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RESEARCH ARTICLE

Morphometry as a tool in species identification: a study with special reference to species of the genus *Mycalesis* (Lepidoptera: Nymphalidae)

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Abstract: Morphological variability among four species of *Mycalesis* in Sri Lanka that are difficult to discriminate due to their morphological similarity was investigated to identify characters that distinguish species more accurately. Using traditional morphometrics, 90 variables from the wing, forelegs and genitalia of *M. perseus typhlus*, *M. mineus polydecta*, *M. subdita* and *M. rama* were measured and analysed. A set of 19 characters of the wing, male genitalia and forelegs were identified to discriminate species. Results of the analysis showed that male specimens were discriminated with nine wing characters and five characters of genitalia. Females could be discriminated with three wing characters and two foreleg characters. Male specimens of *M. p. typhlus* and *M. m. polydecta* showed the greatest morphological differentiation, while females of *M. subdita* and *M. rama* were the most similar species. These results were used to improve the currently available identification key. Two instances of possible hybridisation were discovered: one between *M. p. typhlus* and *M. m. polydecta* and the other between *M. p. typhlus* and *M. subdita*. Hence, the species of *Mycalesis*, particularly *M. p. typhlus*, *M. m. polydecta* and *M. subdita* may not be strictly reproductively isolated in Sri Lanka. Preliminary comparisons of *M. p. typhlus* in Sri Lanka with *M. p. tabitha* of India indicated that the Sri Lankan subspecies is unlikely to be a synonym.

Keywords: Discriminant analysis, morphology, *Mycalesis*, traditional morphometrics.

INTRODUCTION

Many species of butterflies share close similarities which, coupled with individual variation and phenotypic plasticity, make identification difficult (Ormiston, 1924; Woodhouse, 1949). Identification keys use objective and clearly observable differences as well as minute differences to distinguish between taxa (Bingham, 1905; 1907; Talbot, 1939; 1947). They also commonly use objective characters such as 'purplish-white' or 'infused with purple' to describe the appearance of the wing (Bingham, 1905; Talbot, 1947). Such descriptions are sometimes inadequate for identification, especially when the characters show a great deal of variation. Examination of older keys has also shown errors and discrepancies in the accounts given (van der Poorten & van der Poorten, 2016). Due to these drawbacks, there is a need for a more reliable key to distinguish morphologically similar species.

The current trend is to use tools based on DNA analysis. Molecular information has re-classified Lepidoptera (Aduse-Poku *et al*., 2015; Lukhtanov *et al*., 2015), uncovered cryptic species (Seraphim *et al*., 2014; Dincă *et al*., 2015), mapped phylogenetic relationships (Kreuzinger *et al*., 2015; Wells *et al*., 2015), determined

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past biogeographic events (Carnicer *et al*., 2012) and the radiation and diversification of species (Müller & Beheregaray, 2010). The use of traditional methods employing morphology to reconstruct phylogenies is considered tedious although morphology has been useful in resolving ambiguous relationships (Warren *et al.,* 2009; Heikkilä *et al*., 2014) and increasing the reliability of phylogenetic data (Wahlberg *et al*., 2005; Garzón-Orduña *et al*., 2013). Morphological data has also provided 'hidden support' to certain relationships (Garzón-Orduña *et al*., 2013). The combination of morphological data together with molecular data has resulted in more robust clades (Wahlberg *et al*., 2005; Warren *et al*., 2009; Tóth *et al*., 2014), and has also allowed the use of a smaller number of morphological characters for analysis reducing the tedium of measuring several hundred characters (Wahlberg *et al*., 2005; Warren *et al*., 2009; Ruhfel *et al*., 2013).

Two methods of carrying out an extensive morphological study are, with traditional morphometry and geometric morphometry. In traditional morphometry, linear measurements made directly on the voucher specimen (Gillespie *et al*., 2013) or on a digital photograph (Hill *et al*., 2013; Sañudo-Restrepo *et al*., 2013) are used for multivariate statistical analyses. Geometric morphometry quantifies shape in 2D and 3D space with measurements taken on digital photographs with the use of stereomicroscopy and specialised software packages (Dincă *et al*., 2011a; Habel *et al*., 2012).

Instances of morphologically similar species which are difficult to identify are common among butterflies (Hajibabaei *et al*., 2006; Bonebrake *et al*., 2011; Dincă *et al*., 2011b; Sourakov & Zakharov, 2011), and traditional morphometry or geometric morphometry has been reliably used to aid in their identification. In the Lycaenid butterfly, *Tongeia fischeri*, geometric morphometry showed that the three subspecies could be differentiated on the basis of wing shape, which had taxonomic implications (Jeratthitikul *et al*., 2014). Using traditional morphometrics, Prieto *et al*. (2009) showed that wing length and genitalia provided useful characters to discriminate species of *Cupido*. The present study has employed methods in traditional morphometrics to identify characters to establish a more reliable identification key of the morphologically similar species of *Mycalesis* recorded in Sri Lanka.

Genus *Mycalesis* is a species rich group of butterflies in the subfamily Satyrinae. Five species of *Mycalesis* are recorded in Sri Lanka: *M. rama* (Moore, 1892); *M. patnia patnia* Moore, 1857; *M. subdita* (Moore, 1892), *M. perseus typhlus* (Fruhstorfer, 1908) and *M. mineus polydecta* (Cramer, 1777) (MOERE, 2014; van der Poorten & van der Poorten, 2016). *M. rama* and *M. patnia patnia* are endemics to Sri Lanka while *M. perseus* (Fabricius, 1775) and *M. mineus* (Linnaeus, 1758) are distributed throughout the Oriental region. The National Red List of Sri Lanka has documented *M. subdita* of Sri Lanka also as an endemic species to the island (van der Poorten, 2012). However, *M. subdita* is unlikely to be endemic to Sri Lanka, as it has been reported in both Sri Lanka and India (Moore, 1890 – 1892; Bingham, 1905; Evans, 1920; 1932; Talbot, 1947; Mathew & Soumya, 2013; Anonymous, 2016). Since Mathew and Soumya (2013) described the morphology of male genitalia of *M. subdita* from India, a comparison could be made to the Sri Lankan populations and the results are reported in this study.

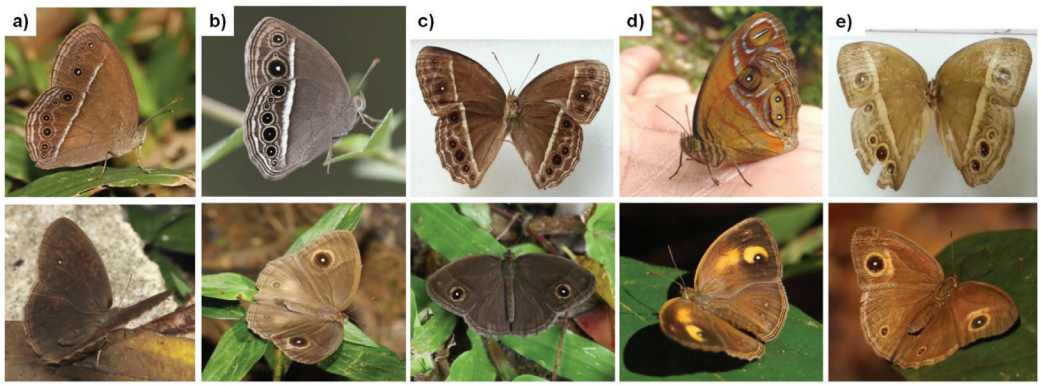

Figure 1: Ventral and dorsal surfaces of the five species of *Mycalesis* recorded in Sri Lanka: (a) *M. perseus typhlus*; (b) *M. mineus polydecta*; (c) *M. subdita*; (d) *M. patnia patnia*; (e) *M. rama*

Apart from *M. p. patnia*, the four remaining species share a close morphological similarity and are difficult to distinguish, especially the females (Ormiston, 1924; Woodhouse, 1949) (Figure 1). Males are distinguished by the differences in morphology of the sex brands present on the dorsal hindwing (DHW) and the ventral forewing (VFW) (van der Poorten & van der Poorten, 2016) and differences in genitalia (Evans, 1932). Females, however, lack species specific characters of the wing or genitalia (Ormiston, 1924; Evans, 1932; Talbot, 1947). Females of *M. p. typhlus*, *M. m. polydecta* and *M. subdita* are particularly difficult to differentiate (Ormiston, 1924; Woodhouse, 1949). Identification is further complicated by the presence of individual variation, geographical variation and seasonal variation in wing pattern and colour.

