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Phylogenetic Relationships among 12 Species of Tetrigidae (Orthoptera: Tetrigoidea) Based on Partial Sequences of 12*S* and 16*S* Ribosomal RNA

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Abstract: Mitochondrial 12*S* and 16*S* ribosomal RNA genes sequences were sequenced using dye-labeled terminator on an ABI 377 automated sequencer in 11 individuals and 1 species' sequences were gained from GenBank, representing 6 genera of family Tetrigidae. The collated sequences were aligned using Clustal X version 1.81 and then, the sequence variability and heredity distances based on Kimura 2-parameter model were calculated using Mega 2.1. In obtained sequences (736 bp), the average A + T content is 73.9%, ranging from 71.2% to 77.5%; the overall G + C content is 26.1%, ranging from 22.5% to 28.8%. Based on alignment of the combined sequences, 185 parsimony-informative sites were revealed in 755 available base pairs. Phylogenetic trees were reconstructed using NJ, MP and ML methods with *Cylindraustralia kochii* as outgroup. The results indicated that the monophyletic nature of *Tetrix* is questioned and suggest that *T. tubercarina* may be given tribal rank. Our results also show that *Coptltettix huanjiangensis* and *C. gongshanensis* are the same species, *i.e. Coptltettix gongshanensis* Zheng, and *C. huanjiangensis* is the synonyms of *C. gongshanensis*.

Key words: Tetrigidae; Phylogeny; 12*S* rRNA gene; 16*S* rRNA gene

基于线粒体 12*S* 和 16*S* rRNA 基因部分序列探讨蚱科 12 种的系统发育关系

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摘要：用 ABI 377 自动测序仪测定了蚱科 5 属 11 个种的 12*S* 和 16*S* rRNA 基因部分序列，并从 GenBank 获得 1 属 1 种的同源序列；用 Clustal X 1.81 比较其同源性，用 Mega 2.1 计算序列变异性和遗传距离。在获得的 736 bp 序列中，A + T 含量为 71.2% ~ 77.5%，平均为 73.9%；G + C 含量为 22.5% ~ 28.8%，平均为 26.1%。经 Clustal X1.81 软件比对，共得到 755 个位点，其中简约信息位点 185 个。以 *Cylindraustralia kochii* 为外群，构建 NJ、MP 和 ML 分子系统树，结果表明：(1) 蚱属并非一个单系群，而是一个并系群；(2) 环江柯蚱 *Coptltettix huanjiangensis* 和贡山柯蚱 *C. gongshanensis* 为同一个种，即贡山柯蚱，而环江柯蚱是贡山柯蚱的同物异名。

关键词：蚱；系统发生；12*S* rRNA 基因；16*S* rRNA 基因
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The Tetrigoidea comprises eight families (Liang & Zheng, 1998). The pronotum is greatly extended. The elytra are reduced to small scales, but the wings are usually functional. The anterior edge of the

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prosternum forms a collar around the mouthparts, and the tarsi of the fore and middle legs have only two segments. The male genitalia are entirely membranous and concealed under paired chitinous plates, absent from other superfamilies of Caelifera of Orthoptera. The Tetrigoidea has been regarded as one of the oldest groups in Caelifera and more related to Tridactyloidea (Flook & Rowell, 1997).

The reported studies were concentrated on the descriptions of new species and morphological taxonomy (Liang & Zheng, 1998; Jiang & Zheng, 1998; Zheng et al, 2000; Zheng & Li, 2001; Zheng & Jiang, 2002). Due to the subtlety and insufficient information from morphological data, the phylogenetic relationship in Tetrigoidea is controversial. The molecular technique has been extensively applied to systematics. Unfortunately, the studies on Tetrigoidea using molecular technique are few (Lu et al, 2002; Jiang et al, 2002). The 12*S* and 16*S* ribosomal genes appear to be promising markers that have been useful in estimating relationships in genus and family levels of divergence (Yin et al, 2003; Hwang et al, 1999; Marco et al, 2004). The purpose of the present study is to discuss the utility of 12*S* and 16*S* genes in phylogenetic reconstruction at the levels of genus.

1 Materials and Methods

1.1 Materials

The 12 species from 6 genera were analyzed in this paper (Table 1). All specimens were preserved in 100% ethanol and stored at −20 ℃.

