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# Phylogeny of Libellulidae: are there relationships between molecular phylogenetics and morphological analysis of wing shape of dragonflies? 

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Background: Establishing the species limits and resolving phylogenetic relationships are primary goals of taxonomists and evolutionary biologists. At present, a controversial question is about interspecific phylogenetic information in morphological features. Are the interspecific relationships established based on genetic information consistent with the traditional classification system? To address these problems, this study analyzed the wing shape structure of 10 species of Libellulidae, explored the relationship between wing shape and dragonfly behavior and living habits, and established an interspecific morphological relationship tree based on wing shape data. By analyzing the sequences of mitochondrial COI gene and the nuclear genes 18S, 285 rRNA and ITS in 10 species of dragonflies, the interspecific relationship was established. Method: The wing shape information of the male forewings and hindwings was obtained by the geometric morphometrics method. The inter-species wing shape relationship was obtained by principal component analysis (PCA) in MorphoJ1.06 software. The inter-species wing shape relationship tree was obtained by cluster analysis (UPGMA) using Mesquite3.2 software. The COI, 18S, ITS and 28S genes of 10 species dragonfly were blasted and processed by BioEdit v6 software. The maximum parsimony (MP) tree was established by Puap4.0 software. The Bayes inference (BI) tree was established by MrBayes 3.2.6 in Geneious software. Results: The main difference in forewings among the 10 species of dragonfly was the apical, radial and discoidal regions dominated by the wing nodus. In contrast, the main difference among the hindwings was the apical and anal regions dominated by the wing nodus. The change in wing shape was closely related to the ability of dragonfly to migrate. The interspecific relationship based on molecular data showed that the species of Orthetrum genus branched independently of the other species. Compared to the molecular tree of 10 species, the wing shape clustering showed some phylogenetic information on
the forewing shape (with large differences in the forewing shape tree vs. molecular tree), and there was no interspecific phylogenetic information of the hindwing shape tree vs. molecular tree. Conclusion: The dragonfly wing shape characteristics are closely related to its migration ability. Species with strong ability to migrate have the forewing shape that is longer and narrower, and have larger anal region, whereas the species that prefer shortdistance hovering or standing still for a long time have forewing that are wider and shorter, and the anal region is smaller. Integrating morphological and molecular data to evaluate the relationship among dragonfly species shows there is some interspecific phylogenetic information in the forewing shape and none in the hindwing shape. The various regions of the forewing and hindwing are inconsistent, which may be due to their different functions.

# Phylogeny of Libellulidae: are there relationships between molecular phylogenetics and morphological analysis of wing shape of dragonflies? 

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#### Abstract

Background: Establishing the species limits and resolving phylogenetic relationships are primary goals of taxonomists and evolutionary biologists. At present, a controversial question is about interspecific phylogenetic information in morphological features. Are the interspecific relationships established based on genetic information consistent with the traditional classification system? To address these problems, this study analyzed the wing shape structure of 10 species of Libellulidae, explored the relationship between wing shape and dragonfly behavior and living habits, and established an interspecific morphological relationship tree based on wing shape data. By analyzing the sequences of mitochondrial COI gene and the nuclear genes $18 S$, $28 S r R N A$ and ITS in 10 species of dragonflies, the interspecific relationship was established. Interspecific phylogenetic information regarding wing shape structure was analyzed. Method: The wing shape information of the male forewings and hindwings was obtained by the geometric morphometrics method. The inter-species wing shape relationship was obtained by principal component analysis (PCA) in MorphoJ1.06 software. The inter-species wing shape relationship tree was obtained by cluster analysis (UPGMA) using Mesquite3.2 software. The COI, $18 S$, ITS and $28 S$ genes of 10 species dragonfly were blasted and processed by BioEdit v6


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Results: The main difference in forewings among the 10 species of dragonfly was the apical, radial and discoidal regions dominated by the wing nodus. In contrast, the main difference among the hindwings was the apical and anal regions dominated by the wing nodus. The change in wing shape was closely related to the ability of dragonfly to migrate. The interspecific relationship based on molecular data showed that the species of Orthetrum genus branched independently of the other species. Compared to the molecular tree of 10 species, the wing shape clustering showed some phylogenetic information on the forewing shape (with large differences in the forewing shape tree vs. molecular tree), and there was no interspecific phylogenetic information of the hindwing shape tree vs. molecular tree.

Conclusion: The dragonfly wing shape characteristics are closely related to its migration ability. Species with strong ability to migrate have the forewing shape that is longer and narrower, and have larger anal region, whereas the species that prefer short-distance hovering or standing still for a long time have forewing that are wider and shorter, and the anal region is smaller. Integrating morphological and molecular data to evaluate the relationship among dragonfly species shows there is some interspecific phylogenetic information in the forewing shape and none in the hindwing shape. The various regions of the forewing and hindwing are inconsistent, which may be due to their different functions.

Subjects Entomology, Biodiversity, Taxonomy, Zoology
Keywords Dragonflies, Molecular taxonomy, Morphological taxonomy, Libellulidae, Interspecific relationship, Wing, Clustering, Phylogeny.