Seasonal variation as an adaptive response to changes in the environment, also known as seasonal polyphenism, is common among butterflies. The most prominent seasonal forms are the dry season forms (DSFs) and the wet season forms (WSFs). Each form is characterised by a change in the wing pattern and colouration that optimises survival during the Dry and Wet Seasons, respectively (Braby, 1994; Roskam & Brakefield, 1999). Although the morphological variations are a strategy to increase fitness of these insects, it creates a considerable problem in identification. While WSFs are easily identified due to the conspicuous wing patterns, DSFs are characterised by much reduced ocelli and dull colourations (Braby, 1994; Roskam & Brakefield, 1999). Apart from DSFs and WSFs the species of *Mycalesis* in Sri Lanka also display intermediate season forms (IntSFs), which occur in the intervening periods between the Dry and Wet Season.

Taxonomic issues also surround *M. perseus* in Sri Lanka. Some authors contend that the Sri Lankan subspecies *M. p. typhlus*, is synonymous with the Indian subspecies, *M. p. tabitha* (K. Kunte in van der Poorten & van der Poorten, 2012). Based on morphological data of the wing and male genitalia, Evans (1920; 1932) classified the Sri Lankan subspecies as *M. p. typhlus*, whereas Talbot (1947) using morphological data of the wing considered it as *M. p. tabitha*. However, the descriptions of the wing given in both accounts (Evans, 1920; Talbot, 1947) are the same. Genetic data has shown that the Sri Lankan population is a subspecies of *M. perseus* but there was insufficient data to determine if it is *M. p. typhlus* or *M. p. tabitha* (unpublished). The morphology of male genitalia of the Indian *M. p. tabitha* described by Mathew and Soumya (2013) were utilised in this study to resolve this question.

Thus, in the present study the morphological variations in the species of *Mycalesis* in Sri Lanka were investigated with the following objectives: 1) test the suitability of using methods in traditional morphometrics with multivariate analysis in order to discriminate morphologically similar species; 2) identify a set of morphological characters through morphometry, that are unbiased and not subjected to the experience of the identifier to improve identification of the species, including species specific characters to differentiate females; and 3) resolve taxonomic issues surrounding the Sri Lankan species of *Mycalesis* by comparing morphology to the corresponding conspecifics in India.

This study has used characters of the wing, forelegs and genitalia for analysis. The most commonly used characters of the wing to distinguish among the species of *Mycalesis* in Sri Lanka are the following: 1) the presence or absence of an ocellus above vein CuA2 on the dorsal forewing (DFW); 2) the size of the ocellus on the DFW; 3) the presence or absence of an ochreous yellow ring on the DFW ocellus; 4) the size of the discal band on the ventral surface of the forewing (FW) and hindwing (HW); and 5) the position of the ocellus above vein CuA2 on the ventral hindwing (VHW) in relation to the ocellus above it and the two ocelli below it (Ormiston, 1924; Woodhouse, 1949; van der Poorten & van der Poorten, 2016). Forelegs are the most variable of the legs. However, apart from the presence of reduced forelegs, especially in the males, intra-specific variations have not been previously described (Evans, 1932; Talbot, 1939; 1947). Male genitalia are of taxonomic importance and have been described by past researchers with sketches and line drawing showing variations in shape (Evans, 1920; Ormiston, 1924; Woodhouse, 1949). However, the genitalia of females have not been previously described.

METHODOLOGY

Sample collection and identification

Adult specimens of the 5 species of *Mycalesis* were collected in Sri Lanka from 2012 to 2014 using field nets and baited traps. Field collections of *M. rama* were restricted due to the endangered status of the species (van der Poorten, 2012). Hence, museum specimens of the 5 species of *Mycalesis* stored at the Colombo National Museum were also included.

Morphological characters of wings and genitalia were used for preliminary identification of the specimens with the aid of descriptions from Bingham (1905), Ormiston (1924), Evans (1932) and Talbot (1947). Each

identification was then verified by a taxonomic expert (G. van der Poorten).

Laboratory preparations of field specimens

A prothoracic leg was removed for morphometric measurements and placed in 30 % ethanol. The abdomen was separated for dissection of genitalia. The specimen was then relaxed using a new alcohol relaxing method (modified from the method used by M. Schenck, *Personal communication*, *26 January 2012*) in which the thorax of the specimen was sprayed with a small quantity (approx. 0.5 mL) of 40 % ethanol, avoiding soaking of the wings. The specimens were left to dry for 10 min on tissue paper. Once relaxed, the insect was pinned, left to set at room temperature for two days and removed to a storage box and stored in a humidity controlled dry box maintained at 50 % relative humidity.

The forelegs were washed in distilled water and cleaned of hair and scales. They were then mounted with Euparal Mounting Medium (BioQuip, USA). The genitalia of females and males were dissected as described by Cribb (1972). In the males, one clasper was removed for measurement before the genitalia were mounted on a permanent slide. Forelegs and genitalia of museum specimens were not examined.

Measurement of characters

The right wing (fore and hind) was used for wing characteristics and was measured in millimetres (mm) or converted to millimetres. All continuous characters of the wing were measured with a vernier caliper (0.05 mm accuracy). Continuous characters of genitalia and forelegs were measured with a calibrated eyepiece micrometre (0.03 mm accuracy). Discrete characters were counts of ocelli on wings and number of spines on forelegs. All measurements of characters were made by specifying landmarks on the wings, forelegs and genitalia to minimise errors.

The same vernier caliper was used for every measurement and the same observer measured all collected specimens. These measures allowed the standardisation of measurements in order to minimise equipment error and human error (Van Hook *et al*., 2012). Measurements of each character were repeated 3 times and an average was used (Yezerinac *et al*., 1992).

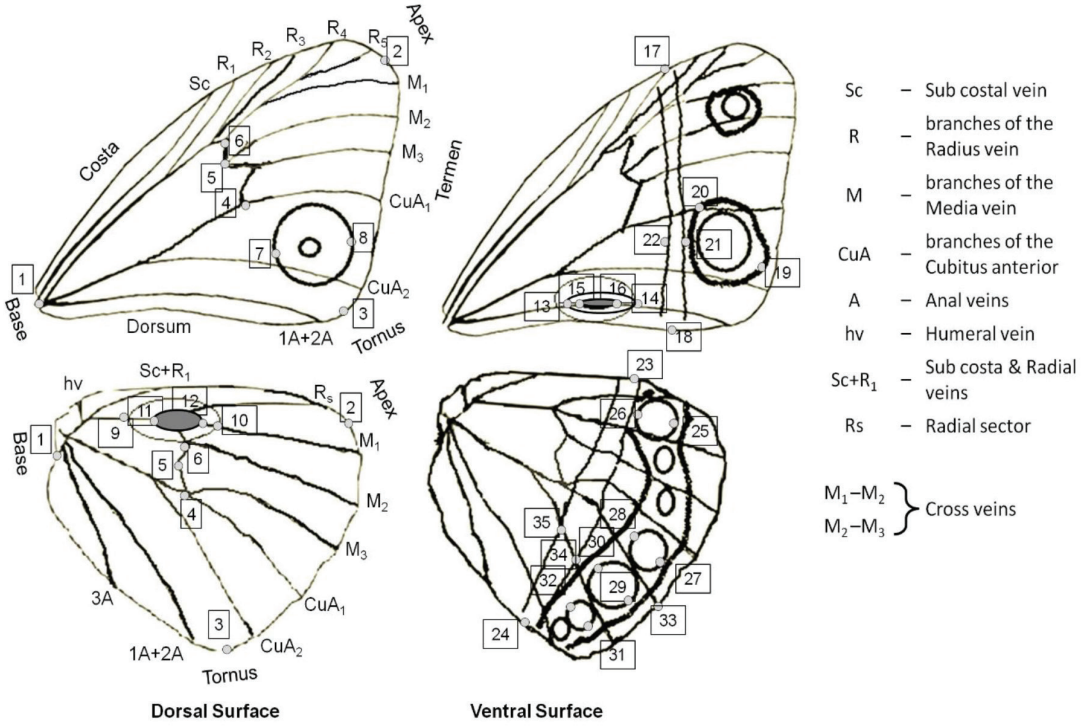

Figure 2: Illustration of the dorsal and ventral surfaces of the forewing and hindwing of *Mycalesis* displaying landmarks used to measure characters

