Phylogeny of the Chinese Cantatopidae (Orthopter: Acridoidea) Inferred from Mitochondrial DNA Sequences*

ZHAO Ling[1], *ZHENG Zhemin*[2], *HUANG Yuan*[2], *ZHANG Ling*[2], *YANG Liang*[3]
(1. College of Agronomy, Xinjiang Agricultural University, Urumqi 830052, China;
2. Institute of Zoology, Shaanxi Normal University, Xi'an 710062, China;
3. College of Life Sciences, Yan'an University, Yan'an 716000, China)

Abstract: DNA sequence comparisons of the mitochondrial COI, COII, and Cytb genes were used to infer phylogenetic relationships of partial genera and species of Cantatopidae, including 7 new species (*Apalacris eminifronta*, *Caryanda pseudodentata*, *Caryanda bannaensis*, *Caryanda ruiliensis*, *Longchuanacris fui*, *Longchuanacris xiaoheishanensis*, *Podismodes dabieshanensis*). Phylogenetic trees were constructed using maximum likelihood and Bayesian inference. The combined results suggest several phylogenetic relationships including: ① *Longchuanacris* is placed within the subfamily Caryandinae; ② *Sinopodisma* and *Yunnanacris* are both synonyms of *Podismodes*; ③ *Yupodisma* is still a separate genus within Podisminae instead of a synonym of *Anapodisma*; 4) the molecular phylogeny of 7 new species is consistant with previous morphological phylogeny. This finding suggests a combination of molecular and morphological approaches are necessary for accurate species identification.

Key words: Cantatopidae; mtDNA; COI; COII; Cytb; molecular systematics

中国斑腿蝗科线粒体基因的分子系统学研究

赵 玲[1]，郑哲民[2]，黄 原[2]，张 陵[2]，杨 亮[3]
（1. 新疆农业大学农学院，新疆 乌鲁木齐 830052；
2. 陕西师范大学动物所，陕西 西安 710062；3. 延安大学生命科学学院，陕西 延安 716000）

摘 要：使用线粒体基因 COI，COII，Cytb 分析并建立中国斑腿蝗科部分种属的系统发育关系，其中对 7 个新种（*Apalacris eminifronta*, *Caryanda pseudodentata*, *Caryanda bannaensis*, *Caryanda ruiliensis*, *Longchuanacris fui*, *Longchuanacris xiaoheishanensis*, *Podismodes dabieshanensis*）的归属从分子角度也做了讨论。本研究将实验所获得的 56 条序列与 NCBI 中所收录的斑腿蝗科序列进行联合分析，采用最大似然法、贝叶斯推论等系统分析方法并综合 P 距离进行分析，最终得出以下几点结论：①龙川蝗属 *Longchuanaceis* Zheng et Fu 划归卵翅蝗亚科 Caryandinae；②蹦蝗属 *Sinopodisma* Chang、云秃蝗属 *Yunnanacris* Chang 均为华秃蝗属 *Podismodes* Ramme 的同物异名；③不支持 Storozhenko 将豫蝗属 *Yupodisma* Zhang *et* Xia 并入安秃蝗属 *Anapodisma* Dovnar-Zapolskii，从本研究所建的系统发育树来看，豫蝗属仍应作为秃蝗亚科 Podisminae 的一个独立属；④前人利用形态特征鉴定的七个新种与分子系统发育分析结果一致，新种独立有效。

关键词：斑腿蝗科；线粒体基因；COI；COII；Cytb；分子系统学
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作者简介：赵玲（1977 年生），女，讲师，博士；通讯作者：杨亮；E-mail：yangliang@163.com

Catantopidae is the largest family of Acridoidea with world-wide distribution. The group includes *ca.* 4 000 known species in more than 760 genera, which are mostly distributed in the tropics and subtropics. China is the species rich Catantopidae country with *ca.* 430 in more than 100 genera and endemism is very high. Many are Oriental species, with only a few species occurring in Palearctic region. Many species of Catantopidae are pest of agriculture, husbandry and forestry. The harmful species usually belong to the best studied insects in China. Thus, a reliable and accessible classification of these species is fundamental to research in pest control, ecology, evolutionary biology and biodiversity. Meanwhile, the systematic researches on Catantopidae are also very important. To date, there is no consensus system of Catantopidae in China. Some subfamilies, genera and species are still contentious.