## Introduction

The morphological evolution of insects and the formation of species have been the scientific issues that taxonomists, evolutionary biologists and ecologists are constantly exploring (Misof et al., 2014; Crispo, 2008; Ho \& Zhang, 2018). In natural selection and adaptation, insects have formed diverse phenotypic characteristics and genetic structure (Lundsgaard-Hansen, Matthews \& Seehausen, 2014; Schneider, 2000). With the continuous development and improvement of modern molecular biology technology, establishing reliable inter-species ancestry from a genetic perspective has been well documented (Mack \& Nachman, 2017; Soria-Carrasco et al., 2014;

Gompert et al., 2014). However, the traditional classification system is based on the morphological characteristics of the species. Currently, the hot issue to be explored is whether the interspecific relationships established by morphological features can be supported by the molecular data or, in other words, to what extent the current classification system is supported (Virgilio et al., 2010; Lukhtanov, Sourakov \& Zakharov, 2016; Renaud, Savage \& Adamowicz, 2012). Because morphological characteristics involve ecological adaptation and behavioral problems, such as living in the same ecological environment, similar feeding behaviors and patterns of movement lead to morphological similarities, whereas genetic structures may be those of completely different species. According to the decade-old literature, the difference in genetic structure between species does not necessarily appear in phenotype (Stern \& Orgogozo, 2008). Alternative views are that different genetic structures can produce similar phenotypic patterns (Robertson, 1959; Wlikens, 1971). Therefore, genetic diversity may not necessarily be related to morphological differences. However, in recent decades, studies in molecular biology and developmental biology have suggested that mutations in gene expression regulation may promote phenotypic evolution, especially the change in morphological characteristics (Kaessmann, 2010; Rabosky, 2012; Crispo, 2008). It indicates that the differences in the genetic structure are predictable, and to a certain extent, they will result in differences in the morphological structure. These contradictory views are common in evolution of the related species of insects (Víctor \& Zúñiga, 2016; Heikkilä et al., 2015). In recent years, with the constant development and improvement of morphological measurement technology, the study of population evolution law and systematic generation relationship based on morphological characteristics has been published (Klingenberg \& Marugán-Lobón, 2013). Geometric morphometrics is an advanced method of morphological analysis in biology; based on the curve, landmark point and semi-landmark point data of the homologous locus concept, it can accurately quantify the phenotypic traits of organisms and explore the morphological evolution of populations (Cooke \& Terhune, 2015; Baylac, Villemant \& Simbolotti, 2003).

A large number of studies have shown that the morphology-based interspecific relationship is basically consistent with the interspecific relationship established by molecular data when the morphological characteristics are selected judiciously (Grzywacz et al. 2017; Noguerales, Cordero \& Ortego, 2018). Marin et al. (2017) study showed that the interspecific relationship of Nymphalidae based on wing shape and wing vein was consistent with that based on molecular
data. It indicated the existence of phylogenetic information in the insect wing morphology. Francischini et al. (2017) used COI gene and female genital structure to illustrate the interspecific relationship of Diatraea by the molecular and morphological methods, and the results obtained by the two methods were consistent in the classification of the interspecific relationships. Similar studies were conducted by Ortego, Aguirre \& Cordero (2012) on the population differentiation of Mioscirtus wagneri locust in different geographical regions by using quantitative morphological features (anterior and posterior plates) and mtDNA, suggesting that morphology-based geographical differentiation correlated with geographical differentiation at the molecular level. Therefore, in many animals, the law of genetic differences can be reflected in morphology. However, the establishment of interspecific phylogenetic relationships based on morphology and molecular data can also lead to inconsistent results. For example, Bocek \& Bocek (2017) showed the morphology of beetle pronotum cannot fully support interspecific phylogenetic relationships. Bapst, Schreiber \& Carlson (2018) used molecular and morphological data to study the interspecific relationship of Branchiopoda and found that the morphological data did not have interspecific phylogenetic information. Due to the common phenomenon of coevolution in nature, the morphological features may only reveal some difference in phenotype of different research objects, but to accurately reflect the phylogenetic relationships among species the morphological data need to be combined with the molecular data for synthesis.

This research selected 10 species of dragonfly from the same habitat to study their interspecific relationships. Libellulidae belong to Odonata, and have two pairs of large and transparent membranous wings, with the wing veins clearly visible; the shape and direction of the wing veins are often used as an important classification basis for dragonflies (Fauziyah et al., 2014). Geometric morphometrics was used to analyze the morphological differences among species. The mitochondrial gene COI and nuclear genes $18 S$, $I T S$ and $28 S$ were used to analyze the phylogenetic relationships among the species. We analyzed the phylogenetic relationships based on wing-type features as well as on molecular data. Accordingly, this study addressed the following questions: 1) what was the relationship between the characteristics of the wing shape and the behavior of the dragonfly? 2) Did the wing shape and wing vein contain interspecific phylogenetic information? 3) Did forewings and hindwings exhibit a consistent pattern of morphological changes in different species?

## Materials \& Methods

## 1 Materials and Data acquisition

1.1 Specimen collection and image acquisition

In order to explore the relationship between the wing shape of different species of dragonfly in Odonata, we collected dragonflies from May to October 2018 in Taizhou City(121.3495E, 28.6522 N ) and its surrounding areas. After classification and identification, the males of 10 species of dragonfly were selected for this research. A total of 84 individuals were studied. The species names and numbers are shown in Table 1. The wings of all specimens were spread; then, the left forewing and the left hindwing were taken and pressed between two slides to make slide specimens. The forewings and hindwings were photographed with a Nikon 5100 camera, fixed on a stand. A ruler and a slide specimen were placed on the same horizontal plane for photographing. All photographs were made using the identical camera settings and were saved in a picture format for later use.

Ethics statement
All collected dragonflies under anesthesia to death. Dragonfly is not a legally protected species according to China and international conservation legislation. Under China legislation, there are no ethical policies that apply to experiments on wild insects like dragonflies.

Table 1 Species name, genus, subfamily, family and the number of specimens of each species
1.2 Landmark data acquisition

The TPSdig2 software (Rohlf, 2000) was used to digitize wing images of 10 species of Libellulidae, identifying 26 landmarks on the forewing and 27 on the hindwing (in each case, including two on a ruler) (Fig. 1). The landmark-based geometric morphometrics method was applied to study the morphological diversity in wing size and shape. We set landmarks at the intersections of wing veins with the wing margin and intersections of cross veins with major veins and vein branch points (Table 2), which was according to Rohlf \& Corti (2000).

