chiang Mai	CMI1	18° 92.74 N, 98° 88.04 E	6(60)	18/02/2015
2. Hangdong, ChiangMai	CMI2	18° 73.87 N, 98° 92.23 E	7(70)	01/05/2017
3. Sanpatong, ChiangMai	CMI3	18° 62.86 N, 98° 89.58 E	6(60)	26/04/2017
4. Sanpantong, ChiangMai	CMI4	18° 53.23 N, 98° 89.69 E	6(60)	28/04/2017
5. Thoeng, ChiangRai	CRI	19° 57.27 N, 99° 97.30 E	7(70)	24/04/2017
6. Chiang Kham, Phayao	PYO	19° 45.67 N, 100° 30.41 E	6(60)	24/04/2017
7. Mueang, Lampun	LPN1	18° 59.59 N, 99° 02.80 E	7(70)	27/04/2017
8. Mueang, Lampun	LPN2	18° 62.15 N, 99° 04.37 E	6(60)	27/04/2017
9. Pasang, Lampun	LPN3	18° 45.09 N, 98° 94.37 E	6(60)	27/04/2017
10. Pasang, Lampun	LPN4	18° 45.59 N, 98° 94.53 E	6(60)	27/04/2017
11. MaeTha, Lampun	LPN5	18° 40.86 N, 98° 96.70 E	6(60)	27/04/2017
12. Wangthong, Phitsanulok	PSK	16° 75.38 N, 100° 56.58 E	6(60)	23/04/2017
13. Mueang, Uttaradit	UTT	17° 36.45 N, 100° 65.24 E	6(60)	02/05/2017
14. Mueang, Khon Kane	KKN	16° 44.19 N, 102° 83.60 E	7(70)	13/10/2015
15. Thabo, Nongkhai	NKI	17° 81.11 N, 102° 58.12 E	7(70)	14/10/2015
16. Poogradeung, Loie	LOI	16° 89.70 N, 101° 84.36 E	6(60)	13/10/2015
17. Prathumrat, Roi Et	RET	15° 63.53 N, 103° 34.23 E	6(60)	15/10/2015
18. Mueang, Nakhon Ratchasima	NKA	14° 57.24 N, 102° 05.22 E	7(70)	09/05/2017
19. Mueang, Udon Thani	UDI	17° 41.38 N, 102° 78.73 E	6(60)	17/05/2017
20. Wang muang, Saraburi	SRI1	14° 83.06 N, 101° 13.91 E	7(70)	26/10/2016
21. Banmor, Saraburi	SRI2	14° 36.56 N, 100° 43.38 E	6(60)	26/10/2016
22. Thasae, Chumporn	CPN	10° 75.64 N, 99° 10.13 E	7(70)	26/09/2016
23. Thepha, Songkhla	SKA	6° 79.07 N, 100° 91.81 E	7(70)	28/11/2016
24. Khaokichakoot, Chantaburi	CTI	12° 48.02 N, 102° 07.18 E	6(60)	12/06/2016
25. Saiyok, Kanjanaburi	KJI	14° 12.05 N, 99° 13.69 E	6(60)	10/05/2017
Total			**160(1600)**	

superimposed using Procrustes analysis to remove unrelated non-shape variations of these landmarks such as size, position, and orientation (Dryden and Mardia, 1998). In order to reduce the measurement error, each wing was measured twice by the same person and the two measurements were then averaged by MorphoJ version 1.06d software (Klingenberg, 2011; Miguel et al., 2011).

