



Evaluating the significance of wing shapes in inferring phylogenetic proximity among the generic taxa: an example of Cantharinae (Coleoptera, Cantharidae)

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<https://zoobank.org/BDC58E34-55FC-4081-AB4E-E039C1EBA54D>

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Received 03 February 2023

Accepted 18 February 2023

Published 17 March 2023

Academic Editors André Nel, Marianna Simões

Citation: Zhao W, Liu HY, Ge XY, Yang YX (2023) Evaluating the significance of wing shapes in inferring phylogenetic proximity among the generic taxa: an example of Cantharinae (Coleoptera, Cantharidae). *Arthropod Systematics & Phylogeny* 81: 303–316. <https://doi.org/10.3897/asp.81.e101411>

Abstract

The resolution of phylogenetic relationship among animals is still one of the most challenging problems in systematic zoology. Insect wing is a highly valued morphological character in the systematics, but few studies have been conducted to quantify wing shape variations for phylogenetic reconstruction. In this study, with Cantharinae as the subject, we conducted the GM analyses from hindwings of 16 representative genera. Further, we conducted the UPGMA based on Procrustes distance and Euclidean similarity measure of Mahalanobis distance, respectively, and NJ analysis of the Mahalanobis distance, as well as MP analysis using merged landmark dataset. In the meantime, we constructed the phylogenetic relationships among these genera based on the mitochondrial genomes, with a total of 41 sequences novel to Cantharinae, by BI and ML analyses. As a result, the CVA analysis demonstrated that the hindwing shapes of the cantharid genera are significantly different from one another. All the topologies produced by the GM data partially correspond with that of mitogenomic data. The close relationships of some genera are frequently recovered, including *Cyrebion* + *Themus*, *Cantharis* + *Taiwanocantharis* + *Taocantharis*, *Stenothemus* + *Falsopodabrus* + *Habronychus*. These results prove the importance and potential application of the hindwing shapes in recovering the relationships among the sibling genera.

Keywords

Geometric morphometric, genera, hindwing, mitochondrial genome, phylogeny, Soldier beetles

1. Introduction

Knowledge of how living (and extinct) taxa are related to one another underpins much of evolutionary biology (Kapli et al. 2020). The resolution of phylogenetic relationship among animals is one of the most challenging problems in systematic zoology (Field et al. 1988). In the pre-molecular age, organismal phylogenies were generally

created based on morphological character states. However, there are very few homologous morphological characters that can be compared among all organisms. With the arrival of DNA sequencing, molecular phylogenetic has become the standard for inferring evolutionary relationships (Ziemert and Jensen 2012). Since that a number of

genes with fundamental biochemical functions are found in all species and they can be sequenced, aligned, and analyzed to study phylogenetic relationships at the deepest part of the tree of life (Hillis and Dixon 1991). Moreover, based on the analyses of ribosomal gene sequences, this relationship appeared to be robust to tree-building methods. In recent years, mitochondrial genome has become the most popular molecular marker in inferring the phylogenetic relationships among the animals, especially for various groups of insects (Bajpai and Tewari 2010).

Although many advantages of molecular over morphological phylogenetic have been recognized, morphological analysis still cannot be replaced or neglected in the construction of phylogenetic relationships. For many groups of poorly known organisms, the only known specimens of many species are represented merely by the holotype or type series. Collecting additional material can be prohibitive because of rarity of the species, inaccessibility of the habitat, destruction of known collection localities, legal protection of the habitat or species, or high costs of procurement. A high percentage of recently extant species have been exterminated in this century by human activities, especially through the destruction of tropical rainforests (Myers 1986). Because of this high extinction rate, a majority (or at least a large fraction) of described species may never be collected again and will remain known only from traditionally preserved specimens. So this is the reason why paleontology always has been primarily a morphological endeavor, and the fossils at least represent a set of taxa that provide potential information about evolution (e.g. Patterson and Rosen 1977; Schaeffer et al. 1972), which is nearly limited to morphological analysis. Moreover, an understanding of morphological variation in fossils requires an understanding of the morphology of living species.

It is well-known that wing shape of insects exhibits a high heritability in nature (Bitner-Mathé and Klaczko 1999; Moraes et al. 2004), and wing morphology is of a primary importance to entomologists interested in systematics (Su et al. 2015). It was Comstock (1893) who first popularized the use of insect wing venation for traditional classification (Kunkel 2004). Wing veins and their intersections are unambiguously homologous (Ross 1936), so since the 1970's, several authors have begun to use the insect wings of morphometrical studies in systematics and phylogeny (Plowright and Stephen 1973; Rohlf 1993; Klingenberg 2003; Gumiel et al. 2003). Geometric morphometrics (GM) utilizes powerful and comprehensive statistical procedures to analyze shape differences of a morphological feature, using either homologous landmarks or outlines of the structure (Rohlf and Marcus 1993; Marcus and Corti 1996; Adam et al. 2004), and it is considered to be the most rigorous morphometric method (Gilchrist et al. 2000; Debat et al. 2003). Compared with other organs, the wing venation is unique and the examination of wing venation pattern shows many methodological advantages, because they are basically 2-dimensional and the venation provides many well-defined morphological landmarks (Gumiel et al. 2003), the interactions of the veins, which are easy for identification and

