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Classification and Morphology of *Rhinocypha* spp. (Odonata): A Comprehensive Taxonomic Study Within the Females

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Studies on Odonata have gained attention worldwide as well as locally in Malaysia. Although there is a wealth of data available to be utilized for solving taxonomic problems, ecological and behavioural research areas are more favoured than taxonomy and systematics. Thus, there are confusions over how to correctly identify closely related and sympatric species, especially in female odonates. One such example is in the genus *Rhinocypha*. Consequently, the present study focuses on taxonomic work, employing multi-approaches in the form of morphological (morphological diagnostics, Field Emission Scanning Electron Microscope (FESEM) and geometric morphometric analysis), applying the molecular technique. Seventeen morphological characteristics were created to differentiate between the females of *Rhinocypha* spp. A FESEM was used on the female's ovipositor to focus on the anal appendages and sheathing valve (V3). Also, the phylogenetic patterns expressed by *COI* and 16S rRNA genes, and canonical variate analysis for the wing geometric morphometric revealed three clusters that supported the distinction of the *Rhinocypha* group. In summary, this study effectively developed an integrated approach of classic morphological and trendy molecular, combined with FESEM microscopy techniques, which provided corroborative evidence and resolved taxonomic uncertainties.

Key words: Dragonflies, Female's ovipositor, Geometric morphometric, Mitochondrial *COI*, 16S rRNA.

BACKGROUND

In scientific fields, taxonomy is very important and correct identification of organisms establishes an essential infrastructure for other research areas (Dijkstra et al. 2013). The numerous high-throughput technologies currently available allow for the characterization of the genome, transcriptome, proteome and even the morphology of an organism; for instance, CT scans, (Busse et al. 2015). The application of such technologies to taxonomic research in dragonflies and damselflies could increase the quality and quantity of data that can be applied, not only to help describe new species, but also to provide new perspectives for the correct identification of specimens (Raupach et al. 2015).

However, according to Jisha and Sebastian (2015), identification using traditional taxonomy is problematic due to the external changes in the organisms caused by seasonal and geographical variations. Numerous organisms can adapt themselves physiologically and morphologically to unfavorable conditions in the environment. Therefore, the implementation of manual taxonomy frequently leads to a wrong identification of the species. This problem has influenced the development of the molecular taxonomic studies for the conformation and the improvement in the identification of species.

Formerly, the main guide for classifying Odonata

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has used the wing venation; however, as similar characters evolved multiple times, this frequently does not reliably indicate close relationships (*e.g.*, Dijkstra and Vick 2006; Ware et al. 2007; Carle et al. 2008; Fleck et al. 2008; Pilgrim and von Dohlen 2008; Dijkstra et al. 2013). Furthermore, as any potential outgroup of winged insects lacks wings, wing-based phylogenies and classifications (*e.g.*, Bechly 1996; Trueman 1996) depend on prior assumptions about wing evolution, and must hence be treated carefully (Trueman 2007). Studies integrating other morphological characteristics, such as those of the genital organs and larvae, can help to overcome this problem (*e.g.*, von Ellenrieder 2002; Rehn 2003; Fleck et al. 2008; Pessacq 2008); in addition, genetics are increasingly being used in these studies (*e.g.*, Bybee et al. 2008; Dumont et al. 2010).

DeSalle et al. (2005) proposed a framework requiring corroboration from more than one line of evidence: a taxonomic circle that serves as a bridge between morphological and molecular approaches and provides sufficient rigor for species identification and discovery. The taxonomic circle contains the components of a modern taxonomic system: hypothesis testing, corroboration, reciprocal illumination and revision. In this scheme more than one of the five components of the circle—DNA, morphology, reproduction, ecology or geography—has supported the hypothesis of a new species. The DNA based identification will provide an initial decision while non-DNA data can complement the dataset. Accordingly, the DNA based information can be associated with biological information to include both the evolutionary and taxonomically backgrounds (Vogler and Monaghan 2007).

For examples, odonates species have been identified using morphometric studies (Bookstein 1991; Dryden and Mardia 1998; Adams et al. 2004), geometric morphometry of the wing shape (Rohlf and Marcus 1993; Adams et al. 2004), a combination of DNA sequences and morphology (Pilgrim et al. 2002; Stoks et al. 2005; Pilgrim and von Dohlen 2007), and the ovipositor—such as on the skeleton and musculature, cuticular microstructures and functional aspects of the endophytic ovipositor (Matushkina and Lambret 2011; Matushkina and Klass 2011).

Although there is a wealth of data available to be utilized for solving taxonomic problems, ecological and behavioral research areas are more favored than taxonomy and systematics. Thus, it is difficult to correctly identify closely related and sympatric species, especially in female odonates. In Malaysia, a considerable number of taxonomic studies were performed in the early part of the last century by M.A. Lieftinck in particular, but many areas are still entirely unexplored (Orr 2004). The present study aims to better understand the taxonomy of *Rhinocypha*, one understudied genus in this region.

Rhinocypha spp. (suborder Zygoptera) is the most abundant species found in the forest reserve (Wahizatul Afzan et al. 2006) and the most abundant damselflies in Selangor (Noorhidayah 2013). Mapi-ot et al. (2013) found that this species can adapt to and tolerate disturbed habitats, while Villanueva (2012) observed that this species can be found even in areas with significant human activity and can tolerate streams that have agricultural and domestic runoffs.

However, *Rhinocypha* spp. can be challenging to study. The females are more cryptic at the species level, and identifying the females is troublesome. They are difficult to differentiate from other females of the same genus even though the males of *Rhinocypha* are conspicuous and easy to identify with their distinct blue thoracic marks.

Additionally, the phylogeny of the Anisoptera has been reasonably well studied and its classification is fairly settled (Ware et al. 2007; Fleck et al. 2008); however, recent studies of Zygoptera rely on rather incomplete molecular data sets (Bybee et al. 2008; Carle et al. 2008; Dumont et al. 2010). Besides the morphological studies, mitochondrial gene region cytochrome *c* oxidase subunit 1 (*COI*) and 16S ribosomal RNA (16S rRNA) can be used to confirm species of the Malaysian taxon and preliminary interspecific phylogeny of the *Rhinocypha* group.

Female-limited colour polymorphism in damselflies is a counter-adaptation to male mating harassment; therefore, it is expected to alter population dynamics through relaxing sexual conflict (Takahashi et al. 2014). Such female-limited colour polymorphisms are widespread among damselflies. Typically, females have two or more morphs, where one 'andromorph' shows a male-like colour pattern and one or two 'gynomorph(s)' express colour patterns that are different from the males.

Additionally, according to Bechly et al. (2001), in this group of insects (Odonata), the endophytic oviposition is expected to be a plesiomorphic feature. The odonate females deposit their eggs within plant tissues as a result of a well-developed ovipositor composed of the genitals appendages of the 8th and 9th abdominal segments (Matushkina 2011).

Throughout more than 20 years, extensive work has been done on the comparative and functional morphology of the plesiomorphic well-developed ovipositor in Odonata. For instance, previously specific studies have been focused on the skeleton and musculature (Klass 2008; Matushkina 2004 2008a b; Matushkina and Gorb 1997; Matushkina and Klass

2011; Matushkina and Lambret 2011), cuticular microstructures (Matushkina 2008b; Matushkina and Lambret 2011; Matushkina and Klass 2011), and functional aspects of the endophytic ovipositor (Matushkina and Gorb 2002 2007; Matushkina and Lambret 2011; Matushkina and Klass 2011).

In addition, it is becoming apparent that the majority of phylogenetic reconstructions of higher-level relationships in Odonata suffer from the absence of a common morphological character system apart from the wing venation (Pritykina 1980; Bechly 1996; Lohmann 1996; Trueman 1996). This highlights the importance of a search for new phylogenetically informative characters, and according to Matushkina (2005), the ovipositor is expected to provide such characters. The three species of *Rhinocypha* as well as all Zygoptera and aeshnid Anisoptera have a cutting ovipositor, used for egg deposition within plant tissues (St. Quentin 1962).

In addition, insect wings have been the subject of geometric morphometric analysis in the past many years (Rohlf and Slice 1990; Baylac and Daufresne 1996). They are especially attractive because they can be treated with biological realism in only two dimensions. Morphometry is the study of variation and covariation of the biological form (Bookstein 1991; Dryden and Mardia 1998; Adams et al. 2004). According to Rohlf and Marcus (1993), the morphometric methods are important for the description and statistical analysis of the shape of an organism, while the term 'geometric morphometric' was introduced to distinguish it from the measurement-based techniques of 'traditional' morphometric.

The geometric morphometric approach uses morphometry, in which shapes are expressed as geometric coordinates and the representation and comparison of these shapes are subject to mathematical and statistical techniques (Zelditch et al. 2004). This method allows shapes to be visualized independent of their size (Rohlf and Marcus 1993; Adams et al. 2004) and often proves useful in phylogenetic investigations (Monteiro 1999; Pierce et al. 2008). Moreover, the geometric morphometric method is a relatively innovative technique that has generated valuable results in many fields of classic morphometry. A major advantage of the geometric framework is the complete use of information about the shape that available from a set of landmarks (Bookstein 1996).

In consequence, wing morphometry can help characterize populations within a species, as shown by previous studies such as the analyses of geographic variation in populations of *Drosophila lummei* (Haas and Tolley 1998), *Drosophila serrata* (Hoffman and Shirrifs 2002) and *Scythris obscurella* (Lepidoptera) (Roggero and d'Entrèves 2005). In addition, wings also prove useful when studying complexes of species—for example, in Diptera (De La Riva et al. 2001)—and examining the effects of hybridization, such as in *Apis melifera* subspecies (Smith et al. 1997).

Traditionally, taxonomy is based on phenotypic analyses; although several researchers found that in many taxa this approach is impossible due to the lack of sufficient morphological characters (Wilkerson et al. 1993; Chilton et al. 1995; Floyd et al. 2002). For several aquatic insect orders such as Ephemeroptera (Ball et al. 2005; Williams et al. 2006; Alexander et al. 2009), Diptera (Pfenninger et al. 2007), Coleoptera (Balke et al. 2007; Dutton and Angus 2007) and Trichoptera (Pauls et al. 2010), morphological characters only do not allow reliable distinction. Therefore, molecular genetic techniques have become widespread in taxonomic studies. Though there an increasing number of studies combining DNA sequences and morphology, relatively few studies have been focused on odonates (Pilgrim et al. 2002; Stoks et al. 2005; Pilgrim and von Dohlen 2007). Expectedly, there is still much debate regarding the taxonomic connections in this order (Schmidt 2001; Dijkstra 2003; Dijkstra and Lewington 2006).