Table 1: Results of discriminant analysis for characters of the wing and genitalia of male specimens of *Mycalesis*

Characters of the wing				Characters of genitalia		
	Correlation coefficients				Correlation coefficients	
Character	DF1	DF2	DF3	Character	DF1	DF2
DFWocBA	0.85 **	- 0.31 **	- 0.16	LdTcl	1.00 **	1.00 **
DFWcol	0.43 **	- 0.49 **	0.80 **	WdTcl	1.00 **	1.00 **
VFWSxNec	0.51 **	0.65 **	0.31 **	WclTcl	1.00 **	1.00 **
VFWNecBA	0.66 **	0.33 **	- 0.12	AedSp	0.62 **	- 0.01
Vsxcol	0.76 **	- 0.18 *	- 0.23 **	LDDTcl	1.00 **	1.00 **
DFWdis	0.81 **	- 0.12	- 0.28 **	LdTcl	1.00 **	1.00 **
VHW5	0.78 **	- 0.17 *	- 0.18 *			
Dsxcol	0.81 **	0.06	0.28 **			
DsxVsx	- 0.47 **	- 0.42 **	- 0.14			
AntLBA	- 0.27 **	- 0.02	- 0.25 **			
% Var.	57.44	28.17	14.39		85.75	14.25
Cum. %	57.44	85.61	100		85.75	100.00
Wilks' λ	0.001	0.024	0.205		0.88	0.60
Chi-squared	772.42 *	441.41 *	187.28 *		216.28 *	50.38 *
Canonical correlation	0.97	0.94	0.89		0.14	0.64

Analysis for wing characters conducted for specimens of *Mycalesis perseus typhlus*, *M. mineus polydecta*, *M. subdita* and *M. rama*. Analysis for characters of genitalia did not include specimens of *M. rama.* Correlation coefficients of the characters with respect to the discriminant functions (DF) indicated. Characters are in hierarchical order. Significance * $p < 0.05$, ** $p < 0.01$

A total of 90 discrete and continuous characters of the wing (62), forelegs (12) and genitalia (16) were measured (Appendix, Table I). Figure 2 shows 33 of the characters measured on the wing.

Colour measurements of the wing were made on digital photographs taken from a digital camera with 3264 × 2448 (8 megapixel) resolution. Photographs were captured using natural light when sunlight was brightest (12 – 2 pm). White balance, shutter speed and ISO, which control the instrument's sensitivity to light and light exposure were set to the auto setting; aperture size described as exposure value was kept at zero. Colour variations in the proximal half of the discal cell of the dorsal forewing (DFW) and ventral forewing (VFW) were quantified in hexadecimal scale (an alphanumeric code) using ColorPic software (ICONICO, 2010). The hexadecimal values were converted to decimal values before being used in the analysis. The colour of the sex brand was identified visually rather than by a digital measurement because a photograph depicting the colour was not obtainable. Hence, the colour of the sex brand was scored 0 for black, 1 for grey, 2 for silver, 3 to 11 for variations from pink and maroon to yellow, and 12 for bronze.

On the foreleg, 9 characters were measured in the male and 12 characters in the female. Landmarks used to measure the characters are shown in Figure 3. On the genitalia, 12 characters were measured on the claspers of the male using 10 landmarks (Figure 4) and in the female, the length of the corpus bursa and ductus bursa was measured.

To compensate for size differences in the specimens, the value of each continuously variable character was expressed as a ratio. For the continuously variable characters of the wing, the value of the measured character was expressed as a ratio of the length of the wing from base to apex. Similarly, each measured character of male genitalia was expressed as a ratio of the length of the clasper and each measured character of female genitalia was expressed as a ratio of the total length of the corpus bursa and ductus bursa. Each measured character of the foreleg was expressed as a ratio of the total length of the foreleg. Several other characters were expressed as ratios between characters of similar length. For example, FWclHW is the ratio between the length of the discal cell of the FW and the similar length of the discal cell of the HW.

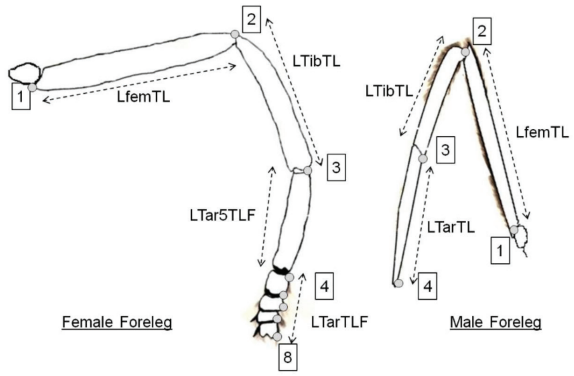

Figure 3: Illustrations of female and male foreleg of *Mycalesis* displaying landmarks used to measure characters

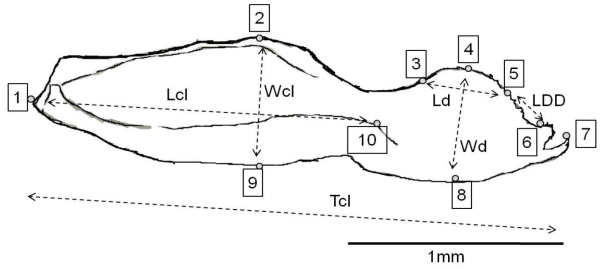

Figure 4: Illustration of the clasper of male genitalia of *Mycalesis* displaying landmarks used to measure characters

Statistical analysis of morphometric data

All statistical analyses were performed with SPSS 20.0 for Windows Software (IBM Corporation, 2011) and R statistical package (R Core Team, 2013). To determine if a significant difference existed between field-captured and museum specimens, the characters of the wing were first subjected to Shapiro-Wilk's test to determine normality followed by one way analysis of variance (ANOVA). Only seven characters of the wing were normally distributed and none of the characters showed a significant difference ($p > 0.05$) between field-captured and museum specimens.

Because all variables were not normally distributed, non-parametric tests were employed for further analyses. Stepwise canonical linear discriminant analysis was performed on the morphometric data of the wing, forelegs and genitalia for males and females separately. *M. p. patnia* was excluded from the discriminant analyses as it is morphologically distinct and the most easily identifiable species. The preliminary identification of the specimens was used as *a priori* criterion in discriminant analysis. The stepwise method with Wilk's lambda was selected assuming all groups as equal and using the covariance within group matrix for the calculation. Spearman's correlation was used to test the significance between selected characters and the discriminant functions.

Multivariate analysis of variance (MANOVA) was selected to compare means of the multivariate sample. The species character was selected as the independent variable. The significant discriminant characters from each discriminant analysis were chosen as the dependent variables.*Post-hoc* test was conducted applying Hochberg's GT2 test when assuming equal variance or Games-Howell test when variance was not similar (Field, 2000). The Levene test was utilised to verify homogeneity of variance (Glass, 1966).