able to capture the general shape of the wing (Bookstein 1991), as well as their rigidity and good conservation in either living or fossil specimens (Pavlinov 2001). Among insects, the use of GM analysis to study wing venation has been useful in identification at the individual level (Baylac et al. 2003; Dujardin et al. 2003; Sadeghi et al. 2009), in distinguishing sibling species (Matias et al. 2001; De la Riva et al. 2001; Villegas et al. 2002; Roggero and Dentrèves 2005; Aytekin et al. 2007; Francuski et al. 2009; Tüzün 2009) and in delimitation among the genera (Baracchi et al. 2011; Su et al. 2015) and higher taxonomic category (Bai et al. 2012, 2013). However, few studies have been conducted to quantify such wing variations for phylogenetic reconstruction. Thanks to the advent of the phylogenetic morphometric (PM) analysis method (Díaz-Cruz et al. 2021), it makes possibility to explore the relationships among the organisms based on the morphometric data.

The beetle family Cantharidae, commonly known soldier beetles, is a large group with approximately 6,000 species in the world (Delkeskamp 1977; Kazantsev and Brancucci 2007). It is divided into five subfamilies (Brancucci 1980), based on a comprehensive comparative morphological study. In this classification, hindwing venation is one of the highly valued characters in the subfamilial level, and different from one another in the number of vein, cells and their length. In comparison, within each subfamily, the venation is stable and only exhibits quantitative variations among genera (Lanham 1951). Therefore, it is an ideal material to explore the relationships among the genera based on the hindwing variation through PM analysis.

In the present study, taking the cantharid beetles as the subject, we are going to apply GM to analyze the hindwing shapes of 16 representative genera of Cantharinae, based on which to explore their relationships by the PM analysis. Meanwhile, we shall reconstruct the phylogeny among these genera based on the (nearly) complete mitochondrial genomes by both Maximum-likelihood (ML) and Bayesian inference (BI) analyses. Finally, we will examine the accuracy of PM of hind wing shapes, by comparison with the produced topology of mitogenomes. Based on the results, we are able to evaluate the reliability of the hindwing shapes in inferring phylogenetic relationships among the generic taxa, also shed new lights on reconstruction of phylogenetic relationships, especially for those taxa rare, inaccessible or extinct organism relying on the morphology.

2. Materials and methods

2.1. Studied material

Hind wings of the following Cantharinae species (Table 1) are used in this study. Prior to geometric morphometric analysis, identification of specimens was performed using other morphological characters of adults

Table 1. The species of subfamily Cantharinae used in the GM analysis and information for the representative species' mitogenomes used for phylogenetic analysis.