## Table 2 Definition and numbering of the landmarks

### 1.3 Wing shape analysis

The forewing and hindwing shape information was input into CoordGen software (Rohlf \& Slice, 1990) in the IMP series package. Based on the ruler, the errors caused by the focal length of the photograph were eliminated, and the datum line was set. To examine wing-shape
variation, digitized landmark data were subjected to generalized procrustes superimposition to standardize the size of the landmark configurations and eliminate differences caused by translation and rotation (Adams, Rohlf \& Slice, 2004). All standardized data were converted into a two-dimensional data format.

Figure 1 Landmarks on the forewing and hindwing of Libellulidae. A. Landmarks 1 to 24 on forewing, 25 to 26 are ruler; B. Landmarks 1 to 25 on hindwing, 26 to 27 are ruler

## 2 Methods and Analysis

2.1Statistical analysis of morphological data

The standardized morphological information data were imported into MorphoJ1.06d software (Klingenberg, 2009), and the morphological changes of 10 species of dragonfly were analyzed by principal component analysis (PCA), Procrustes analysis and Discriminant analysis. The first two main components were extracted as scatter plots of forewings and hindwings. In the MorphoJ1.06d software, the thin-plate spline analysis (Bookstein, 1989) was performed, and the difference in landmark points was analyzed. The visualized legend was used to show the variation in forewings and hindwings in the first two principal components.
2.2 Acquisition of molecular data

The DNA barcode data of 10 species of dragonfly was obtained from the NCBI website. We obtained Cytochrome oxidase subunit $I(C O I)$ gene of each species with length of 349 bp and $18 S$ $r R N A$, Internal Transcribed Space1 (ITS1) $+5.8 s r R N A+$ Internal Transcribed Space2 (ITS2) and $28 S r R N A$ of each species with length of 747 bp . All data were imported into BioEdit v6 software for editing, and the built-in clustalw was used to blast sequences (Hall TA, 1999). Total obtained $C O I+18 s+I T S+28 s$ gene data with a length of 1096 bp was used to construct the maximum parsimony (MP) tree and the Bayesian inference (BI) tree. The gene sequence numbers and related information are shown in Table 3.

Table 3 Family, subfamily and GenBank number of 10 species of Libellulidae

### 2.3Establishment of morphological and molecular phylogenetic trees

In this study, Mesquite 3.2 software (Maddison \& Maddison, 2009) was used to cluster the morphological characteristics of forewings and hindwings of 10 dragonfly species. The cluster analysis was based on the landmark data for forewings and hindwings of each species established as a matrix. The distance among the taxa represented uncorrected distance. Then, the
relationships among the populations were further summarized based on the unweighted pairgroup method with arithmetic averages (UPGMA) to build forewing and hindwing shape trees (Ramirez-Sánchez, Luna \& Cramer, 2016).

The sequence data were analyzed using maximum parsimony method (MP) and Bayesian inference method (BI). For the maximum parsimony reconstruction, a tree bisectionreconnection (TBR) branch swapping heuristic search was run using Geneious and PAUP 4.0 with 10,000 random additions (Swofford, 2002). Gaps were treated as missing data. To estimate branch support, 500 bootstrap pseudoreplicates were performed using 10 random addition searches per pseudoreplicate (Felsenstein, 1981).

Bayesian Inference (BI) using MrBayes 3.2.6 (Huelsenbeck JP \& Ronquist F, 2001) was executed from within Geneious (Kearse M et al., 2012). Both programs suggested a GTR + I + G model (Yang Z, 1994) for the mitochondrial gene COI and nuclear gene $18 S+I T S+28 S$. All BI analyses consisted of $1.1 \times 10^{6}$ generations of Markov Chain Monte Carlo searches containing 4 chains, heated chain temperature of 0.2 and burn-in of 100,000 generations. Compound Dirichlet priors for branch lengths were assigned to avoid branch-length overestimation using the following: prset brlenspr =unconstrained, gammadir(1.0,0.1,1.0,1.0) shapepr=exponential(10.0). Trees were saved every 1,000 generations. The confidence values of the BI tree were presented as the Bayesian posterior probabilities (BPP) in percentages. Phylogenetic analyses were performed for each of the gene sequences.

## Results

## 1. Morphological differences in forewings and hindwings of 10 species of dragonflies

## (Libellulidae)

The wing shape data were analyzed by PCA and centroid size to find out the shape variation (Fig. 2A). The first two PCs accounted for $35.09 \%$ and $21.77 \%$ of the variation, with the cumulative variation explaining $56.86 \%$ of the total shape variation in forewings. Procrustes analysis (Table 4) of forewings showed Deielia phaon and Pantala flavescens to have the smallest distance (0.006), suggesting their forewing shape differences was small. Trithemis aurora and Tramea virginia had the largest distance (0.120), meaning their forewing shape differences were large. Discriminant analysis results showed no significant difference in forewing shapes between Deielia phaon and Pantala flavescens ( $P=1.000$ ), significant
differences between Crocothemis servilia and Orthetrum albistylum ( $P=0.023$ ) and Crocothemis servilia and Orthetrum melania( $P=0.042$ ), and strongly significant differences among the other species ( $\mathrm{P}<0.01$ ). A scatter plot (Fig. 2A) of the first and second principal components showed that on the $\mathrm{PC1}$ axis, Trithemis aurora, Pseudothemis zonata and Orthetrum testaceus were mainly distributed on the negative direction, whereas the other seven species were mainly distributed on the positive direction. Taking into account the profile plots of the wing veins (Fig. 3), the differences mainly occurred in the apical region (LM6-8) and the discoidal region (LM1114). On the PC2 axis, Orthetrum melania, Tramea virginia, Trithemis aurora and Pseudothemis zonata were positioned mainly on the negative direction, and the other six dragonfly species were distributed mainly on the positive direction. The forewing profiles (Fig. 3) showed that the differences occurred mainly in the apical (LM6-8) and the radial region (LM8-10). Centroid Size Analysis (Fig. 2B) results showed that Deielia phaon, Pantala flavescens and Acisoma panorpoides had smaller, and Tramea virginia and Orthetrum melania had larger forewings.