Data analysis

Procrustes ANOVA was performed using MorphoJ version 1.06d software to ensure that the observed variability was not influenced by measurement error (Klingenberg and Mclntyre, 1998). We also calculated the repeatability index (R) to examine quality of the measurements following the method of Arnqvist and Martensson (1998). Since we had no information about which subspecies of *A. mellifera* was present in each apiary, we performed hierarchical cluster analysis (CA) based on the squared Euclidian distance to classify groups of bee samples using PAST version 3.15 software (Hammer et al., 2001). A multivariate analysis of variance (MANOVA) was applied to compare the wing shape differences among groups. The groups indicated by the cluster analysis was used in both wing size and shape analysis. The wing size of each group were estimated by centroid size (CS) (Bookstein, 1991). Wing size differences among honeybee groups were analyzed through box plots and one-way ANOVAs of CS using PAST version 3.15 software (Hammer et al., 2001). In addition, canonical

variate analysis (CVA) was conducted to indicate the best characters to use for group separation (Gumiel et al., 2003; Villemant et al., 2007). Moreover, Mahalanobis distances between the groups of *A. mellifera* were calculated with significant differences by the permutation test (10,000 iterations). Additionally, a cross-validation test in discriminant function analysis (DFA) was used to assess the accuracy of classification based on Mahalanobis distances in a permutation test with 10,000 rounds using MorphoJ version 1.06d software (Klingenberg, 2011). Wing deformation grids and wireframe graph of shape variations were generated.

RESULTS

Procrustes ANOVA revealed that variation among individuals was significant ($P < 0.0001$; Table 2) and R index in the repeatability measures was very high (R = 0.98). This indicated that the variability arose initially from the wing shape rather than from measurement errors. Group classification based on wing shape using a hierarchical cluster analysis revealed that the *A. mellifera* populations in Thailand were divided into five major groups (Fig. 3). The CA results showed that the bee samples in each group were originated from various apiaries (Fig. 3). Moreover, the MANOVA of all forewing landmarks indicated that the groups of *A. mellifera* were significantly different from each other (Wilk's $\lambda = 0.0101$; $P < 0.001$). The variations in wing
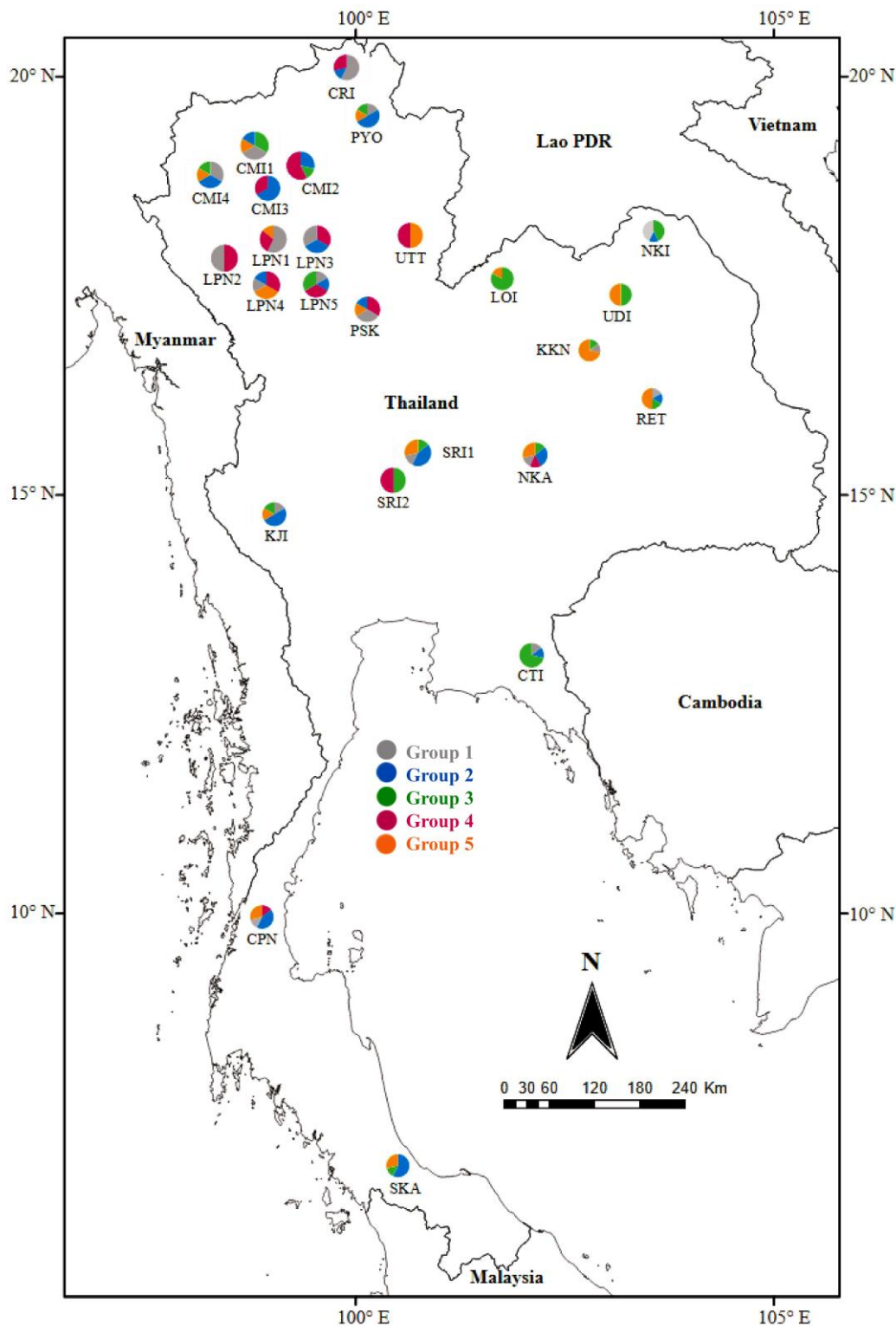
FIGURE 1. Sampling locations and morphotype distribution of *Apis mellifera* in Thailand.