Genus	Species for GM analysis	Number	Species for phylogenetic analysis	GenBank Accession	Voucher number	Locality of molecular material
<i>Asiopodabrus</i>	<i>Asiopodabrus cheni</i>	3	<i>Asiopodabrus cheni</i>	OQ221889	2CA132	China: Zhejiang, Fengyang Mts
			<i>Asiopodabrus satoi</i>	OQ221851	2CA39	China: Guangxi, Mao'er Mts
<i>Cantharis</i>	<i>Cantharis rufa</i>	3	<i>Cantharis jindraii</i>	OQ221852	2CA70	China: Beijing, Xiaolongmen
	<i>Cantharis brunneipennis</i>	3	<i>Cantharis brunneipennis</i>	OQ221875	CAN197	China: Shaanxi, Foping
	<i>Cantharis (Cyrtomoptila) plagiata</i>	3	<i>Cantharis (Cyrtomoptila) plagiata</i>	MT364421	CAN74	China: Shaanxi, Yangxian
<i>Cyrebion</i>	<i>Cyrebion subrufolineatus</i>	3	<i>Cyrebion subrufolineatus</i>	OQ221853	2CA65	China: Xizang, Mangkang
	<i>Cyrebion gracilicornis</i>	3	<i>Cyrebion gracilicornis</i>	OQ221870	CAN24	China: Hubei, Shennongjia
<i>Falsopodabrus</i>	<i>Falsopodabrus tridentatus</i>	3	<i>Falsopodabrus tridentatus</i>	OQ221854	2CA161	China: Xizang, Cona
	<i>Falsopodabrus rolciki</i>	3	<i>Falsopodabrus rolciki</i>	OQ221876	2CA81	China: Xizang, Bomé
<i>Cephalomalthinus</i>	<i>Cephalomalthinus</i> sp.1	3	<i>Cephalomalthinus</i> sp.1	OQ221871	CAN182	China: Hainan, Jianfengling
	<i>Cephalomalthinus</i> sp.2	3	<i>Cephalomalthinus</i> sp.2	OQ221877	2CA24	China: Guangxi, Daming Mts
<i>Habronychus</i>	<i>Habronychus</i> (s. str.) sp.1	3	<i>Habronychus</i> (s. str.) sp.1	OQ221855	CAN27	China: Hubei, Shennongjia
	<i>Habronychus</i> (s. str.) sp.2	3	<i>Habronychus</i> (s. str.) sp.2	OQ221878	CAN210	China: Hubei, Huangbaoping
	<i>Habronychus (Monohabronychus)</i> sp.1	3	<i>Habronychus (Macrohabronychus)</i> sp.	OQ221884	2CA3	China: Xizang, Medog
	<i>Habronychus (Monohabronychus)</i> sp.2	3	<i>Habronychus (Macrohabronychus) chaoi</i>	OQ221859	2CA162	China: Xizang, Cona
	<i>Habronychus (Macrohabronychus) chaoi</i>		3	<i>Habronychus (Monohabronychus)</i> sp.	OQ221873	CAN98
		<i>Habronychus (Monohabronychus)</i> sp. 3		OQ221880	CAN83	China: Shaanxi, Yangxian
<i>Lycocerus</i>	<i>Lycocerus bilineatus</i>	3	<i>Lycocerus inopaciceps</i>	OQ221874	CAN198	China: Shaanxi, Foping
	<i>Lycocerus inopaciceps</i>	3	<i>Lycocerus curvatus</i>	OQ221857	CAN36	China: Hubei, Shennongjia
	<i>Lycocerus orientalis</i>	3	<i>Lycocerus hubeiensis</i>	OQ221858	CAN123	China: Hubei, Yichang
	<i>Lycocerus limbatus</i>	3	<i>Lycocerus orientalis</i>	OQ221882	2CA44	China: Jiangxi, Jinggang Mts
			<i>Lycocerus limbatus</i>	OQ221883	CAN16	China: Hubei, Shennongjia
<i>Micropodabrus</i>	<i>Micropodabrus coleatus</i>	3	<i>Micropodabrus oudai</i>	OQ221860	CAN201	China: Shaanxi, Foping
<i>Podabrus</i>	<i>Podabrus annulatus</i>	3	<i>Podabrus annulatus</i>	OQ221861	2CA47	China: Beijing, Yanqing
<i>Pseudopodabrus</i>	<i>Pseudopodabrus atripes</i>	3	<i>Pseudopodabrus atripes</i>	OQ221885	2CA27	China: Guangxi, Daming Mts
<i>Prothemus</i>	<i>Prothemus kiukianganus</i>	3	<i>Prothemus semimetallicus</i>	OQ221862	CAN102	China: Hunan, Wulingyuan
	<i>Prothemus sanguineus</i>	3	<i>Prothemus sanguineus</i>	OQ221872	CAN96	China: Hubei, Yi'en
<i>Rhagonycha</i>	<i>Rhagonycha nigroimpressa</i>	3	<i>Rhagonycha nigroimpressa</i>	OQ221863	CAN100	China: Hunan, Yongshun
	<i>Rhagonycha przewalskii</i>	3	<i>Rhagonycha przewalskii</i>	OQ221886	CAN108	China: Hebei, Xiaowutai Mts
<i>Stenothemus</i>	<i>Stenothemus grahami</i>	3	<i>Stenothemus fukienensis</i>	OQ221864	2CA137	China: Zhejiang, Fengyang Mountain
	<i>Stenothemus biimpres-siceps</i>	3	<i>Stenothemus biimpres-siceps</i>	OQ221887	2CA99	China: Zhejiang, Tianmu Mts
<i>Taiwanocantharis</i>	<i>Taiwanocantharis parasatoi</i>	3	<i>Taiwanocantharis parasatoi</i>	OQ221865	2CA28	China: Guangxi, Daming Mts
	<i>Taiwanocantharis chumbiensis</i>	3	<i>Taiwanocantharis</i> sp.	OQ221881	2CA150	China: Yunnan
<i>Taocantharis</i>	<i>Taocantharis businskae</i>	3	<i>Taocantharis businskae</i>	OQ221888	CAN206	China: Hubei, Huangbaoping

Genus	Species for GM analysis	Number	Species for phylogenetic analysis	GenBank Accession	Voucher number	Locality of molecular material
<i>Themus</i>	<i>Themus (Telephorops) coelestis</i>	3	<i>Themus (Telephorops) coelestis</i>	OQ221866	CAN1	China: Hubei, Shennongjia
	<i>Themus (Telephorops) cavipennis</i>	3	<i>Themus (Telephorops) cavipennis</i>	OQ221867	2CA73	China: Xizang, Medog
	<i>Themus (Themus) stigmaticus</i>	3	<i>Themus (Themus) stigmaticus</i>	OQ221868	CAN104	China: Hebei, Xiaowutai Mts
	<i>Themus (Themus) luteipes</i>	3	<i>Themus (Themus) luteipes</i>	OQ221869	CAN69	China
	<i>Themus (Haplothemus) hedinii</i>	3	<i>Themus (Haplothemus) hedinii</i>	OQ221879	CAN148	China: Qinghai, Menyuan
	<i>Themus (Haplothemus) bimaculicollis</i>	3	<i>Themus (Haplothemus) bimaculicollis</i>	OQ221856	2CA110	China: Sichuan, Liziping

(Yang 2010). The materials of the representative species are deposited in the Museum of Hebei University, Baoding, China (MHBU) and the Institute of Zoology, Chinese Academy of Sciences, Beijing, China (IZAS) respectively. The left hind wing of each specimen (a total of 111 wings, see Supplemental material) was removed from the body and mounted in neutral balsam between a microscope slide and a cover slip.

2.2. Landmark acquisition and digitalization

The structure analyzed was the shape of hind wings, which was directly photographed by a stereomicroscope Nikon SMZ1500 and attached video camera Canon 450D connected to a HP computer. For the hind wings, a total of 13 landmarks of type II (Fig. 1) were placed. Digital photographs or scanned images were input to tps-UTIL 1.43 software (Rohlf 2008a). Cartesian coordinates of landmarks were digitized with tps-DIG 2.12 software (Rohlf 2008b). Landmark configurations were scaled, translated and rotated against the consensus configuration using the GLS Procrustes superimposition method (Bookstein

1991). The coordinates were analyzed using tps-RELW 1.44 (Rohlf 2006) to calculate eigenvalues for each principal warp. The digitalization procedure was repeated three by the same observer on different days to evaluate landmark measurement error.

