The Significance of Wing and Body Morphometry in Discriminating Six Species of *Eurema* Butterflies (Lepidoptera: Pieridae) of Peninsular Malaysia

(Kepentingan Morfometri Sayap dan Badan dalam Membezakan Enam Spesies Rama-rama *Eurema* di Semenanjung Malaysia)

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ABSTRACT

Six species of Eurema *butterflies comprised of 261 adult individuals collected from various sampling sites in Peninsular Malaysia were identified. The morphometric measurement of the specimens was carried out by using seven characters measured from the wing and body regions. Morphometric analysis was determined through the multivariate analysis of Discriminant Function Analysis (DFA) and Cluster Analysis. The results of DFA showed that two out of seven characters utilized have the lowest values of Partial's Wilks Lambda; basal length and hindwing length. These characters therefore are significant for the discrimination of the* Eurema *species. The classification matrix shows that both* E. hecabe *and* E. blanda *were completely discriminated by the characters set. Moreover, there was great overlaps occurred between the species groups when plotting a conical graph except for species* E. blanda *that formed homogenous group. Basal length was also suggested as the species-specific character for grouping the species* E. blanda. *In Cluster Analysis,* E. hecabe *tends to be closer to* E. sari *with the lowest value of agglomeration. The results from dendogram generated from average linkage also showed several relationship patterns among the six species.*

Keywords: Discrimination; Eurema; *morphometric; multivariate analysis*

ABSTRAK

Enam spesies rama-rama Eurema *telah dikenal pasti daripada 261 individu dewasa yang ditangkap dari pelbagai lokasi di Semenanjung Malaysia. Kajian morfometri telah dijalankan dengan menggunakan tujuh ukuran sifat daripada bahagian sayap dan badan. Analisis morfometri ditentukan berdasarkan analisis multivariat iaitu Analisis Diskriminasi Fungsi (DFA) dan Analisis Kelompok. Keputusan DFA merungkaikan dua daripada tujuh sifat yang digunakan mempunyai nilai Partial's Wilks Lambda yang terendah; panjang basal dan panjang kepak belakang. Justeru, sifat ini bermanfaat untuk membezakan antara spesies* Eurema. *Klasifikasi matriks pula menunjukkan kedua-dua* E. hecabe *dan* E. andersonii *sepenuhnya didiskriminasikan oleh set sifat. Seterusnya, terdapat pertindihan yang ketara berlaku antara spesies dalam analisis pemboleh ubah konikal kecuali bagi spesies* E. blanda *yang dilihat membentuk kelompok homogen. Panjang basal juga dicadangkan sebagai spesies khusus sifat untuk pengelasan spesies* E. blanda. *Analisis Kelompok pula menunjukkan* E. hecabe *lebih cenderung rapat dengan* E. sari *dan mempunyai nilai jadual aglomerasi yang terendah. Keputusan daripada dendogram juga menunjukkan beberapa corak hubungan antara enam spesies dalam kajian ini.*

Kata kunci: Analisis multivariat; diskriminasi; Eurema; *morfometri*

INTRODUCTION

The butterflies of the genus *Eurema* are classified under family Pieridae and typically recognized by the bright to pale yellow lemon coloured bordered with black margin on the apical site of forewings. The *Eurema* butterflies in Malaysia were discovered by Yata in 1989. Since their discovery, there were nine species recorded in Malaysia which were identified using taxonomic keys developed by Corbet and Pendlebury (1992) that relied on the morphological characteristics of butterfly's wings including the pattern, structure and colouration.

Even though this genus was developed well taxonomic keys, however, the *Eurema* is still one of the most burdensome species for taxonomists in doing identification and classification due to its cryptic species complexes. Cryptic species complexes are defined as assemblages of closely related species that have been classified as one broadly delimited species due to the difficulty of identifying the species on the basis of visible phenotype (Bickford et al. 2006; Collins & Paskewitz 1996). Such species complexes present a worst case challenge for the use of morphological characteristics in species identification. Species identification was difficult due to high intra-specific variation of form and colour (Heim 2003). This problem sometimes may leads to the misidentification of the species.

Comparative morphological analysis is one of the useful tools for the separation of problematic groups of insects. Numerical taxonomy or known as morphometric have been used to assist in identifying many insect species (Baylac et al. 2003; Daly 1985). In insect study, the use of morphometric technique relies mainly on variation in size and shape of the insects (Daly 1985). Moreover, wing morphology has historically been a major focus in taxonomic and evolutionary studies of Lepidopterans.

The uses of statistical techniques are nowadays routinely applied to the morphometric study. The multivariate analysis of the butterflies' body parts for instance has shown a significant element for resolving the complex taxonomy. A combination of morphometric studies and multivariate analysis has been used in the last few years and is growing in importance as an approach (Hernández-Roldán & Munguira 2008; Prieto et al. 2008; Simonsen 2006; Wakeham-Dawson et al. 2004). The product from numerical taxonomy is often taken to be unbiased indicators of the similarity or difference between the taxa, which were in turn used to arrange taxa in hierarchy (Quike 1993).