In this study, four contrasting tools—morphological diagnostics, ovipositor characteristics, geometric morphometric of the wings, and phylogenetic patterns—of adult females of three congeneric damselfly species, *R. biforata*, *R. fenestrella*, and *R. perforata*, were used to discover the problems in differentiating these species from other females in the same genus.

MATERIALS AND METHODS

Collection of the Specimens

Adult damselflies, *Rhinocypha* spp. (Odonata: Zygoptera) were collected within Peninsular Malaysia in 2015 with the permission of the Forestry Department Peninsular Malaysia (Permit Number: JH/100 Jld.7 (12)). Methods for sampling and preserving Odonata were based previously described standard methods by Orr and Hämäläinen (2003) and Borror and White (1970). Adult females of *Rhinocypha fenestrella* (Rambur 1842), *Rhinocypha biforata* (Selys 1859), and *Rhinocypha perforata* (Percheron 1835) collected from peninsular Malaysia were used.

Morphological Description of Female *Rhinocypha* spp.

Five females from each species of *Rhinocypha* were investigated and examined to create dichotomous

keys. To ensure correct pairs of species, the female individuals were collected during pairings or matings.

Several characters were highlighted in order to identify females of *Rhinocypha* spp. using the morphological nomenclature by Djikstra et al. (2014) and modified from Gunther (2009): 1) wings, to observe the wing venation; 2) pterostigma; 3) nodus; 4) thorax in dorsal view to see the metepimeron, metanepisternum, mesanepisternum, and etc; 5) abdominal segments (S1–10) length, and width and 6) length of the wing (Fig. 1).

Field Emission Scanning Electron Microscope (FESEM)

Three air-dried females of each species of *Rhinocypha* were first examined with a stereo microscope and then with a field emission scanning electron microscope (FESEM). This study focused on the ovipositor part of the adult females of *Rhinocypha* spp. For FESEM, the female's abdomen was cut at the S7 and mounted using carbon tape on a stub. All the specimens were then examined with a FEI QUANTA 450 FEG field emission scanning electron microscope. A general description of the odonate endophytic ovipositor was provided by Matushkina (2008).

To record the morphometric data, eleven continuous characters were measured from the FESEM images captured. The mean and standard deviation values of the characters were calculated. These characters were: length of 8th, 9th and 10th segments, the length of the anal appendages, basal width of the anal appendages, length of the stylus, width of V3 (third valves of ovipositor, valvulae 3), peak of the tooth to the median base, the space between the tooth, the width of the distal tooth, and width of the stylus. Detailed images with the measurements are given in figure 2.

Geometric Morphometric Analysis of the Wings

A total of 30 females of each *Rhinocypha* species were used in this analysis. The right wing of each individual was carefully removed from the specimen and placed on a white paper with the dorsal side of the wing facing upwards. A ruler with minimum scales of 1 mm was placed on the white paper to calibrate of the measurement and a digital image of each specimen was taken with a Dino-lite EDGE AM7115MZT attached with RK-10 Stand. Images were imported into tpsDig (Rohlf 2005) to digitize landmarks. Fifteen homologous landmarks were chosen in this study to quantify wing shape variation, as shown in figure 3, which uses *Rhinocypha biforata* as an example.

The coordinates of all the samples were superimposed to remove the information on size, position and orientation to standardize each specimen according to centroid size. To analyze wing shape

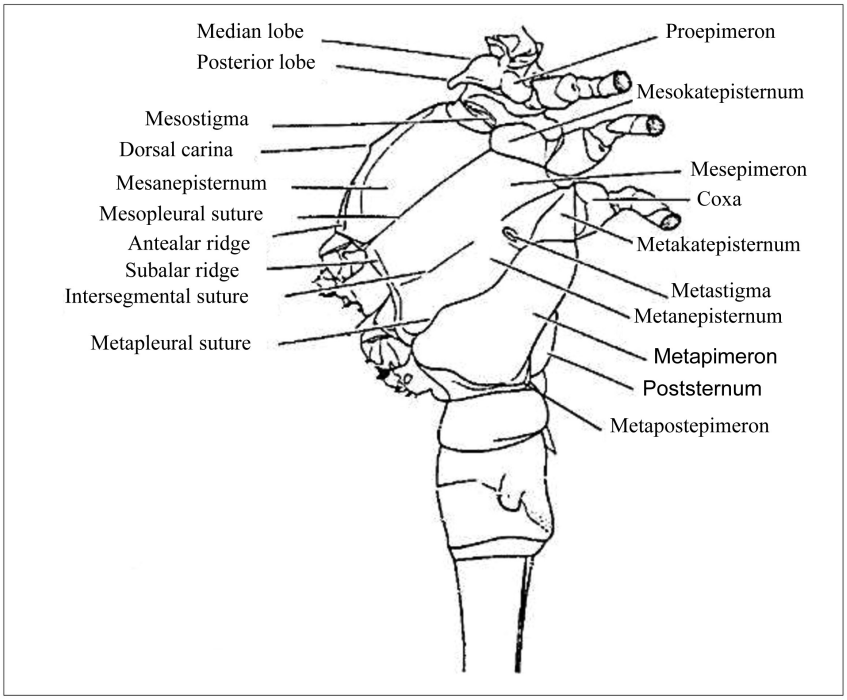


Fig. 1. Lateral view of thorax and anterior abdomen. Characters used in order to create the key identification for females of *Rhinocypha* spp. using the morphological nomenclature by Djikstra et al. (2014) and modified from Gunther (2009).

variation within the females of the three species of *Rhinocypha*, principle component analysis (PCA) were conducted on the landmark coordinates data set, while to examine the amount of symmetric variation and shape dimorphism, Procrustes ANOVA were used. Thin plate spline deformation grids were generated and used to visualize shape variation along PC axes (Bookstein 1991). On the other hand, canonical variate analyses (CVA), a multivariate statistical method, was conducted to determine the shape characteristics that best distinguished the groups of specimens from each other by using these coordinates. All analyses were then run using MorphoJ software version 1.06d (Klingenberg 2011).

Phylogeny Comparison

A total of five individuals for each *Rhinocypha* species were used to estimate a phylogenetic tree from this group. Another chlorocyphid, *Rhinocypha bisignata* Hagen, 1853 (MF358830), was used as an outgroup.

Genomic DNA was extracted from four to six legs of each fresh specimen using the i-genomic CTB DNA Extraction Mini Kit (iNtRON Biotechnology Inc., Seongnam, South Korea) (see Appendix 1). The DNA amplifications of both *COI* and 16S rRNA genes were conducted using an Applied Biosystems Veriti 96-Well Thermal Cycler (Applied Biosystems Inc., Foster City, CA, USA), with the amplification protocol consisting of 300 sec at 94°C followed by 35 cycles of 50 sec at 94°C, 50 sec at 50°C and 50 sec at 72°C, and a final 7 min at 72°C.

Primers amplifying the mitochondrial-encoded *COI* gene were adopted from Folmer et al. (1994) (forward primer: 5'- GGT CAA CAA ATC ATA AAG ATA TTG G – 3') and Barrett and Hebert (2005) (reverse primer: 5'- GGA TGG CCA AAA AAT CAA AAT AAA TG –3'). For the 16S rRNA gene, ODO 12852

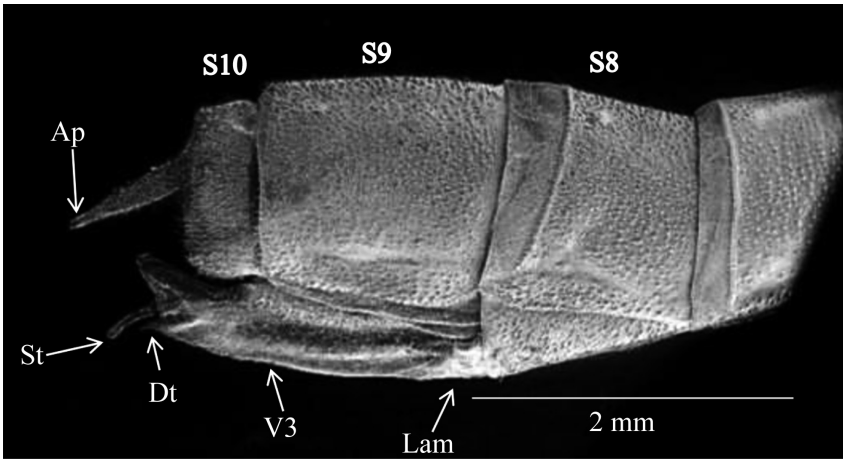


Fig. 2. Lateral view of the external morphology of the ovipositor of *Rhinocypha* spp. Ap: anal appendages; St: stylus; Dt: distal tooth; V3: third valves of ovipositor (valvulae 3); Lam: basal plate of ovipositor (lamina valvarum).