2.3. Geometric morphometric (GM) analyses

The GM method based on landmark data in inferring phylogenetic relationships among the generic taxa considering UPGMA, Maximum Parsimony and Neighbor-Joining as the optimality criterion (Champakaw et al. 2021; Goloboff and Catalano 2016). In applying the GM method, we selected the cantharid beetles as the experimental subject.

The tps files produced in tps-DIG was used to perform GM analysis. To examine the shape variation, the digitized landmark data is analyzed using MorphoJ software (Klingenberg 2011). Principal component analysis (PCA) was employed to test how well the genera can be distinguished by the shape of hind wings. Frequently the characters with high loading value in PCAs correspond

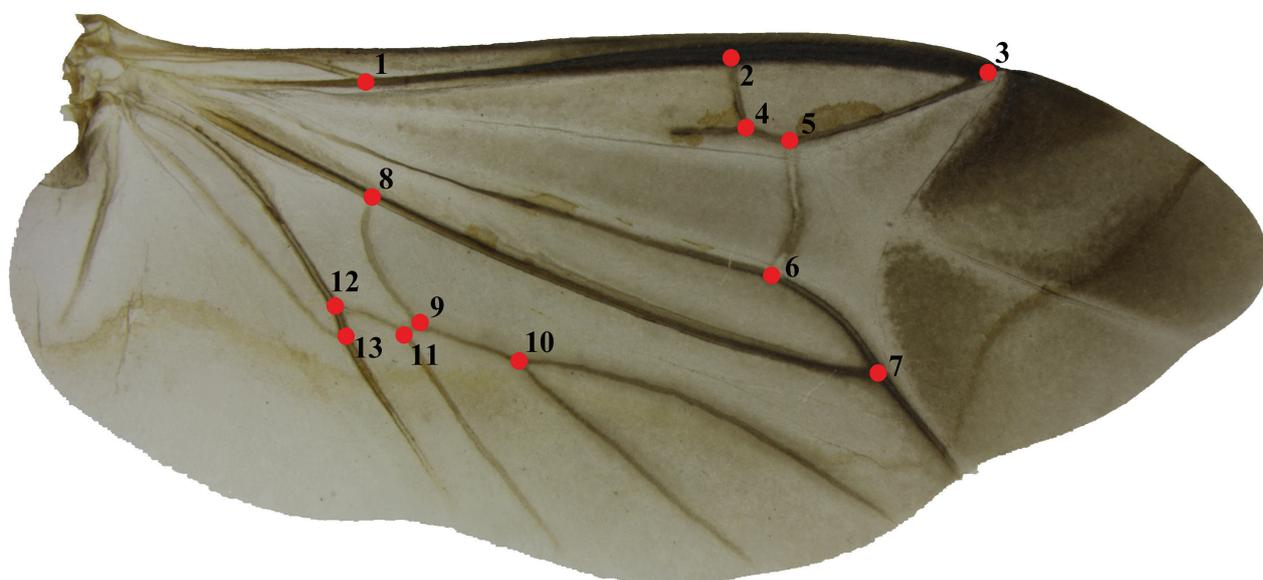


Figure 1. Position of the 13 landmarks (recorded from the 1st to the 13th respectively) on a *Cantharis brunneipennis* Heyden, 1889 hindwing.

to the observed variation patterns among genera. The relative similarity and discrimination of the test groups is analyzed using Canonical Variates Analysis (CVA). CVA finds shape values that maximize group means relative to variation within groups, by assuming that covariate matrices are identical (Klingenberg 2010). Procrustes distances and Mahalanobis distances (the square root of the sum of squared differences between corresponding points) between each of the genera were computed and the matrix was produced by the MorphoJ software (Klingenberg 2011). Procrustes distance matrix was subjected to UPGMA (unweighted pair group method using arithmetic averages) and cluster analyses to determine the phonetic relationships among genera. The most important advantage of using Procrustes distances to capture shape variation was that these distances were considered the best method for measuring shape differences among taxa (Chapman 1990; Goodall 1991; Goodall and Bose 1987; Marcus et al. 1993; Pretorius and Scholtz 2001; Rohlf 1990). This is an effective method for detecting differences among taxa. The Mahalanobis distance score matrix was posteriorly loaded in PAST 2.17 (Hammer et al. 2001) to determine the phonetic relationships among genera using Euclidean similarity measure.

2.4. Phylogenetic morphometric (PM) analysis

The tps files produced in tps-DIG was also used to perform MP analysis in TNT 1.5 (Goloboff and Catalano 2016). The search strategy followed a heuristic (traditional search), using random addition sequences, tree bisection reconnection (TBR) as branch swapping algorithm, holding one tree per replicate and 1000 runs (mult = r, tbr hold 1 rep 1000) (Díaz-Cruz et al. 2021). In addition to, the Neighbor-Joining (NJ) trees (Sneath and Sokal 1973) were constructed to display the Mahalanobis distances between populations using PAST 2.17 with 1000 bootstrap replicates. Geometric morphometrics can be used to determine shape differences, and the resulting phenograms can effectively indicate phenetic relationships between the samples, summarizing overall patterns of similarity (Pretorius and Scholtz 2001).