size (CS) among five *A. mellifera* groups are illustrated by box plot (Fig. 4). The ANOVA result revealed that centroid sizes among five *A. mellifera* morphotypes were significantly different (ANOVA: $F_{4,\ 1595} = 33.38$; $P < 0.0001$). The largest wings were found in Group 4 (mean wing CS = 975.98 ± 20.22), followed by Group 1 (mean wing CS = 970.27 ± 19.50), Group 2 (mean wing CS = 966.25 ± 14.84), Group 3 (mean wing CS = 962.90 ± 21.23) and Group 5 (mean wing CS = 959.54 ± 13.06). The shape differences among five *A. mellifera* morphotypes were scattered on the first two canonical variate axes (CV1 and CV2). The first two
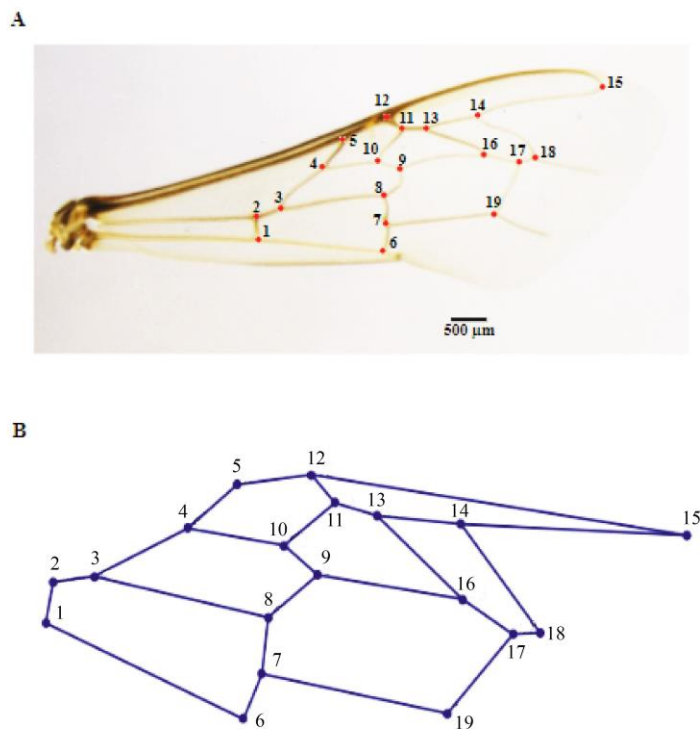
FIGURE 2. A right forewing of *Apis mellifera*. (A) The 19 landmarks. (B) Wireframe representation of the 19 landmarks. Scale bar: A= 500 µm.