In the study of *Eurema* butterflies, though, attempts had been made to separate the *Eurema* species by using morphological characteristics and observation on male genetalia (Corbet & Pendlebury 1992; Jeratthitikul et al. 2009; Yata 1989), but no morphometric techniques have been applied to investigate the status of the group. Thus, the present work was designed to investigate the possible morphological differences using the morphometric characters from wing and body region to determine the significant characters for discriminating the *Eurema* species of Peninsular Malaysia. Moreover, data from multivariate analyses were used to determine the relationship pattern between the species members in order to clarify the relationship status and exact placement of species within taxa to produce strong and well supported classification.

Materials and Methods

Sampling Sites

The samples were collected at various sites comprising of four main areas which were North, West, South and East of Peninsular Malaysia. Samplings to obtain as many individuals as possible were conducted at several sites that were chosen to maximize the geological and ecological coverage of the area since the distribution and abundance of *Eurema* species is almost unpredictable and irregular. The distribution of various sampling sites is shown in Figure 1. Sampling was done around the forest reserves and recreational forests since disturbance of the forest environment is minimal, thus ensuring efficient collection of samples. Moreover, the vegetations here are mostly abundant and provide many host-plants for butterflies as well as flowering plants. Sampling was also conducted along the periphery of the forests since the studied butterflies can be found abundantly in opened areas especially those with direct exposure to sunlight.

Sample Collection and Storage

Sampling is preferably refrained when weather is rainy or relatively cloudy as the butterflies are only active during hot sunny days. The time for sampling ranged from dawn until just before dusk because studied butterflies are only active at day time. Opportunistic sampling strategy was employed to capture as many available butterflies that were sighted, without any quantitative method of sampling. Method of butterfly collection is based on Orr (2003). Butterflies were caught using insect sweep net throughout the sampling sites. Caught butterfly was grasped by its body and stunned by pinching the thorax. Then, the sample was placed temporarily in the insect envelope by folding the wings together above the body to avoid damaging their fragile scaly wings. All the information of collected sample was written on the opened flap of the envelope for recording purposes.

After that, the collected sample were preserved before it can be stored permanently. The best way to preserve butterfly is by pinning as it will retain the normal appearance of butterfly especially for the use in morphometic study. The forewings were spread forward until the dorsum of inner margin is perpendicular to the thorax. The hindwings were spread backward until the position is in right angle normally about 45° towards the abdomen. The antenna was arranged in position parallel to the costa of forewing and held by cross-pin. The abdomen also was crossed-pin to hold it in a central horizontal position.

Then, the pinned butterflies were dried up to remove the excess water body content. When drying was completed, the samples were transferred into the insect storage box. A proper label contains all needed information was placed on each sample. The specimens later were being identified to its respective species based on morphological characteristics on wings with reference from classification keys derived by Corbet and Pendlebury (1992). All the voucher specimens later were deposited in the Museum of Zoology, University of Malaya.