1.2 DNA preparation

Total genomic DNA was extracted from hind femora using a simple proteinase K/SDS method. Before incubation the samples were marinated in ddH_2O for 2 days. Scissored tissue was re-suspended in 4 mL 0.01 mol/L Tris (pH 8.0), 0.1 mol/L EDTA (pH 8.0), 0.05 mol/L NaCl, 1% SDS, 10 μL Proteinase K and incubated at 52 ℃ for 12 − 16 h. The digested samples were phenol-extracted, ethanol-precipitated once more, and redissolved in 10 mmol/L Tris-HCl, pH 8.0. All DNA samples were stored at 4 ℃.

1.3 DNA amplification

Two mitochondrial DNA fragments (portions of 12*S* rDNA, 16*S* rDNA) were amplified from the same individual. Standard insect mtDNA primers (Simon et al, 1994) SR-J-14233 5′-AAGAGCGACGGGCGATGTGT-3′ and SR-N-14588 5′-AAACTAGGATTAGATACCCTATTAT-3′ for the 12*S* rDNA fragment; and LR-J-12887 5′-CCGGTCTGAACTCAGATCACGT-3′ and LR-N-13398 5′-CGCCTGTTTAACAAAAACAT-3′ for 16*S* rDNA fragment. PCR reactions were carried out in 30 μL volumes containing 10 × reaction buffer 3 μL, 25 mmol/L $MgCl_2$ 2 μL, 2 mmol/L dNTPs 2 μL, primers 10 μmol/L per 1 μL, ddH_2O 19.8 μL, 1 U Tag DNA Polymerase, template 1 μL

Table 1 Species, localities and number of 12 Tetrigidae and *C. kochii* used in the present study

	Genus and Species	Collecting locality	Accession number (12*S*/16*S*)
	Teredorus		
1	*Ter. carmichaeli*	Shangsi, Guangxi	AY590154/AY590165
2	*Ter. prominemarginis*	Fulong, Guangxi	AY590155/AY590166
	Coptotettix		
3	*C. huanjiangensis*	Fangcheng, Guangxi	AY590156/AY590167
4	*C. gongshanensis*	Fangcheng, Guangxi	AY590157/AY590175
	Tetrix		
5	*T. bolivari*	Tianlin, Guangxi	AY590158/AY590168
6	*T. japonica*	Tianlin, Guangxi	AY590159/AY589169
7	*T. tubercarina*	Tianlin, Guangxi	AY590160/AY590170
8	*T. subulata*	Tianlin, Guangxi	AY590161/AY590171
	Formosatettix		
9	*F. yuanbaoshanensis*	Jiuwanshan, Guangxi	AY590162/AY590172
	Euparatettix		
10	*E. bimaculatus*	Fangcheng, Guangxi	AY590163/AY590173
11	*E. variabilis*	Shangsi, Guangxi	AY590164/AY590174
	Paratettix		
12	*P. cucullatus*		Z93273/Z93311*
	Cylindraustralia		
13	*C. kochii*		Z93277/Z93315*

* Sequences obtained from Genbank.

(containing DNA 20 – 50 ng). Amplification were performed under the following conditions: an initial denaturation step at 94 ℃ for 4 min; 30 cycles of 30 s 94 ℃, 40 s 49 ℃, 30 s 72 ℃; and a final extension step at 72 ℃ for 7 min.

1.4 Purification of PCR products and sequencing

Amplification products were examined on 1.5% agarose gels and purified using PCR clean-up kit or DNA gel extraction kit according to conditions and then sequenced using Dye-labeled terminator on an ABI 377 automated sequencer.

New sequences were deposited in GenBank under accession numbers from AY590154 to AY590175 (Table 1). Sequences were collated manually and ambiguous parts were deleted. The collated sequences were aligned using Clustal X version 1.81 (Thompson et al, 1997) and then, the sequence variability and heredity distances based on Kimura 2-parameter model were calculated using Mega 2.1 (Kumar et al, 2001).

1.5 Phylogenetic analysis

Prior to phylogenetic reconstruction, nucleotide compositions were analyzed for the two data sets; the results showed the trend of divergence between the two genes is similar. The lengths of the parsimonious trees for the small-subunit (SSU), large-subunit (LSU) and combined data sets were 249, 284 and 538 respectively (consistency indices = 0.7068, 0.8028 and 0.7509). These results indicated that combination of the data was justifiable and we therefore based subsequent analyses on a single alignment of the two sequences.