TABLE 2. Procrustes ANOVA for measurement error for shape of *Apis mellifera*.

Effect	SS	MS	df	F	P
1. Individuals (variation among individuals)	0.21751163	4.048×10^{-5}	5372	5.76	< .0001
2. Measurement error (error due to digitizing landmarks)	0.03707386	7.034×10^{-6}	5270	0.78	0.9861

Note: SS: sum of squares; MS: mean squares; df: degree of freedom; F: F-criterion; *P*: P-value

canonical variates accounted together for 66.48% of the total variation (CV1 = 39.90%, CV2 = 26.38%) and the scatter plot showed a partial overlap in all groups, except Group 2 which was clearly separated from the others (Fig. 5A). Although there was overlap among groups, it is possible to observe separation among five *A. mellifera* groups from the scatter plot. The three most powerful landmarks of CV1 were landmarks 14, 13 and 16 (Fig. 5B), whereas the three most influencing landmarks to the CV2 were landmarks 15, 13 and 2 (Fig. 5B). The wireframe graph shows the changed shape variation among samples (Fig. 5C). Relationships among the groups based on Mahalanobis distances indicated that all group pairs represented highly significant differences in wing shape (Table 3). The Mahalanobis distances values ranged from 3.7683 (Group 1 vs. Group 3) to 5.2356 (Group 1 vs. Group 5). The cross-validation test based on shape indicated

85.63% correct classification of the samples to their respective group, while cross-validated classifications on shape + size reached 87.50 % (Table 4).

DISCUSSION

In this research, we reported for the first time the application of a geometric morphometric approach to examine and visualize the morphological variation of the commercial honey bee species, *A. mellifera* in Thailand. High morphological variation on the right forewings was found among *A. mellifera* populations in the country. Our cluster analysis based on wing shape of *A. mellifera* populations from 25 apiaries throughout Thailand indicated that they were separated into 5 distinct groups. Interestingly, CA revealed that all localities (apiaries) had a mixture of populations.
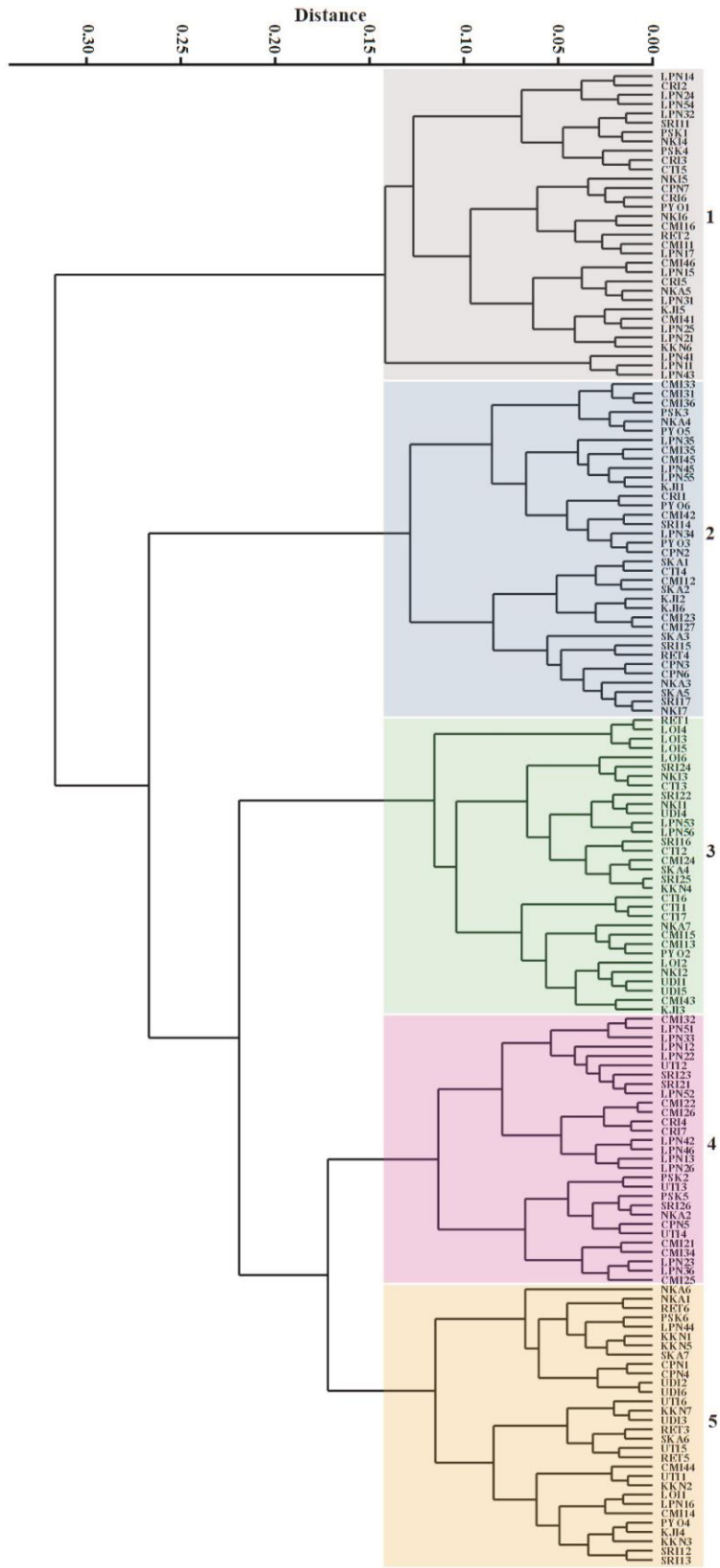
FIGURE3. Relationships among 5 morphotypes of *Apis mellifera* in Thailand based on the squared Euclidian distance using a hierarchical cluster analysis (CA).