RESULTS AND DISCUSSION

A total of 221 specimens of *Mycalesis* were collected from the field: *M. p. typhlus* (N = 75, ♂ = 60, ♀ = 15), *M. m. polydecta* (N = 63, ♂ = 42, ♀ = 21), *M. subdita* (N = 30, ♂ = 20, ♀ = 10), *M. p. patnia* (N = 51, ♂ = 39, ♀ = 12) and *M. rama* (♂ = 1, ♀ = 1). Twenty-eight museum specimens were included: *M. m. polydecta* (N = 8, ♂ = 2, ♀ = 6), *M. subdita* (N = 1, ♀ = 1), *M. p. patnia* (N = 9, ♂ = 4, ♀ = 5) and *M. rama* (N = 7, ♂ = 4, ♀ = 3).

Discrimination of male specimens

Characters of the wing and genitalia discriminated the male specimens of *M. p. typhlus*, *M. m. polydecta*, *M. subdita* and *M. rama* (Table 1). Three discriminant functions and a set of ten characters of the wing differentiated 94.5 % (96.4 % cross validation) of specimens. The analysis successfully grouped 100 % of the specimens of *M. p. typhlus*, 88.9 % of *M. m. polydecta*, 100 % of *M. subdita* and 80 % of *M. rama*. Small values of Wilk's lambda, significant ($p < 0.05$) Chi-squared tests and > 89 % canonical correlation coefficients of all three discriminant functions showed that the significantly correlated characters were best able to discriminate the four species.

Colour of the DFW (DFWcol); three characters of wing pattern: diameter of the DFW ocellus (DFWocBA), presence of a DFW discal band (DFWdis) and the position of the ocellus above vein CuA2 on the VHW (VHW5); five secondary sexual characters: ratio of sex brand to nacreous patch on the VFW (VFWSxNec), length of the nacreous patch on the VFW (VFWNecBA),

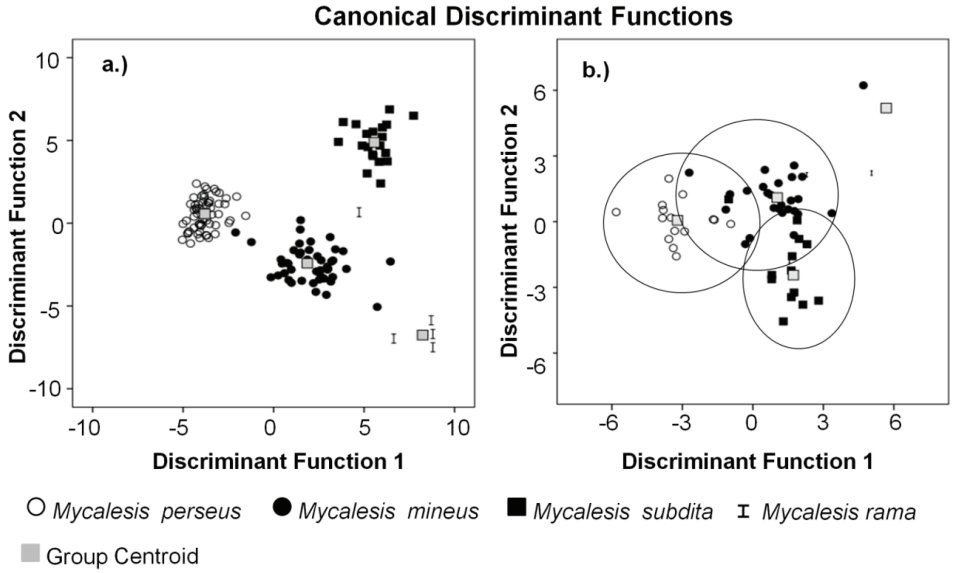

Figure 5: Scatter plot of the first two discriminant functions of characters of the wing discriminating specimens of *Mycalesis perseus typhlus*, *M. mineus polydecta*, *M. subdita* and *M. rama*: (a) discrimination of male specimens; (b) discrimination of female specimens

colour of the VHW sex brand (Vsxcol), colour of the DFW sex brand (Dsxcol) and ratio of the VHW and DFW sex brands (DsxVsx); and antenna length (AntLBA) were the most discriminatory characters to differentiate male specimens. A scatter plot between the first two discriminant functions accounting to 85.61 % variance, showed males were easily discriminated using these wing characters (Figure 5a).

The *M. rama* was not included in the analyses with characters of male genitalia and forelegs as only a single specimen was available. The analysis showed a set of six characters of genitalia (Table 1) that were significantly correlated to three discriminant functions that separated 89.3 % (86.9 % cross validation) of the specimens to their respective species. The analysis grouped 98.3 % of the specimens of *M. p. typhlus*, 88.1 % of *M. m. polydecta* and 65 % of *M. subdita*. The success of discriminating males with characters of forelegs was < 50 % and thus not suitable to differentiate the species.

To determine if the characters of the wing and genitalia which discriminated the four species were able to significantly differentiate the species, a further analysis using MANOVA was performed. The results indicated that the characters were able to significantly differentiate the species (Wilks' lambda 0.002 and 0.005, $p < 0.05$, respectively). As males are easily identified with secondary sexual characters and differences in genitalia, the successful discrimination of males to their respective species confirmed the suitability of using discriminant analysis and traditional morphometrics for the differentiation of morphologically similar species.

Of the 16 characters, antenna length (AntLBA) and ratio of the width of dorsal process to width of clasper (WdWcl) were the only two characters, which did not significantly differentiate the specimens ($p > 0.05$). *Post-hoc* test of multiple comparisons showed a single character, the length of VFW sex brand (VFWSxNec), to be significantly different ($p < 0.05$) among all four species.

Of the characters of wing pattern, the position of the ocellus above vein CuA2 on the VHW in relation to the ocellus above it and the two ocelli below (VHW5), and size of the DFW ocellus (DFWocBA) are common characters used to identify the species of *Mycalesis*. However, in the present analysis both distinguished only *M. p. typhlus*. Colour of the DFW was the only character significantly different ($p < 0.05$) in *M. rama* whereas the character identifying the presence of a DFW discal band was significantly different ($p < 0.05$) among *M. p. typhlus*, *M. m. polydecta* and *M. subdita*.

Of the secondary sexual characters, the colour of the sex brands (Dsxcol and Vsxcol) is a commonly used character for the identification of *Mycalesis* (van der

Poorten & van der Poorten, 2016). The present study also identified species specific variations in the size of the sex brands and nacreous patches (VFWSxNec, VFWNecBA and DsxVsx); the colour of the DHW sex brand (Dsxcol) was significantly different ($p < 0.05$) between *M. m. polydecta* and *M. subdita*; the colour of the VFW sex brand (Vsxcol) was common to both *M. p. typhlus* and *M. rama*; and the ratio of length of sex brands on the DHW to VFW (DsxVsx) and length of VFW nacreous patch (VFWNecBA) was significantly different ($p < 0.05$) in *M. subdita*.

Hence, with the use of these nine characters, an unknown male specimen of *M. p. typhlus*, *M. m. polydecta*, *M. subdita* or *M. rama* can be correctly identified and may not require genital dissections for the confirmation of identity. Nevertheless, five very discriminatory characters of the claspers and aedeagus were also identified; three characters of genitalia (LdTcl, WdTcl, AedSp) were significantly different ($p < 0.05$) among *M. p. typhlus*, *M. m. polydecta* and *M. subdita*; the distance between the distal and dorsal process of the clasper (LDDTcl) was significantly different ($p < 0.05$) in *M. m. polydecta*; and the width of the clasper (WclTcl) was significantly different ($p < 0.05$) in *M. p. typhlus*.