Figure 2 PCA (A) and Centroid Size Analysis (B) of forewings of 10 dragonfly species (Libellulidae). Ap: Acisoma panorpoides; Pz: Pseudothemis zonata; Pf: Pantala flavescens; Tv: Tramea virginia; Om: Orthetrum melania; Dp: Deielia phaon; Cs: Crocothemis servilia; Ot: Orthetrum testaceus; Ta: Trithemis aurora; Oa: Orthetrum albistylum.

## Figure 3 Thin-plate spline analysis of forewing profiles of $\mathbf{1 0}$ dragonfly species

(Libellulidae). Each profile represents the deformations in wing shape in extreme conditions for each PC.

Table 4 The Procrustes distance of forewing and hindwing shape among 10 species of Libellulidae

The hindwing shape data were analyzed via PCA and Centroid size to find out the shape variation (Fig. 4A). The first two PCs accounted for $37.08 \%$ and $21.41 \%$ of the variation, with the cumulative variation explaining $58.49 \%$ of the total shape variation in hindwings. Procrustes analysis (Table 4) on hindwings showed Crocothemis servilia and Orthetrum testaceus with the smallest distance (0.026), suggesting their hindwing shapes were similar. The Acisoma panorpoides and Tramea virginia had the largest distance (0.132), indicating relatively large differences in their hindwing shapes. Discriminant analysis showed no significant difference in
hindwing shapes between Orthetrum melania and Pseudothemis zonata $(\mathrm{P}=0.111)$, significant differences between Crocothemis servilia and Orthetrum testaceus ( $P=0.034$ ), Crocothemis servilia and Orthetrum albistylum $(\mathrm{P}=0.046)$ and Crocothemis servilia and Orthetrum melania ( $P=0.014$ ), and strongly significant differences between Crocothemis servilia and Pseudothemis zonata $(P=0.001)$ and among the other species ( $P<0.01$ ). A scatter plot of PC 1 vs. PC2 (Fig. 4A) showed that on the PC1 axis, Orthetrum testaceus, Orthetrum melania, Crocothemis servilia, Deielia phaon, and Acisoma panorpoides were positioned mainly on the positive direction, and the other five dragonfly species were distributed mainly on the negative direction. Taking into account the profile plot of the wing vein (Fig. 5), the differences in hindwings occurred mainly in the anal region (LM13-16). On the PC2 axis, Tramea virginia, Acisoma panorpoides, Orthetrum testaceus, and Deielia phaon were distributed mainly on the positive direction, and the other six species were positioned mainly on the negative direction. The profiles of hindwing veins (Fig. 5) showed that the differences occurred mainly in the apical (LM6-8) and the anal region (LM1316). Centroid Size Analysis (Fig. 4B) showed that Deielia phaon and Acisoma panorpoides had smaller hindwings, whereas Tramea virginia and Orthetrum melania had larger hindwings.

Combining the results of the two analyses (PCA and Centroid size), the forewing shape change law among species was different to that of hindwing shape. For example, the forewing and hindwing shape analysis of Trithemis aurora showed large differences on the PC1 axis. In Centroid size analysis, Orthetrum melania had the biggest forewings, but Tramea virginia had the biggest hindwings.

Figure 4 PCA (A) and Centroid Size Analysis (B) of hindwings of 10 dragonfly species (Libellulidae). Ap: Acisoma panorpoides; Pz: Pseudothemis zonata; Pf: Pantala flavescens; Tv: Tramea virginia; Om: Orthetrum melania; Dp: Deielia phaon; Cs: Crocothemis servilia; Ot: Orthetrum testaceus; Ta: Trithemis aurora; Oa: Orthetrum albistylum.

Figure 5 Thin-plate spline analysis of hindwing profiles of 10 dragonfly species
(Libellulidae). Each profile represents the deformations in wing shape in extreme conditions for each PC.

## 2. Analysis of interspecific relationships based on molecular data

Analysis of the interspecific relationship among 10 species of dragonfly by the BI method (Fig. 6) divided them into two main branches, with Orthetrum species (subfamily Libellulinae)
in one branch, having a distant relationship with other species. The remaining seven species were divided into four branches, forming a paraphyletic group. Deielia phaon was on a separate branch, whereas Acisoma panorpoides and Crocothemis servilia were clustered into a branch with a high degree of support (all three species belonging to subfamily Sympetrinae).

Pseudothemis zonata was on a separate branch (subfamily Trithemistinae). Pantala flavescens, Tramea virginia and Trithemis aurora were clustered into a branch with a high degree of support (Pantala flavescens and Tramea virginia belonging to subfamily Trameinae, and Trithemis aurora to subfamily Trithemistinae).

The phylogenetic tree obtained by the MP method was basically consistent with the relationship tree obtained by the BI method. Although the MP tree divided further the relationship among the seven species in the four paraphyletic groups, the support was not high, so the interspecific relationships obtained by the Bayesian Inference method were only considered in this study.

