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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, 28S 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 Morphol1.06 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.

- 1 Phylogeny of Libellulidae: are there relationships between
- 2 molecular phylogenetics and morphological analysis of wing

### **3 shape of dragonflies?**

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### 16 Abstract

17 **Background:** Establishing the species limits and resolving phylogenetic relationships are

18 primary goals of taxonomists and evolutionary biologists. At present, a controversial question is

- 19 about interspecific phylogenetic information in morphological features. Are the interspecific
- 20 relationships established based on genetic information consistent with the traditional
- 21 classification system? To address these problems, this study analyzed the wing shape structure of
- 22 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*,
- 25 28S rRNA and ITS in 10 species of dragonflies, the interspecific relationship was established.
- 26 Interspecific phylogenetic information regarding wing shape structure was analyzed.
- 27 Method: The wing shape information of the male forewings and hindwings was obtained by the
- 28 geometric morphometrics method. The inter-species wing shape relationship was obtained by
- 29 principal component analysis (PCA) in MorphoJ1.06 software. The inter-species wing shape
- 30 relationship tree was obtained by cluster analysis (UPGMA) using Mesquite3.2 software. The
- 31 COI, 18S, ITS and 28S genes of 10 species dragonfly were blasted and processed by BioEdit v6

- 32 software. The maximum parsimony (MP) tree was established by Puap4.0 software. The Bayes
- 33 inference (BI) tree was established by MrBayes 3.2.6 in Geneious software.
- 34 **Results:** The main difference in forewings among the 10 species of dragonfly was the apical,
- 35 radial and discoidal regions dominated by the wing nodus. In contrast, the main difference
- 36 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
- 38 relationship based on molecular data showed that the species of Orthetrum genus branched
- 39 independently of the other species. Compared to the molecular tree of 10 species, the wing shape
- 40 clustering showed some phylogenetic information on the forewing shape (with large differences
- 41 in the forewing shape tree vs. molecular tree), and there was no interspecific phylogenetic
- 42 information of the hindwing shape tree vs. molecular tree.
- 43 **Conclusion:** The dragonfly wing shape characteristics are closely related to its migration ability.
- 44 Species with strong ability to migrate have the forewing shape that is longer and narrower, and
- 45 have larger anal region, whereas the species that prefer short-distance hovering or standing still
- 46 for a long time have forewing that are wider and shorter, and the anal region is smaller.
- 47 Integrating morphological and molecular data to evaluate the relationship among dragonfly
- 48 species shows there is some interspecific phylogenetic information in the forewing shape and
- 49 none in the hindwing shape. The various regions of the forewing and hindwing are inconsistent,
- 50 which may be due to their different functions.
- 51 Subjects Entomology, Biodiversity, Taxonomy, Zoology
- 52 Keywords Dragonflies, Molecular taxonomy, Morphological taxonomy, Libellulidae,
- 53 Interspecific relationship, Wing, Clustering, Phylogeny.
- 54

