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Morphometrics as a tool for species and localities discriminating of two *Ambulyx* species (Lepidoptera: Sphingidae)

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ABSTRACT

The hawkmoth genus *Ambulyx* is one of the complex genera in the family Sphingidae (Subfamily: Smerinthinae). *Ambulyx siamensis* and *Ambulyx pryeri* are found in many parts of Thailand. Both species exhibit very similar morphological characteristics, making identification challenging, especially for non-experts. Geometric morphometrics is an inexpensive tool that has been developed and widely applied for size and shape analyses in various fields including taxonomy and evolutionary studies. This study aimed to examine the possibility of using a geometric morphometric approach to discriminate two species of *Ambulyx* and distinguish the geographical locality of the specimens. A total of 50 pinned specimens, including 32 specimens of *A. siamensis* and 18 specimens of *A. pryeri,* from the Natural History Museum, National Science Museum, Thailand were imaged and digitized. Fourteen landmarks on the right forewing were chosen and used as the primary morphometric dataset to represent wing shape variations. A canonical variate analysis was performed to examine variation between species and localities. The results indicated a high accuracy of species identification between *A. siamensis* (83.33%) and *A. pryeri* (71.88%). Interestingly, specimens localities could potentially be specified using geometric morphometrics, with total an accuracy of 70.97% and 87.50% for *A. siamensis* and *A. pryeri*, respectively. These findings suggest that geometric morphometrics is an effective approach for determining species and localities of *A. siamensis* and *A. pryeri*, potentially supporting future studies on hawkmoths.

Keywords: *Ambulyx pryeri*, *Ambulyx siamensis*, geometric morphometrics, species identification, Sphingidae

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INTRODUCTION

Hawkmoths or Sphingidae, are one of the most attractive insect groups that are found worldwide (Chiquetto-Machado *et al*., 2018; Van *et al*., 2011). Although hawk moths are not primary pollinators for food crops, they are crucial pollinators and vital for the survival and diversity of many other flora (Martins and Johnson, 2007; Fox *et al*., 2013; Macgregor *et al*., 2014; Danaher *et al*., 2020). They also serve as food resources for many organisms (Vaughan, 1997; Young *et al*., 2017). The hawkmoths, as well as other pollinators, are threatened by excessive use of pesticides, climate change, invasive species, and habitat loss due to deforestation and human activities (Beck *et al*., 2006; Young *et al*., 2017). Moths are one of the insect groups which are sensitive to ecological stress, habitat alterations, and climate changes (Thomas, 2005; Van Dyck *et al*., 2009; Fox, 2012). Thus, monitoring their populations is important for detecting temporal changes in biodiversity, climate change, and making decisions for conservation (Jaroensutasinee *et al*., 2011; Chiquetto-Machado *et al*., 2018). To study ecology and conservation, fundamental data including taxonomy and distribution are required.

Ambulyx siamensis and *Ambulyx pryeri* are hawkmoths in the family Sphingidae (Subfamily: Smerinthinae). These two species are found in many parts of Thailand and occur broadly in the same habitat. Both species have very similar phenotypic traits and morphological identification is quite complicated. DNA barcoding is an effective genetic species identification tool. However, this approach is expensive, requires specialized skills, and specific laboratory equipment. Alternatively, wing morphology, including wing shape and wing size, has been widely used for ecological, taxonomic, and evolutionary studies in insects (Soto *et al*., 2007; Wells *et al*., 2018). Interestingly, studies on wing shape and size variations in moths are very limited, primarily focusing on intraspecific variations (Nath and Devi, 2009; Hernández-L *et al*., 2010) and are rarely conducted in Smerinthinae. Apart from interspecific variation, in Lepidoptera, wing shape and size can be related to environmental variables, dispersal ability, and predators (Hoffmann *et al*., 2002; Beck and Kitching, 2007). Many previous studies in Lepidoptera found significant regional differences in wing shape (Hernández-L *et al*., 2010; Wells *et al*., 2018).