Number of groups (morphotypes) found in each locality ranged from 2-5 groups (Fig. 1), each colony represented 1 group. This result suggested that the population in the same locality (apiary) was mixed,
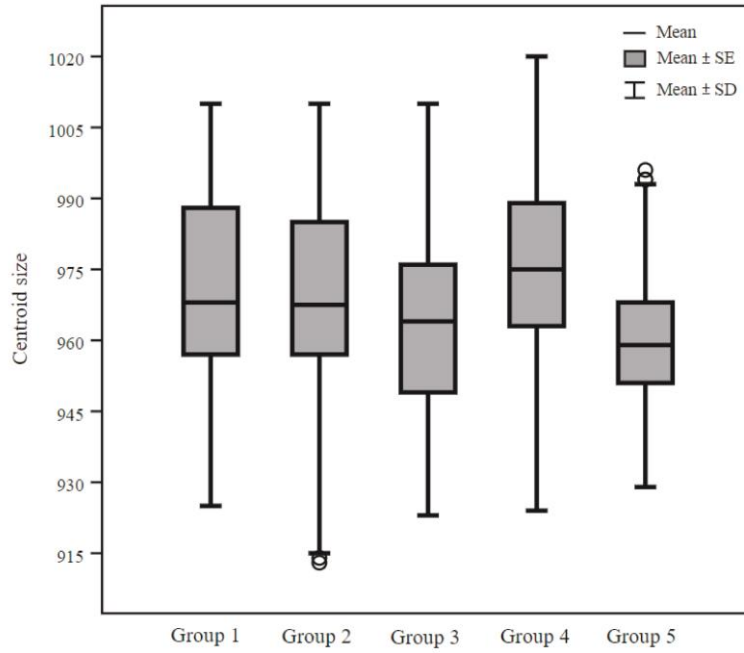
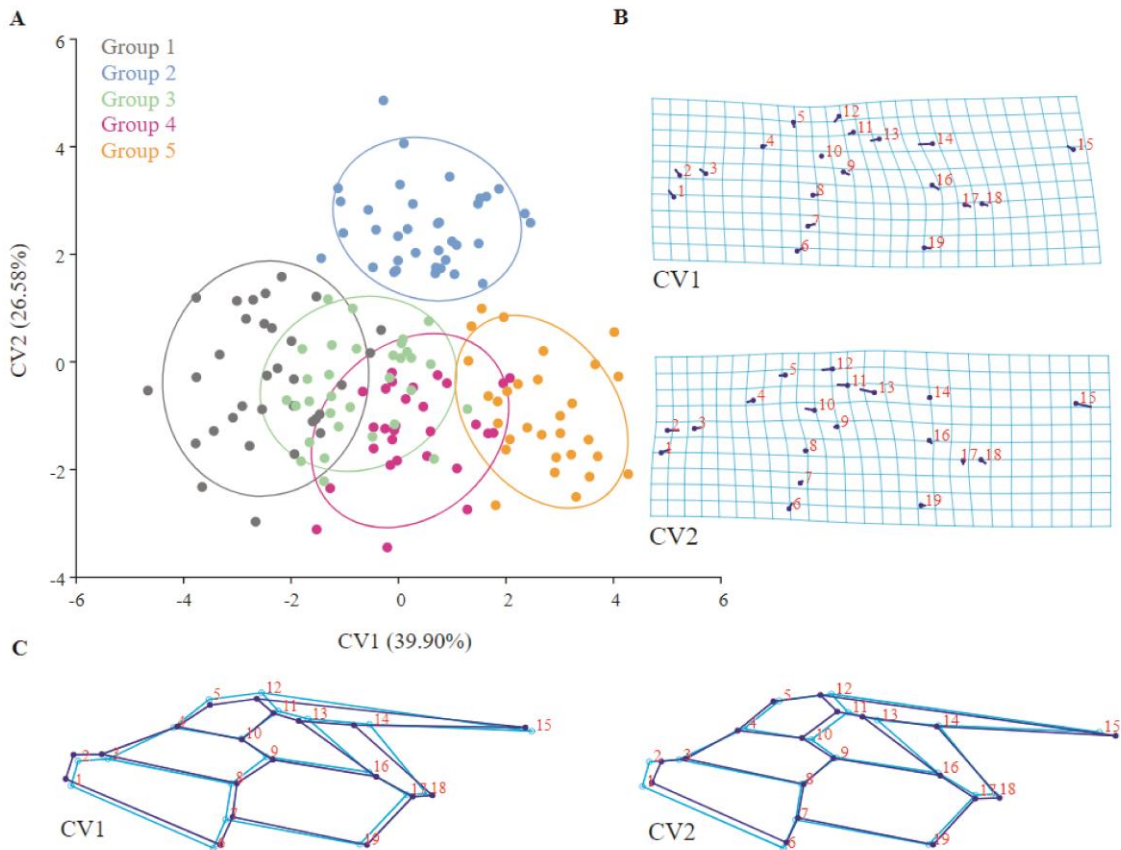**FIGURE 4.** Box plot showing mean centroid sizes among five *Apis mellifera* morphotypes.