Morphometric Measurement

Morphometric measurements were taken for seven quantitative characters representing the wings and body regions. The characters used are wingspan (WS), body length (BL), forewing width (FW), forewing length (FL), hindwing length (HL), hindwing width (HW) and basal length (SL). All measurements were taken using a manual calliper in millimeter (mm) unit and were measured to the nearest 0.5 mm. The details of the characters used in morphometric measurement are given in Figure 2. Except for the damaged samples, the measurements were made on the left side of both forewing and hindwing of each sample to avoid the problem of asymmetry.
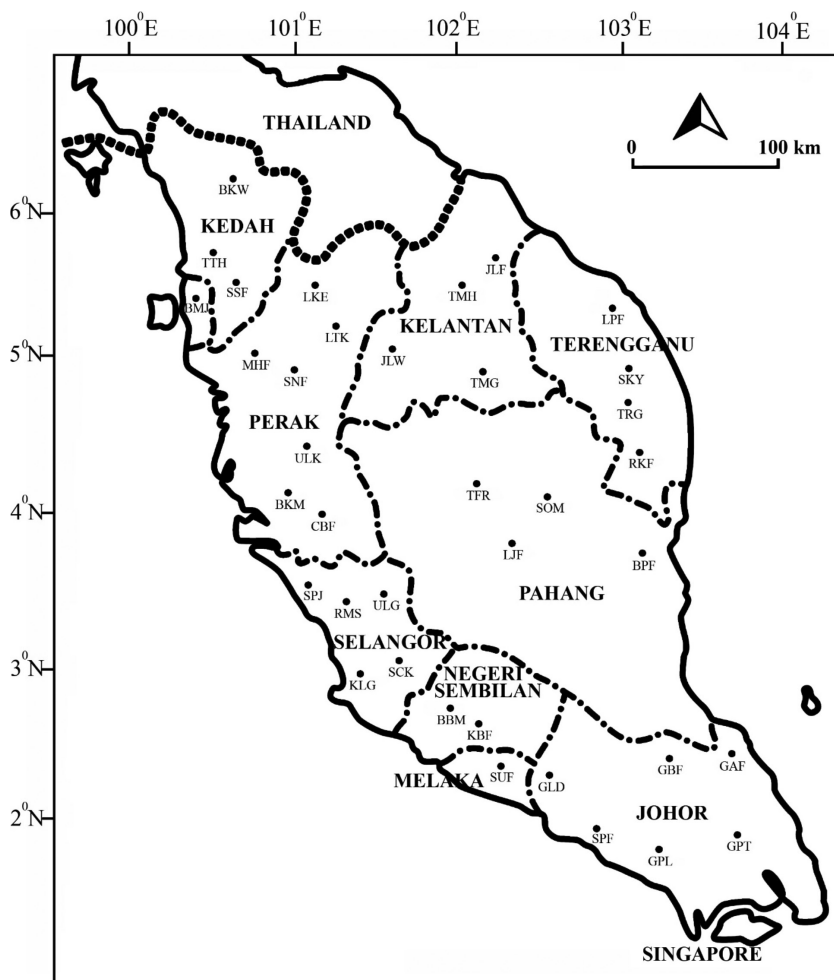
FIGURE 1. The distribution of various sites (indicated by black dot) where the samplings have been conducted in Peninsular Malaysia. Triplet letter represent the site code (not given)
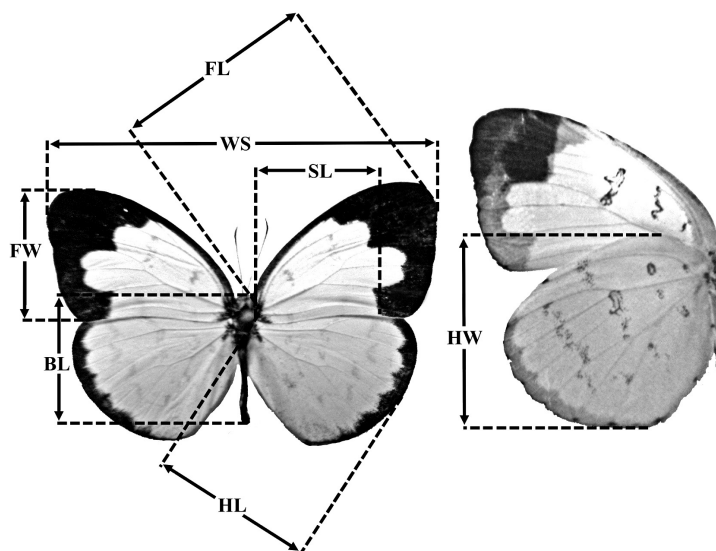

FIGURE 2. Adult *Eurema* butterfly showed the measurements of morphometric characters used. WS - Wingspan: the distance measured across a butterfly's wings; BL - Body length: the distance from tip of the head to the end of the abdomen; FL - Forewing length: the distance from base of forewing articulation to tip of wing; FW - Forewing width: the greatest width of forewing; SL - Basal length: the distance from the base of forewing articulation along dorsum to the end of the black apical border marking; HW - Hindwing width: the greatest width of hind wing; HL - Hindwing length: the distance from articulation part until the base of the wing

STATISTICAL ANALYSIS

The analysis of morphometric data was conducted using the SPSS 16.0 (SPSS Inc. 1999) and STATISTICA 7.0 (StatSoft Inc. 2004) software package. Univariate analyses were initially used to estimate means, standard deviations, maximum and minimum values. The range of each morphometric character was compared between the species with box-plot. Then, multivariate analysis was carried out for Discriminant Function Analysis (DFA) and Cluster Analysis.

DFA was conducted to test the capacity to discriminate the species by the selected characters. The characters were selected with the Partial Wilks' Lambda statistic which measures how each function separates cases into groups. Smaller value in Partial Wilks' Lambda indicates the greater discriminatory ability of the function, thus being as the most significant character for discriminating the species (Simões & Quartau 2009). Moreover, scatterplots were also generated from the DFA conical variables data to investigate the pattern of species grouping based on the measured characters.

In Cluster Analysis, agglomeration schedule was generated using average linkage with the variables related to the measured characters. This matrix was studied with the dendogram which allows the determination of relationship among the species according to the pattern shown by the variables.