Geometric morphometrics is a powerful, inexpensive, and comprehensive approach for examining shape and size variations (Tatsuta *et al*., 2017). This study primarily aimed to evaluate the possibility of using landmark-based geometric morphometrics to discriminate between two hawk moth species based on their wing size and wing shape, and to examine variations in wing shape across different geographical localities.

MATERIALS AND METHODS

This study used a total of 50 specimens, including 32 specimens of *Ambulyx siamensis* and 18 specimens of *Ambulyx pryeri,* from the Natural History Museum, National Science Museum, Thailand. These specimes were collected from 2001−2020. To obtain the morphological data, all specimens was imaged using Fujifilm GFX 100s, Fujinon GF 120mm f/4 Macro R LM OIS WR and Laowa 25mm F/2.8 2.5-5X Ultra Macro, on the Cognisys StackShot stacking system. Then the right front wings were used to extract data of wing shape and wing size.

Data acquisition. All specimens were imaged using a standardized template with specimen label(s) and scale bar (see Figure 1). Collection locations were manually extracted from specimen labels. Fourteen landmarks on the junctions of veins were chosen based on the certainty of these landmarks being present on every wing of both species (see Figure 2) and generally used in the study of Sphingidae (de Carmago *et al*., 2015). These landmark data were recorded as *x,y* coordinates and used as the primary morphometric dataset. This dataset was digitized using standard image processing software, tpsDig2 (Rohlf, 2005), available at https://life.bio.sunysb.edu/ee/rohlf/software.html.

Figure 1. Specimen photograph along with specimen labels and scale bar.

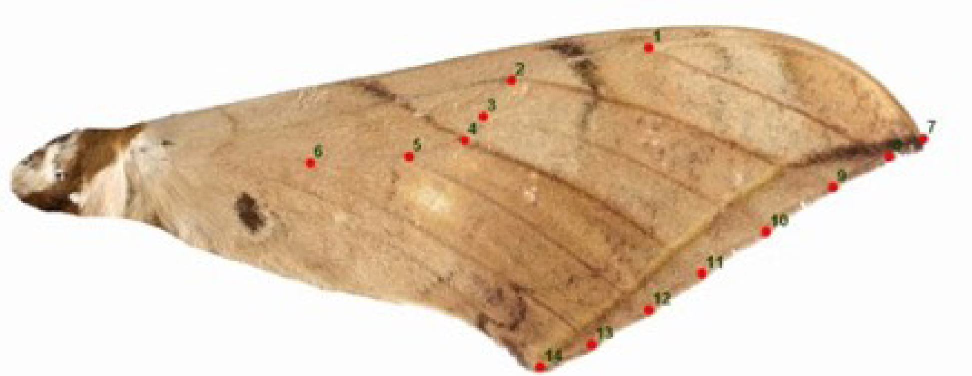

Figure 2. Fourteen landmarks on the right front wing of *Ambulyx siamensis*

Data analyses. A primary morphometric dataset for each species was processed using Procrustes Superposition to minimize shape differences caused by position, scaling, and rotation (Lele, 1999). To examine wing size of both species, the centroid size which is the square root of the sum of squared distance of all landmarks of the wing from their centroid were calculated (Zelditch *et al*., 2004). Boxplots were generated to illustrate wing centroid size of both species and wing centroid size among different localities for each species. The significant difference in mean centroid size between both species was compared using Independent Sample *t-test* (Ross and Willson, 2017), and the significant difference of centroid size among different localities for each species was examined using one-way ANOVA. Then, to reduce the dimensionality of the Procrustes coordinates to the minimum number of independent variables that needed to retain 95% of the original wing shape variability, Procrustes superposition coordinates were processed through a principal component analysis (PCA) (Hotelling, 1993; Smith, 2002).

The wing shape variation between species and among different localities within the same species was assessed using a multivariate analysis of variance (MANOVA). A canonical variate analysis (CVA) was performed to maximize the separation of species and localities. Then a bootstrapped (1,000 iteration) log-likelihood ratio test (Woolf, 1957) was performed to estimate the statistical significance of the separation between species and localities.