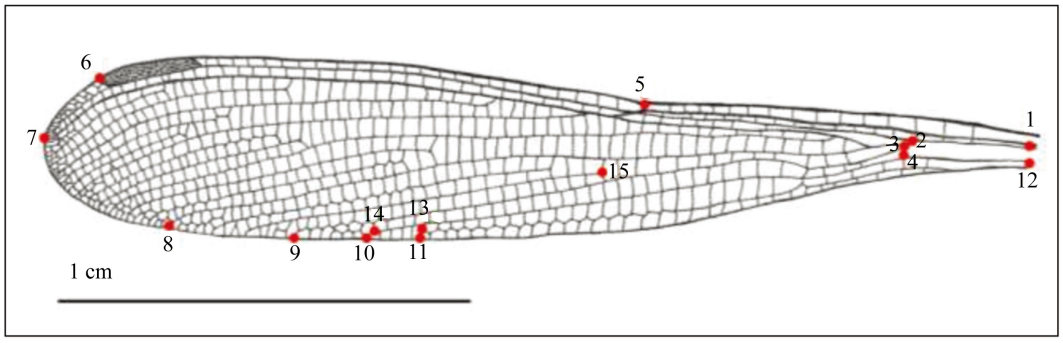


Fig. 3. Landmark configuration of *Rhinocypha* spp. Fifteen landmarks were used in geometric morphometric analysis. Landmarks represent: (1) costa – subcostal connection, (2, 3 & 4) distal angles of arculus, (5) the nodus, (6) posterior intersection of the pterostigma and radius 1 (R1), (7) end of vein radius 2 (R2), (8) posterior end of the radius 4 (R4), (9) posterior end of the anterior media (MA), (10) posterior end of the Cubital Vein (CuP), (11) posterior end of the Anal Vein 1 (A1), (12) proximal apex of anal triangle, (13) anterior end of the cubital vein supplementary (Cupspl); (14) anterior end of the anterior media supplementary (Mspl); and (15) anterior end of the radius 4 supplementary (R4spl).

and ODO 13393 primer set (forward primer, 5'- AGA AAC CGA CCT GGC TTA AA -3'; reverse primer, 5'- CGC CTG TTT ATC AAA AAC AT -3') was adopted from Dijkstra et al. (2014). Each PCR amplification was performed in a reaction mixture containing 50–100 ng of genomic DNA, 25 µL of NEXpro e-PCR 2x Master Mix (Genes Labs Inc., Gyeonggi-do, South Korea), and 10 pmol of each forward and reverse primer.

The amplified samples were then electrophoresed on 2% agarose gel pre-stained with SYBR Safe™ (Invitrogen Corp., Carlsbad, CA, U.S.A.) and the PCR products were sent to a commercial company for DNA sequencing in both forward and reverse directions. The samples were sequenced using the BigDyeH Terminator 3.1 Sequencing Kit.

All the five sequences for each *Rhinocypha* spp. using *COI* gene (614 bp) and 16S rRNA (533 bp) were assembled and edited using Molecular Evolutionary Genetics Analysis (MEGA) software Version 6.0 (Tamura et al. 2013) and BioEdit 7.0.9.0 (Hall 1999) and preliminarily aligned using MUSCLE (Edgar 2004a b). The step was further analysed using Hasegawa-Kishino-Yano model for *COI* and combined *COI* + 16S rRNA genes, while the General Reversible Chloroplast model for the 16S rRNA gene based on the the best DNA/Protein Models (ML) suggested by MEGA was used to build the Maximum Likelihood (ML) phylogenetic tree with a bootstrap replicate of $n = 2000$.

These representative sequences were deposited into the GenBank database under accession numbers MZ229751-MZ229765 for *COI* and MZ230039-MZ230053 for 16S rRNA genes. To compare their phylogenetic relationships, *Rhinocypha bisignata* (MF358830) *COI* sequences publicly available in the GenBank database were included in the analyses.

For data analysis, the step was further analyzed to build a Neighbor-joining phylogenetic tree of *Rhinocypha* species based on combined *COI* + 16S rRNA sequences with the bootstrap replicate of $n = 1000$. The evolutionary distances were computed using the Maximum Composite Likelihood method. The evolutionary analyses were conducted and performed using MEGA version 6 (Tamura et al. 2013).

RESULTS

Morphological Description of Female *Rhinocypha* spp.

The male of *Rhinocypha* spp. is easy to identify by the distinct blue thoracic marks on its thorax or abdomen. However, identifying the females is challenging, and it is difficult to differentiate them from other females of the same genus (Hämäläinen and Divasiri 1997). The *Rhinocypha* spp. can be a challenge to the studies on odonates, although the males are conspicuous with established key identification (Lahiri and Sinha 1985; Orr 2002; Hämäläinen et al. 2009), females are more cryptic at species level.

Generally, the female species in the *Rhinocypha* group that can be identified within each species among other features is the pterostigma. The coloration of the pterostigma was distinct from each species, although it looks very similar with the naked eye (Fig. 4), besides the apparent brown marking at the tip of the *R. biforata* wing. In addition, the markings on the thorax seen in lateral view (Fig. 5) suggests that the species has different yellow marking and some sort of blue marking in *R. biforata* species at the thorax. The following is a detailed key for describing dichotomous females of *Rhinocypha* spp.

Key to species for the genus *Rhinocypha* Rambur (Females)

1a. Tiny yellow stripe at below of mesopleural suture 2a
1b. Widened yellow stripe at below of mesoplueral suture 2b
2a. Yellow marking on metanepisternum, occupying approximately half of width with extend to mesepimeron at anterior part 3a
2b. Much more extensive of yellow marking on metanepisternum, occupying more to two third of width with extend to mesepimeron at anterior part 3b
3a. Tiny yellow stripe at the tip of intersegmental suture, tiny yellow stripe at upper part of mesanepisternum which form a circle to near of mesostigma segment ..... 5 (Fig. 5a)
3b. Bellow intersegmental suture, widened yellow mark at the tip 4
4a Tiny of yellow stripe at upper part of mesanepisternum segment with yellow stripe extending down in front of mesoplueral suture ..... 6a (Fig. 5c)
4b Long yellow stripe at the upper part of mesanepisternum which form a yellow lobe to near of mesostigma segment and broadened at the tip ..... 6b (Fig. 5b)
5. Metakatepisternum dark-brown with pale yellow at below segment; no marking at the wing; yellow at the centre of the pterostigma distinct, Wing length : Wing width (24–26 mm : 5 mm), Abdomen length 16.6–18.8 mm ..... *fenestrella* Rambur
6a. Metakatepisternum dark-brown and surrounded by yellow pale; brown marking at the tip of the wing; pterostigma generally markedly paler of black colour, Wing length : Wing width (23–24.5 mm : 4 mm), Abdomen length 14.8–17.7 mm .... *biforata* Seyls
6b. Metakatepisternum dark-brown with pale yellow at upper segment; no marking at the wing; pterostigma with brownish color, Wing length : Wing width (24–25 mm : 4.5 mm), Abdomen length 15.6–17.8 mm ..... *perforata* Percheron

Description of the Female's Ovipositor in Three Species of *Rhinocypha* using FESEM

Figure 2 shows the lateral view of the external morphology of the ovipositor of *Rhinocypha* spp.

generated from the field emission scanning electron micrograph. The basal plate of the ovipositor (Lam) connects the first valves with the sternite of the 8th segment (S8) and tergite of the 9th segment (S9). The sheathing valves (V3) showed ensheathing cutting valves laterally in a resting position. In addition, the anal appendages (Ap) were connected with the 10th segment (S10), and the stylus (St) and distal tooth (Dt) were connected at the end of the V3.

Below the figure (Fig. 6), showed the images of the 8th, 9th and 10th segments, together with ovipositor parts representatives of each species of the *Rhinocypha* group, *Rhinocypha biforata*, *Rhinocypha fenestrella* and *Rhinocypha perforata* generated by FESEM in lateral view. In addition, it showed the morphometric measurements taken for each part of each individual of the samples.

Based on high-resolution images generated in this study, the ovipositor of females *Rhinocypha* species were categorized in the following morphological types:

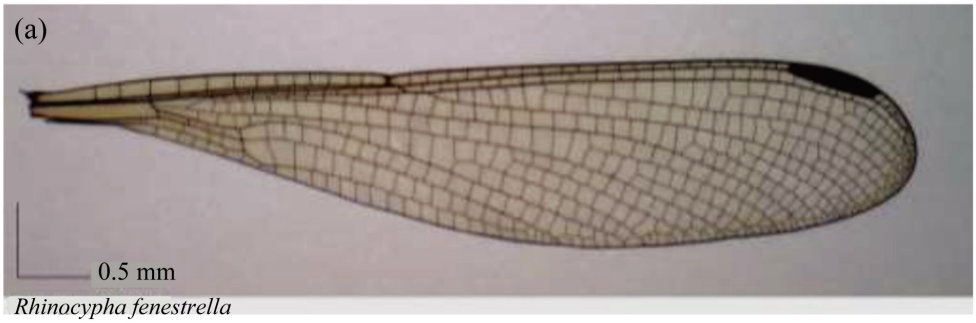
1) The sensilla and the setae of the anal appendages.

2) The characteristics of the sheathing valve (V3) and distal tooth.

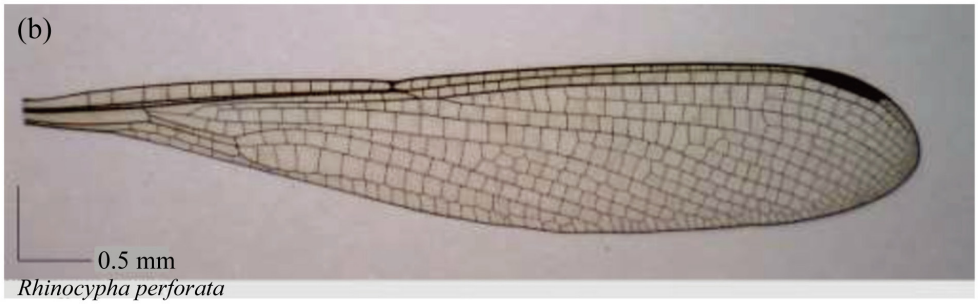
3) The hair sensilla of the stylus.

Anal appendages

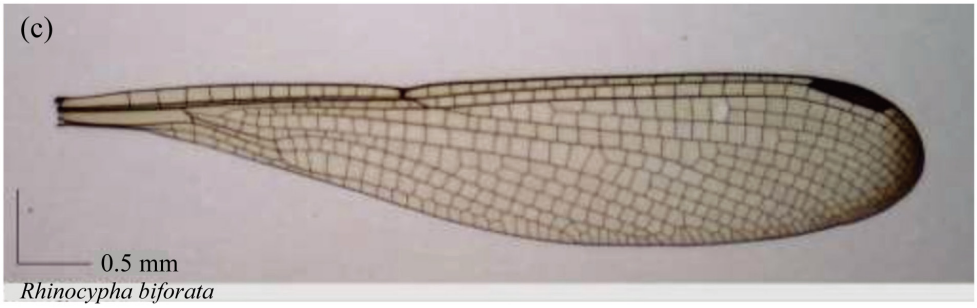
After visualization of the anal appendages for each species of *Rhinocypha*, it showed three primary characteristics (Fig. 7). For the species of *R. biforata*, they had few long articulated setae (Fig. 7a), compared to species of *R. fenestrella* (Fig. 7b). However, for the species of *R. perforata*, they had short articulated setae (Fig. 7c). On the other hand, the distribution of the basiconic sensilla in *R. biforata*, they had a compact of the basiconic sensilla in their anal appendages, whereas in *R. fenestrella* anal appendages, they had more space



Rhinocypha fenestrella



Rhinocypha perforata



Rhinocypha biforata

Fig. 4. Wing of *Rhinocypha* spp. (a) *Rhinocypha fenestrella*, (b) *Rhinocypha perforata*, and (c) *Rhinocypha biforata*.

between the basiconic sensilla, and as the articulated setae, the *R. perforata* had a compact of short basiconic sensilla. Instead, *R. fenestrella* had a lot of coeloconica-like sensilla compared to the other two species.