#### 55 Introduction

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

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63 *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 64 65 the interspecific relationships established by morphological features can be supported by the 66 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, 67 68 2012). Because morphological characteristics involve ecological adaptation and behavioral problems, such as living in the same ecological environment, similar feeding behaviors and 69 patterns of movement lead to morphological similarities, whereas genetic structures may be 70 those of completely different species. According to the decade-old literature, the difference in 71 72 genetic structure between species does not necessarily appear in phenotype (Stern & Orgogozo, 73 2008). Alternative views are that different genetic structures can produce similar phenotypic 74 patterns (*Robertson, 1959; Wlikens, 1971*). Therefore, genetic diversity may not necessarily be 75 related to morphological differences. However, in recent decades, studies in molecular biology 76 and developmental biology have suggested that mutations in gene expression regulation may 77 promote phenotypic evolution, especially the change in morphological characteristics 78 (Kaessmann, 2010; Rabosky, 2012; Crispo, 2008). It indicates that the differences in the genetic 79 structure are predictable, and to a certain extent, they will result in differences in the 80 morphological structure. These contradictory views are common in evolution of the related species of insects (Victor & Zúñiga, 2016; Heikkilä et al., 2015). In recent years, with the 81 82 constant development and improvement of morphological measurement technology, the study of 83 population evolution law and systematic generation relationship based on morphological 84 characteristics has been published (*Klingenberg & Marugán-Lobón, 2013*). Geometric 85 morphometrics is an advanced method of morphological analysis in biology; based on the curve, 86 landmark point and semi-landmark point data of the homologous locus concept, it can accurately 87 quantify the phenotypic traits of organisms and explore the morphological evolution of 88 populations (Cooke & Terhune, 2015; Baylac, Villemant & Simbolotti, 2003). 89 A large number of studies have shown that the morphology-based interspecific relationship 90 is basically consistent with the interspecific relationship established by molecular data when the 91 morphological characteristics are selected judiciously (*Grzywacz et al. 2017; Noguerales*, Cordero & Ortego, 2018). Marín et al. (2017) study showed that the interspecific relationship of 92

93 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. 94 Francischini et al. (2017) used COI gene and female genital structure to illustrate the 95 96 interspecific relationship of Diatraea by the molecular and morphological methods, and the 97 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 98 99 population differentiation of *Mioscirtus wagneri* locust in different geographical regions by using quantitative morphological features (anterior and posterior plates) and mtDNA, suggesting 100 101 that morphology-based geographical differentiation correlated with geographical differentiation 102 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 103 based on morphology and molecular data can also lead to inconsistent results. For example, 104 105 *Bocek & Bocek (2017)* showed the morphology of beetle pronotum cannot fully support interspecific phylogenetic relationships. Bapst, Schreiber & Carlson (2018) used molecular and 106 107 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 108 109 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 110 111 relationships among species the morphological data need to be combined with the molecular data for synthesis. 112

113 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 114 115 transparent membranous wings, with the wing veins clearly visible; the shape and direction of 116 the wing veins are often used as an important classification basis for dragonflies (*Fauziyah et al.*, 117 2014). Geometric morphometrics was used to analyze the morphological differences among 118 species. The mitochondrial gene COI and nuclear genes 18S, ITS and 28S were used to analyze the phylogenetic relationships among the species. We analyzed the phylogenetic relationships 119 120 based on wing-type features as well as on molecular data. Accordingly, this study addressed the 121 following questions: 1) what was the relationship between the characteristics of the wing shape 122 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 123 124 morphological changes in different species?

#### 125 Materials & Methods

#### 126 1 Materials and Data acquisition

127 1.1 Specimen collection and image acquisition

128 In order to explore the relationship between the wing shape of different species of dragonfly 129 in Odonata, we collected dragonflies from May to October 2018 in Taizhou City(121.3495E, 130 28.6522N) 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 131 132 species names and numbers are shown in Table 1. The wings of all specimens were spread; then, 133 the left forewing and the left hindwing were taken and pressed between two slides to make slide 134 specimens. The forewings and hindwings were photographed with a Nikon 5100 camera, fixed 135 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 136 137 a picture format for later use. Ethics statement 138 139 All collected dragonflies under anesthesia to death. Dragonfly is not a legally protected species according to China and international conservation legislation. Under China legislation, 140 141 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 142 1.2 Landmark data acquisition 143 The TPSdig2 software (*Rohlf, 2006*) 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, 144 including two on a ruler) (Fig. 1). The landmark-based geometric morphometrics method was 145 146 applied to study the morphological diversity in wing size and shape. We set landmarks at the 147 intersections of wing veins with the wing margin and intersections of cross veins with major

148 veins and vein branch points (Table 2), which was according to *Rohlf & Corti (2000)*.