RESULTS

Wing centroid size variation between species and among localities. On average, the wing centroid size of *Ambulyx pryeri* and *Ambulyx siamensis* was 2691.64+-240.47 and 2282.97+-120.67, respectively (Table 1, Figure 3). The Independent sample *t-test* was highly significant for mean wing centroid size between *Ambulyx pryeri* and *Ambulyx siamensis* (t = 6.9324, *p*<0.001). For each species, one-way ANOVA indicated a non-significant difference in centroid size among localities (F = 2.381, *p* = 0.132 for *A. pryeri* and F = 0.829, *p* = 0.56 for *A. siamensis*).

Table 1. Number of specimens of two *Ambulyx* species used in the study, and the meanSD, minimum and maximum wing centroid size of each species.

Species	*n*	MeanSD	Min-Max
A. siamensis	32	2282.97+-120.67	2024.24-2530.82
A. pryeri	18	2691.64+-240.47	2420.76-3404.53

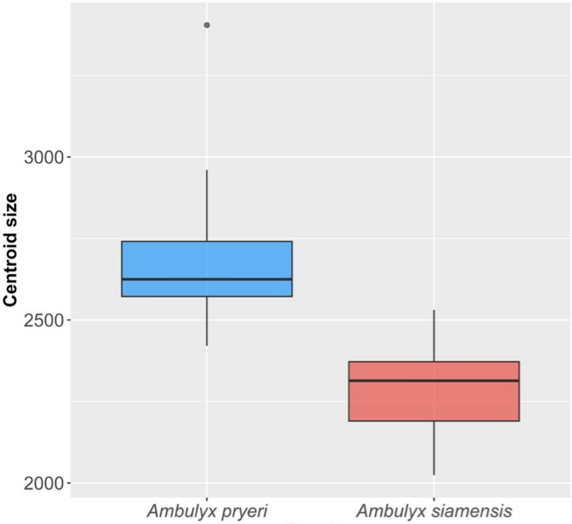

Figure 3. Centroid size of *Ambulyx pryeri* and *Ambulyx siamensis*. Independent sample *t-test* indicated significant difference in mean centroid size between *Ambulyx pryeri* and *Ambulyx siamensis* (t = 6.9324, *p*<0.001).

Wing shape variation for species discrimination. The first three principal components accounted for 27.67, 18.75, and 11.81 percent of all shape variation, respectively (Figure 4). The histogram produced by Canonical Variate Analysis (CVA) of wing shape indicated some overlapping between two species (Figure 5). However, wing shape was significantly different between both species (1000 bootstraps; $p<0.01$) (Figure 6). The overall percentage of individuals correctly assigned to their original species is 76% (Table 2).

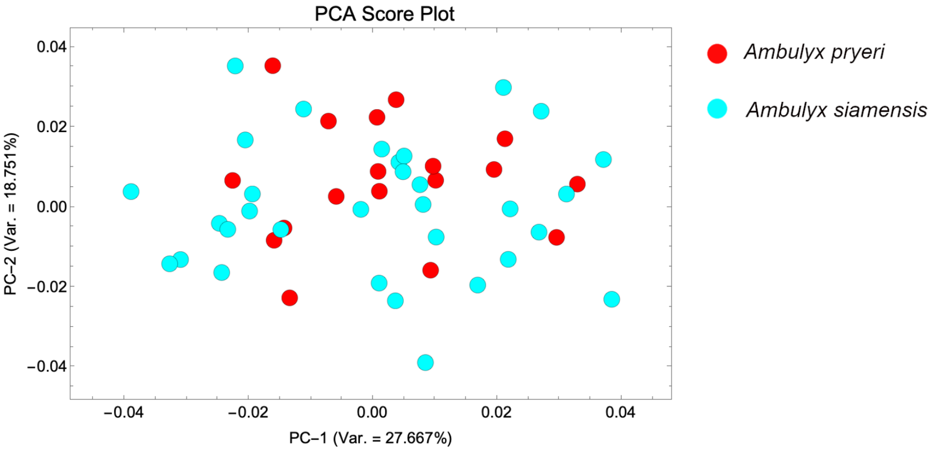

Figure 4. The first two principal component analysis (PCA) plots of wing shape variations which accounted for 27.67, 18.75 percent of overall shape variation.