2.5. Phylogenetic analysis based on mitogenomes

Meanwhile, both Maximum-likelihood and Bayesian inference analyses of mitochondrial genomes to examine the accuracy of phylogenetic morphometrics of hind wing shapes, we newly sequenced 41 species mitochondrial genomes and the detailed information was provided in Table 1. Specimens were stored at -80°C in anhydrous alcohol. The DNA was extracted from a single individual of each species, using the Qiagen DNAeasy™ extraction kit. Library (150-bp insert size) was prepared and sequenced on the Illumina Novaseq 6000 platform (Illumina, Alameda, CA, USA). About 6 Gb of clean data were obtained and

assembly was conducted through IDBA-UD, with k -mer length of 40 and 160 bp. The partial sequences of gene *cox1* for each species were amplified via polymerase chain reaction and used as ‘reference sequences’ to target the assemble scaffolds and acquire the best-fit, which achieves at least 98% similarity (Peng et al. 2012).

The individual genes were aligned and concatenated using PhyloSuite version 1.2.2 (Zhang et al. 2020). ModelFinder (Kalyaanamoorthy et al. 2017) was used to select the best-fit partition model. ML analyses were conducted using IQ-TREE version 1.6.8 (Nguyen et al. 2015) with 1000 SH-aLRT replicates. BI analyses were carried out using MrBayes 3.2.6 (Ronquist et al. 2012) with two independent Markov Chain Monte Carlo (MCMC) chain runs of 2×10^6 generations, in which the initial 25% of sampled data were discarded as burn-in. The phylogenetic tree was visualized and edited using Interactive Tree of Life (Letunic and Bork 2019; iTOL, <https://itol.embl.de>).

3. Results

3.1. Geometric morphometric analysis

The first three principal components of the shape of hind wings explain 76.847% of the micromesh variation, which were 56.097%, 13.314% and 7.436%, respectively (see Supplementary material: Table S1). They were plotted to indicate variation along the first two relative warp two axes, which were shown as deformations of the least squares reference using thin-plate splines (Fig. 2). The statistical test performed by TpsSmall showed that the correlation (uncentred) between the tangent space, Y, regressed onto Procrustes distance (geodesic distances in radians) were 0.999999, which suggested that the data is acceptable for further geometric morphometric analysis (Pretorius and Scholtz 2001). Further, the CVA analysis of the hind wing shapes demonstrated that all genera of Cantharinae are significantly different in both Mahalanobis distances ($p < 0.0001$) and Procrustes distances ($p < 0.0001$) (Table 2).

3.2. Phylogenetic analyses of mitogenomes

The phylogenies of Cantharinae based on the mitochondrial genome data by both ML and BI analyses produced highly congruent topologies (Fig. 3). In general, it was divided into two large branches with high supporting values. One was composed of *Asiopodabrus* and *Podabrus* (PP = 1, BS = 100), and the other consisted of the remaining ones (PP = 1, BS = 100). Within the latter, it was subdivided into two clades, one of which was recovered as (*Cyrebion* + *Themus*) + (*Prothemus* + (*Falsopodabrus* + (*Habronychus* + *Stenothemus*))) (PP = 1, BS = 83), the other was (*Lycocerus* + (*Cantharis* + (*Taiwanocantharis* + *Taocantharis*))) + (*Rhagonycha* + (*Micropodabrus* +