Discrimination of female specimens

Earlier authors were unable to conclusively identify distinguishing female specific species characters (Ormiston, 1924; Evans, 1932; Talbot, 1947). In the present analysis, a set of five characters of the wing that were significantly correlated to three discriminant functions separated 84.5 % of the specimens (77.6 % cross validation) (Table 2). Three characters of wing pattern (DFWocBA, VFWwDisBA and 1VHW4) and colour of the FWs (DFWcol and VFWcol) were the discriminatory characters for females. However, unlike the male specimens which were separated clearly based on wing morphometrics, in females there exist a degree of overlap among the species of *Mycalesis* even when using the five discriminatory characters identified (Figure 5b). The analysis successfully grouped 93.3 % of the specimens of *M. p. typhlus*, 88.5 % of *M. m. polydecta*, 84.6 % of *M. subdita* and 25 % of *M. rama*. To determine if characters of the wing were more distinct in males in comparison to females the analysis was repeated for male specimens without including secondary sexual characters. The results showed that a set of eight characters of the wing (DFWocBA, DFWcol, DFWdis, VHW5, DHWLoOc, VHW6ocBA, FWfdcAgl and BA) significantly correlated to three discriminant functions separated 89.2 % of specimens (89.2 % cross validation). However, as the percentage of discrimination of females (84.5 %) and males (89.2 %) was similar it was not considered that wing characters of males have a greater significance.

Two foreleg characters that were significantly correlated to two discriminant functions separated only 58.6 % of the specimens with 53.4 % cross validation (Table 2). The foreleg characters discriminated 80 % of

Table 2: Results of discriminant analysis for characters of the wing and forelegs of female specimens of *Mycalesis*

Characters of the wing				Characters of the foreleg		
	Correlation coefficients				Correlation coefficients	
Character	DF1	DF2	DF3	Character	DF1	DF2
DFWocBA	0.96 **	0.04	0.20	FemTar	0.79 **	-. 061 **
VFWwDisBA	0.23	0.87 **	- 0.12	TL	0.69 **	0.72 **
VFWcol	0.30 *	0.33 *	- 0.71 **			
DFWcol	0.27 *	0.08	- 0.41 **			
1VHW4	- 0.36 **	0.47 **	0.19			
% Var.	60.60	30.70	8.70		92.60	7.40
Cum. %	60.60	91.30	100		92.60	100
Wilks' λ	0.023	0.15	0.56		0.59	0.95
Chi-squared	163.71 *	82.71 *	25.20 *		22.04 *	2.005
Canonical correlation	0.92	0.86	0.66		0.61	0.21

Analysis conducted for specimens of *Mycalesis perseus typhlus, M. mineus polydecta, M. subdita* and *M. rama*. Correlation coefficients of the characters with respect to the discriminant functions (DF) indicated. Characters are in hierarchical order. Significance * $p < 0.05$, ** $p < 0.01$

M. p. typhlus and 80.8 % of *M. m. polydecta*. However, only 7.7 % of the specimens of *M. subdita* and no specimens of *M. rama* were differentiated, which reflects the poorer ability of foreleg characters to discriminate the species. A single character of genitalia (TLBur) was selected to separate the female specimens. However, the success of differentiation was < 50 %. Hence, this character was not used in discrimination of females.

MANOVA performed on the characters selected to differentiate females indicated that the characters of the wing and forelegs were able to significantly differentiate the species (Wilks' lambda 0.059 and 0.595, $p < 0.05$, respectively). Of the seven characters, only colour of the wing (DFWcol and VFWcol) did not significantly differentiate the specimens ($p > 0.05$). *Post-hoc* test showed a single character, width of the VFW discal band (VFWwDisBA), to be significantly different ($p < 0.05$) among all four species. The diameter of the DFW ocellus (DFWocBA) was significantly different ($p < 0.05$) in *M. p. typhlus* and *M. rama*. The ratio of the diameter of the VHW ocellus above vein M1 to that above vein CuA1 (1VHW4) was significantly different among *M. p. typhlus*, *M. m. polydecta* and *M. subdita*. Hence, we could improve the identification of an unknown female specimen based on these wing characters even though some difficulties could arise due to the presence of seasonal forms.

Of the characters of the forelegs, ratio of femur to tarsus (FemTar) was significantly different ($p < 0.05$) between *M. p. typhlus* and *M. m. polydecta*, and total length of the foreleg (TL) was significantly different ($p < 0.05$) among *M. p. typhlus*, *M. m. polydecta* and *M. subdita*. The study also showed that the length of the corpus bursa and ductus bursa (TLBur) cannot be used to identify the species of *Mycalesis*. However, characters in the female genitalia may exist that can be used to identify the species of *Mycalesis* (e.g., Braby & Zwick, 2015). Forelegs and female genitalia of *M. rama* were not compared as the field collected specimen was too damaged for measurement. Hence, a further sampling of *M. rama* is necessary to determine the inter-specific variations that may exist.

Descriptive statistics for species of *Mycalesis* in Sri Lanka

Tables 3 and 4 represent the most discriminatory characters of the wing, male genitalia and forelegs that facilitate the identification of males and females of the species of *Mycalesis* in Sri Lanka. All selected characters were unbiased and not subject to the experience of the identifier as is often the case with morphological characters. The analysis was conducted on a mixed sample including DSFs, WSFs, and IntSFs of each species, except *M. patnia*: *M. p. typhlus* (N = 75, DSF = 14, WSF = 34, IntSF = 27), *M. m. polydecta* (N = 71, DSF = 14, WSF = 39, IntSF = 18), *M. subdita* (N = 31, DSF = 14, WSF = 9, IntSF = 8), *M. p. patnia* (N = 63, DSF = 13, WSF = 50, IntSF = 0) and *M. rama* (N = 9, DSF = 1, WSF = 2, IntSF = 6). As seasonal variation adds considerably to the difficulty of identification it was important to include all three morphs of each species so that the range of variation in each character could be recorded. Although variations in wing pattern and fecundity have been extensively recorded in Lepidoptera (Brakefield, 1987; Roskam & Brakefield, 1999; Garcia-Barros, 2000), studies have not looked at the structural variation in appendages. Hence, the variation recorded in secondary sexual characters, male genitalia and female forelegs may represent variation as a result of polyphenism. A larger sample will help to verify these results.

The characters were also used to produce an improved identification key (Figure 6). As the key does not use subjective characters it provides a more direct approach to identification. The inclusion of quantified characters presents the user with an understanding of the dimensions of the character, allowing the user a better understanding of each character. Such descriptive statistics will have limited use in the field, but will be useful for laboratory or museum studies. Colour of the DEW, although identified as a character for the identification of species, the failure to use reference photographs and using standard settings in the camera may have introduced bias. Nevertheless, this character was included in the identification key as an indication of average wing colour.

M. m. polydecta was the only species to show variation in secondary sexual characters and male genitalia. The colour of the dorsal DHW sex brand (Dsxcol) is a commonly used character to identify *M. p. typhlus*, *M. m. polydecta*, *M. subdita* and *M. rama* (van der Poorten & van der Poorten, 2016). However, the colour in the specimens of *M. m. polydecta* examined in this study varied from pink to shades of yellow, grey and black. The claspers also displayed variation especially in the shape and length of the distal and dorsal processes (Figure 7b). Hence, the differentiation of *M. m. polydecta* may become more difficult due to the greater degree of variation shown.