Sheathing valves (V3)

Internal view of the apical part of carina showed that the three species of *Rhinocypha* had a different shape from each other (Fig. 8). For the species of *R. biforata*, they had sharply pointed of the carina and they are more diagonal in projection (Fig. 8a), while for the species of *R. fenestrella*, they had evenly sharply pointed of the carina and they are more vertical in projection (Fig. 8b). In contrast in *R. perforata* species, they had the blunt and rounded tip of carina in the sheathing valves (V3) (Fig. 8c). In addition, the three species had no differences in the distal tooth (Fig. 8e, f, g).

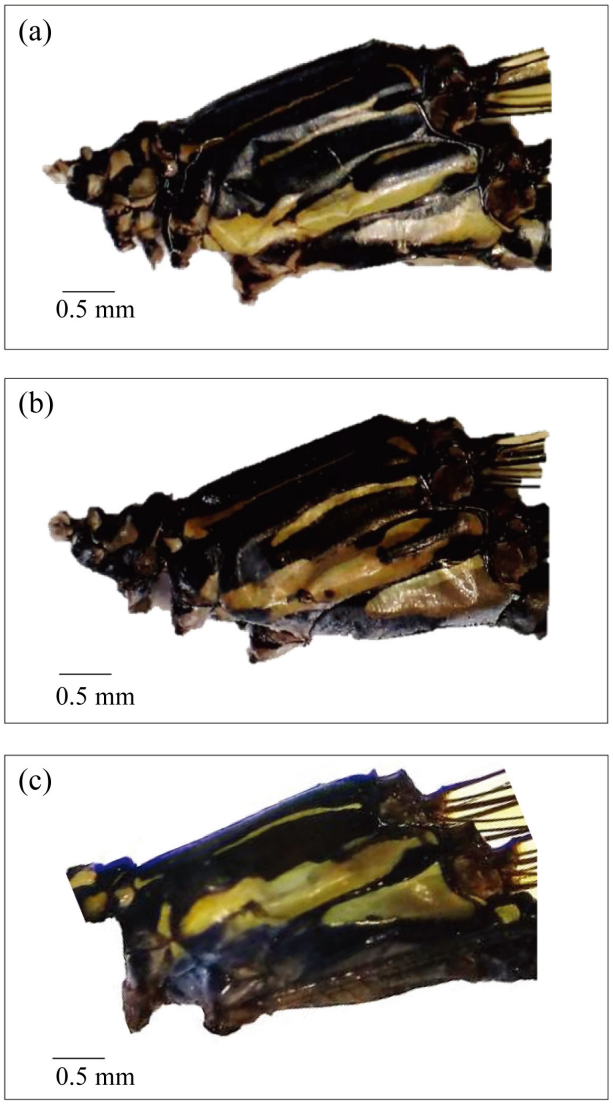


Fig. 5. Thorax of the females of *Rhinocypha* spp. (a) *R. fenestrella*, (b) *R. perforata*, and (c) *R. biforata*.

Stylus

Examining the apex of the stylus showed they had different shapes and distribution of sensilla and knobble (Fig. 9). For the species of *R. biforata*, the sensilla were gathered at the tip of the stylus, while *R. fenestrella* species, the sensilla scattered throughout the stylus. In addition, for *R. perforata* species, the sensilla were scattered at the tip of the stylus. On the other hand, figure 9d, e, and f showed the base of the stylus of the three studies species where the knobbles of *R. biforata* were rounded and not compact as in the species of *R. fenestrella* that had rounded, more compact of the knobbles and evenly distributed at the base of the stylus. Conversely, the base of the stylus of the species of *R. perforata* showed they had flat and scattered knobbles.

On top of that, from the micrographs generated by using the FESEM, the morphometric measurements were taken in several parts of the female ovipositor (Table 1). The table below shows that species of *R. fenestrella* had longer of 8th and 9th segment compared to the two species, *R. biforata* and *R. perforata*, but had the shortest 10th segment. In addition, *R. biforata* species had the longest anal appendages, while *R. perforata* had the widest base of the anal appendages and stylus, and the widest stylus compared to the other two species.

Moreover, in terms of V3, *R. biforata* had the widest of V3, and also showed they had more space between the distal tooth. As well, consistent with the micrograph taken from the FESEM, the distal tooth of *R. fenestrella* was wider and more in upright protrusion likened to the species of *R. biforata* and *R. perforata.*

Geometric Morphometric Analysis of the Wings

The landmark configuration of the Procrustes superimposed coordinates for the wings are presented in figure 10. Overall, the landmarks 6, 7, and 15 of the forewings of *Rhinocypha* spp. are more variable than the other landmarks. Between the three *Rhinocypha* damselflies, the landmarks of the *R. biforata* demonstrated more shape variation than the other species, suggested by the percentage of the variance of the principal component analysis.

The wireframe in figure 11 visualizes the shape variation on the axes. PC1 of *R. biforata* species accounted for 72.92% of the total variance, besides *R. fenestrella* accounted for 40.53% and 52.29% for

R. perforata. The species with high scores on PC1, *R. biforata*, have a shorter wing length compared to the other species; the species with the lowest PC1 scores have a longer wing length.

The PCA plot graph (Fig. 12), showed considerable dispersion across morphospace among species. The first five principal components explaining 88.68% of total variation accounted for 48.64%, 17.45%, 9.96%, 8.33% and 4.30% respectively. A total of up to six axes were required to cover more than 90% of the shape variation.

Accordingly, the PCA analysis of the three species explained 66.09% of shape variation within samples by the two first PCA axes extracted from the variance-covariance matrix (PC1 explains 48.64% and PC2, 17.45%). A plot of PC1 and PC2 demonstrated overlapping of wing shapes between the three species of *Rhinocypha.*

Differences in shape among species were described in terms of thin-plate deformation grids and the coordinates of landmarks were used for estimating the overall size of the wing known as centroid size, an isometric estimator defined as the square root of the sum of the squared distances of all landmarks from their centroid. Figure 13 showed the thin-plate spline deformation grids of wing shape variation and the species-specific differentiation was evident in the forewing in the three species of *Rhinocypha.*

From the thin-plate deformation grids, *Rhinocypha biforata* presented narrower wings, whereas *Rhinocypha fenestrella* had broader wings. On the other hand, the species of *Rhinocypha perforata* had a broader elongated apex.

In contrast to PCA, the differences between species well illustrated by a canonical variate analysis (CVA) plot. The CVA was applied to the Procrustes coordinates extracted from the fore wings of all the samples. A scatter plot of CV1 (eigenvalue 8.887) vs. CV2 (eigenvalue 2.150) showed that the wing shapes of the three species of *Rhinocypha* were not overlapping each other and well clustered according to species (Fig. 14). This suggested that the geometric morphometric of the wing shapes successfully differentiate between the species of *Rhinocypha* group.

Phylogeny Comparison

The phylogenetic relationships of the investigated damselflies were recovered using two different DNA regions; *COI* and 16S rRNA (Fig. 15). The Maximum Likelihood (ML) analysis revealed that the phylogeny of all the samples was separated into three clades in the both regions, *COI* (Fig. 15a) and 16S rRNA (Fig. 15b),

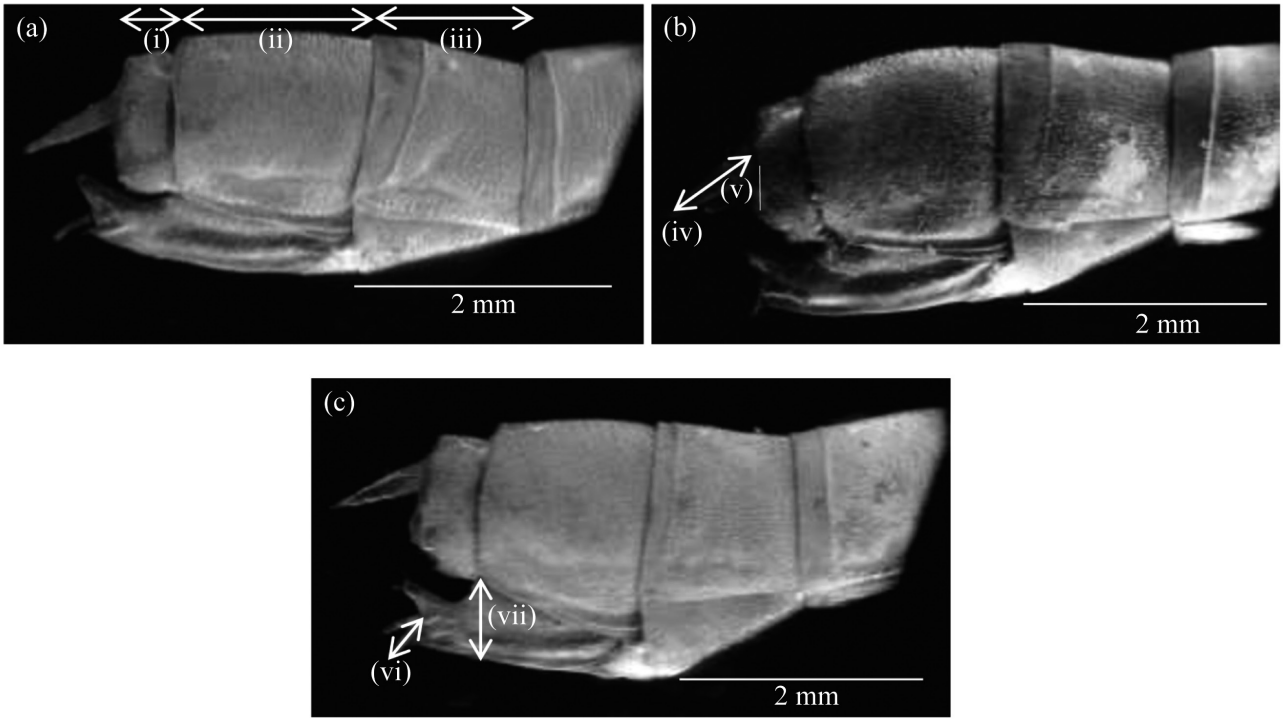


Fig. 6. Scanning electron micrographs of the morphometric measurements of the ovipositor for the females of *Rhinocypha* spp. (lateral view). (a) *Rhinocypha biforata*, (b) *Rhinocypha fenestrella*, (c) *Rhinocypha perforata.* (i, ii & iii) length of each segment; (iv) length of anal appendages; (v) basal width of anal appendages; (vi) length of stylus; (vii) width of the V3.

and the combined dataset (Fig. 15c) that were supported by high bootstrap values of > 50%. The species of *R. biforata* and *R. perforata* formed a separate monophyletic clade with a high bootstrap support for 16S rRNA region, and for *COI* and combined both regions respectively.