RESULTS

A total of 261 adult individuals were studied. From the total individuals, there were six *Eurema* species have been identified and examined morphologically. The species list and corresponding morphometric characters employed for discrimination of the species with their means and standard deviations are given in Table 1. The box-plot comparing the range of each morphometric character by the species is shown in Figure 3. From Figure 3, the value of each character showed little differences among the species members. However, the result showed that *E. simulatrix* was the biggest species in this study which dominated the highest average values for all characters except for basal

length character. In contrast, *E. ada* was the smallest species in this study which dominated the lowest average values for all characters. The highest average value for basal length character however was dominated by the species *E. blanda*.

DISCRIMINANT FUNCTION ANALYSIS

The DFA shows that basal length and hindwing length were two characters with the lowest Partial Wilks' lambda values (0.306578 and 0.682419, respectively) (Table 2) and therefore being as the best characters for discriminating of six *Eurema* species in this study. Only the first three roots were considered from the 10 canonical roots generated since the successive roots have reduced discriminatory power. The first, second and third roots of the conical variables extracted eigenvalues of 4.35930, 0.900400 and 0.64719, respectively (Table 3) and were marked by the most negative coefficients value for basal length in Root 1 (-1.93727) and in Root 2 (-0.932741) while hindwing length in Root 3 (-2.64953).

The scatterplot of Root 1 against Root 2 (Figure 4(a)) shows the formation of six groups corresponding to the six *Eurema* species used in this study. However, each group shows overlapping pattern between each other. Group consists of *E. blanda* tends to be clustered separately and forming a homogenous group with few overlapping with the species *E. hecabe* at periphery side. The group consists of species *E. andersonii* clustered on the right side of the graph but still shows great overlapping with several species of *E. sari, E. hecabe* and *E. ada*. Moreover, species of *E. simulatrix* forms a nearly distinct group in the up-right quadrant but overlapping with a few other species.

In contrast, when Root 1 was plotted against Root 3 (Figure 4(b)), instead of a clear group formed by species *E. blanda*, the graph also showed that species of *E. ada* and *E. hecabe* have reduced the overlapping and forming nearly distinct groups on the bottom part of the graph. The other species however highly mixed with each other in both graphs forming undefined groups.

From a total specimen used, overall 87.65% were correctly attributed into their respective species (Table 4).

TABLE 1. The mean (left) and standard deviation (right) of seven morphometric characters representing major body region used in this study. WS - Wingspan; BL - Body length; FL - Forewing length; FW - Forewing width; HL - Hindwing length; HW - Hindwing width; SL - Basal length