#### Table 2 Definition and numbering of the landmarks

- 149 1.3 Wing shape analysis
- 150 The forewing and hindwing shape information was input into CoordGen software (*Rohlf &*
- 151 *Slice*, *1990*) in the IMP series package. Based on the ruler, the errors caused by the focal length
- 152 of the photograph were eliminated, and the datum line was set. To examine wing-shape

- 153 variation, digitized landmark data were subjected to generalized procrustes superimposition to
- 154 standardize the size of the landmark configurations and eliminate differences caused by
- 155 translation and rotation (Adams, Rohlf & Slice, 2004). All standardized data were converted into
- 156 a two-dimensional data format.

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

- 157 2 Methods and Analysis
- 158 2.1 Statistical analysis of morphological data

159 The standardized morphological information data were imported into MorphoJ1.06d

160 software (*Klingenberg, 2009*), and the morphological changes of 10 species of dragonfly were

161 analyzed by principal component analysis (PCA), Procrustes analysis and Discriminant analysis.

162 The first two main components were extracted as scatter plots of forewings and hindwings. In the

163 MorphoJ1.06d software, the thin-plate spline analysis (*Bookstein, 1989*) was performed, and the

164 difference in landmark points was analyzed. The visualized legend was used to show the

165 variation in forewings and hindwings in the first two principal components.

166 2.2 Acquisition of molecular data

167 The DNA barcode data of 10 species of dragonfly was obtained from the NCBI website. We

- 168 obtained *Cytochrome oxidase subunit I (COI)* gene of each species with length of 349bp and *18S*
- 169 *rRNA*, Internal Transcribed Space1 (ITS1) + 5.8s rRNA + Internal Transcribed Space2 (ITS2)
- and *28S rRNA* of each species with length of 747bp. All data were imported into BioEdit v6
- 171 software for editing, and the built-in clustalw was used to blast sequences (*Hall TA, 1999*). Total

172 obtained COI + 18s + ITS + 28s gene data with a length of 1096bp was used to construct the

- 173 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

175 2.3Establishment of morphological and molecular phylogenetic trees

176 In this study, Mesquite 3.2 software (*Maddison & Maddison, 2009*) was used to cluster the

177 morphological characteristics of forewings and hindwings of 10 dragonfly species. The cluster

- 178 analysis was based on the landmark data for forewings and hindwings of each species established
- 179 as a matrix. The distance among the taxa represented uncorrected distance. Then, the

- 180 relationships among the populations were further summarized based on the unweighted pair-
- 181 group method with arithmetic averages (UPGMA) to build forewing and hindwing shape trees
- 182 (Ramírez-Sánchez, Luna & Cramer, 2016).
- 183 The sequence data were analyzed using maximum parsimony method (MP) and Bayesian
- 184 inference method (BI). For the maximum parsimony reconstruction, a tree bisection-
- 185 reconnection (TBR) branch swapping heuristic search was run using Geneious and PAUP 4.0
- 186 with 10,000 random additions (*Swofford, 2002*). Gaps were treated as missing data. To estimate
- 187 branch support, 500 bootstrap pseudoreplicates were performed using 10 random addition
- 188 searches per pseudoreplicate (*Felsenstein, 1981*).
- 189 Bayesian Inference (BI) using MrBayes 3.2.6 (*Huelsenbeck JP & Ronquist F, 2001*) was
- 190 executed from within Geneious (*Kearse M et al., 2012*). Both programs suggested a GTR + I + G
- 191 model (*Yang Z, 1994*) for the mitochondrial gene *COI* and nuclear gene *18S+ITS+28S*. All BI
- 192 analyses consisted of  $1.1 \times 10^6$  generations of Markov Chain Monte Carlo searches containing 4
- 193 chains, heated chain temperature of 0.2 and burn-in of 100,000 generations. Compound Dirichlet
- 194 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).
- 196 Trees were saved every 1,000 generations. The confidence values of the BI tree were presented
- 197 as the Bayesian posterior probabilities (BPP) in percentages. Phylogenetic analyses were
- 198 performed for each of the gene sequences.
- 199