As observed in the ML tree, *R. biforata* and *R. fenestrella* together formed a monophyletic group clearly separated from the investigated *R. perforata* species based on the *COI* gene and both combined regions. Additionally, *R. biforata* species were recovered as a sister taxon to *R. fenestrella.* The relationships among *Rhinocypha* seemed clearly resolved and one consistent finding across all analyses was that the three

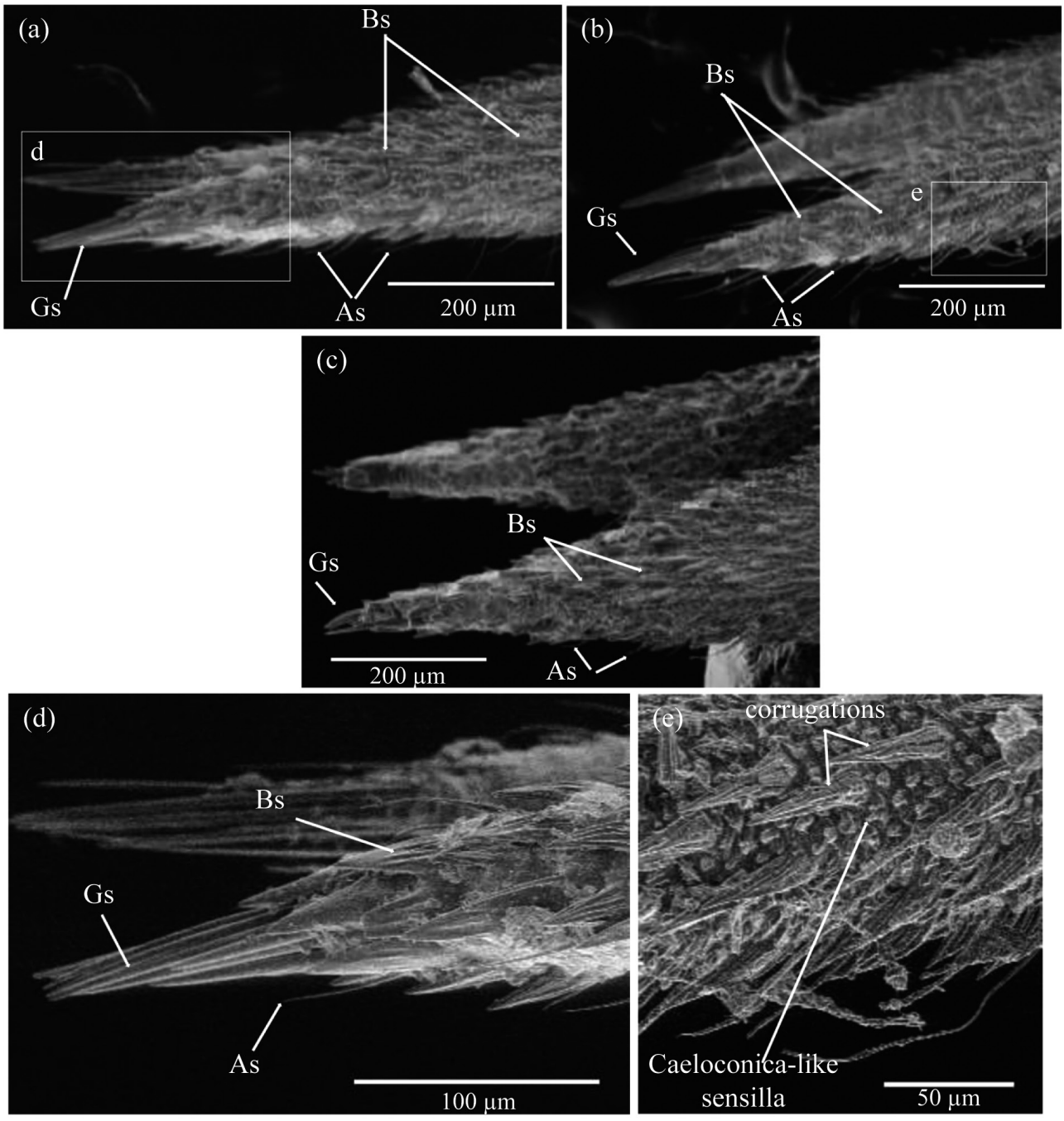


Fig. 7. Scanning electron micrographs of anal appendages of *Rhinocypha* spp. (a) *Rhinocypha biforata* – inset indicates the group of sensilla; (b) *Rhinocypha fenestrella* – inset shows the caeloconica-like sensilla; (c) *Rhinocypha perforata*; (d) group of sensilla on the tip of anal appendages; (e) caeloconica-like sensilla on the surface of the anal appendages. Gs: group of sensilla; Bs: basiconic sensilla; As: articulated setae.

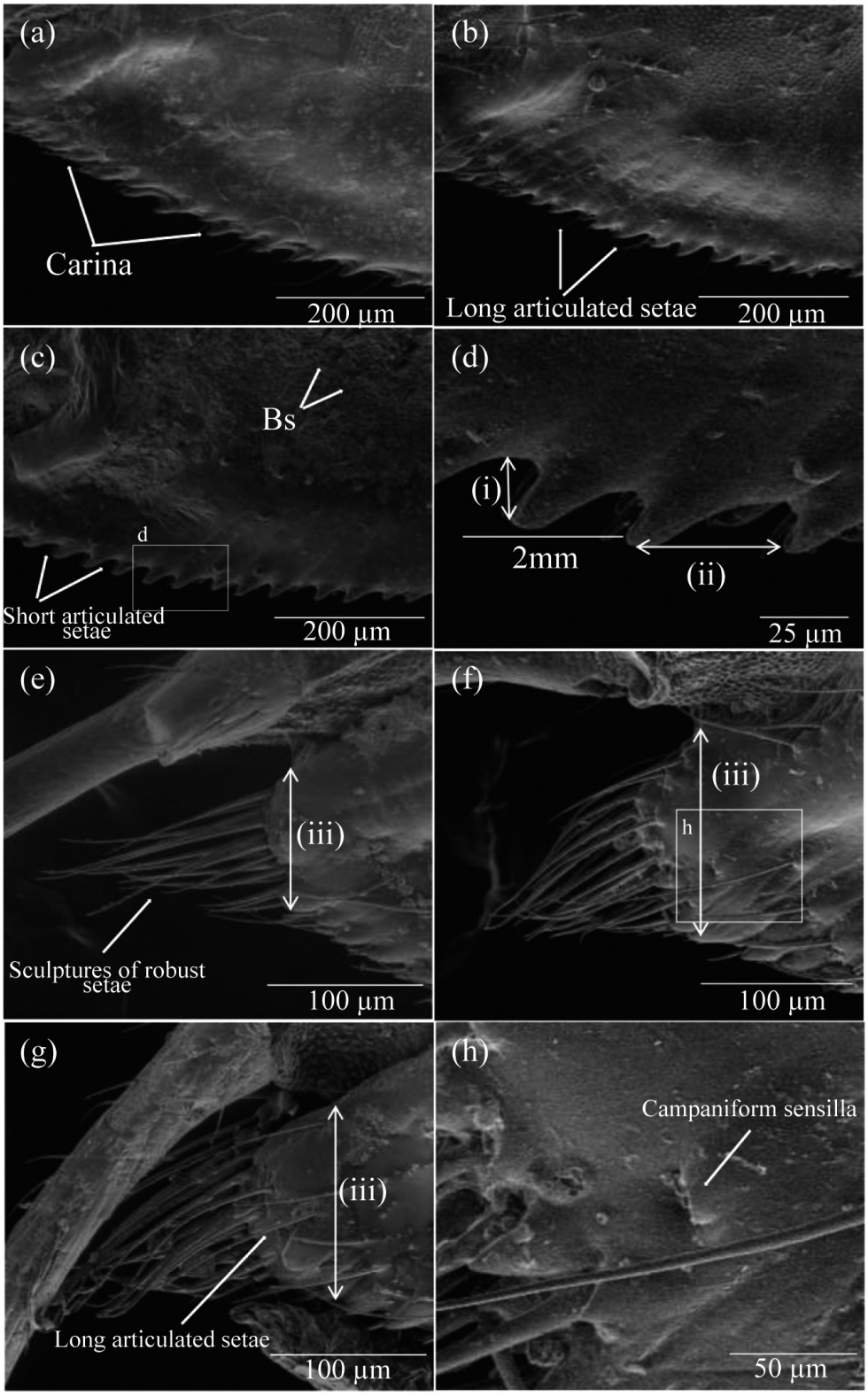


Fig. 8. Scanning electron micrographs of sheathing valve (V3) and distal tooth of *Rhinocypha* spp. (a) *Rhinocypha biforata*, (b) *Rhinocypha fenestrella*, (c) *Rhinocypha perforata* – inset shows the carina, (d) measurement of (i) the peak of the tooth to the median base (ii) the space between the tooth. (e, f & g) shows the scanning electron micrographs of distal tooth of *Rhinocypha* spp.: (e) *Rhinocypha biforata* - (iii) indicates the width of the distal tooth, (f) *Rhinocypha fenestrella* – inset shows the campaniform sensilla, (g) *Rhinocypha perforata*, (h) campaniform sensilla at the distal tooth surface.