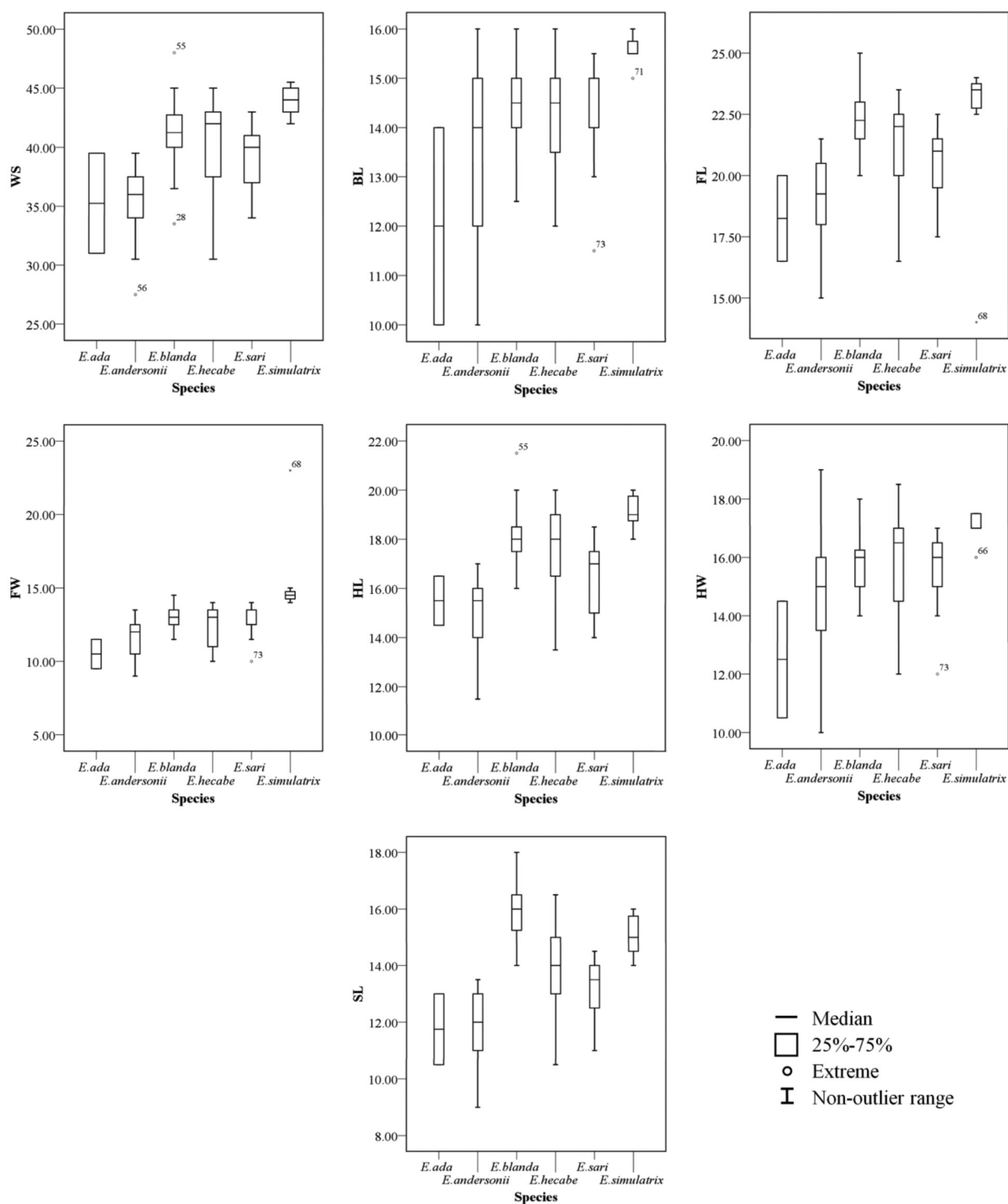| Species	Morphometric measurement (mm)						
	WS	BL	FL	FW	HL	HW	SL
E. hecabe (*n*= 39)	40.11/±4.70	14.17/±1.44	21.11/±2.29	12.39/±1.45	17.61/±2.07	15.94/±2.08	13.89/±1.90
E. blanda (*n*= 58)	41.23/±2.88	14.55/±0.72	22.20/±1.19	13.05/±1.45	18.09/±1.12	15.82/±0.95	16.05/±1.07
E. andersonii (*n*= 56)	35.60/±2.86	13.69/±1.62	19.23/±1.68	11.62/±1.05	15.19/±1.41	14.46/±1.92	11.85/±1.19
E. simulatrix (*n*=37)	43.93/±1.30	15.57/±0.35	22.07/±3.60	15.64/±3.26	19.14/±0.75	17.07/±0.53	15.07/±0.79
E. sari (*n*=39)	39.22/±2.87	14.28/±1.33	20.56/±1.61	12.56/±1.21	16.56/±1.59	15.39/±1.62	13.17/±1.22
E. ada (*n*=32)	35.25/±6.01	12.00/±2.83	18.25/±2.47	10.50/±1.41	15.50/±1.41	12.50/±2.83	11.75/±1.77

FIGURE 3. Box-plot comparing the measurement of seven morphometric characters representing major body region for each species of *Eurema* from a total of 261 individuals. WS - Wingspan; BL - Body length; FL - Forewing length; FW - Forewing width; HL – Hindwing length; HW - Hindwing width and SL - Basal length. Non-outlier range is a value below the upper outlier limit and above the lower limit; Outlier is a value outside 1/5 box length range from upper and lower value of the box; Extreme is a value outside of 3 box length range from upper and lower value of the box

TABLE 2. Summary for the Discriminant Function Analysis of the measurement of seven morphometric characters of the *Eurema* species. WS - Wingspan; BL - Body length; FL - Forewing length; FW - Forewing width; HL - Hindwing length; HW - Hindwing width; SL - Basal length

$n = 261$	Wil k's Lambda	Partial Wilks' Lambda	F-remove (5,69)	p-level	Toler.	1-Toler. (R-sqr.)
WS	0.060668	0.858660	2.27155	0.056826	0.133958	0.866042
BL	0.054929	0.948361	0.75143	0.587879	0.201625	0.798375
FL	0.069829	0.746004	4.69857	0.000946	0.077974	0.922026
FW	0.075984	0.685577	6.32903	0.000069	0.149036	0.850964
HL	0.076336	0.682419*	6.42220	0.000059	0.084839	0.915161
HW	0.061405	0.848346	2.46694	0.040868	0.185070	0.814930
SL	0.169917	0.306578*	31.21305	0.000000	0.200075	0.799925

*The two lowest value of Partial Wilks' Lambda indicate greater discriminatory ability of the character and were selected as the best characters for discriminating of *Eurema* butterflies

TABLE 3. Standardized coefficients for canonical variables of Root 1, Root 2 and Root 3. WS - Wingspan; BL - Body length; FL - Forewing length; FW - Forewing width; HL - Hindwing length; HW - Hindwing width; SL - Basal length