This is the first study that describes inter-specific variation in the aedeagus including the presence of spines, which are laterally placed from close to the tip

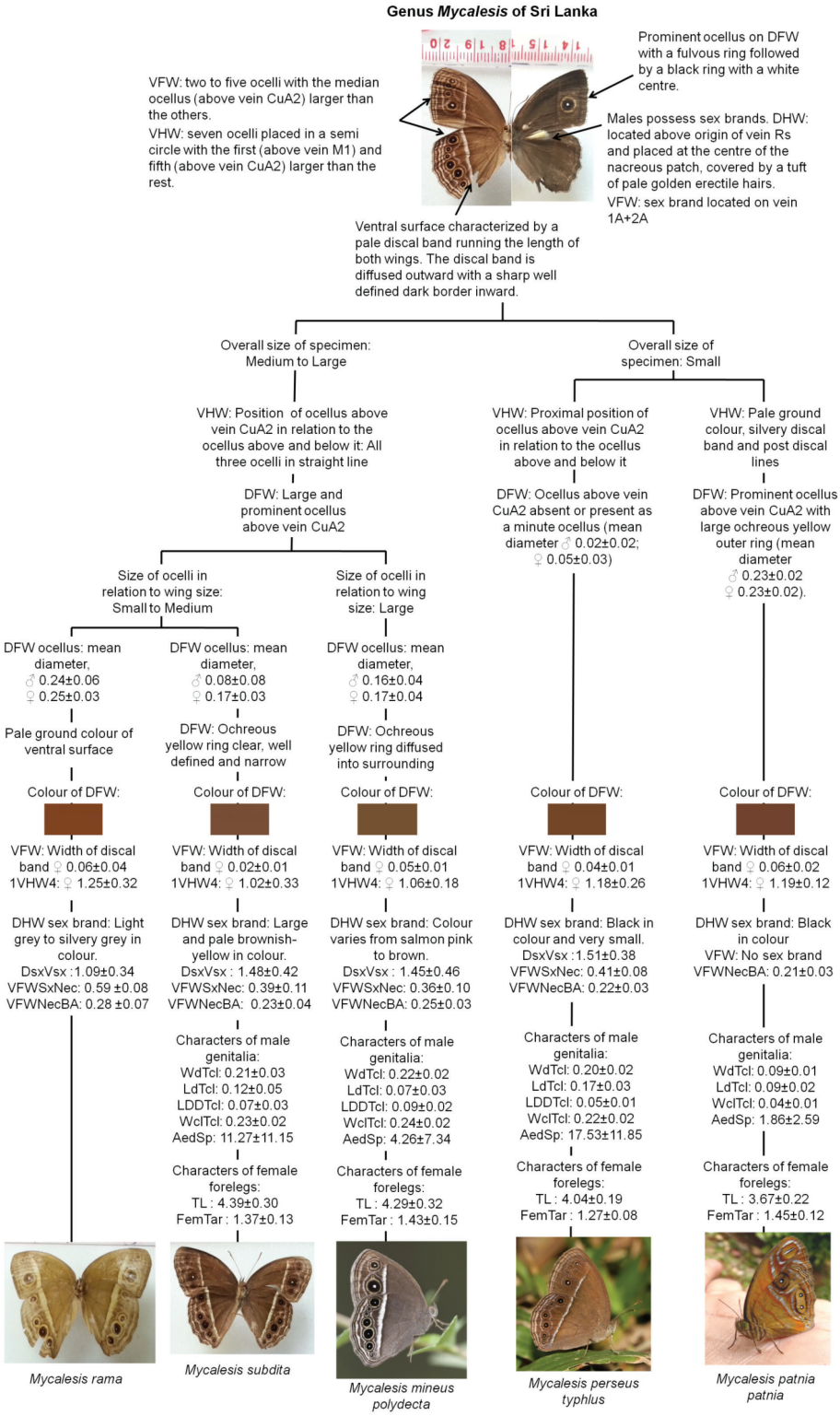

Figure 6: Identification key characterising the species of *Mycalesis* recorded in Sri Lanka

Table 3: Descriptive statistics of the wing and genitalia that differentiate males of the species of *Mycalesis*

		Wing pattern elements				Secondary sexual characters					Characters of male genitalia				
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
M. perseus typhlus	Min	4.18	absent	0.00	0.00	Black	Black	0.81	0.27	0.16	0.14	0.12	0.03	0.18	0.00
	Max	13.88	present	1.00	0.08	Black	Black	2.42	0.59	0.35	0.25	0.23	0.08	0.28	48.00
	Mean	8.83	0.02	0.08	0.02	Black	Black	1.51	0.41	0.22	0.20	0.17	0.05	0.22	17.53
	Stdev	± 2.26	± 0.13	± 0.28	± 0.02	± 0.00	± 0.00	± 0.38	± 0.08	± 0.03	± 0.02	± 0.03	± 0.01	± 0.02	± 11.85
M. mineus polydecta	Min	4.56	absent	0.00	0.00	Black	Black	0.63	0.15	0.20	0.16	0.04	0.04	0.18	0.00
	Max	12.83	present	1.00	0.23	Bronze	Bronze	2.67	0.58	0.35	0.28	0.24	0.12	0.29	27.00
	Mean	9.36	0.87	0.87	0.16	4.11	8.46	1.45	0.36	0.25	0.22	0.07	0.09	0.24	4.26
	Stdev	± 2.12	± 0.34	± 0.34	± 0.04	± 3.40	± 5.16	± 0.46	± 0.10	± 0.03	± 0.02	± 0.03	± 0.02	± 0.02	± 7.34
M. subdita	Min	3.52	absent	0.00	0.00	Grey	Golden	0.47	0.53	0.24	0.14	0.07	0.04	0.21	4.00
	Max	13.12	present	1.00	0.23	Pale yellow	Bronze	2.67	0.93	0.35	0.28	0.24	0.12	0.29	48.00
	Mean	8.96	0.43	0.48	0.08	2.53	4.62	1.48	0.39	0.23	0.21	0.12	0.07	0.23	11.27
	Stdev	± 2.15	± 0.50	± 0.50	± 0.08	± 2.82	± 5.15	± 0.42	± 0.11	± 0.04	± 0.03	± 0.05	± 0.03	± 0.02	± 11.15
M. rama	Min	6.17	absent	1.00	0.19	Silver	Silver	0.58	0.50	0.23	0.21	0.14	0.05	0.21	0.00
	Max	10.37	present	1.00	0.33	Bronze	Bronze	1.50	0.69	0.40	0.21	0.14	0.05	0.21	0.00
	Mean	7.30	0.60	1.00	0.24	10.20	10.20	1.09	0.59	0.28	0.21	0.14	0.05	0.21	0.00
	Stdev	± 1.73	± 0.55	± 0.00	± 0.06	± 4.02	± 4.02	± 0.34	± 0.08	± 0.07	-	-	-	-	-
M. patnia patnia	Min	5.51	absent	0.00	0.20	Black	NA	NA	NA	0.14	0.08	0.04	NA	0.03	0.00
	Max	13.59	present	1.00	0.27	Black	NA	NA	NA	0.28	0.11	0.12	NA	0.06	11.00
	Mean	9.94	0.05	0.77	0.23	1.00	NA	NA	NA	0.21	0.09	0.09	NA	0.04	1.86
	Stdev	± 1.76	± 0.22	± 0.43	± 0.02	± 0.00	NA	NA	NA	± 0.03	± 0.01	± 0.02	NA	± 0.01	± 2.59

1 = DFWcol; 2 = DFWdis; 3 = VHW5; 4 = DFWocBA; 5 = Dsxcol; 6 = Vsxcol; 7 = DsxVsx; 8 = VFWSxNec; 9 = VFWNecBA; 10 = WdTcl; 11 = LdTcl; 12 = LDDTcl; 13 = WclTcl; 14 = AedSp
Refer Appendix, Table I for description of characters.

Note: *M. p. patnia* do not have a sex brand on the VFW and the claspers do not have a distal process (Figure 7d). Thus, the characters Vsxcol, DsxVsx, VFWSxNec and LDDTcl were not applicable. Characters of genitalia were not measured on museum specimens and the values for characters of genitalia of *M. rama* were from a single field collected specimen.