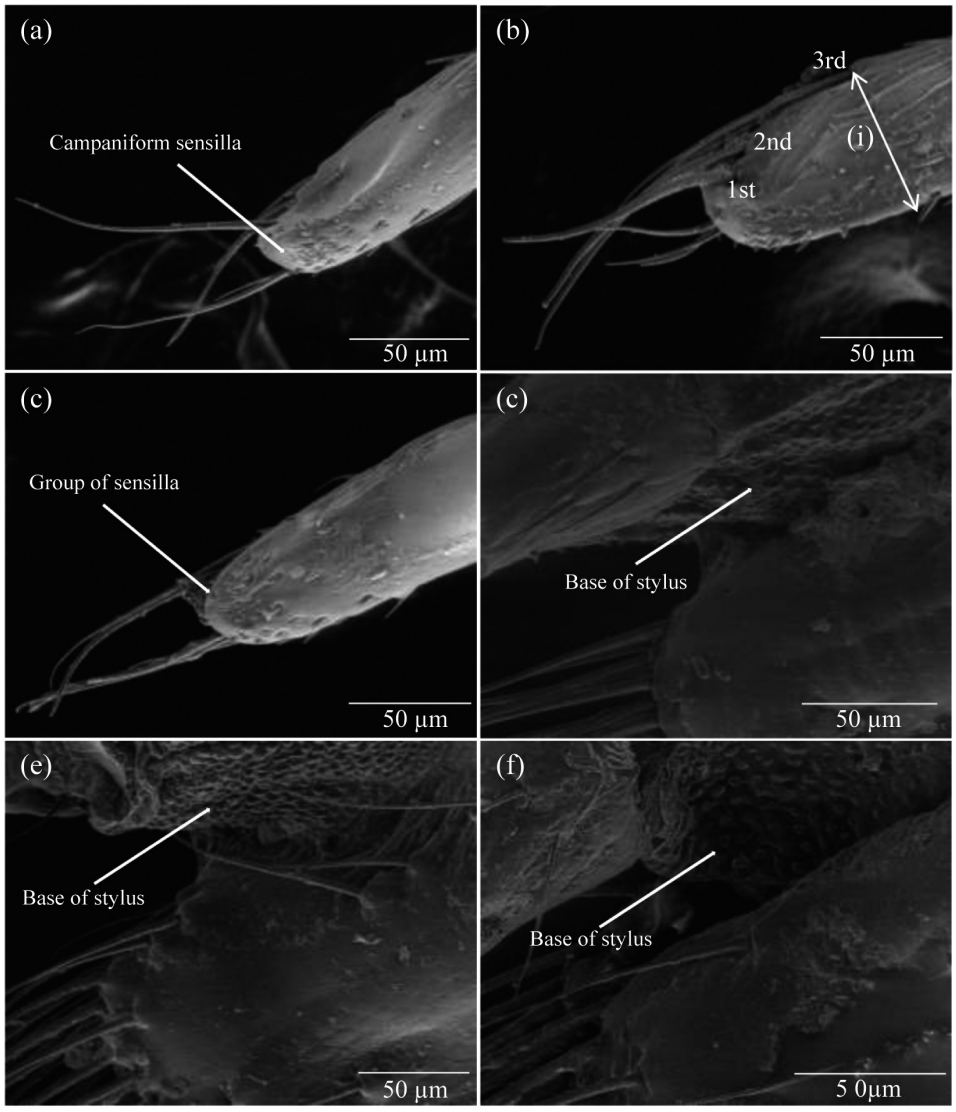


Fig. 9. Scanning electron micrographs of the stylus and the base of stylus of *Rhinocypha* spp. (a) *Rhinocypha biforata*, (b) *Rhinocypha fenestrella* – (i) width of the stylus from the third of hair sensilla, (c) *Rhinocypha perforata.* (d, e & f) scanning electron micrographs of the base of stylus. (d) *Rhinocypha biforata*, (e) *Rhinocypha fenestrella*, (f) *Rhinocypha perforata.*

Table 1. Morphometric measurements calculated from the ovipositor of the females' of *Rhinocypha* spp. The values show the mean ± standard deviations of each characteristic

Character of female's ovipositor	*R. biforata*	*R. fenestrella*	*R. perforata*
Length of 8th segment	1.205 ± 0.088	1.383 ± 0.002	1.215 ± 0.122
Length of 9th segment	1.512 ± 0.044	1.563 ± 0.023	1.446 ± 0.160
Length of 10th segment	0.375 ± 0.062	0.318 ± 0.023	0.359 ± 0.076
Length of anal appendages	0.877 ± 0.094	0.789 ± 0.103	0.846 ± 0.067
Basal width of anal appendages	0.221 ± 0.018	0.241 ± 0.062	0.256 ± 0.062
Length of stylus	0.215 ± 0.082	0.158 ± 0.009	0.287 ± 0.024
Width of V3	0.625 ± 0.093	0.620 ± 0.038	0.620 ± 0.039
Peak of tooth to the median base	0.017 ± 0.003	0.017 ± 0.003	0.016 ± 0.002
Space between the tooth	0.042 ± 0.019	0.040 ± 0.016	0.032 ± 0.008
Width of the distal tooth	0.119 ± 0.023	0.160 ± 0.008	0.141 ± 0.005
Width of the stylus	0.040 ± 0.007	0.043 ± 0.008	0.050 ± 0.005

species were clustered well in their own specific cluster, together with *R. bisignata* which was used as the outgroup. On the whole, the phylogenetic relationships support the generic status of *Rhinocypha* spp.

DISCUSSION

Adults of odonates are conspicuous, easy to record, and taxonomically well studied (Brown 1991). Although there is a wealth of data available to be utilized for solving taxonomic problems, there remain existing confusions around how to correctly identify closely related and sympatric species, especially in female odonates. However, the similarities in the appearance of odonates, their behaviour and their body size support the view that at least two species could not live in the same habitat (Khelifa et al. 2013). The female of the three studied species belonged to the *Rhinocypha* genus had broadly the same appearance and similar body size, which made it difficult to distinguish between the species.

The detailed morphological studies from this work revealed that *Rhinocypha fenestrella* has a slightly longer and broader wing compared to *Rhinocypha perforata*, followed by *Rhinocypha biforata*. Similarly, the abdominal length in the order of longest to shortest; where *R. fenestrella* > *R. perforata* > *R. biforata*. Although all three species had enfumed wings, *Rhinocypha biforata* had a brown marking at the tip of the wing while *Rhinocypha perforata* had more extensive yellow color at the thorax. It has been shown that coloration (Andrew 1966), apart from flight pattern (Pajunen 1966), affects visual recognition of adult Odonata.

The ovipositor structures are known to play an important role in determining species differences. According to Matushkina (2011), a well-developed ovipositor in Odonata is represented by three main elements: (1) the shaft of the ovipositor, including paired cutting 1st and 2nd valves; (2) paired large plates, the 3rd valves; the distal edges of the 3rd valves that bear moveable stick-like appendages, the styli (gonostyli of 9th segment); and (3) several sclerites associated with the ovipositor valves (paired gonocoxites of 8th segment and gonanguli, unpaired internal sclerite).

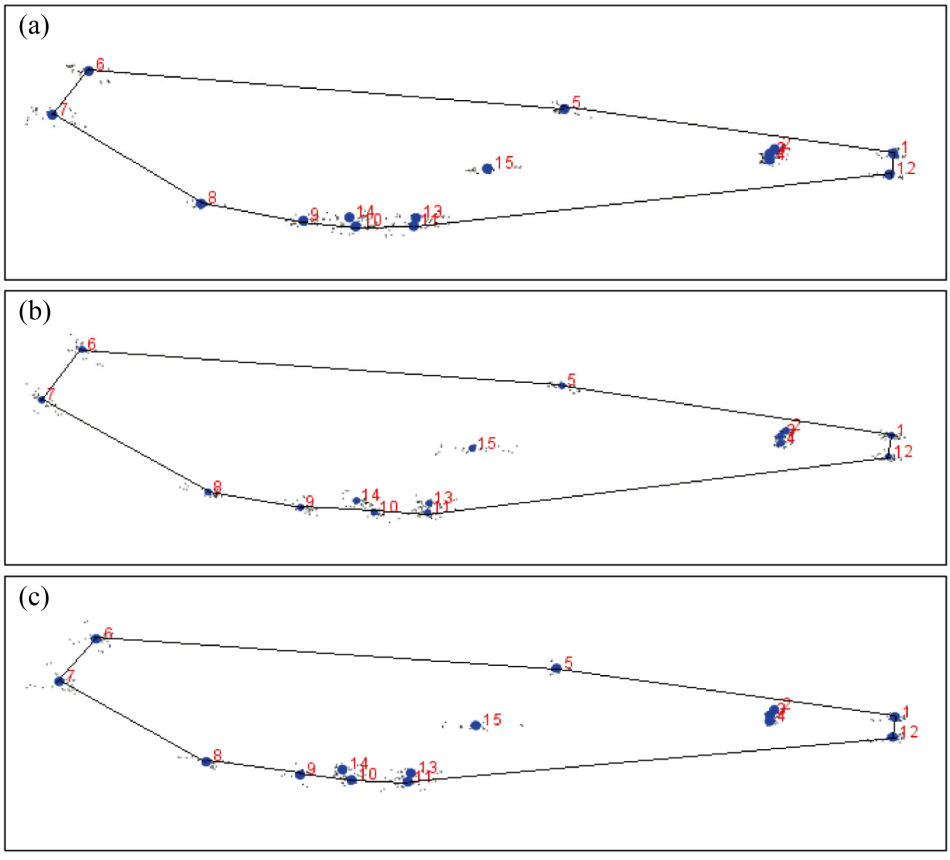


Fig. 10. Scatterplot of all 15 landmarks configurations after Procrustes superimposition. The plotted line and blue dots represent the mean shape for the respective species; (a) *Rhinocypha biforata*, (b) *Rhinocypha fenestrella*, (c) *Rhinocypha perforata.*

The ovipositor of *Rhinocypha* spp. belonged to the endophytic type that occurred in all Zygoptera, the anisozygopteran, *Epiophlebia superstes*, and most aeshnids (Asahina 1954; St. Quentin 1962; Pfau 1985; Matushkina and Gorb 1997; Matushkina 2004 2008). This study examined the ovipositor in *Rhinocypha* spp. using the FESEM, focusing on three structural parts; the sensilla and setae of the anal appendages, the V3 and