Characters	Root 1	Root 2	Root 3
WS	0.78726	0.586534	-0.57172
BL	0.18136	-0.670058	0.00819
FL	0.03328	-0.400673	2.84373
FW	0.03118	0.547080	2.21720
HL	-0.15330	1.352508	-2.64953*
HW	0.65947	0.143713	-0.71873
SL	-1.93727*	-0.932741*	0.02957
Eigenvalue	4.35930	0.900400	0.64719
Cum. Prop	0.72093	0.869830	0.97686

*The lowest coefficient value indicating the domination of the character for each root

Both *E. blanda* and *E. andersonii* were 100% correctly discriminated into their group while other species were 85.71% for *E. simulatrix*, 77.78% for *E. hecabe*, 50.00% for *E. ada* and 33.33% for *E. sari*.

CLUSTER ANALYSIS

Table 5 shows average linkage between the groups on agglomeration schedule. The cluster that exists between species 1 (*E. hecabe*) and species 5 (*E. sari*) is having the lowest coefficient value (3.059) whereas that which exists between species 5 (*E. sari*) and species 6 (*E. ada*) is having the highest coefficient value (79.556) showing great degree of variation in their morphometry. Dendogram generated (Figure 5) from average linkage has grouped six *Eurema* species into two distinct clusters; cluster-I consists of species *E.hecabe*, *E. sari*, *E. blanda* and *E. simulatrix* while cluster-II consists of *E. andersonii* and *E. ada*.

DISCUSSION

Although the wide samplings have been applied, but only six *Eurema* species were identified from the total collected samples in this study. Some of the reported *Eurema* species from the previous study by Corbet and Pendlebury (1992) were not found. The species includes species of *E. lacteola*, *E. brigitta* and *E. tilaha*. All these three species were considered as rare or least commonly encountered species by this study because of their difficulty to find or capture during field sampling, thus were not included in this morphometric study.

The methods of numerical taxonomy have been widely used in classifying many species as well as interpreting results of taxonomic studies (Sneath & Sokal 1973). Morphometric analysis adds a quantitative element to descriptions, allowing more rigorous comparisons (Soladoye et al. 2010). Even though this study used only a traditional morphometric analysis which will be less powerful analysis as compared to geometric morphometric analysis, but this method was seem sufficiently evident in the first approach.

From this study, the box-plot indicates that all the morphometric characters used were not influenced by the geographical factors of sampling sites. Even though the individuals were collected from the wide range of locations, the wing and body size for individuals of the same species were almost consistent. This finding proved that all the characters used have no effect toward the different geographical areas, therefore can ideally and confidently being used in discrimination of the *Eurema* species.
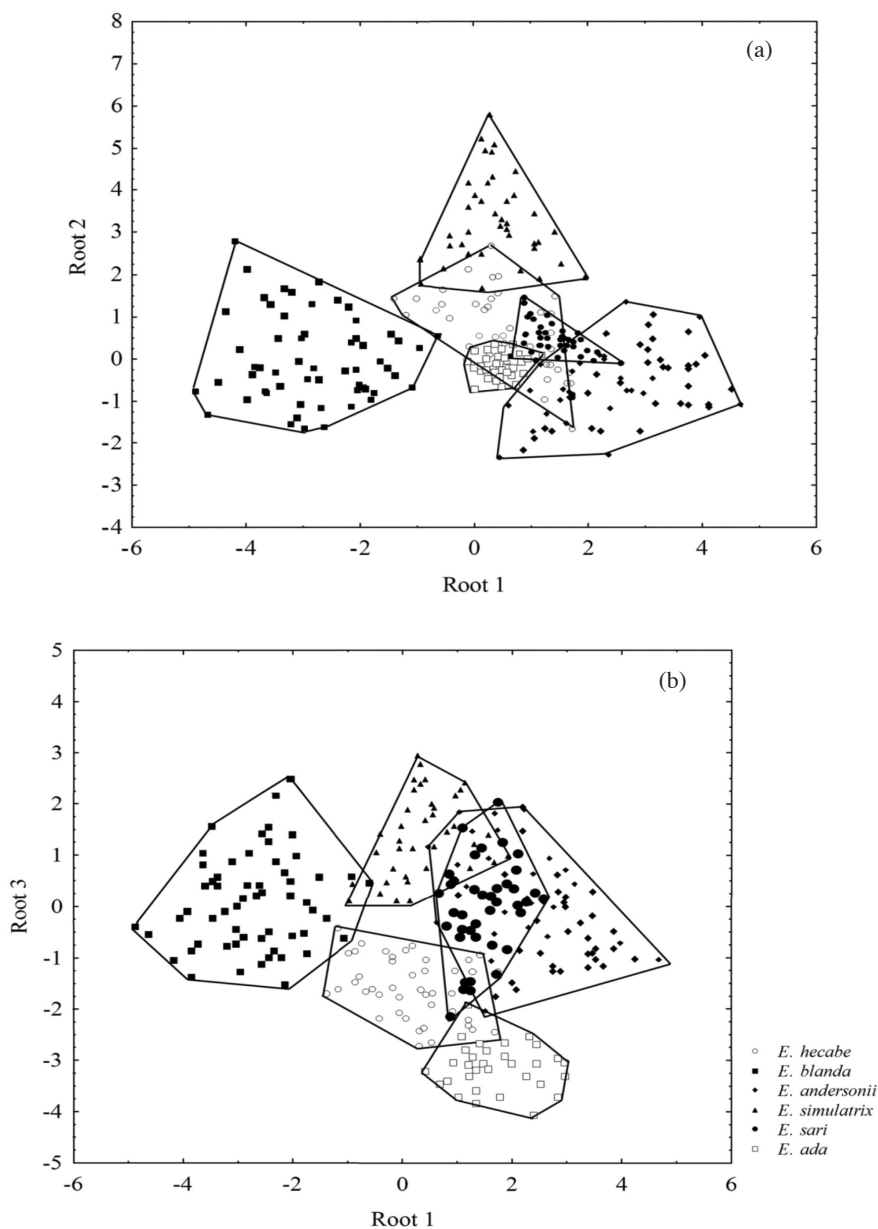
FIGURE 4. (a) Scatterplot of the Root 1 and Root 2 and (b) scatterplot of Root 1 and Root 3
of DFA for seven morphometric characters of 261 specimens