## 3. Comparative analysis between the morphological relationship tree of forewings and

 hindwings obtained based on UPGMA method and the interspecific relationship tree based on Bayesian Inference methodThe analysis of forewings (Fig. 7) showed that (based on the wing shape) the individuals of each species clustered together first, then clustered with the other species with relatively close morphological relationships. In the morphological tree, the species of genus Orthetrum were grouped together, but were mixed with Crocothemis servilia and Deielia phaon; also, Pantala flavescens and Trithemis aurora were clustered together. These groupings were consistent with the results of molecular-based genetic analysis. However, for some other species, the results of morphological clustering based on forewings were completely different from those based of the molecular relationships.

The hindwing shape analysis also showed that individuals within the species could be clustered first (Fig. 8). Compared with the results of the forewing shapes, many similarities were found. For example, Crocothemis servilia and Deielia phaon were also clustered first with Orthetrum, Tramea virginia was a separate branch, and Pseudothemis zonata and Trithemis aurora were clustered into a branch. However, the hindwing shape clustering was completely different from that based on the molecular relationships. In general, even though there was some phylogenetic information in the forewing shape, the relationships based on the molecular data
were still substantially different. In contrast, there was no interspecific phylogenetic information in the hindwing shape.

Figure 6 Bayesian Inference tree (A) and Maximum parsimony tree (B). The phylogenetic trees were constructed based on molecular data of the mitochondrial COI and nuclear $18 \mathrm{~S} r \mathrm{RNA}$ $+I T S 1+5.8 S r R N A+I T S 2+28 S r R N A$ genes.

Figure 7 The Morphological tree of forewings (A) vs. Bayesian Inference phylogram obtained from the molecular dataset (mitochondrial COI + nuclear 18S rRNA + ITS1 + $5.8 S r R N A+I T S 2+28 S r R N A(B)$. The clustering of the forewing morphological tree on the left was (.....) or was not (..x..) consistent with the clustering based on the phylogenetic analysis using the molecular data on the right.

Figure 8 The Morphological tree of hindwings (A) vs. Bayesian Inference phylogram obtained with the molecular dataset (mitochondrial COI + nuclear $18 S$ rRNA + ITSI + $5.8 S r$ RNA + ITS $2+28 S r$ RNA (B). The clustering of the hindwing morphological tree on the left was (...) or was not (..x..) consistent with the clustering based on of the phylogenetic analyses using the molecular data on the right.

## Discussion

## 1. Wing shape and migratory habits

The application of geometric morphometrics method to study wing shape diversity of dragonflies can effectively reveal the relationships among related species (Breuker et al., 2010; Klingenberg, 2016). The PCA results of the forewing shape in this study showed the main difference between the 10 species of dragonfly was in the apical and radial regions as well as the discoidal region dominated by the wing nodus. In contrast, the main difference in the findwing shape was in the apical region and the anal region dominated by the wing nodus. Based on the dynamic load in flight, the wing nodus of dragonfly is the basis of the whole wing structure, with all the wing veins centered on the wing nodus; hence, the wing nodus is the main load-bearing region during flight (Rajabi et al., 2017). The surface of the dragonfly's wings forms various hollow and ridge regions (Nakamura, Osonoi \& Terauchi, 2016), so the wing nodus may be
affected by bending as well as twisting deformations during flight. The 10 species of dragonfly in this study exhibit large differences in flight behavior, and these differences in behavior might have led to differences in the wing shape. From the perspective of the wing function, the characteristics of the apical region of dragonfly wing are related to its forward dive and fast flight, playing an important role in long-distance migration, territorial patrol and courtship competition (Rajabi et al., 2018). Therefore, the difference in wing shape among different species tested in the present study was expressed prominently at the apical region of the wing. Regardless of the forewing or hindwing, the cubital region and the anal region differed greatly among species. From a functional point of view, these two regions are closely related to the migratory ability of dragonflies. It is generally considered that dragonflies with strong migratory ability have larger cubital and anal regions than non-migrating dragonflies.

In this study, the five species dragonflies of Crocothemis servilia, Orthetrum melania, Orthetrum albistylum, Orthetrum testaceus as well as Acisoma panorpoides were distributed mainly on the positive axis of PC1 and PC2. These species had wide and short forewing, with the small anal region of the hindwing. Tramea virginia, Trithemis aurora, Pseudothemis zonata, and Pantala flavescens were distributed mainly on the negative axis of PC1 and PC2. Their forewings were long and narrow, and the anal region of the hindwing was large. According to the research by Rajabi et al. (2018), the species of dragonfly with long and narrow wing were more suitable for migration, whereas those with wide and short wings were more suited to standing still. Among the dragonflies tested in the present study, from the behavioral point of view, Tramea virginia, Trithemis aurora, Pseudothemis zonata, and Pantala flavescens were all species with strong flying ability, conducting stagnation flight and territory patrols, whereas the species Crocothemis servilia, Orthetrum melania, Orthetrum albistylum, Orthetrum testaceus and Acisoma panorpoides would prefer hovering around ponds or standing still for long periods. The results of this study were in good agreement with those of Rajabi et al. (2018), further confirming the relationship between wing shape and migration.