#### 200 **Results**

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

#### 202 (Libellulidae)

203 The wing shape data were analyzed by PCA and centroid size to find out the shape variation 204 (Fig. 2A). The first two PCs accounted for 35.09% and 21.77% of the variation, with the 205 cumulative variation explaining 56.86% of the total shape variation in forewings. Procrustes 206 analysis (Table 4) of forewings showed *Deielia phaon* and *Pantala flavescens* to have the 207 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 208 209 differences were large. Discriminant analysis results showed no significant difference in 210 forewing shapes between *Deielia phaon* and *Pantala flavescens* (P=1.000), significant

211 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 (P<0.01). A scatter plot (Fig. 2A) of the first and second principal components showed
- that on the PC1 axis, *Trithemis aurora*, *Pseudothemis zonata* and *Orthetrum testaceus* were
- 215 mainly distributed on the negative direction, whereas the other seven species were mainly
- 216 distributed on the positive direction. Taking into account the profile plots of the wing veins (Fig.
- 217 3), the differences mainly occurred in the apical region (LM6-8) and the discoidal region (LM11-
- 218 14). On the PC2 axis, Orthetrum melania, Tramea virginia, Trithemis aurora and Pseudothemis
- 219 *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
- 222 Analysis (Fig. 2B) results showed that Deielia phaon, Pantala flavescens and Acisoma
- 223 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.

#### 224

**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.

#### 225

 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
- 231 *panorpoides* and *Tramea virginia* had the largest distance (0.132), indicating relatively large
- 232 differences in their hindwing shapes. Discriminant analysis showed no significant difference in

233 hindwing shapes between Orthetrum melania and Pseudothemis zonata (P=0.111), significant

- 234 differences between Crocothemis servilia and Orthetrum testaceus (P=0.034), Crocothemis
- 235 servilia and Orthetrum albistylum (P=0.046) and Crocothemis servilia and Orthetrum melania
- 236 (*P*=0.014), and strongly significant differences between *Crocothemis servilia* and *Pseudothemis*
- 237 *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,
- 239 Deielia phaon, and Acisoma panorpoides were positioned mainly on the positive direction, and
- 240 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*
- 243 *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 (LM13-
- 246 16). Centroid Size Analysis (Fig. 4B) showed that *Deielia phaon* and *Acisoma panorpoides* had
  247 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.

#### 253

### **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.

#### 254 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)

257 in one branch, having a distant relationship with other species. The remaining seven species were

258 divided into four branches, forming a paraphyletic group. *Deielia phaon* was on a separate

259 branch, whereas Acisoma panorpoides and Crocothemis servilia were clustered into a branch

260 with a high degree of support (all three species belonging to subfamily Sympetrinae).

261 Pseudothemis zonata was on a separate branch (subfamily Trithemistinae). Pantala flavescens,

262 Tramea virginia and Trithemis aurora were clustered into a branch with a high degree of support

263 (*Pantala flavescens* and *Tramea virginia* belonging to subfamily Trameinae, and *Trithemis* 

264 *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.

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

271 hindwings obtained based on UPGMA method and the interspecific relationship tree based
272 on Bayesian Inference method

273 The analysis of forewings (Fig. 7) showed that (based on the wing shape) the individuals of 274 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 275 276 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 277 278 the results of molecular-based genetic analysis. However, for some other species, the results of 279 morphological clustering based on forewings were completely different from those based of the 280 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

- 288 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 *18S rRNA* + *ITS1* + 5.8S rRNA + *ITS2* + 28S rRNA genes.

290

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 + 28S rRNA (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.

291

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.