Three different methods of phylogenetic analysis were performed. First, we obtained NJ tree based on Kimura 2-parameter model using Mega 2.1 (Kumar et al, 2001). Second, maximum parsimony (MP) tree was reconstructed using PAUP* 4.0 b10 (Swofford, 2001). Heuristic searches were performed using 100 replicates of random addition sequences and the tree-bisection-reconnection (TBR) option for branch swapping. Each base was treated as unordered character with equal weight, and gaps were treated as missing data. Third, maximum likelihood (ML) method was employed and ML tree was reconstructed under the HKY85 model, using base frequencies estimated by PAUP and the default number of substitution types, HKY85 variant) as well as transition/transversion ratio 2. Heuristic searches were used with 10 replicates of random addition sequences and TBR branch swapping. The confidence values of all trees were evaluated by bootstrap analysis with 1 000 replicates.

2 Results and Discussion

2.1 Nucleotide composition

The lengths of 12*S*, 16*S* sequences are about 306 bp and 430 bp respectively, 736 bp in total. The nucleotide compositions of the sequences are similar, and have the high A + T content both in 12*S* and 16*S* sequences. The average A + T content is 73.9%, ranging from 71.2% to 77.5%. The overall G + C content is 26.1%, ranging from 22.5% to 28.8%. Based on alignment of the sequences, 185 parsimony-informative sites were revealed in 755 available base pairs. The substitutions (transition + transversion) among the species (except outgroup) ranged from 0 to 15.82%, with an average of 11.94%. The differentiation of the sequences ranged from 0 to 2.15% (except *T. tubercarina*) at the interspecific level, which was much lower than at the intergeneric level (from 5.69% to 15.82%).

2.2 Phylogenetic relationships

The resulting MP tree is presented in Fig.1. With

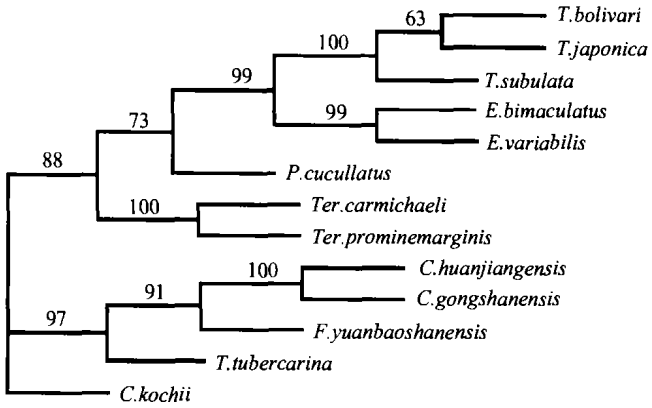

Fig.1 Phylogenetic tree reconstructed using MP methods with *C. kochii* as outgroup
Numbers above branches are confidence values (%)

confidence values obtained from the identical analysis on each branches, and those reconstructed with NJ and ML analysis have the same branching order in the main groups (the trees were not presented here).

From all trees, the species referred here can be divided into two groups: *C. huanjiangensis* and *C. gongshanensis* first clustered with *F. yuanbaoshanensis*, then with *T. tubercarina* to form the first group branching from the base of tree. *T. bolivari*, *T. japonica* and *T. subulata* clustered with *E. bimaculatus* and *E. variabilis* firstly, then with *P. cucullatus*, at last with *Ter. carmichaeli* and *Ter. prominemarginis* to form the second group. It is indicated the reasonable phylogenetic relationships of the 6 genera was: ((*Formosatettix* + *Coptotettix*) + (*Teredorus* + (*Paratettix* + (*Euparatettix* + *Tetrix*)))). But this was not well supported by morphological characters. *Euparatettix* and mains of *Tetrix* clustered together on the top of the trees with a confidence value of 94 - 100 percent. According to morphological characters, there were so much difference between the two genera (Zheng & Jiang, 2001; Jiang & Zheng, 1998) and they should not cluster together first.

The relationship among *Formosatettix*, *Coptotettix*, *Teredorus*, and *Euparatettix* has ever been researched based on RAPD (Lu et al, 2002), which suggested that *Formosatettix* and *Teredorus* were more related. Our results are different from theirs. We thought genus *Formosatettix* was more related to *Coptotettix*, and *Teredorus* to *Euparatettix*. It is suggested that *T. japonica* was a relatively primitive species in genus *Tetrix* and other species of *Tetrix* originated from it (Jiang et al, 2002), but our results did not support this opinion.