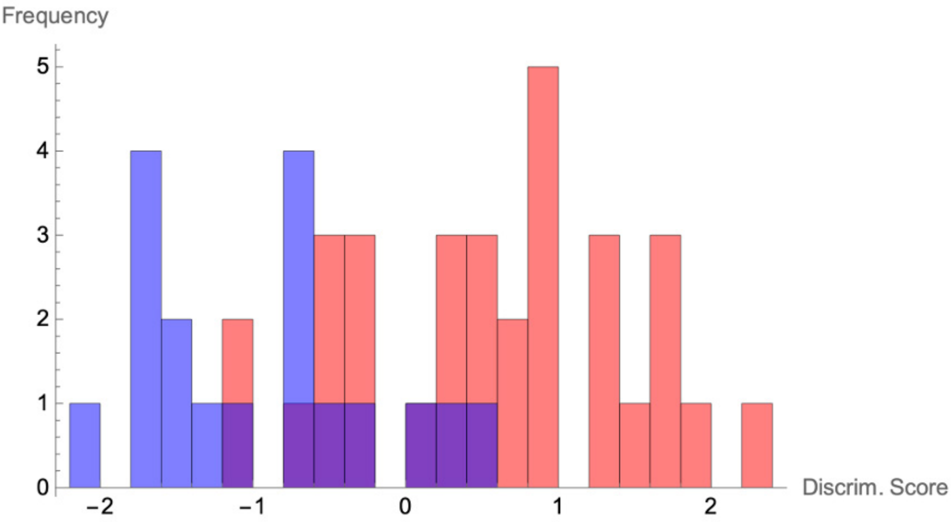

Figure 5. Histogram produced by CVA of wing shape variations of *Ambulyx pryeri* (red) and *Ambulyx siamensis* (blue). Percentage of individuals correctly assigned to their original species is 76%.

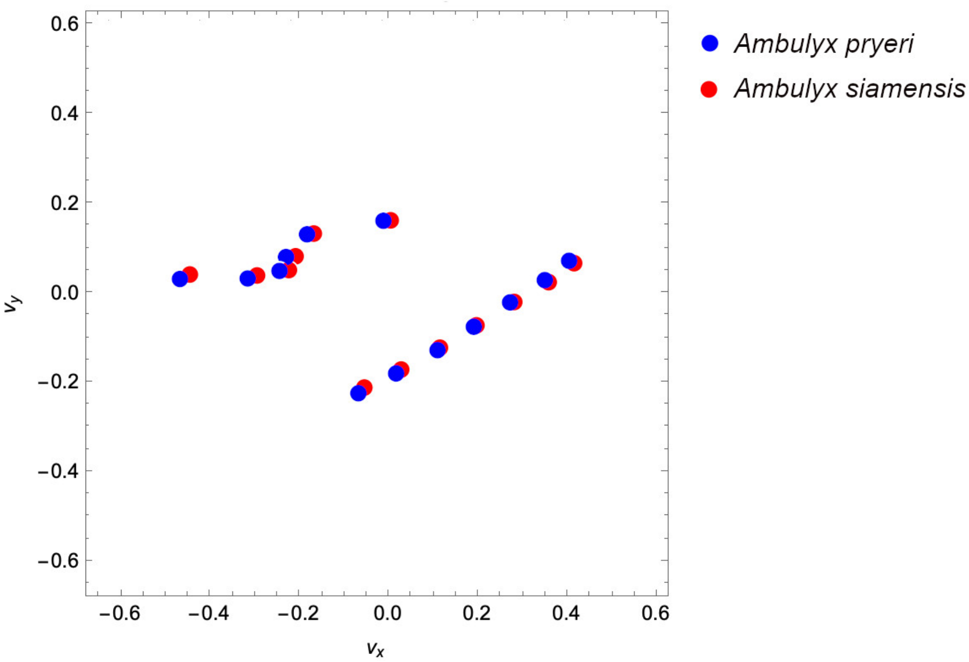

Figure 6. Mean wing shape of *Ambulyx pryeri* (blue) and *Ambulyx siamensis* (red).