Longchuanaceis was traditionally considered a genus within the subfamily Oxyinae[1]. Niu[2] removed *Longchuanaceis* to Caryandinae by morphological characters. On the basis of his results *Sinopodisma* Chang and *Yunnanacris* Chang are both synonymized with *Podismodes* Ramme. He also support that *Yupodisma* Zhang *et* Xia is synonymized with *Anapodisma* Dovnar-Zapolskii[3]. In his dissertation, one genus, one subgenus and forty-two species in Cantatopidae, all from China, are new to science. Species groups are proposed in genera: *Oxya* Serville, *Caryanda* Stål, *Longchuanacris* Zheng *et* Fu, *Podismodes* Ramme, *Traulia* Stål[2].

Systematic research on Cantatopidae by morphology alone can not resolve these contentions completely. Recent studies have demonstrated a great potential for DNA sequence analysis in species identification, phylogeny, and gene flow[4-6]. Sequences of the mitochondrial genes cytochrome oxidase subunit I (COI), cytochrome oxidase subunit II (COII), and cytochrome *b* (Cytb) have been extensively applied in rapid species identification, phylogenetic reconstructions for a variety of taxa[7-10]. In this study, our objectives were to: ① obtain nucleotide sequences of the COI, COII, and Cytb mitochondrial genes from Cantatopidae species as a basis for further species identification and phylogenetic analyses, ②conduct phylogenetic analyses using these nucleotide sequences (alone and in combination), ③examine the species and genera status by molecular phylogeny, and ④compare molecular findings with morphological assessments of the contentious genera and 7 new species.

1 Materials and Methods

1.1 Taxon samples, DNA extraction, and GenBank sequences

All Cantatopidae samples, including one outgroup species (*Atractomorpha sinensis* I. BoI.) were collected at 17 sites in six provinces of China with majority from Yunnan Province, and were preserved in 100% ethanol prior to DNA extraction. Total genomic DNA was extracted from single specimens using a standard phenol-chloroform extraction with slight modifications. Extracted genomic DNA was stored at −20 ℃ until it was used as polymerase chain reaction (PCR) template. Published sequences from 33 species, including the other two outgroup species (*Filchnerella sunanensis and Haplotropis brunneriana*) obtained from GenBank were also included in our phylogenetic analyses besides the COI sequence of *Oxya hyla intricata* (Accession NO. AF385191). All samples information from this study and GenBank are not present in this paper, but available on request.

1.2 PCR and sequencing

Fragments of the mitochondrial genes COI, Cytb, and the complete sequence of COII gene were amplified by PCR, using established primer pairs[11-12] and reaction conditions.

PCR products were purified with DNA Gel purification kit (U-Gene). Direct sequencing of PCR products was performed by the ABI PRISM™ 3100-Avant Genetic Analyzer.

1.3 Sequence alignments and phylogenetic inferences

Sequences were trimmed by removing ambiguously resolved parts of the 3′ and 5′ ends and edited using the Contig1 software package (Cexpress. exe). DNA sequences were aligned using the program Clustal X with default values.

Uncorrected p-distances were calculated with MEGA 4.0. Gaps and missing data were excluded from each pairwise calculation. A maximum likelihood[13]

was carried out in PAUP * 4.0b10[14] using GTR + I + G model with parameter values as estimated by Modeltest 3.7[15]. PAUP searches consisted of TBR heuristic searches. The three data sets, COI, COII and Cytb, were analyzed separately and in combination of COI and Cytb. The robustness of the trees was tested using the bootstrap method. All bootstrap values are based on 100 replicates for COI and Cytb, 200 for combination of COI and Cytb, and 500 for COII.

Bayesian analyses were also conducted with MrBayes V3.1.2[16], using a GTR + I + G model. We used the default priors starting with random trees, and ran four Markov chains for 150 000 generations, sampled at intervals of 10 generations. To determine the point at which the Markov chains reached stationarity, the log-likelihood scores were plotted against generation time, to determine when the log-likelihood values stabilize. After burn-in samples were discarded, trees were combined in a single majority consensus topology, and the percentage of the nodes were taken as *a posteriori* probabilities[16].

2 Results

2.1 Nucleotide analyses

The PCR products of COI, COII, and Cytb are approx. 708, 684 and 763 bp, respectively for the Cantatopidae species examined. For the COI gene, the multiple sequence alignment (excluding outgroup) has 648 characters, of which 364 are constant, 279 variable, and 220 are parsimony-informative. For COII, multiple sequence alignments (excluding the outgroup but including sequences obtained from GenBank) had 585 characters, of which 257 are constant, 328 variable, and 293 are parsimony-informative. For Cytb, there are 711 characters excluding the outgroup but including GenBank sequences, of which 377 are constant, 332 variable, and 283 are parsimony-informative.