TABLE 4. Classification matrix for Discriminant Function Analysis of the measurement of seven morphometric characters of
Eurema species. Rows: observed classification, Columns: predicted classification. [1] - *E. hecabe*;
[2]- *E. blanda*; [3]- *E. simulatrix*; [4] - *E. andersonii*; [5] - *E. sari*; [6] - *E. ada*

Species	% of correct	[1] $p=0.1111$	[2] $p=0.3457$	[4] $p=0.3210$	[3] $p=0.0864$	[5] $p=0.1111$	[6] $p=0.0247$	TOTAL
[1]	77.78	31	4	4	0	0	0	39
[2]	100.00	0	58	0	0	0	0	58
[3]	85.71	0	0	0	32	5	0	37
[4]	100.00	0	0	56	0	0	0	56
[5]	33.33	4	0	22	0	13	0	39
[6]	50.00	16	0	0	0	0	16	32
TOTAL	87.65	51	62	82	32	18	16	261

TABLE 5. Agglomeration schedule of average linkage between the groups.[1] - *E. hecabe*;   [2]- *E. blanda*;
[3]- *E. simulatrix*; [4] - *E. andersonii*; [5] - *E. sari*; [6] - *E. ada*

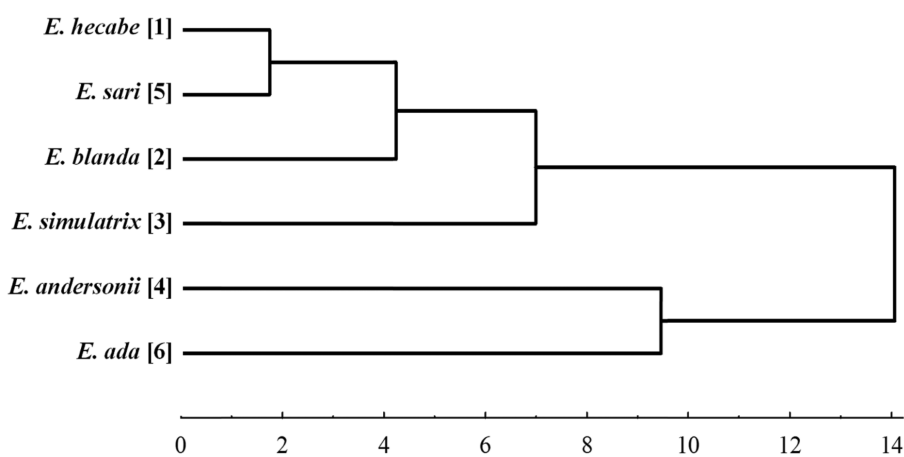| Stage | Cluster combined | | Coefficients | Stage cluster first appears | | Next stage |
	Cluster 1	Cluster 2		Cluster 1	Cluster 2	
1	[1]	[5]	3.059	0	0	3
2	[2]	[3]	9.141	0	0	5
3	[4]	[6]	12.898	0	1	4
4	[2]	[4]	33.477	3	0	5
5	[5]	[6]	79.556	2	4	0

FIGURE 5. Dendogram showing the relationship of six *Eurema* species based on seven morphometric characters

From multivariate analyses, they were significant variations among the studied morphometric characters that could be used taxonomically in discriminating of *Eurema* species in this study. From seven characters used, basal length has the lowest value of Partial Wilks' Lambda affirming their usefulness for discrimination purpose. This character therefore was the most effective and useful to be employed in the taxonomic treatment of the other *Eurema* species that were not included in this analysis.