2.3 Toxonomic position of *Tetrix tubercarina*

Among the genus of *Tetrix* our results showed strong support for the paraphyletic nature of this group. *T. tubercarina* was not clustered firstly with other species of *Tetrix*, but with genera *Formosatettix* and *Coptotettix*. The *Tetrix* can be roughly divided into two clades, one consisting of *T. bolivari* + *T. japonica* + *T. subulata*, the other of *T. tubercarina*. The distances between *T. tubercarina* and other species of *Tetrix* were 0.175 - 0.180 but 0.006 - 0.008 between other species of *Tetrix* each other, which confirmed our results further (Table 2). The morphological characters were also support our results (Zheng, 1998; Zheng & Xie, 2000; Zheng et al, 2000). So we consider that *T. tubercarina* may be given tribal rank when more evidence is available to support this branch in future.

In this study, we reconstructed the phylogeny of the 6 genera using sequences of 12S and 16S ribosomal genes and supported with high confidence values (though there were conflicts with morphological characters). We considered that 12S and 16S ribosomal genes were promising markers in reconstructing the phylogeny on genus level in family Tetrigidae.

2.4 Toxonomic position of *Coptotettix huanjiangensis*

In genus *Coptotettix*, we found the 12S and 16S sequences gained from *C. huanjiangensis* and *C. gongshanensis* are identical. From morphological characters, *C. huanjinangensis* is allied to *C. gongsh-*

Table 2 Mitochondrial 12S and 16S rRNA genes sequence variations in 12 species (the ratio of transitions/transversions and distances are shown above and below the diagonal, respectively)

	1	2	3	4	5	6	7	8	9	10	11	12	13
1 *Ter. carmichaeli*		2.000	0.676	0.676	0.478	0.470	0.667	0.470	0.506	0.541	0.565	0.516	0.413
2 *Ter. prominemarginis*	0.004		0.685	0.685	0.435	0.435	0.648	0.426	0.494	0.492	0.516	0.455	0.396
3 *C. huanjiangensis*	0.174	0.180		n/c	0.623	0.623	1.243	0.590	1.700	0.739	0.714	0.627	0.589
4 *C. gongshanensis*	0.174	0.180	0.000		0.623	0.623	1.243	0.590	1.700	0.739	0.714	0.627	0.589
5 *T. bolivari*	0.141	0.141	0.183	0.183		4.000	0.537	5.000	0.580	1.300	1.143	0.439	0.508
6 *T. japonica*	0.138	0.138	0.183	0.183	0.006		0.519	n/c	0.588	1.190	1.091	0.464	0.528
7 *T. tubercarina*	0.167	0.170	0.117	0.117	0.180	0.175		0.519	1.068	0.622	0.680	0.653	0.580
8 *T. subulata*	0.138	0.138	0.182	0.182	0.008	0.008	0.175		0.588	1.238	1.091	0.464	0.528
9 *F. yuanbaoshanensis*	0.183	0.187	0.115	0.115	0.188	0.187	0.129	0.187		0.667	0.618	0.588	0.624
10 *E. bimaculatus*	0.134	0.134	0.176	0.176	0.063	0.062	0.181	0.064	0.184		4.667	0.646	0.552
11 *E. variabilis*	0.138	0.138	0.176	0.176	0.061	0.062	0.186	0.062	0.181	0.023		0.660	0.548
12 *P. cucullatus*	0.143	0.141	0.186	0.186	0.119	0.119	0.181	0.119	0.195	0.115	0.113		0.459
13 *C. kochii*	0.332	0.329	0.355	0.355	0.320	0.322	0.318	0.322	0.350	0.330	0.332	0.336	

n/c representing denominator is zero.

anensis, but different from *C. gongshanensis* in: 1) the width of vertex wider than one eye; 2) hind process of pronotum not reaching the knee of hind femur; 3) median carina of pronotum interrupted in the posterior part; 4) posterior angles of the lateral lobes of the pronotum round or obliquely truncated; 5) frontal ridge distinctly sinuate at the median ocellus (Zheng & Jiang, 1994). But the differences between the two are so small that we consider them the same species, *i.e.* *C. gongshanensis* Zheng = *C. huanjiangensis* Zheng & Jiang, and *C. huanjiangensis* is the synonyms of *C. gongshanensis*.

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