Average nucleotide composition among Cantatopidae species (excluding the outgroup but including GenBank sequences) is as follows: COI [31.6% (A), 34.2% (T), 15.9% (G), and 18.3% (C)]; COII [37.1% (A), 32.3% (T), 13.7% (G), and 16.9% (C)]; and Cytb [39.3% (A), 34.0% (T), 11.5% (G), and 15.2% (C)].

2.2 Uncorrected "p" distance analyses

The sequence divergence between *Longchuanacris fui* Niu *et* Zheng and *Longchuanacris xiaoheishanensis* Niu *et* Zheng, based on uncorrected "p" distance, was 0.027 across the COI fragment, and 0.021 across the Cytb fragment. Within *Caryanda*, mean COI and Cytb divergences were 0.032 and 0.030, respectively. The pairwise sequence divergence among *Caryanda* four species was 0.014 (*Caryanda pseudodentata* Niu *et* Zheng and *Caryanda bannaensis* Niu *et* Zheng) in COI, 0.006 in Cytb, 0.006 (*Caryanda ruiliensis* Niu and *Caryanda quadrata* Bi *et* Xia) in COI, 0.009 in Cytb, 0.040 (*Caryanda pseudodentata* Niu *et* Zheng and *Caryanda quadrata* Bi *et* Xia) and 0.041 (*Caryanda bannaensis* and *Caryanda quadrata* Bi *et* Xia) in Cytb. Mean pairwise divergence among three genera was 0.001 (*Pedopodisma* and *Podismodes*) for COI, 0.002 for Cytb, and 0.003 (*Sinopodisma* and *Podismodes*) for Cytb. Furthermore, the divergence values were also calculated for *Podismodes dabieshanensis* Niu *et* Zheng and *Yunnanacris yunnaneus* Ramme in COI (uncorrected "p" distance = 0.056); *Apalacris varicornema* Walker and *Apalacris eminifronta* Niu et Zheng in the COII gene (0.001).

2.3 Phylogenetic relationships inferred from COI, COII, and Cytb

Phylogenies derived using maximum likelihood and Bayesian analyses were generally congruent, and four typical trees are shown in the results (Fig. 1 - 4). Based on the COI gene sequence with *Atractomorpha sinensis* as the outgroup, the Bayesian tree (not present) showed an overall similar topology to the likelihood tree (Fig. 1). Three major clades within the two trees are apparent, the major differences being the position of *Traulia* two species (*Traulia szetschuanensis* and *Traulia minuta*) and Catantopinae three species (*Catantops pinguis pinguis*, *Stenocatantops splendens* and *Xenocatantops brachycerus*), which formed separate clades with likelihood. In the likelihood tree, one clade includes *Caryanda* six species and *Longchuanacris* two species with strong support. The second clade is composed of two sub-clades. These two sub-clades include *Yunnanacris yunnaneus*, *Podismodes dabieshanensis* and *Pedopodisma funiushana* in one sister group, and *Qinlingacris choui*, *Anapodisma miramae*

and *Prumna* three species in another. The third clade consists of *Epistaurus aberrans*, *Apalacris varicornema*, *Ecphanthacris mirabilis* and *Apalacris eminifronta*.