#### 292

#### 293 Discussion

#### 294 1. Wing shape and migratory habits

295 The application of geometric morphometrics method to study wing shape diversity of 296 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 297 difference between the 10 species of dragonfly was in the apical and radial regions as well as the 298 299 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 300 301 dynamic load in flight, the wing nodus of dragonfly is the basis of the whole wing structure, with 302 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 303 304 hollow and ridge regions (*Nakamura, Osonoi & Terauchi, 2016*), so the wing nodus may be

305 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 306 307 have led to differences in the wing shape. From the perspective of the wing function, the 308 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 309 310 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. 311 312 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 313 migratory ability of dragonflies. It is generally considered that dragonflies with strong migratory 314 ability have larger cubital and anal regions than non-migrating dragonflies. 315

316 In this study, the five species dragonflies of *Crocothemis servilia*, *Orthetrum melania*, Orthetrum albistylum, Orthetrum testaceus as well as Acisoma panorpoides were distributed 317 318 mainly on the positive axis of PC1 and PC2. These species had wide and short forewing, with the 319 small anal region of the hindwing. Tramea virginia, Trithemis aurora, Pseudothemis zonata, and 320 Pantala flavescens were distributed mainly on the negative axis of PC1 and PC2. Their 321 forewings were long and narrow, and the anal region of the hindwing was large. According to the 322 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 323 324 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 325 326 species with strong flying ability, conducting stagnation flight and territory patrols, whereas the 327 species Crocothemis servilia, Orthetrum melania, Orthetrum albistylum, Orthetrum testaceus 328 and Acisoma panorpoides would prefer hovering around ponds or standing still for long periods. 329 The results of this study were in good agreement with those of *Rajabi et al. (2018)*, further 330 confirming the relationship between wing shape and migration. 331 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
- 334 mitochondrial COI gene and the nuclear genes 18S, 28S rRNA and ITS. Deielia phaon and
- 335 *Pantala flavescens* showed a close relationship, even though they belong to different subfamilies;

- 336 moreover, they formed a paraphyletic group with *Acisoma panorpoides* and *Crocothemis*
- 337 servillia, belonging to subfamily Sympetrinae. This result is similar to the results of Ware, May
- 338 & *Kjer (2007)* based on the nuclear genes *16S* and *28S rRNA*, and also similar to the results of
- 339 *Kim et al. (2014)* based on the mitochondrial *COI* gene and the nuclear genes *16S* and *28S rRNA*.
- 340 In the phylogenetic tree, Pantala flavescens and Trithemis aurora formed a paraphyletic group,
- 341 indicating a close relationship despite belonging to different subfamilies; this result was similar
- 342 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
- 344 were consistent with the results of *Kim et al. (2014)*, *Carle, Kjer & May (2015)* and *Yong et al.*
- 345 (2016), indicating that the interspecific phylogenetic relationships based on the mitochondrial

346 COI gene and the nuclear genes 18S, 28S rRNA and ITS were reliable, and that these genes can

- 347 be used as barcode genes for interspecies classification.
- 348 3. Evaluation of genetic information on wing shape
- 349 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 350 351 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 352 353 by numerous studies (Ramírez-Sánchez, Luna & Cramer, 2016; Fouquet et al., 2012; Gvoždík, Moravec & Kratochvíl, 2008; Limsopatham et al., 2018). The UPGMA method is effective in 354 355 interspecies morphological analysis, although Robinson & Terhune (2017) suggested the UPGMA method in subspecies analysis, such as morphological relationship between subspecies 356 357 or geographical populations, might obscure the patterns among individuals by the interobserver 358 and intermethod errors. However, in the interspecific relationship analysis, this method is ideal. 359 Using the UPGMA method in the present study to analyze forewing and hindwing shapes, the 360 individuals of each species were clustered initially into one branch. Then, topological relationships were established with other species. Compared to the intra-species relationships, 361 362 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) 367 had a distant relationship with Orthetrum (subfamily Libellulinae). However, from the 368 369 behavioral point of view, Crocothemis servilia, Deielia phaon and the species of Orthetrum have 370 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 371 372 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 373 374 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 375 migrating ability that might have influenced clustering based on the morphology. In terms of the 376 molecular data, Pantala flavescens and Trithemis aurora were clustered together as a branch, 377 378 and were distant from *Pseudothemis zonata*. These findings showed there was some genetic 379 information in the wing shape, but it was influenced more by the behavior and life habits. Hence, 380 for dragonflies, establishing inter-species relationships based directly on wing shape may be 381 unreliable. *Pilgrim & Vondohlen (2008)* studied the phylogenetic relationships of Sympetrinae 382 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 383 384 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 385 386 homoplasy. However, morphological and genetic structure may undergo synchronous evolution in other insects, such as the pronotum and genital segments of grasshopper genus Zoniopoda 387 388 (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 389 390 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.