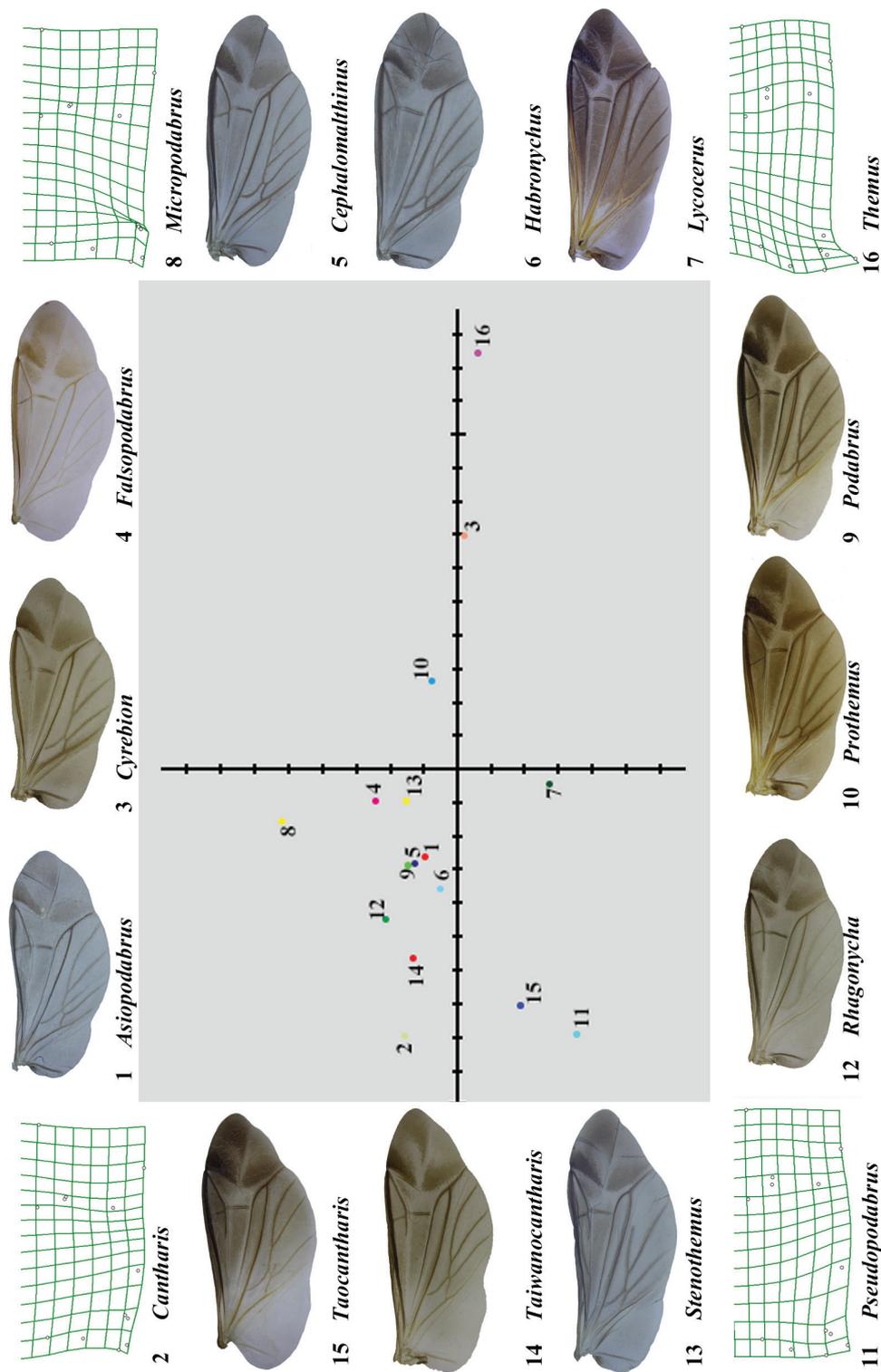


Figure 2. Shape differences of hind wings among 16 genera of Cantharinae. Shape changes among genera are implied by variations along the first two relative warp axes. Splines indicate deformation of the landmarks in comparison with the reference configuration.

(*Cephalomalthinus* + *Pseudopodabrus*))) (PP = 0.99, BS = 77). This result would be used as the standard reference to make comparisons with the phylogenetic morphometric analysis below.

3.3. Phylogenetic morphometric analysis

In comparison with the above mitophylogenetic topologies (Fig. 3), the phonograms based on both Procrustes

distance (Fig. 4A) and Euclidean similarity metrics of Mahalanobis distance (Fig. 4B) showed that some genera were consistently grouped together, including *Falsopodabrus* + *Habronychus* + *Stenothemus* (clade I), *Cantharis* + *Taocantharis* + *Taiwanocantharis* (clade II), and *Cyrebion* + *Themus* (clade III), whose average shapes were displayed near the clades respectively (Figs 4A–C). Unlikely, another two pairs of genera, including *Cephalomalthinus* + *Podabrus*, and *Lycocerus* + *Prothemus*, were grouped into separate clades respectively by using both two methods, but were not recovered in the mitophylogeny.

Table 2. Difference in the hindwings shapes among the groups. Mahalanobis distances p-values (above) from permutation tests (10000 permutation rounds); Procrustes distances p-values (below) from permutation tests (10000 permutation rounds).

	<i>Asiopodabrus</i>	<i>Cantharis</i>	<i>Cyrebion</i>	<i>Falsopodabrus</i>	<i>Cephalomathinus</i>	<i>Habronychus</i>	<i>Lycocerus</i>	<i>Micropodabrus</i>	<i>Podabrus</i>	<i>Prothemus</i>	<i>Pseudopodabrus</i>	<i>Rhagonycha</i>	<i>Stenothemus</i>	<i>Taiwanocantharis</i>	<i>Taocantharis</i>	<i>Themus</i>
<i>Asiopodabrus</i>	—	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
<i>Cantharis</i>	<0.0001	—	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
<i>Cyrebion</i>	<0.0001	<0.0001	—	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
<i>Falsopodabrus</i>	<0.0001	<0.0001	<0.0001	—	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
<i>Cephalomathinus</i>	<0.0001	<0.0001	<0.0001	<0.0001	—	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
<i>Habronychus</i>	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	—	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
<i>Lycocerus</i>	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	—	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
<i>Micropodabrus</i>	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	—	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
<i>Podabrus</i>	0.0001	0.0002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	—	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
<i>Prothemus</i>	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0083	<0.0001	<0.0001	—	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
<i>Pseudopodabrus</i>	<0.0001	0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	—	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
<i>Rhagonycha</i>	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	<0.0001	<0.0001	—	<0.0001	<0.0001	<0.0001	<0.0001
<i>Stenothemus</i>	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	<0.0001	0.0005	<0.0001	<0.0001	<0.0001	—	<0.0001	<0.0001	<0.0001
<i>Taiwanocantharis</i>	0.0001	0.0015	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0036	<0.0001	<0.0001	<0.0001	<0.0001	—	<0.0001	<0.0001
<i>Taocantharis</i>	0.0001	0.0044	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	—	<0.0001
<i>Themus</i>	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	—

Furthermore, the phylogeny of Cantharinae was reconstructed by MP analysis based on the two landmark configurations shown in Fig. 4C. Similar to the mitophylogenetic tree (Fig. 3), *Podabrus* (but not coupled with *Asiopodabrus*) was the first to be separated into a single branch, and the remaining ones were grouped together into a large branch. Within the latter, only the sister relationships of *Cantharis* + (*Taiwanocantharis* + *Taocantharis*) (clade II) was recovered.