## 2. Genus, Subfamily and Family relationships

This study illustrated the preliminarily relationships among species, genera, subfamilies, and families based on the phylogenetic relationships of 10 species of dragonfly based on the mitochondrial COI gene and the nuclear genes $18 S, 28 S$ rRNA and ITS. Deielia phaon and Pantala flavescens showed a close relationship, even though they belong to different subfamilies;
moreover, they formed a paraphyletic group with Acisoma panorpoides and Crocothemis servillia, belonging to subfamily Sympetrinae. This result is similar to the results of Ware, May \& Kjer (2007) based on the nuclear genes $16 S$ and $28 S r R N A$, and also similar to the results of Kim et al. (2014) based on the mitochondrial COI gene and the nuclear genes $16 S$ and $28 S r$ RNA. In the phylogenetic tree, Pantala flavescens and Trithemis aurora formed a paraphyletic group, indicating a close relationship despite belonging to different subfamilies; this result was similar to those of Ware, May \& Kjer (2007). In this study, the three species of Orthetrum were independent as a branch, and far away from other species. In general, the results of this study were consistent with the results of Kim et al. (2014), Carle, Kjer \& May (2015) and Yong et al. (2016), indicating that the interspecific phylogenetic relationships based on the mitochondrial COI gene and the nuclear genes $18 S, 28 S r R N A$ and $I T S$ were reliable, and that these genes can be used as barcode genes for interspecies classification.

## 3. Evaluation of genetic information on wing shape

This study constructed the interspecific relationship trees for the morphological information on forewings and hindwings based on the UPGMA method, and compared them with the phylogenetic trees obtained based on the molecular data by the BI method. The establishment of interspecific relationships using the UPGMA method in morphological analysis can be supported by numerous studies (Ramírez-Sánchez, Luna \& Cramer, 2016; Fouquet et al., 2012; Gvoždik, Moravec \& Kratochvil, 2008; Limsopatham et al., 2018). The UPGMA method is effective in interspecies morphological analysis, although Robinson \& Terhune (2017) suggested the UPGMA method in subspecies analysis, such as morphological relationship between subspecies or geographical populations, might obscure the patterns among individuals by the interobserver and intermethod errors. However, in the interspecific relationship analysis, this method is ideal. Using the UPGMA method in the present study to analyze forewing and hindwing shapes, the individuals of each species were clustered initially into one branch. Then, topological relationships were established with other species. Compared to the intra-species relationships, the interspecific morphological relationship was farther, so they clustered later.

Comparing the morphological relationship tree based on the wing shape and the phylogenetic tree based on the molecular data, some relationships, but also many differences, were found. Regarding the forewing shape, the three species of Orthetrum were clustered into a branch, but had Crocothemis servilia and Deielia phaon mixed in. The phylogenetic tree based
on molecular data showed that Crocothemis servilia and Deielia phaon (subfamily Sympetrinae) had a distant relationship with Orthetrum (subfamily Libellulinae). However, from the behavioral point of view, Crocothemis servilia, Deielia phaon and the species of Orthetrum have many similarities, generally living around ponds or streams, resting on grasses and dead branches, or hovering over grass and ponds. Their territorial consciousness is weak and they can coexist with other species. Similar behaviors and habits may be associated with similar forewing and hindwing shapes. In terms of forewing morphology, Pantala flavescens, Trithemis aurora and Pseudothemis zonata were clustered together as one branch, but could not be combined into one branch based on hindwings. From the behavioral point of view, these dragonflies have strong migrating ability that might have influenced clustering based on the morphology. In terms of the molecular data, Pantala flavescens and Trithemis aurora were clustered together as a branch, and were distant from Pseudothemis zonata. These findings showed there was some genetic information in the wing shape, but it was influenced more by the behavior and life habits. Hence, for dragonflies, establishing inter-species relationships based directly on wing shape may be unreliable. Pilgrim \& Vondohlen (2008) studied the phylogenetic relationships of Sympetrinae based on molecular data (mitochondrial loci 16S and 12S rRNA) and morphological traits (38 wing venation characters); even though the study did not involve direct comparison of phylogenetic trees based on the two types of information, its conclusion was that the characteristics of the wing veins might be useless in the analysis of relationships due to the trait homoplasy. However, morphological and genetic structure may undergo synchronous evolution in other insects, such as the pronotum and genital segments of grasshopper genus Zoniopoda (Pocco et al., 2018), the wing veins and genital segments of Euptychiina butterflies (Marin et al., 2017), indicating that phylogenetic information may be contained in morphological features of some insects.

Because the sample size selected in this study was relatively small and limited to Libellulidae so the results need to be confirmed on a larger and more diverse collection of species. In the future and using a larger sample size, additional morphological features (such as genital segments) need to be examined to achieve a deeper understanding of the relevance among dragonfly interspecific phylogenetic relationships, morphological evolution and genetic differentiation.

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## Figure 1

Landmarks on the forewing and hindwing of Libellulidae
A. Landmarks 1 to 24 on forewing, 25 to 26 are ruler; B. Landmarks 1 to 25 on hindwing, 26 to 27are ruler


## Figure 2

PCA (A) and Centroid Size Analysis (B) of forewings of 10 dragonfly species (Libellulidae) Ap: Acisoma panorpoides; Pz: Pseudothemis zonata; Pf: Pantala flavescens; Tv: Tramea virginia; Om: Orthetrum melania; Dp: Deielia phaon; Cs: Crocothemis servilia; Ot: Orthetrum testaceus; Ta: Trithemis aurora; Oa: Orthetrum albistylum


B


Figure 3

Thin-plate spline analysis of forewing profiles of 10 dragonfly species (Libellulidae)

Each profile represents the deformations in wing shape in extreme conditions for each PC


## Figure 4

PCA (A) and Centroid Size Analysis (B) of hindwings of 10 dragonfly species (Libellulidae)

Ap: Acisoma panorpoides; Pz: Pseudothemis zonata; Pf: Pantala flavescens; Tv: Tramea virginia; Om: Orthetrum melania; Dp: Deielia phaon; Cs: Crocothemis servilia; Ot: Orthetrum testaceus; Ta: Trithemis aurora; Oa: Orthetrum albistylum

A


B


## Figure 5

Thin-plate spline analysis of hindwing profiles of 10 dragonfly species (Libellulidae)