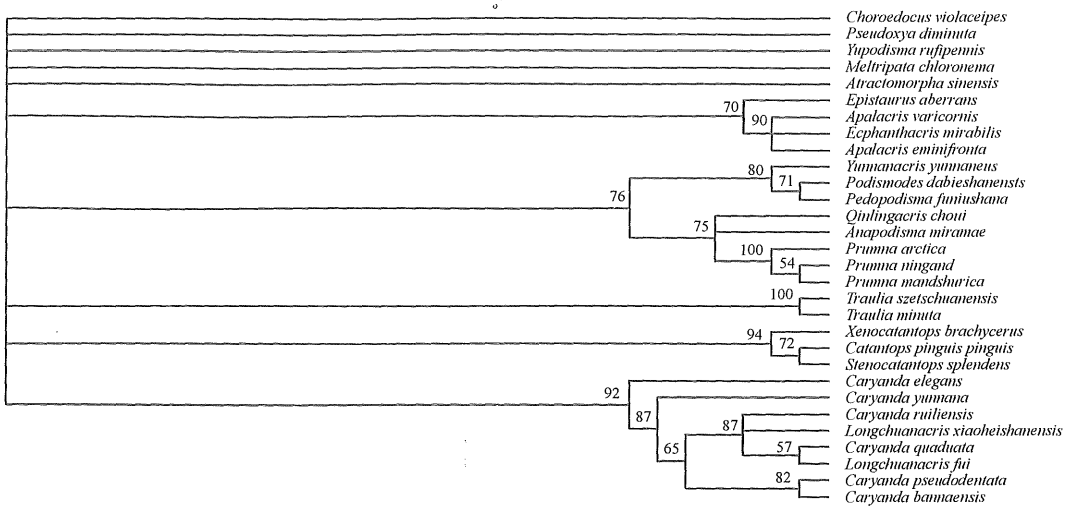

Fig. 1 Maximum likelihood tree of the COI gene for Chinese Catantopidae species. Numbers near nodes represent bootstrap support (100 replicates)

For the COII gene with *Filchnerella sunanensis* as the outgroup, the topology of the Bayesian tree (Fig. 2) was generally identical to that of the likelihood tree (not present), therefore, only Bayesian tree (Fig. 2) is shown in the results. Both analyses for COII support phylogenetic relationships including: ① Coptacrinae species formed a well supported monophyletic group (posterior probability 89%) with the exception of four species, which formed a separate Clade; ② Cyrtacanthacrinae was found sister to Calliptaminae, and both sister to Eyprepocnemidinae; ③ Catantopinae four species also formed a monophyletic group, and have a close relationship to Cyrtacanthacrinae, Calliptaminae and Eyprepocnemid-inae. These four subfamilies constituted a distinct clade; ④ Spathosterninae one species, Oxyinae two species and Caryandinae one species grouped together, and formed another one clade; ⑤ the same truth for Melanoplinae three species and Podisminae two species with highly support with posterior probabilities 100%. Our results support the relationships within the genus level, in agreement with the traditional view of morphological studies.

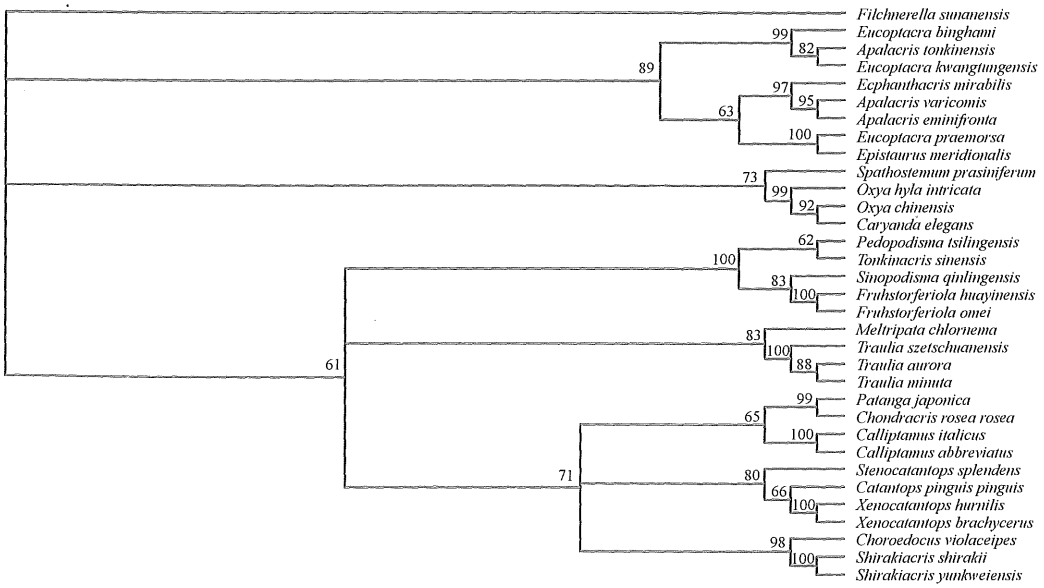

Fig. 2 Bayesian tree of the COII gene for Chinese Catantopidae species. Numbers near nodes represent Bayesian posterior probabilities

For the Cytb gene with *Haplotropis brunneriana* as the outgroup, the Bayesian tree (Fig. 3) showed essential congruence with the likelihood tree (available on request). There are two distinct clades in both trees. One clade includes *Caryanda* six species, *Longchuanacris* two species and Oxyinae three species with well support. Within this clade, *Caryanda* six species and *Longchuanacris* two species grouped together, and paraphyletic with respect to Oxyinae three species. The other clade mainly comprised Podisminae nine species and Melanoplinae three species plus *Spathosternum prasiniferum* and *Qinlingacris choui*. In both trees, Coptacrinae was never recovered as monophyly with *Traulia* always in uncertain position. The remaining taxa plus *Traulia* either composed a separate Clade in a comb-like arrangement (Fig. 3) or split direct from the backbone of the tree in a comb-like arrangement as well.