Overall, from total individuals used, more than 80% were correctly attributed into their respective species with species of *E. blanda* and *E. andersonii* were fully discriminated. The remaining species were not classified correctly into their respective species indicating of the presence of similarities on the morphometric characters. Meanwhile, fully discriminated shows that the species has distinct morphometric characters make them differ from the others.

E. blanda was the only *Eurema* species that has a reduced pattern of black apical border towards the basal part which corresponding to the basal length character. This variant has made *E. blanda* differs in appearance from other *Eurema* species. This was probably the reason for *E. blanda* to be fully discriminated into their species in this analysis. On the other hand, *E. andersonii* was fully

discriminated probably because among the individuals of this species, they have uniform size of wing and body corresponding to the characters used to make them have consistent measurement, thus to be fully discriminated by the analysis.

The scatterplot analysis of Root 1 against Root 2 was able to discriminate the species *E. blanda* by forming a homogenous group. Both roots were dominated by the basal length character which depends on the wing pattern. This can be related with the variant possessed by *E. blanda* as discussed earlier; has a reduced pattern of black apical border on basal part. The variant therefore was a clear evident for *E. blanda* forming a homogenous group. Thus, instead of being as the best character for discriminating of *Eurema* species in this study, basal length character was also suggested as a species-specific character for discriminating of *E. blanda*.

As comparison, the scatterplot analysis of Root 1 against Root 3 was successfully separated the species of *E. ada* and *E. hecabe* into distinct groups (Figure 4(b)) in which, in Figure 4(a), they appeared to hardly mixed with each other. Root 3 was dominated by the hindwing length character that relates to the wing size. This showed that, both species have a uniform size of hindwing which makes them differ from other *Eurema* species, therefore

can be used in discriminating those species. The other species however did not form any clear group on both graphs maybe because the species mostly have overlapped measurement of characters caused DFA unable to separate them into the different groups.

In Cluster Analysis, the lowest value of agglomeration indicates the clusters have shared high similar morphometric characters, thus have close relation with each other. Those with similar morphometric character will be grouped together by the variables used in the analysis. From the result, *E. hecabe* and *E. sari* species are closely related. Morphologically, *E. sari* was obviously differs from *E. hecabe* by possessing the entirely darken brown patch which is species-unique characteristic. But, through morphometric analysis, both species suggested to share similar characters which makes them clustered together. However, the shared character was undetermined from the analysis.

Moreover, the high similarities in morphological appearance between *Eurema* species sometimes may lead to the misidentification of the species for several individuals due to the difficulties of being recognized. This also suggested that the overlapping pattern found in DFA was probably due to the specimens being misidentified morphologically. Even though the normal and standard identification procedures were practiced, but the results still ended up in some identification error especially when identification rely solely on morphological component.

Even though identification of *Eurema* species was made confidently by using taxonomic keys (Corbet & Pendlebury 1992), but still results in species misidentification. Through morphometric study of wing and body region, the results can help in showing any misidentification of the species done morphologically. In this study, the species that were not correctly attributed by the morphometric analysis were being re-identified and surprisingly, some of the individuals that re-identified were not belong to the species that initially being identified. This suggests that the use of morphometry of wing and body region has high significant in detecting any species misidentification through morphology to ensure an accurate classification. Overall, from this case of study, correct assignment of *Eurema* species can confidently made and verified based on the morphological characteristics with the aids of morphometric analysis of wing and body region. It is also important to note that morphometric alone is not good enough in discriminating the species thought it was benefited the systematic. Further studies are therefore recommended especially on the molecular data in order to provide strong and clear evidences on the correct placement and relationship status of the taxa.

From the present study, we have done the preliminary work on phylogeny of *Eurema* species using mtDNA CO1 and rDNA 28S molecular sequences. The data showed that species that was initially identified by morphology with the aid of morphometric analyses has forming the monophyletic group representing their respective species. Thus, in order to show any doubts on the classification made solely through morphological study, morphometric and molecular data should be investigated together in which each component has its own significant in showing any changes that exist in classification and placement of the species within the genus.

CONCLUSION

In conclusion, morphometric analysis of seven characters for six *Eurema* species showed that variations in wing and body size are significant, important and diagnostic that can be used taxonomically in discrimination of these taxa. Among the characters utilized, basal length and hindwing were suggested as the best characters that can be employed in discrimination of *Eurema* species of Peninsular Malaysia. Furthermore, basal length was also a species-specific character for discriminating of *E. blanda* from other species. From morphometric study, *E. hecabe* and *E. sari* were suggested as the most closely related species.

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