Each profile represents the deformations in wing shape in extreme conditions for each PC


## Figure 6

Bayesian Inference tree (A) and Maximum parsimony tree (B)

The phylogenetic trees were constructed based on molecular data of the mitochondrial CO and nuclear $18 S$ rRNA + ITS1 $+5.8 S$ rRNA $+I T S 2+28 S$ rRNA genes


## Figure 7

The Morphological tree of forewings (A) vs. Bayesian Inference phylogram obtained from the molecular dataset (mitochondrial COI + nuclear 18S rRNA + ITS1 + 5.8S rRNA + ITS2 $+28 S$ rRNA (B)
he clustering of the forewing morphological tree on the left was (.....) or was not (..x..) consistent with the clustering based on the phylogenetic analysis using the molecular data on the right


## Figure 8

The Morphological tree of hindwings (A) vs. Bayesian Inference phylogram obtained with the molecular dataset (mitochondrial COI + nuclear 18S rRNA + ITS1 + 5.8S rRNA + ITS2 + 28S rRNA (B)

The clustering of the hindwing morphological tree on the left was (...) or was not (..x..) consistent with the clustering based on of the phylogenetic analyses using the molecular data on the right


## Table $\mathbf{1}_{\text {(on next page) }}$

The Species name, Genus, Subfamily, Family and number of 10 species of Libellulidae

| Table 1 The Species name, Genus, Subfamily, Family and number of 10 species of Libellulidae |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: |
| Species | Genus | Subfamily | Family | Nu <br> mb <br> ers |
| Orthetrum albistylum (Selys) | Orthetrum | Libellulinae | Libellulidae | 8 |
| Orthetrum melania (Selys) | Orthetrum | Libellulinae | Libellulidae | 5 |
| Orthetrum testaceus (Burmeister) | Orthetrum | Libellulinae | Libellulidae | 6 |
| Acisoma panorpoides (Rambur) | Acisoma | Sympetrinae | Libellulidae | 9 |
| Deielia phaon (Selys) | Deielia | Sympetrinae | Libellulidae | 15 |
| Crocothemis servilia (Drury) | Crocothemis | Sympetrinae | Libellulidae | 6 |
| Trithemis aurora (Burmeister) | Trithemis | Trithemistinae | Libellulidae | 13 |
| Pseudothemis zonata (Burmeister) | Pseudothemis | Trithemistinae | Libellulidae | 3 |
| Tramea virginia (Rambur) | Tramea | Trameinae | Libellulidae | 7 |
| Pantala flavescens (Fabricius) | Pantala | Trameinae | Libellulidae | 9 |
| Anotogaster sieboldii (Selys) | Anotogaster |  | Cordulegastridae | 3 |

## Table 2 (on next page)

Definition and numbering of the landmarks

| Table 2 | Definition and numbering of the landmarks |  |  |
| :--- | :--- | :--- | :--- |
| L. of forewing | Definition | L. of hindwing | Definition |
| 1 | Initial of costa | 1 | Initial of costa |
| 2 | End of costa | 2 | End of costa |
| 3 | Left of stigma | 3 | Left of stigma |
| 4 | Right of stigma | 4 | Right of stigma |
| 5 | Midpoint of 4 and 6 | 5 | End of sub-costa |
| 6 | End of RP1 | 6 | End of RP1 |
| 7 | Midpoint of 6 and 8 | 7 | Midpoint of 6 and 8 |
| 8 | End of RP2 | 8 | End of RP2 |
| 9 | Midpoint of 8 and 10 | 9 | End of IRP2 |
| 10 | End of RP3-4 | 10 | Midpoint of 9 and 10 |
| $11-12$ | End of MA | 11 | End of RP3-4 |
| 13 | Midpoint of 12 and 14 | 12 | End of MA |
| 14 | End of the CuP | $13-14$ | End of CuP |
| 15 | End of the anal vein | 15 | Anal angle |
| $16-18$ | triangle region | 16 | End of the anal vein |
| $19-20$ | Sub-nodu | $17-19$ | triangle region |
| $21-24$ | Midvein region | $20-21$ | Sub-nodu |
|  |  | $22-25$ | Midvein region |

## Table 3(on next page)

The Famliy, Subfamily and GenBank number of 10 species of Libellulidae

Table 3 The Famliy, Subfamily and GenBank number of 10 species of Libellulidae
GenBank number