FIGURE 5. Scatter plot of canonical variate analysis (CVA) based on wing shape of *Apis mellifera* (A). Transformation grid shows wing shape changes for each landmark based on CVA results (B). Wireframe graph depicting the average wing shape of *Apis mellifera* (C). The lines in light blue represent the average configuration and those in dark blue represent the average shape from CVA. Direction of these transformation grid and wireframe graph are positive direction (CV+).

possibly because of apiculture practices by beekeepers. Thai beekeepers usually introduced the bee queens

across apiaries for breeding programs (personal communication with beekeepers), while ancestral

TABLE 3. Mahalanobis distance between the centroid among 5 morphotypes of *Apis mellifera* in Thailand.

Group	Group 1	Group 2	Group 3	Group 4
Group 1				
Group 2	4.2967***			
Group 3	3.7683***	4.0049***		
Group 4	3.8564***	4.0602***	3.8581***	
Group 5	5.2356***	4.2111***	4.3676***	3.8189***

***$P < 0.0001$

TABLE 4. Cross-validated classification of *Apis mellifera* morphotypes based on the shape and the form (shape + size) of forewings.

Group	Accuracy (Assigned/Observed)	
	Shape	Form (shape + size)
Group 1	81.81% (27/36)	81.81% (27/33)
Group 2	91.67% (33/36)	94.44% (34/36)
Group 3	84.37% (27/32)	84.37% (27/32)
Group 4	82.76% (24/29)	89.65% (26/29)
Group 5	86.67% (26/30)	86.67% (26/30)
Total	85.63% (137/160)	87.50% (140/160)

origin of the queens was not clear. We suggest that these actions by beekeepers can contribute to hybridization of honey bees across the country. Beekeeping practices such as importation and migratory beekeeping have been found to be important factors that create an admixture population in honey bees (Bouga and Hatjina, 2005; Arias et al., 2006; Charistos et al., 2014; Kükrer et al., 2021). Moreover, queen mating (highly polyandrous) of honey bee is extremely difficult to control (Neumann et al.,1999; Palmer and Oldroyd, 2000), and this coupled with migratory beekeeping, promotes gene flow and hybridization amongst populations (Franck et al., 1998; Jensen et al., 2005; De La Rúa et al., 2009).

The wing shape of *A. mellifera* populations demonstrated 5 groups with highly significant differences in both MANOVA and Mahalanobis distance in all group pairs. The most powerful landmark to CV1 was 14 (the junction of the Rs and 2r-m veins), whereas the most powerful landmark to CV2 was 15 (the junction of the Rs). Dolati et al. (2013) reported similar results in this species, where the junction of the Rs and 2r-m veins (landmark 14) were contributed most to wing shape variation. Based on wing size, the honey bees in 5 morphotypes differed significantly in wing centroid size ($P < 0.0001$). However, wing size can be easily influenced by environmental factors such as food availability and *Varroa* infestation (Nürnberger et al., 2019). Janczyk

and Tofilski (2021) found that the wing size tends to be largest in the middle of the summer, possibly due to food availability differs during the year. Wing size is affected by the environmental factors more than shape (Barour and Baylac, 2016), and therefore, *A. mellifera* group classification in this study was mainly based on wing shape analysis. Wing centroid size was analyzed to confirm differences among groups. We found different groups of *A. mellifera* were distributed in all geographic regions (Fig. 1), presumably owing to beekeeping practices. According to *A. mellifera* is an introduced species to Thailand, the colonies were moved around during the years by beekeepers, therefore differences in both wing shape and wing size among the same group from different geographic regions were not compared. Morphological variation of *A. mellifera* populations in Thailand was more likely affected by ancestral subspecies, which resulted in different wing shape (Węgrzynowicz et al., 2019) than affected by the geographic regions. The percentage of correct classification based on wing shape into each respective group found using a cross validation test was high (85.63%), while the percentage of correctly classified specimens based on the form (wing shape + wing size) was slightly increased to 87.50%. Barour et al. (2011) reported a similar trend in both analyses, they found the percentage of correctly classified specimens based on the form (wing shape + wing size) was slightly higher than the percentage based on the

wing shape. Several authors reported that integrating other characteristics of selection such as a hind wing or number of hamuli (Barour and Baylac, 2016) and combination between contour and landmark methodologies were significant improvement of classification success (Francoy et al., 2012).

In conclusion, our study provided preliminary determination of morphological diversity of *A. mellifera* populations in Thailand and also indicated that the forewings provide necessary information and are sufficient to analyze morphological variation in the bees, as mentioned by Porini et al. (2019). Although, geometric morphometrics cannot be used to trace the ancestry and present subspecies of *A. mellifera* in Thailand, the morphometric results can give an account of its current status that there were 5 morphotypes based on wing morphology. However, to confirm whether these 5 morphotypes are related to the 5 subspecies found in Thailand or not, molecular analysis is required. Information on both morphological variation and genetic variation will complete the picture of biodiversity of this species in Thailand which will benefit beekeepers in breeding programs and the management of their bees.

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