Table 2. Confusion matrix of species identification.

	A. siamensis	*A. pryeri*	Total Correct	Group Totals	Percent Correct
A. siamensis	23	9	23	32	71.88
A. pryeri	3	15	15	18	83.33
Total Correct	23	15	38	50	76.00
Total Estimated	26	24	50		
Total Estimated Correctly	88.46	62.50	76.00		

Wing shape variation among localities. For *A. pryeri,* the CVA plot shows locality discrimination along CV-1, which accounted for 87.75% of wing shape variations among three different locations (Figure 7). For *A. siamensis,* the CVA plot shows more overlapping among different localities with CV-1 which contained 51.93% of wing shape variations (Figure 8). Wing shape showed a statistically significant separation among different localities in both species (1000 bootstraps; p<0.001). The total accuracy for localities discrimination is 70.97% and 87.50% for *A. siamensis* and *A. pryeri,* respectively (Table 3, 4).

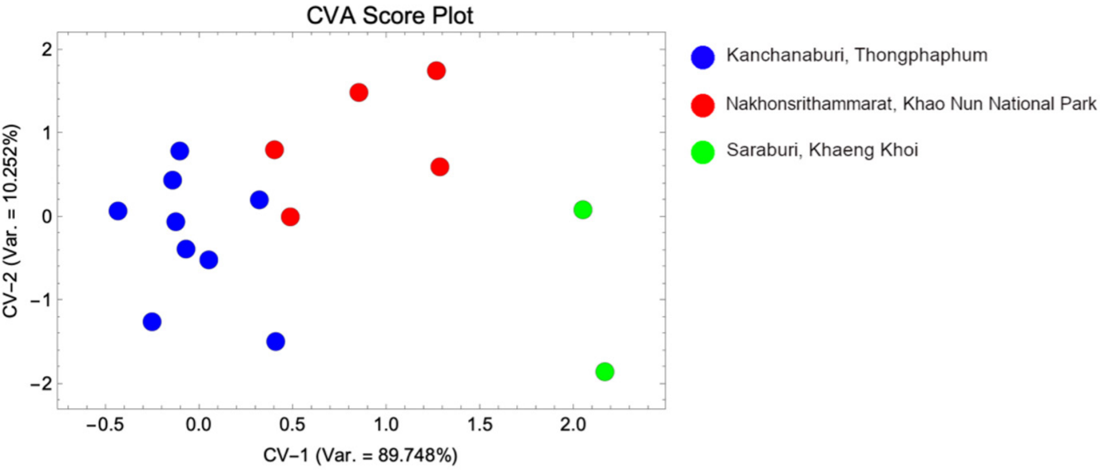

Figure 7. The CVA plot of wing shape variations of *Ambulyx pryeri* among different localities on CV-1 and CV-2. Percentage of individual correctly assigned to their original species is 87.50%.