Moreover, the tree under NJ analysis based on Mahalanobis distance (Fig. 4D) showed that the sister groups of *Stenothemus* + (*Falsopodabrus* + *Habronychus*) (clade I), *Taocantharis* + (*Cantharis* + *Taiwanocantharis*) (clade II), and *Cyrebion* + *Themus* (clade III) were all recovered as those of mitophylogenetic analyses. Besides, like those phenograms of Fig. 4A, B, *Lycocerus* + *Prothemus*, and *Micropodabrus* + (*Rhagonycha* + (*Pseudopodabrus* + *Asiopodabrus*)) (only of Fig. 4B) were shown in separate clades respectively, although some differences exhibited among the four genera within the latter clade, which however was never recovered in the mitophylogeny.

4. Discussion

In the present study, the statistical test performed by TpsSmall suggested that our obtained data of hind wings (3 specimens were collected from each of 37 species amount to 111 samples, 13 landmarks for each sample) is acceptable for the geometric morphometric analysis. Further the CVA analysis suggested that all representative genera (a total of 16 genera) of Cantharinae can be distinguished from one another by the hind wing shapes, which is consistent with the previous study (Su et al. 2015).

In insects, the wing shapes of geometric morphometric analyses are usually applied in distinguishing the sibling species or uncovering the cryptic species (Baylac et al. 2003; Pizzo et al. 2006; Gurgel-Goncalves et al. 2011; Muñoz-Muñoz et al. 2011; Mitrovski-Bogdanović et al. 2013), since that wing GM analysis represents a reliable and rapid alternative that yields satisfactory results when discriminating between morphologically analogous species (Champakaew et al. 2021). Although the geometric morphometric data remains controversial in inferring the relationships among the organisms (Palci and Lee 2019), it has been applied in estimating the evolutionary relationships of some animals (Bogan and Roe 2008; Klingenberg 2015; Siriwut et al. 2015; Püschel and Sellers 2016; Hart et al. 2020; Goharimanesh et al. 2022), especially in the higher grades (tribes or subfamilies or families) of some insect groups, based on the shapes of pronotum and elytra (Acevedo 2015; Eldred et al. 2016; Zhang et al. 2019; Tong et al. 2021), as well as hind wings (Abou-Shaara and Al-Ghamdi 2012; Su et al. 2015; Barour and Baylac 2016). However, none was addressed