| Family | Subfamily | Species | Mitochondria COI | Nuclear 18S $\begin{aligned} & \text { rRNA+ITs1+5.8s } \\ & + \text { ITs2+28S rRNA } \end{aligned}$ |
| :---: | :---: | :---: | :---: | :---: |
| Libellulidae | Libellulinae | Orthetrum albistylum | MF358741.1 | LC366177.1 |
| Libellulidae | Libellulinae | Orthetrum albistylum | MF358740.1 | AB781474.1 |
| Libellulidae | Libellulinae | Orthetrum albistylum | MF358739.1 | AB781473.1 |
| Libellulidae | Libellulinae | Orthetrum melania | LC099937.1 | LC099933.1 |
| Libellulidae | Libellulinae | Orthetrum melania | AB709043.1 | AB707165.1 |
| Libellulidae | Libellulinae | Orthetrum melania | AB709085.1 | AB707187.1 |
| Libellulidae | Libellulinae | Orthetrum testaceus | KU496907.1 | KJ802972.1 |
| Libellulidae | Libellulinae | Orthetrum testaceus | KU496905.1 | KJ802970.1 |
| Libellulidae | Libellulinae | Orthetrum testaceus | MF774527.1 | KJ802969.1 |
| Libellulidae | Sympetrinae | Acisoma panorpoides | KX281827.1 | AB707046.1 |
| Libellulidae | Sympetrinae | Acisoma panorpoides | KX281825.1 | AB707045.1 |
| Libellulidae | Sympetrinae | Acisoma panorpoides | KX281824.1 | FN356030.1 |
| Libellulidae | Sympetrinae | Deielia phaon | AB708961.1 | AB707069.1 |
| Libellulidae | Sympetrinae | Deielia phaon | AB708962.1 | AB707068.1 |
| Libellulidae | Sympetrinae | Deielia phaon | AB708963.1 | AB707066.1 |
| Libellulidae | Sympetrinae | Crocothemis servilia | JN119571.1 | LC366268.1 |
| Libellulidae | Sympetrinae | Crocothemis servilia | MF774561.1 | LC366266.1 |
| Libellulidae | Sympetrinae | Crocothemis servilia | MF774554.1 | LC366265.1 |
| Libellulidae | Trithemistinae | Trithemis aurora | MF358792.1 | AB707343.1 |
| Libellulidae | Trithemistinae | Trithemis aurora | MF358785.1 | AB707342.1 |
| Libellulidae | Trithemistinae | Trithemis aurora | MF358776.1 | GU323038.1 |
| Libellulidae | Trithemistinae | Pseudothemis zonata | MF358738.1 | AB707212.1 |
| Libellulidae | Trithemistinae | Pseudothemis zonata | KF257079.1 | AB707212.1 |
| Libellulidae | Trameinae | Tramea virginia | AB709228.1 | AB707335.1 |
| Libellulidae | Trameinae | Tramea virginia | AB709225.1 | AB707331.1 |
| Libellulidae | Trameinae | Tramea virginia | AB709227.1 | AB707332.1 |
| Libellulidae | Trameinae | Pantala flavescens | KR080133.1 | LC366168.1 |
| Libellulidae | Trameinae | Pantala flavescens | KR080114.1 | AB707211.1 |
| Libellulidae | Trameinae | Pantala flavescens | KR080079.1 | LC366076.1 |
| Cordulegastridae |  | Anotogaster sieboldii | EF155476.1 | AB706931.1 |


| Cordulegastridae | Anotogaster sieboldii | EF155431.1 | AB706930.1 |
| :--- | :--- | :--- | :--- | 1

## Table 4(on next page)

The procustes distance of Forewing and Hindwing shape among 10 species of Libellulidae

Table 4 The procustes distance of Forewing and Hindwing shape among 10 species of Libellulidae

|  |  |  |  |  |  |  |  |  |  |  |  |
| :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- | :--- |
| Hindwing shape <br> distance | Cs | Dp | Ot | Pf | Ta | Ap | Tv | Oa | Om | Pz |  |
| Forewing shape <br> distance |  |  |  |  |  |  |  |  |  |  |  |


| Cs |  | 0.05 | 0.02 | 0.083 | 0.05 | 0.079* | 0.10 | 0.034 | 0.04 |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  | 9** | 6 | ** | 4** | * | 7** |  | 5 | 5 |
| Dp | 0.04 |  | 0.05 | 0.096 | 0.07 | 0.044* | 0.12 | 0.051 | 0.07 | 0.07 |
|  | 2** |  | 1** | ** | 4** | * | 4** | ** | 3** | 4* |
| Ot | 0.03 | 0.04 |  | 0.083 | 0.05 | 0.064* | 0.10 | 0.040 | 0.05 | 0.05 |
|  | 4* | 1** |  | ** | 5** | * | 6** | ** | 1* | 6** |
| Pf | 0.04 | 0.00 | 0.04 |  | 0.06 | 0.116* | 0.11 | 0.073 | 0.06 | 0.05 |
|  | 1** | 6 | 2** |  | 8** | * | 8** | ** | 3** | 1** |
| Ta | 0.08 | 0.08 | 0.06 | 0.085 |  | 0.090* | 0.09 | 0.062 | 0.04 | 0.04 |
|  | 2** | 4** | 9** | ** |  | * | 3** | ** | 9** | 1** |
| Ap | 0.06 | 0.04 | 0.06 | 0.046 | 0.10 |  | 0.13 | 0.078 | 0.09 | 0.09 |
|  | 6** | 5** | 0** | ** | 8** |  | 2** | ** | 5** | 6* |
| Tv | 0.09 | 0.10 | 0.09 | 0.102 | 0.12 | 0.091* |  | 0.116 | 0.08 | 0.11 |
|  | 4** | 1** | 9* | ** | 0** | * |  | ** | 6** | 2* |
| Oa | 0.03 | 0.04 | 0.03 | 0.040 | 0.07 | 0.064* | 0.09 |  | 0.04 | 0.05 |
|  | 2 | 1** | 3** | * | 1** | * | 4** |  | 8** | 4** |
| Om | 0.03 | 0.05 | 0.05 | 0.058 | 0.09 | 0.076* | 0.08 | 0.046 |  | 0.04 |
|  | 3 | 9** | 6* | * | 6** | * | 4** | * |  |  |
| Pz | 0.09 | 0.08 | 0.08 | 0.090 | 0.07 | 0.106* | 0.11 | 0.082 | 0.10 |  |
|  | 4** | 8** | 9** | * | 2* | * | 6* | * | 8* |  |

## Notes:

*represent significance level $<0.01$, ${ }^{* *}$ represent significance level $<0.001$; Ap: Acisoma 3 panorpoides; Pz: Pseudothemis zonata; Pf: Pantala flavescens; Tv: Tramea virginia; Om:
4 Orthetrum melania; Dp: Deielia phaon; Cs: Crocothemis servilia; Ot: Orthetrum testaceus; Ta:
5 Trithemis aurora; Oa: Orthetrum albistylum.
6