Table 4: Descriptive statistics of the wing and forelegs that differentiate females of the species of *Mycalesis*

		Wing pattern elements			Characters of foreleg	
		1	**2**	**3**	**4**	**5**
M. perseus typhlus	Min	0.00	0.02	0.89	3.84	1.14
	Max	0.13	0.06	1.95	4.48	1.41
	Mean	0.05	0.04	1.18	4.04	1.27
	Stdev	± 0.03	± 0.01	± 0.26	± 0.19	± 0.08
M. mineus polydecta	Min	0.03	0.03	0.62	3.65	1.11
	Max	0.22	0.07	1.52	4.94	1.85
	Mean	0.17	0.05	1.06	4.29	1.43
	Stdev	± 0.04	± 0.01	± 0.18	± 0.32	± 0.15
M. subdita	Min	0.10	0.01	0.58	3.99	1.14
	Max	0.23	0.04	1.56	4.79	1.61
	Mean	0.17	0.02	1.02	4.39	1.37
	Stdev	± 0.03	± 0.01	± 0.33	± 0.30	± 0.13
M. rama	Min	0.22	0.00	1.05	-	-
	Max	0.29	0.08	1.73	-	-
	Mean	0.25	0.06	1.25	-	-
	Stdev	± 0.03	± 0.04	± 0.32	-	-
M. patnia patnia	Min	0.19	0.04	0.99	3.31	1.26
	Max	0.26	0.09	1.35	4.10	1.58
	Mean	0.23	0.06	1.19	3.67	1.45
	Stdev	± 0.02	± 0.02	± 0.12	± 0.22	± 0.12

1 = DFWocBA; 2 = VFWwDisBA; 3 = 1VHW4; 4 = TL; 5 = FemTar

Measurement of TL is in millimetres (mm). All other characters are ratios.

Note: Characters of forelegs were not measured on museum specimens and the forelegs of the single field collected female specimen of *M. rama* was too damaged for measurement.

to midway (Figure 7). The most number of spines was found in *M. p. typhlus* (17.53 ± 11.85) whereas 59.52 % of the specimens of *M. m. polydecta* did not have any spines. The least number of spines (1.86 ± 2.59) was found on *M. p. patnia*.

Evidence of hybridisation

The study also showed evidence that the species of *Mycalesis* in Sri Lanka, particularly *M. p. typhlus*, *M. m. polydecta* and *M. subdita* may not be strictly reproductively isolated yet. Two male specimens were found in which each shared characters of two species. One male specimen (specimen ID: 09CMKal062) displayed characters of the wing and secondary sexual characters common to both *M. p. typhlus* and *M. m. polydecta*. The dorsal surface of the wing and the discal band of the ventral surface was typical of *M. p. typhlus*. However, the ocellus above CuA2 on the VHW was in line with the ocellus above it and the two ocelli below it, which is typical of *M. m. polydecta.* The colour of the sex brands on both the VFW and DHW were black, which is again common to both *M. p. typhlus* and *M. m. polydecta*. The shape of the distal and dorsal process of the claspers was similar to those of *M. p. typhlus* although not typical. The specimen was identified as *M. p. typhlus* through the analysis of mitochondrial DNA. However, nuclear DNA data identified the specimen as *M. m. polydecta* (*unpublished data*).

The second ambiguous male specimen (specimen ID: 02BNKur150) displayed characters of the wing specific to *M. p. typhlus*, but secondary sexual characters and characters of male genitalia specific to *M. subdita*. Hence,

the specimen was identified as *M. subdita*. Although the present analysis showed that the variation in the claspers was significantly different among the species of *Mycalesis*, Goulson (1993) showed in *Maniola jurtina* that the distal and dorsal processes of the claspers have no apparent function during copulation, and pointed out that if the differences in the claspers do not interfere with mating and are not constrained by selection, the claspers will not be barriers to cross-fertilisation. Although Goulson (1993) was referring to subspecies, his hypothesis may have some validity even between species based on this evidence of cross-fertilisation in field collected samples of the present study.

Taxonomic inference

The taxonomic question of the status of *M. perseus* in Sri Lanka was resolved to a certain extent. The genitalia of the Sri Lankan taxon (*M. p. typhlus*) (Figure 7a) were compared to those of the Indian subspecies *M. p. tabitha* as illustrated in Mathew and Soumya (2013). The uncus, gnathos, tegument, vinculum and saccus appear similar in both taxa. However, in *M. p. typhlus*, the dorsal process of the claspers is rounded and the distal process has two prominent lobes whereas in *M. p. tabitha* the dorsal process is less rounded, more pointed and the distal process has one lobe. Given this difference and the fact that the claspers of the species of *Mycalesis* in Sri Lanka are significantly different from each other and can be used to discriminate these species (Figure 7), the preliminary examinations suggest that the Sri Lankan subspecies *M. p. typhlus* is unlikely to be conspecific with *M. p. tabitha*.

A comparison of the genitalia of *M. subdita* from Sri Lanka (Figure 7c) and from India (Mathew & Soumya, 2013) showed them to be similar. The only difference found between the two taxa was a single character of the wing: the wet season form of the Indian population was reported to have a single ocellus above vein 1A + 2A on the VHW, whereas the Sri Lankan populations always have two ocelli. The Indian species were identified as common at low elevations to about 914 m asl (Mathew & Soumya, 2013), whereas *M. subdita* in Sri Lanka is common in the Intermediate Zone and Dry Zone below 750 m asl (van der Poorten & van der Poorten, 2016). These findings suggest that *M. subdita* is not endemic to Sri Lanka. Further comparisons of morphological and habitat data of the adult and larval forms as well as a comparison of genetic data is warranted.

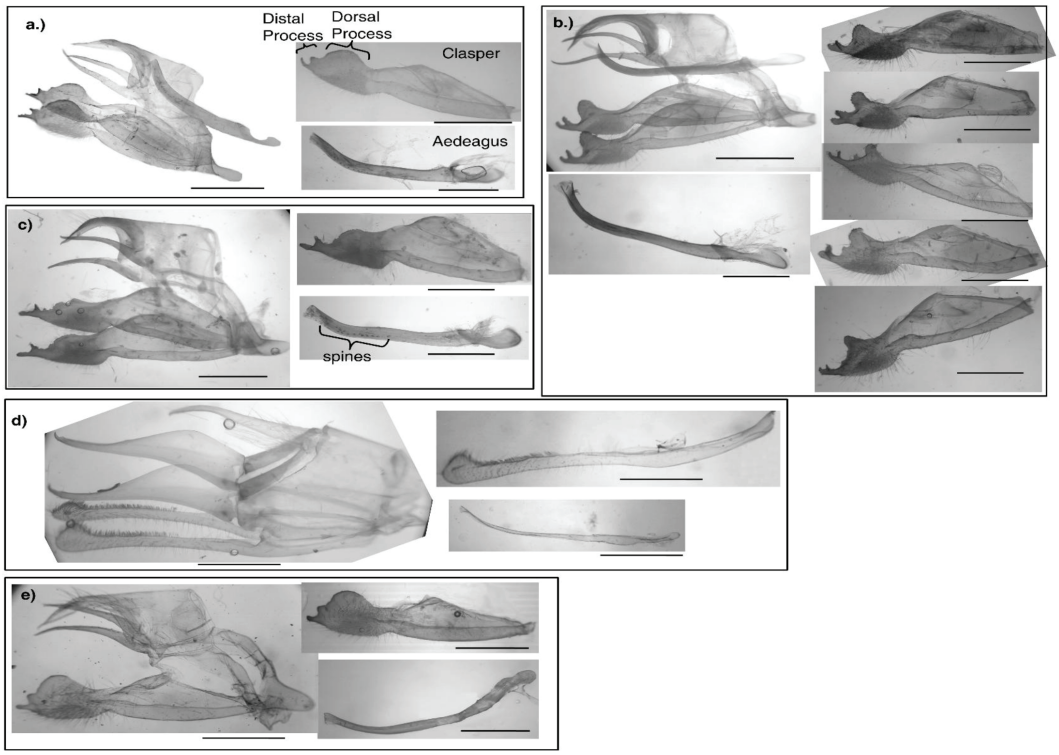

Figure 7: Variations in the morphology of male genitalia of *Mycalesis* in Sri Lanka: (a) *M. perseus typhlus*; (b) *M. mineus polydecta*; (c) *M. subdita*; (d) *M. patnia patnia*; (e) *M. rama*. Scale bar = 1 mm

With respect to *M. patnia*, the genitalia of the Sri Lankan taxon (*M. p. patnia*) (Figure 7d) are the most different from the other species of *Mycalesis* in Sri Lanka but are similar to those of the Indian *M. p. junonia* as illustrated in Mathew and Soumya (2013). These two taxa differ only in the colour of the DFW ocellus ring and the colour of the ventral surface as noted by Evans (1932) and Talbot (1947). In addition, molecular work supports the view that they are conspecific (*unpublished*).