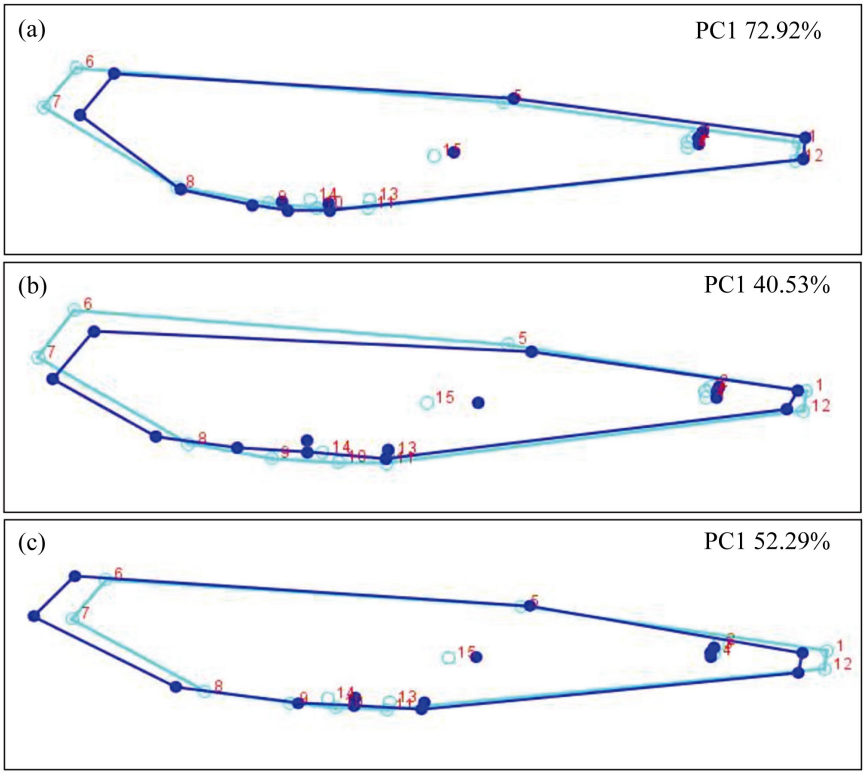


Fig. 11. Wireframe visualization of shape variation along the principal components one (PC1) from geometric morphometric analysis. (a) *Rhinocypha biforata*, (b) *Rhinocypha fenestrella*, (c) *Rhinocypha perforata*. Light blue landmarks represent the configuration of average specimen; dark blue landmarks represent one approximate extreme of the variation on that axis. Percentages indicate the proportion of total variance explained by each axis.

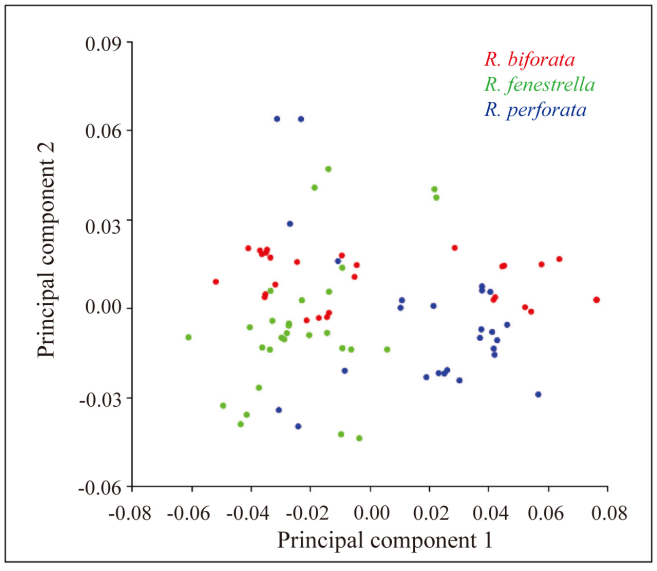


Fig. 12. Results of principal components analysis of all specimens. PC1 = 48.64%, PC2 = 17.45%, accounting for 66.09% of the total variation.

distal tooth, and the stylus.

The ovipositor of *R. biforata* was shown as compact basiconic sensilla and few long setae at the anal appendages, while *R. perforata* had short articulated setae and compact of short basiconic sensilla. This could be differentiated from the species *R. fenestrella* where there were more spaces between basiconic sensilla and a lot of coeloconica-like sensilla in contrast to other species. Previous studies found that the phylogenetically informative characters might be found in microstructural features such as in the position and shape of sensilla, and serrations of valves, but this possibility would require a systematic examination of representatives of other ovipositor-bearing Odonata groups (Matushkina 2007).

Another distinguishing feature is the shape on the carina of V3. *Rhinocypha biforata* had a sharp-pointed carina compared to *R. perforata* with a more diagonal projection while vertical projection in *R. fenestrella.* The teeth of V3 were fused to form a bearing edge, or carina, by which females posturally leaned against oviposition substrates during egg-laying behaviour. A study of *Lestes macrostigma* revealed that the row of teeth on the carina of V3 functions to hold the female abdomen on the plant surface during plant penetration (Matushkina and Lambret 2011). The field of campaniform sensilla on the basis of the stylus on V3 responded to the stylus inclination when the ovipositor contacted a substrate. These two components, located symmetrically on the right and left styli, serve as controllers of spatial characteristics of an egg clutch, such as was previously presumed for *Lestes sponsa* (Matushkina and Gorb 2002). For *Rhinocypha* spp., the three species had a different shape and distribution for the sensilla and knobbles at the stylus. This might relate to the stylus inclination and could imply a chemosensory function.

Furthermore, the robust setae at the apex of the stylus and on the carina of V3 were in contact with the

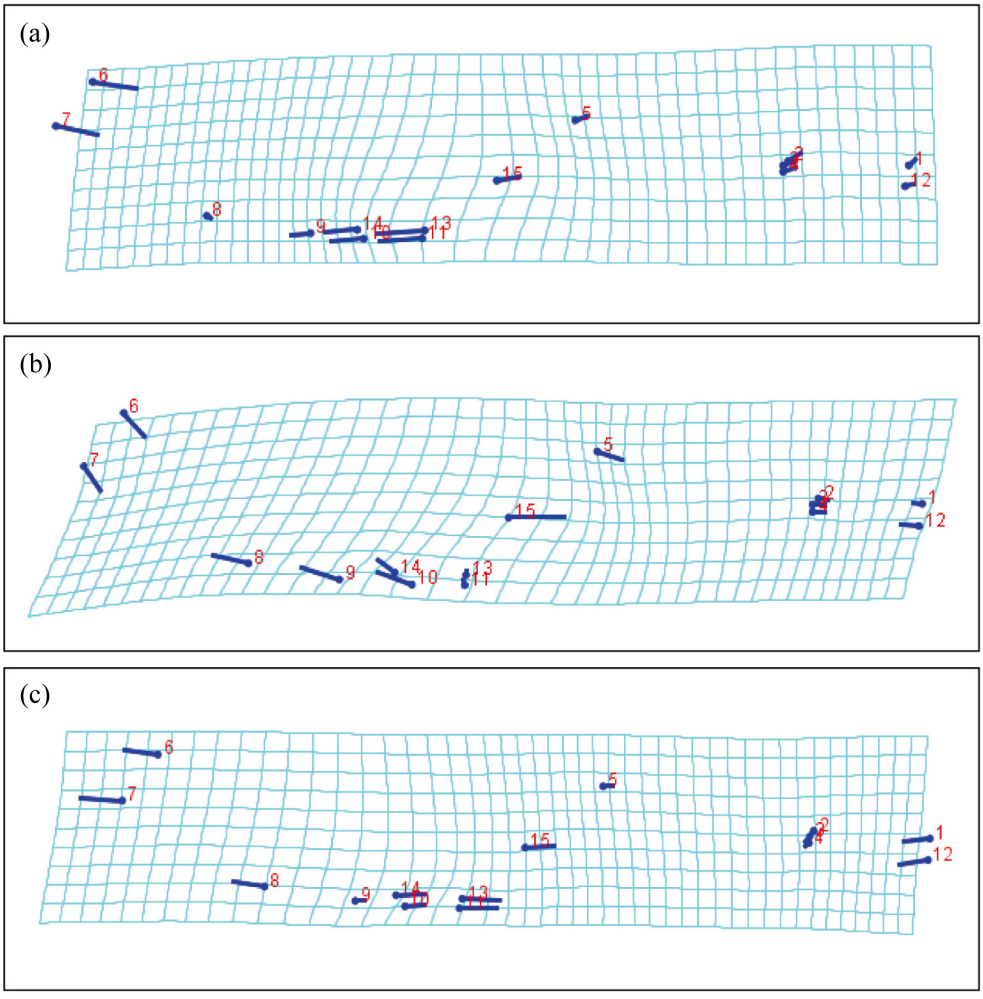


Fig. 13. Thin-plate spline deformation grids of wing shape variation in *Rhinocypha* spp. (a) *Rhinocypha biforata*, (b) *Rhinocypha fenestrella*, (c) *Rhinocypha perforata*, demonstrating the directions (arrows).

plant surface during egg-laying and probably function as mechanoreceptors, since they lack any pore on the surface area. Several knobbles, serrations, and ridges, which were found on the external surface of the cutting valves, probably function in the sawing of plant tissues (Matushkina and Lambret 2011).

While a previous study successfully used different microscopy techniques on the structure of the wings to reveal the flexibility of the wings (Mamat-Noorhidayah et al. 2018), the use of FESEM on the ovipositor now has become one of the techniques used in taxonomy (Matushkina and Gorb 2002 2007; Matushkina and Lambret 2011; Matushkina and Klass 2011). The genitalia is a complex structure that the basis for species discrimination in most families and also in family identification (Powell 2009), and the illustration of the anal appendages of the female would be very helpful (Heckman 2008).

On the other hand, geometric morphometric analysis was able to differentiate the females of *Rhinocypha* spp. and confirm the population differences based on wing shapes. This analysis of wing shape is a useful tool and can be applied to ecological and evolutionary research in odonates (Córdoba-Aguilar 2008). According to Zelditch et al. (2004), an advantage of using wing shape as a discriminating character is that wing with two-dimensional structures, made the alignment of specimens for digitizing landmarks easier and more accurate compared to three-dimensional structural characters, creating possible measuring errors caused by different alignments of individual specimens.