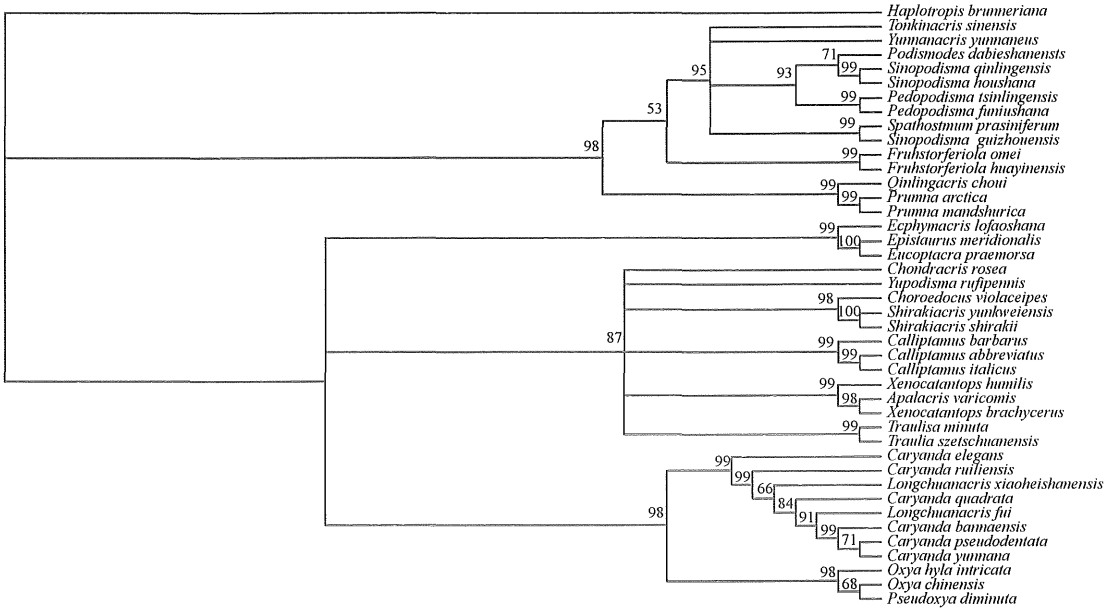

Fig. 3　Bayesian tree of the Cytb gene for Chinese Catantopidae species. Numbers near nodes represent Bayesian posterior probabilities

The combined nucleotide matrix of the COI and Cytb genes generates the two trees topology (only Bayesian tree is present, Fig. 4) similar to that obtained using Cytb DNA sequences. Two clades are suggested: one clade includes *Caryanda* six species, *Longchuanacris* two species and Oxyinae three species with well support, while the other is composed totally of Podisminae seven species except *Qinlingacris choui*, with *Yupodisma rufipennis* at the basal position. Both trees show that *Qinlingacris choui* grouped with *Prumna* two species, formed one sub-clade and *Pedopodisma funiushana* nested in *Podismodes*, constituted another, that *Caryanda* six species and *Longchuanacris* two species consistently grouped together.

3　Discussion

Sinopodisma and *Yunnanacris* are both considered synonyms of *Podismodes*[2]. The result of our investigation of Cytb gene sequence further supports such a classification, and additionally suggests *Pedopodisma* is also a possible synonym of *Podismodes* with Bayesian. However, synonymizing these two genera would be premature because of insufficient taxon and character sampling. *Pedopodisma* has been previously considered a synonyms of *Sinopodisma*[17], but according to Niu[2] *Pedopodisma* was still a separate valid genus with remarkable differences from *Sinopodisma* in forewing. Therefore, further investigations will be needed for addressing their relationship. The relationships inferred from Cytb Bayesian tree are ((((*Pedopodisma funiushana* + *P. tsinlingensis*) ((*Sinopodisma houshana* + *S. qinlingensis*) + *Podismodes dabieshanensis*)) + *S. guizhouensis*) + *Yunnanacris yunnaneus*).