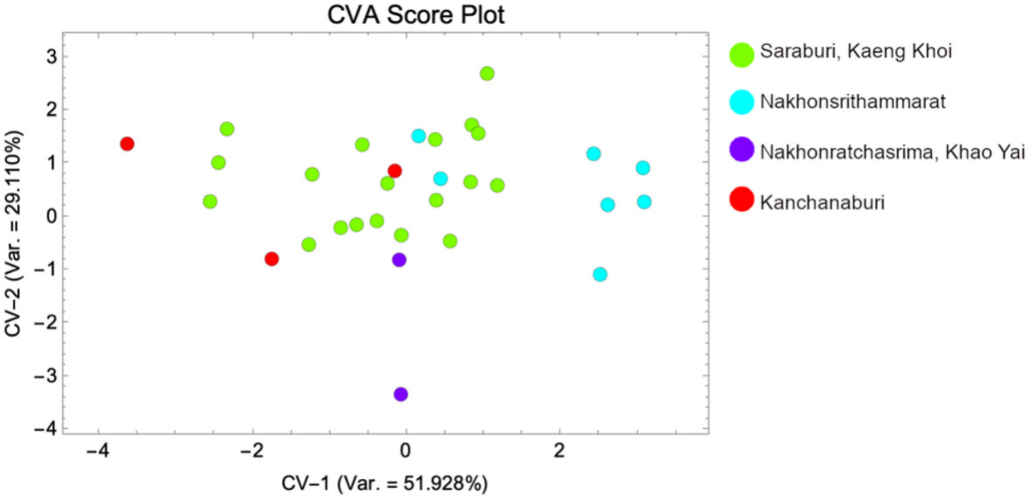

Figure 8. The CVA plot of wing shape variations of *Ambulyx siamensis* among different localities on CV-1 and CV-2. Percentage of individual correctly assigned to their original species is 70.97%.

Table 3. Confusion matrix of localities discrimination for *Ambulyx siamensis.*

	Nakhon-ratchasima, Khao Yai	Kanchanaburi	Nakhon-srithammarat	Saraburi, Kaeng Koi	Total Correct	Group Totals	Percent Correct
Nakhon Ratchasima, Khao Yai	2	0	0	0	2	2	100.00
Kanchanaburi	0	2	0	1	2	3	66.67
Nakhon Sri Thammarat	0	0	5	2	5	7	71.43
Saraburi, Kaeng Koi	0	4	2	13	13	19	68.42
Total Correct	2	2	5	13	22	31	70.97
Total Estimated	2	6	7	16	31		
Total Estimated Correctly	100.00	33.33	71.43	81.25	70.97		

Table 4. Confusion matrix of localities discrimination for *Ambulyx pryeri.*

	Kanchanaburi, Thongphaphum	Nakhonsrithammarat, Khao Nun National Park	Saraburi, Kaeng Khoi	Total Correct	Group Totals	Percent Correct
Kanchanaburi, Thongphaphum	4	1	0	4	5	80.00
Nakhon Sri Thammarat, Khao Nun National Park	1	8	0	8	9	88.89
Saraburi, Kaeng Khoi	0	0	2	2	2	100.00
Total Correct	4	8	2	14	16	87.50
Total Estimated	5	9	2	16		
Total Estimated Correctly	80	88.89	100	87.5		

DISCUSSION

Wing centroid size variation. In this study, the analysis of wing centroid size based on landmarks showed significant differences between two *Ambulyx* species. The wing size of *A. pryeri* was significantly larger than wing size of *A. siamensis*. However, wing size of specimens from different localities was not significantly different. In contrast, a previous study on microlepidoptera indicated wing size-altitude correlation (Sullivan, 2007).

Wing shape variation. We found significant wing shape variations between *A. siamensis* and *A. pryeri.* These findings indicated that both species can be determined using geometric morphometrics based on selected landmarks on their wings. We predicted that the difference in wing shape between these two species was as a result of different life histories and the dispersal ability of species. Previous studies of Lepidoptera (Betts and Wootton, 1988) indicated that dispersal ability contributes to wing shape variations. However, biological and life history data for these two species are required and need further examination to clarify wing shape difference between species.

In both species, wing shape variations among different localities were detected. We assumed that these variations were probably a result of population adaptation to environmental conditions and landscapes. Previous studies of Lepidoptera (Benitez and Vargas, 2017; Wells *et al*., 2018) and other insects (Alves *et al*., 2016; Onder and Aksoy, 2022) suggested that wing shape differences among regions are an adaptive trait related to altitude, local environmental variables or host plant (Benitez and Vargas, 2017). Thus, further studies are needed to clarify the influences of environments factors, distance, and host plant on wing shape variations

In conclusion, overall results highlighted that geometric morphometrics could potentially be used for species determination and locality identification for *A. siamensis* and *A. pryeri.* For further studies, we recommend research with greater sample size that could improve the percentage of total correctness for wing shape determination between species and among localities. Furthermore, more specimens from more localities with environmental data could enlighten the influences of environmental variables on wing shape variations.

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