CONCLUSION

This study shows that morphometrics represent a useful taxonomic tool for the discrimination of the species of *Mycalesis* in Sri Lanka. Although *M. p. typhlus*, *M. m. polydecta*, *M. subdita* and *M. rama* are morphologically similar to each other, discriminant analysis revealed significant differences in twelve morphometric characters of the wing, five characters of male genitalia and two characters of female forelegs that may be used to improve the successful identification of these species.

The use of traditional and geometric morphometric techniques should be further investigated to achieve the maximum degree of discrimination among the species, particularly the females.

The preliminary comparisons of the Sri Lankan species to the similar species in India were helpful in advancing the resolution of taxonomic issues of *M. p. typhlus*, *M. subdita* and *M. p. patnia.* The evidence presented here justifies the continuation of the study to confirm the results with further examination and use of genetic data.

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Appendix

Table I: Characters measured on the wings, forelegs and genitalia of *Mycalesis.*
Landmarks for characters of the wing are in Figure 2, landmarks used to measure characters of male and female forelegs are in Figure 3 and landmarks for male genitalia are in Figure 4.

Forewing characters	**Code**	**Landmark**
Length from base to apex of FW	BA	1 to 2
Length from base to tornus of FW	BT	1 to 3
Length from tornus to apex of FW	TA	2 to 3
Angle at M3-CuA1cross veins of FW	FWdcAgl	at 4
Angle at M2-M3cross veins of FW	FWfdcAgl	at 5
Length of FW discal cell	Fwcl	1 to 4
Length from tip of FW discal cell to apex	FWclTA	4 to 2
Length of FW M1-M2cross veins	FWmdc	5 to 6
Length of FW M3-CuA1cross veins	FWldc	-
Diameter of ocellus above vein CuA1 of DFW	DFWoc	7 to 8
Diameter of ocellus above vein CuA1 of VFW	VFWoc	19 to 20
Width of discal line of VFW	VFWwDis	21 to 22
Width of FW	FWwid	17 to 18
Ratio of DFW to VFW	DFWocVFWoc	-
Ratio of FW cell to HW cell	FWclHW	-
Ratio of cell to termen length of FW to HW	FWcltaHW	-
Ratio of cross vein between M3 and CuA1 to BA	FWfdcBA	-
Presence of discal band on DFW	DFWdis	-
Ratio of FW M2-M1cross vein to BA	FWmdcBA	-
Ratio of FW M3-CuA1 cross vein to BA	FWldcBA	-
Hindwing characters	**Code**	**Landmark**
Length from base to apex of HW	HWBA	1 to 2
Length from base to tornus of HW	HWTA	1 to 3
Length from tornus to apex of HW	HWBT	2 to 3
Angle at M3-CuA1cross veins of HW	HWdcAgl	at 4
Angle at M2-M3cross veins of HW	HWfdcAgl	at 5
Length of HW discal cell	Hwcl	1 to 4
Length from tip of HW discal cell to apex	HWclTA	4 to 2
Length of HW M1-M2 cross veins	HWmdc	5 to 6
Length of HW M2-M3 cross veins	HWldc	4 to 5
Width of HW	HWwid	23 to 24
Diameter of VHW ocellus above M1	VHW1oc	25 to 26
Diameter of VHW ocellus above CuA1	VHW4oc	27 to 28
Diameter of VHW cellus above CuA2	VHW5oc	29 to 30
Hindwing characters	**Code**	**Landmark**
Diameter of cellus above 1A + 2A on VHW	VHW6oc	31 to 32
Distance of ocellus above CuA2 to termen on VHW	VHW5ocT	30 to 33
Width of discal line of VHW	VHWwdis	34 to 35
Number of ocelli on VFW	VFWnoc	-
DHW number of ocelli	DHWnoc	-
Location of DHW ocelli	DHWLoc	-
Presence of discal band on DFW	DFWdis	-
Position of White centre of 5th ocellus on VFW	VHW5	-
Ratio of 1st VHW ocellus to 6th	1VHW6	-
Ratio of 1st VHW ocellus to 4th	1VHW4	-
Ratio of 5th VHW ocellus to 6th	5VHW6	-
Ratio of 4th VHW ocellus to 6th	4VHW6	-
Ratio of HW M1-M2 cross vein to HWBA	HWmdcHWBA	-

Ratio of HW M2-M3 cross vein to HWBA	HWldcHWBA	-
Ratio of HW cross vein between M3 and CuA1 to HWBA	HWfdcHWBA	-
Antenna length from head to clubbed tip	AntLBA	-
Male or female	SEX	-
Polyphenic form	DrWt	-
Secondary sexual characters	**Code**	**Landmark**
Length of the sex brand on DHW	Dsx	11 to 12
Length of the sex brand on VFW	Vsx	15 to 16
Length of the nacreous patch on DHW	Dnec	9 to 10
Length of the nacreous patch on VFW	Vnec	13 to 14
Colour of sex brand on DHW	DSxcol	-
Colour of sex brand on VFW	Vsxcol	-
Ratio of sex brand on DHW to VFW	DsxVsx	-
Ratio of necreous patch on DHW to VFW	DNecVNec	-
Ratio of sex brand to necreous patch on DHW	DHWSxNec	-
Relative size of DHW necreous patch to BA	DHWNecBA	-
Ratio of sex brand to necreous patch on VFW	VFWSxNec	-
Relative size of VFW necreous patch to BA	VFWNecBA	-

Characters of forelegs	**Code**	**Landmark**	
		Male	**Female**
Total length of foreleg	TL	1 to 4	1 to 8
Length of Femur	LFem	1 to 2	1 to 2
Length of Tibia	LTib	2 to 3	2 to 3
Length of Tarsus	LTar	3 to 4	3 to 8
Length of last 4 tarsal segments of females	LTarF	N/A	4 to 8
Length of 1st tarsal segment of females	LTar5F	N/A	3 to 4
Total number of spines on tarsus	Tarsp	N/A	-
Number of Tarsal segments	nTarseg	-	-
Nature of Tarsal tip (pointed/blunt)	pnttip	-	-
Ratio of LfemTL to LTibTL	FemTib	-	-
Ratio of LTibTL to LTarTL	TibTar	-	-
Ratio of LfemTL to LTarTL	FemTar	-	-
Characters of male genitalia	**Code**	**Landmark**	
Width of dorsal process	Wd	4 to 8	
Length of dorsal process	Ld	3 to 5	
Length between 2nd distal process and edge of dorsal process	LDD	5 to 6	
Width of clasper arm	Wcl	2 to 9	
Length of clasper arm	Lcl	1 to 10	
Total length of clasper	Tcl	1 to 7	
Length of the aedeagus	AL	-	
Number of spines on aedeagus	AedSp	-	
Ratio of WdTcl to WclTcl	WdWcl	-	
Ratio of LdTcl to LclTcl	LdLcl	-	
Ratio of WclTcl to LclTcl	WclLcl	-	
Ratio of WdTcl to LdTcl	WdLd	-	
Characters of female genitalia	**Code**	**Landmark**	
Length of the corpus bursa	LCor	-	
Length of ductus bursa	LDuc	-	
Combined length of corpus bursa and ductus bursa	TLBur	-	
Ratio of LcorTL to LDucTL	LCpLDc	-	