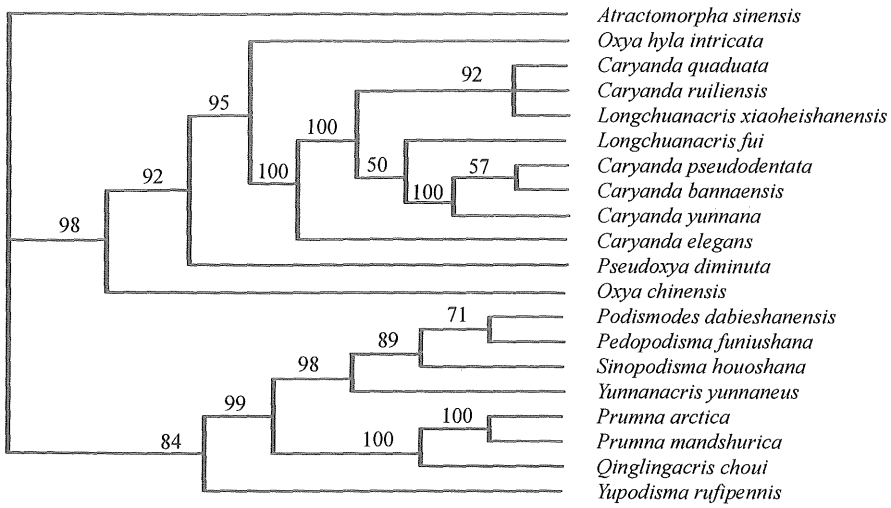

Fig. 4 Bayesian tree of the combination of COI and Cytb gene for Chinese Catantopidae species. Numbers near nodes represent Bayesian posterior probabilities

Sinopodisma houshana Huang and *Podismodes dabieshanensis* are two very similar morphospecies, differing only in the top of subgenital plate and furcula[2]. These two similar species are also identified by molecular data here (one individual Cytb gene sequence, and the combined COI and Cytb gene sequences) and DNA barcodes[18], which as species identifiers has been successfully implemented in the identification of previously described species, and discovery of new and cryptic species[5,8-9,19-21].

Traditionally the *Longchuanacris* was considered a genus within the subfamily Oxyinae[1], but it was recently redefined by Niu[2], who removed it from the Oxyinae and placed it in the subfamily Caryandinae. This revision is well supported by phylogenies reconstructed from Cytb gene sequence. Further, *Longchuanacris* also has shown close relationship with *Caryanda* in the individual genes analyses (COI and Cytb), and the combined analyses.

Caryanda was divided into two subgenera and further 14 species groups by Niu[2], who revised the genus and provided a phylogenetic analysis based on morphology. On the basis of his results, *C. ruiliensis* (*dehongensis*-species group), *C. quadrata* (*quadrata*-species group), *C. pseudodentata* and *C. bannaensis* (*dentate*-species group) in our examined belong to subgenus *Yuncaryanda*, and *C. yunnana* (*yunnana*-species group) and *C. neoelegans* (*neoelegans*-species group) subordinate within subgenus *Caryanda*. The species groups and subgenera of *Caryanda* defined by Niu[2] are in general well supported by our analyses (see Fig. 1). Both show the relationship are (((*C. ruiliensis* + *C. quadrata*) + (*C. pseudodentata* + *C. bannaensis*) + *C. yunnana*) + *C. neoelegans*).

Storozhenko[3] considered *Yupodisma* Zhang *et* Xia was a synonym of *Anapodisma* Dovnar-Zapolskii, and remove *Yupodisma rufipennis* to the genus *Anapodisma*, which supported by Niu[2], but contrary to our result. From the uncorrected "p" distance in COI and Phylogeny inferred from COI gene sequence, we propose that *Yupodisma* is still a separate genus within Podisminae.

Our results also reveal *Ecphanthacris* is close related to *Apalacris*, in agreement with Lu[22]. The molecular phylogeny of seven new species was consistant with recent morphological phylogeny by Niu[2].

In summary, our analyses show that the relationships of lower hierarchical levels are well resolved, and the three mtDNA genes are less useful for resolving deeper nodes, in agreement with general findings in insects[23-24]. So additional markers, especially nuclear markers which proved pivotal to solve the problem of the deeper nodes[25-27], will be required for establishing deeper phylogenetic relationships of Cantatopidae.

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