397

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588

Landmarks on the forewing and hindwing of Libellulidae

A. Landmarks 1to 24 on forewing, 25 to 26 are ruler; B. Landmarks 1to 25 on hindwing, 26 to

27are ruler



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



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



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



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



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

The phylogenetic trees were constructed based on molecular data of the mitochondrial *COI* and nuclear *18S rRNA* + *ITS1* + *5.8S rRNA* + *ITS2* + *28S rRNA* genes



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* + *28S 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



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 1(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				
				mb				
				ers				
Orthetrum albistylum (Selys)	Orthetrum	Libellulinae	Libellulidae	8				
Orthetrum melania (Selys)	Orthetrum	Libellulinae	Libellulidae	5				
Orthetrum testaceus	Orthetrum	Libellulinae	Libellulidae	6				
(Burmeister)								
Acisoma panorpoides	Acisoma	Sympetrinae	Libellulidae	9				
(Rambur)								
Deielia phaon (Selys)	Deielia	Sympetrinae	Libellulidae	15				
Crocothemis servilia (Drury)	Crocothemis	Sympetrinae	Libellulidae	6				
Trithemis aurora	Trithemis	Trithemistinae	Libellulidae	13				
(Burmeister)								
Pseudothemis zonata	Pseudothemis	Trithemistinae	Libellulidae	3				
(Burmeister)								
<i>Tramea virginia</i> (Rambur)	Tramea	Trameinae	Libellulidae	7				
Pantala flavescens	Pantala	Trameinae	Libellulidae	9				
(Fabricius)								
Anotogaster sieboldii (Selys)	Anotogaster		Cordulegastridae	3				

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### 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				

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### Table 3(on next page)

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

	ly, Sublaining and		GenBank number			
			Mitochondria Nuclear 18S			
Family	Subfamily	Species	COI	rRNA+ITs1+5.8s		
			001	+ITs2+28S rRNA		
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		

141100		 and the second	1000	10 10	1000
NO	IP	H-H	$V \rightarrow V$	I-W	
1.01.00	1 B	 1.1.1.1.1	1000	1	L L

Cordulegastridae	Anotogaster sieboldii	EF155431.1	AB706930.1	
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### 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	Cs	Dp	Ot	Pf	Ta	Ар	Tv	Oa	Om	Pz
distance										
Forewing shape										
distance										
Cs		0.05	0.02	0.083	0.05	0.079*	0.10	0.034	0.04	0.05
		9**	6	**	4**	*	7**		5	5
Dn	0.04		0.05	0.096	0.07	0 044*	0.12	0.051	0.07	0.07
~ P	o.₀. ?**		1**	**	Δ <b>*</b> *	*	∆**	**	3**	Δ*
Ot	2 0.02	0.04	1	0.082	0.05	0.064*	т 0.10	0.040	0.05	0.05
0i	0.05	0.04		0.085	0.05	0.004	0.10	0.040	0.05	0.03
	4*	1**		<u> </u>	5**	*	6**	<u>ጥ</u> ጥ	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**
Та	0.08	0.08	0.06	0.085		0.090*	0.09	0.062	0.04	0.04
	2**	4**	9**	**		*	3**	**	9**	1**
Ар	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 1 1 6	0.08	0.11
- ,	Δ**	1**	Q*	**	0**	*		**	6**	0.11 7*
0	т 0.0 <b>2</b>	1	0.02	0.040	0.07	0.064*	0.00		0.04	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**	*		1
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*	

1 Notes:

2 \*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*.

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