In this study, 15 homologous landmarks were used to quantify wing shape variation. The results of the analyses indicated that morphological variation affected different parts of the wing differently, where it was found that landmarks 6, 7, and 15 of the forewings of *Rhinocypha* spp. were more variable compared to the other landmarks. Additionally, the landmarks of the *R. biforata* demonstrated more shape variation than the other species, suggested by the percentage of the variance of the principal component analysis.

The decomposition of variance components according to landmarks showed that the landmarks differed in the amount of variation for each species of *Rhinocypha.* The factor that especially stood out in this respect was directional asymmetry. The previous study suggested that this was not simply a random outcome linked to the subtlety for this effect, and this directional asymmetry was also discovered in two species of flies (Klingenberg et al. 1998).

Wing shape analysis was successful for population differentiation in the European *Calopteryx splendens* (Sadeghi et al. 2009), variation in flight morphology in *Enallagma cyathigerum* (Bots et al. 2009), wing shape evolution (Johansson et al. 2009), and the effects of latitude and selection on wing shape in *Calopteryx virgo meridionalis* (Outomuro and Johansson 2011). This landmark-based wing shape analysis was shown to be useful for discriminating damselflies in the *Euphaea* species group, such as among the *E. guerini* species complex and geographical populations of *E. masoni* on the mainland of Southeast Asia (Van Tol and Rozendaal

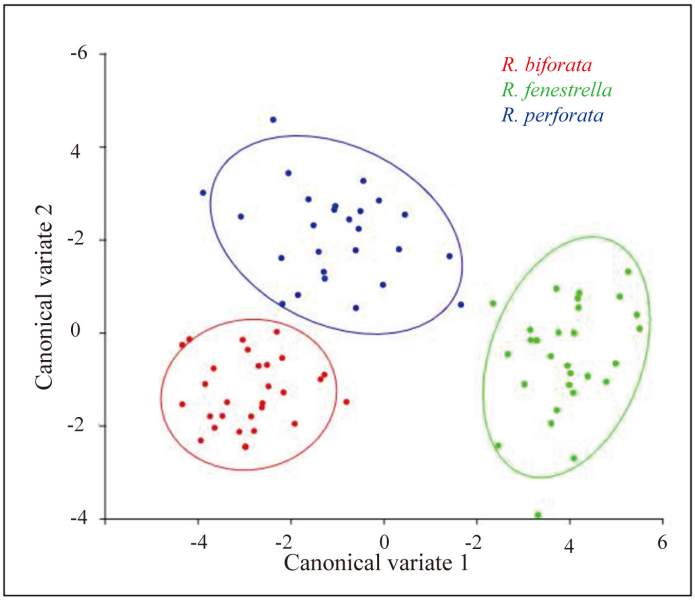


Fig. 14. Canonical Variate analysis (CVA) plot. CV1 (eigenvalue 8.887) vs CV2 (eigenvalue 2.150). 90% confidence ellipses of CVA scores. Colour of ellipses corresponds to the species written alongside.

1995; Hämäläinen and Karube 2001; Toan et al. 2011), and between *E. subcostalis* and *E. subnodalis* in Borneo (Orr and Hämäläinen 2003).

This study concludes that the *R. biforata* has narrower wings compared to *R. fenestrella* which has a broader wing, while *R. perforata* has a broader elongated apex. As a result of the wing shape variation for this damselfly group, the three species were separated in the Canonical Variate Analysis (CVA). Each species created an independent cluster, making a considerable clear separation. Studies have suggested that various selective pressures, including landscape structure (Taylor and Merriam 1995), food and predation stress (Stoks 2001; Svensson and Friberg 2007), latitude and sexual selection (Outomuro and Johansson 2011) can affect the evolution of wing shapes in damselflies. Consequently, the strength of using geometric morphometric for wing analysis was displayed in the technique's ability to pinpoint the location and direction of specific features for the presence of variation. A proper comprehensive

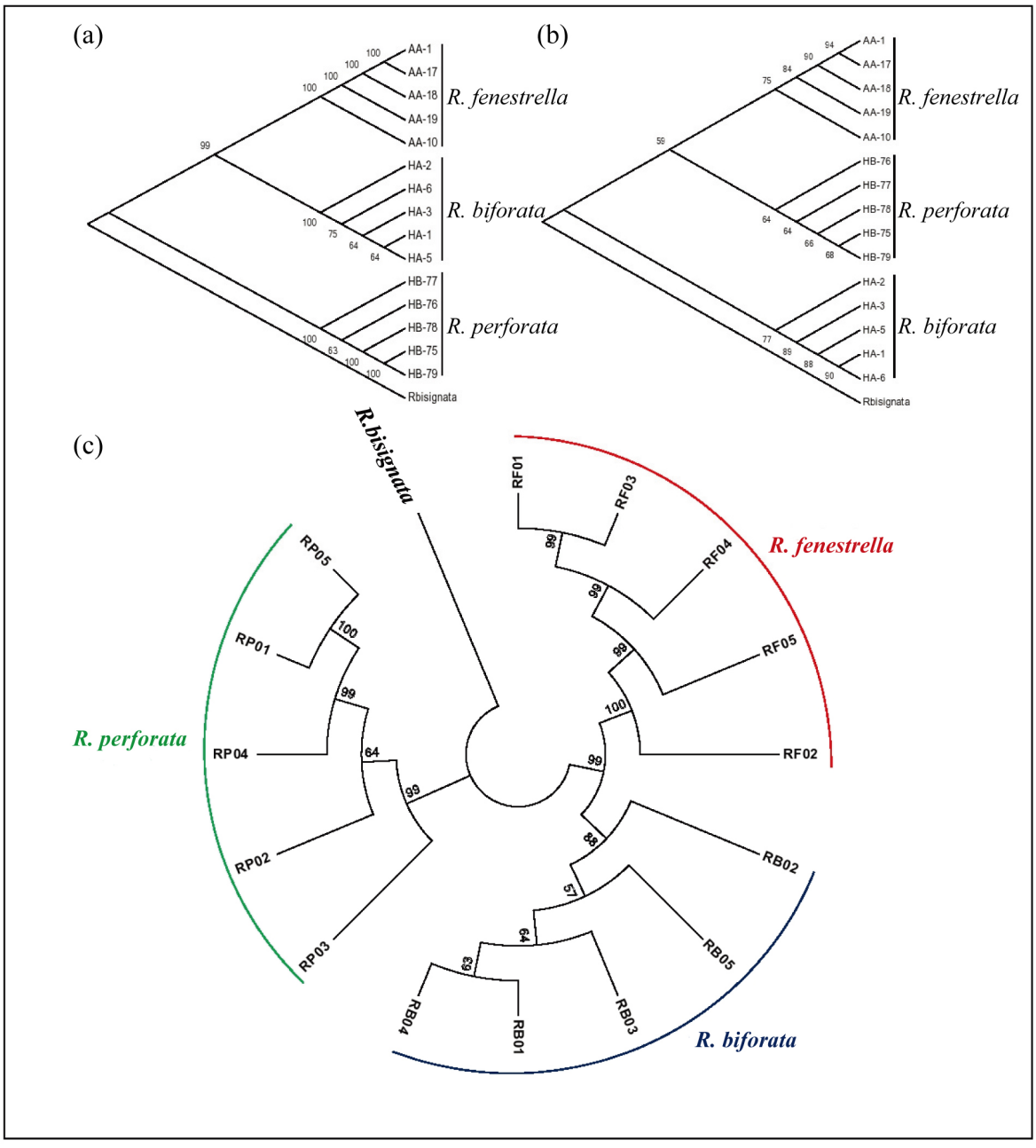


Fig. 15. Maximum Likelihood (ML) phylogenetic tree of *Rhinocypha* spp. based on (a) *COI* gene, (b) 16S rRNA gene, and (c) combined *COI* + 16S rRNA sequences with *R. bisignata* as an outgroup. Bootstrap values are shown on the branches.

analysis of wing shape would thus provide insight into phenotypic variations related to flight performance, a character that should be under selection.

Finally, the molecular analysis revealed distinct interspecific contrasts within the genus. The three *Rhinocypha* taxa formed three different clades groups separated from each species based on two DNA regions: *COI* and 16S rRNA. It could be inferred that the genus Chlorocyphidae contains a monophyly of *R. fenestrella*, *R. biforata*, and *R. perforata*, and *R. bisignata* as an outgroup. To date, the phylogenetic relationships of the genera and species of Chlorocyphidae are poorly understood (Van Tol 1998). However, in 2014, a group of researchers suggested that families within Zygoptera were monophyletic—*e.g.*, Calopterygidae, Euphaeidae, Isostictidae, Lestidae, Lestoideidae, Platystictidae, and Polythoridae, including the family Chlorocyphidae (Dijkstra et al. 2014). This finding further confirmed previous work (Rehn 2003; Bybee et al. 2008; Dumont et al. 2010) that the family Chlorocyphidae is monophyletic and showed reasonable congruence with the classification by Bechly (1996).

CONCLUSIONS

The odonates are now receiving worldwide attention as objects of research, and their phylogenetic position makes them important in comparative studies on the evolution of genomic innovations. Surprisingly, in spite of all the odonate studies, few are taxonomic in nature, especially in Malaysia. This study successfully distinguished the female of sympatric species of *Rhinocypha* group for the first time, using a cohesive approach based on morphology, Field Emission Scanning Electron Microscope, geometric morphometric, and DNA molecular. The present study offers new insights into odonate research, utilizing a combination of classic as well as modern tools and methods. These findings will hopefully prompt more investigations into the potentially vast aspects of such study to promote greater interest in odonates.

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Authors' contributions: MN, YNR designed the study. MN performed the field work and the experiments. MN, AA analysed the data. MN wrote the original draft. All authors contributed to revising the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials: The data and materials that support the findings of this study are available from the corresponding author and University of Malaya upon reasonable request.

Consent for publication: All of the authors consent to the publication of this manuscript.

Ethics approval consent to participate: Not applicable.

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Supplementary materials

Appendix 1. Sequence lengths and accession numbers of the specimens for *COI* and 16S rRNA genes. (download)