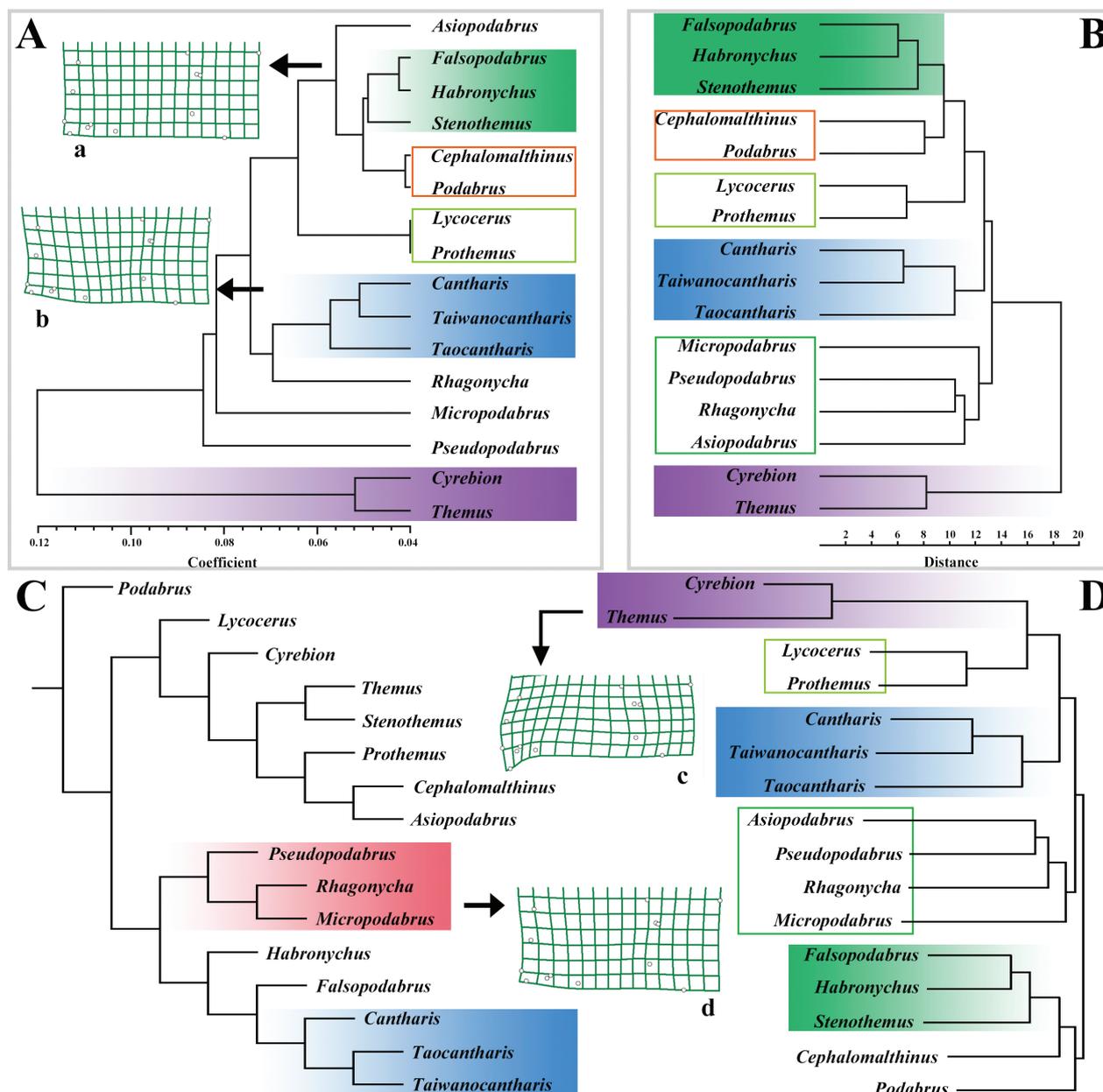


Figure 4. Comparing phylogenetic relationships of Cantharinae. **A** phylogenetic hypothesis based on Procrustes distances using UPGMA. **B** phylogenetic hypothesis based on Mahalanobis distances using Euclidean similarity measure. **C** phylogenetic hypothesis based on two landmark configurations using MP analysis. **D** Neighbor-Joining tree for genera of Cantharinae based on Mahalanobis distances with 1000 bootstrap replicates. The average shape of four groups were displayed near the clades in a, b, c and d.

rus. Except the four genera complex, some genera were always grouped together, including *Lycocerus* and *Prothemus*, *Micropodabrus*, *Rhagonycha*, *Pseudopodabrus* and *Asiopodabrus*, which are never recovered as sister groups by the mitophylogeny. This suggested that hindwing shape may be also convergent in the evolution, although which was usually considered as a character of high value in systematics, like other external morphological characters, such as antennae, pronotum, elytra, etc.

Conflict between morphological and molecular studies of phylogeny may be also resulted from differences in assumptions about the evolutionary process and differences in methods of analysis. The reasons for these differences may be allometric effects, homoplasy, accelerated evolution, genetic drift and, of course possible sampling or

measurement errors (Cardini and O’Higgins 2004). In the case of such conflicting results, molecular sequence data are often favoured, as they are typically much more numerous and/or arguably perceived as being more objective (e.g. Jin et al. 2020). Given the strong statistical support and most groupings in all molecular analyses, and the quantity and suitability of mitochondrial data to elucidate phylogenetic relationships of closely related taxa, in this case we favor the results of molecular analyses to estimate the dependability of the PM analyses. Although no topology was produced in the PM analyses congruent with the mitophylogenetic tree, the close relationships of the allied genera were recovered in the phylogenetic geometric morphometrics, which suggested that the latter was helpful in inferring the relationships of sister groups.

Thus, we propose the GM analyses should be extended to other morphological structures as well in future insect taxonomy research.

5. Conclusions

In the present study, taking the Cantharinae as an example, we evaluated the taxonomic value of the hindwing shapes in inferring phylogenetic relationships among the generic taxa of subfamily Cantharinae. A total of 111 hindwing samples representing 37 species belonging to 16 genera of Cantharinae were analyzed by GM analysis. The statistical test performed by TpsSmall suggested that our obtained data is acceptable for the geometric morphometric analysis, and the CVA analysis demonstrated that all representative genera of Cantharinae can be well separated by the hind wing shape. With the constructed mitophylogeny as reference, the PM analyses of the hindwing shapes data using different methods (MP analysis of the two landmark configurations, NJ analysis of Mahalanobis distance, phonograms of both Procrustes distance and Euclidean similarity metrics of Mahalanobis distance) showed that the sister relationships of allied genera or morphologically defined genera complex are always recovered. However, some genera in distant relationships sometimes are grouped together under PM analysis, probably due to the convergent evolution in the hindwing shapes. No matter how, the landmark-based hindwings shape GM analyses prove to be feasible in phylogenetic reconstruction and be helpful in recovering the sister relationships of allied genera.

6. Funding

The present study was supported by the National Natural Science Foundation of China (Nos. 32270491, 31772507), the Natural Science Foundation of Hebei Province (No. C2022201005), the Interdisciplinary Research Program of Natural Science of Hebei University (No. DXK202103) and the Excellent Youth Scientific Research and Innovation Team of Hebei University (No. 605020521005).

7. Acknowledgements

We wish to express our deepest thanks to Prof. Xingke Yang (Institute of Zoology, Chinese Academy of Sciences, Beijing, China) for his guidance to the senior corresponding author in studying on the taxonomy of Cantharidae. We are very grateful to the editors and anonymous reviewers for careful scrutiny and useful comments for improving the manuscript.

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Supplementary material 1

Table S1

Authors: Zhao W, Liu H, Ge X, Yang Y (2023)

Data type: .pdf

Explanation note: Eigen values and contributions of the principal components analysis in hindwings shape.

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Link: <https://doi.org/10.3897/asp.81.e101411.suppl1>