The comparative mitogenomics and phylogenetics of the two grouse-grasshoppers (Insecta, Orthoptera, Tettigoidea)

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Abstract

Objective: This study aimed to reveal the mitochondrial genomes (mtgenomes) of Tetrix japonica and Alulatettix yunnanensis, and the phylogenetics of Orthoptera species.

Methods: The mtgenomes of A. yunnanensis and T. japonica were firstly sequenced and assembled through partial sequences amplification, and then the genome organization and gene arrangement were analyzed. Based on nucleotide/amino acid sequences of 13 protein-coding genes and whole mtgenomes, phylogenetic trees were established on 37 Orthoptera species and 5 outgroups, respectively.

Results: Except for a regulation region (A+T rich region), a total of 37 genes were found in mtgenomes of T. japonica and A. yunnanensis, including 13 protein-coding genes, 2 ribosomal RNA genes, and 22 transfer RNA genes, which exhibited similar characters with other Orthoptera species. Phylogenetic tree based on 13 concatenated protein-coding nucleotide sequences were considered to be more suitable for phylogenetic reconstruction of Orthoptera species than amino acid sequences and mtgenomes. The phylogenetic relationships of Caelifera species were Acridoidea and Pamphagoidea > Pygromorphoidea > Pneumoroidea > Eumastacoidea > Tettigoidea > Tridactyloidea. Besides, a sister-group relationship between Tettigonioidae and Rhaphidophoroidea was revealed in Ensifera.

Conclusion: Concatenated protein-coding nucleotide sequences of 13 genes were suitable for reconstruction of phylogenetic relationship in orthopteroid species. Tridactyloidea was a sister group of Tettigoidea in Caelifera, and Rhaphidophoroidea was a sister group of Tettigonioidae in Ensifera.

Keywords: Mitogenome, Orthoptera, Tettigoidea, Phylogenetic

Introduction

Mitochondrial genome (mtgenome) is a kind of small circular molecule in most of metazoans, which evolves semi-independently from nuclear genomes and plays an important role in the process of metabolism, programmed cell death, illness, and aging. Generally, the closed circular mtDNA was 14–39 kb in length, which consists of a major non-coding region (regulation region, A+T rich region) and a canonical set of 37 genes, including 13 protein-coding genes, 2 ribosomal RNAs (rRNA) and 22 transfer RNAs (tRNA). The distribution of these genes is always compact with infrequent introns and intergenic space [1, 2]. As low frequency of intermolecular genetic recombination and relatively rapid evolutionary rate, mtgenome has been extensively used for researching on population structures, phylogeography and phylogenetic relationships at various taxonomic levels [3, 4].

Recently, mtgenome has been widely used in phylogenetic analyses. It has been reported, mtgenomes could
provide rich information's in phylogenetics [5]. Phylogenetic analyses based on complete mtgenome sequences could improve the statistical confidence of inferred phylogenetic trees with better resolution than analyses only based on partial mtgenes [6]. The evolution of mtgenomes, instead of mtgenes, was a new instrument for studying biological speciation and lineage divergence [7]. In addition, mtgenome may partly represent the whole genome, and be used as a phylogenetic marker in investigation of structural genomic features easily and systematically [8]. All these features of mtgenome greatly promoted the researches on evolutionary trends and relationships of phylogenetically distant organisms [9].

With the growing interest in mtgenomes, a rapid increase of published complete mtgenome sequences was revealed [10]. Despite insects were the most species-rich class animals, the sequenced mtgenomes are majorly vertebrates. Until now, more than 8634 complete metazoan mtgenomes have been sequenced, and only 337 are from insects and 39 are from Orthoptera (http://www.ncbi.nlm.nih.gov). Besides, two mtgenomes of Tetrigoidea were announced by our previous studies [10]. Orthoptera is a kind of primitive hemimetabolous insects, contains approximately 20,000 described species in two suborders of equal size (Caelifera and Ensifera) [11]. A preliminary phylogenetic analyses of Orthoptera based on the mtgenome data have been performed, while the superfamily Tettigoidea was not involved. Tettigoidea is a moderately diverse group of basal Caelifera comprising approximately 1400 species in 8 families and 270 genera [12]. As a monophyletic group supported by molecular data, Tettigoidea was regarded as one of the oldest groups in Caelifera, which closely related to Tridactyloidea [13, 14]. Researches on the mtgenome sequences of Tettigoidea may contribute to the revelation of phylogenetic relationships in Orthoptera. In this study, the mtgenomes of two Tettigoidea species, A. yunnanensis and T. japonica were firstly revealed, and the genome organization and gene arrangement were then analyzed. Meanwhile, phylogenetic trees were established to evaluate the phylogenetics of Orthoptera species. Our findings may enrich our knowledge on mtgenomes of Tettigoidea, and provide an efficient strategy for biodiversity exploring on Orthoptera species.

Materials and methods
Samples and DNA extraction
Specimens of A. yunnanensis and T. japonica were collected from a public land (not a protected area or a national park) in Nanjing, Jiangsu, China. Total genomic DNA was extracted from the femoral muscle of fresh specimens by the standard proteinase K and phenol/chloroform extraction method. Simply, the tissues were firstly disintegrated with 20 mg/ml proteinase K (Gentbase Gene-Tech Co., Ltd) at 37 °C for 2–3 h. Then, the samples were incubated with extraction solution, and V/2 of phenol and V/2 of chloroform was added. After centrifugation, the supernatant was obtained, and 1/10 volume of 3 M NaOAc and 2 volumes of 100% ethanol were used to precipitate the DNA. Finally, the precipitate (DNA) was dissolved in Tris–EDTA buffer solution, and quantified with spectrophotometer. The isolated DNA samples were stored at −20 °C and used as a template for subsequence PCR reactions.

 Primer design and PCR amplification
Some partial sequences were firstly amplified and sequenced using general primers based on Simon et al. [15]. Then, new primers were designed based on determined sequences, and each amplified segments could overlap the adjacent segments (Primers were shown in Table 1). The fragments of mtgenomes were amplified by PCR using Takara LA Taq™ (Takara Bio, Otsu, Shiga, Japan). The PCR program included an initial denaturation at 94 °C for 3 min, followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 52–59 °C to 0.3 °C/ cycle (depending on primer combinations) for 30 s, elongation at 68 °C for 60–180 s (depending on putative length of the fragments); then followed by another PCR program included 20 cycle of 30 s denaturation at 94 °C, 30 s annealing at 49–56 °C, 60–180 s elongation at 68 °C and a final extension at 68 °C for 8 min. The PCR products were identified by electrophoresis on 1% agarose gel.

Sequencing and sequence assembly
The PCR products with single band were purified using a V-gen PCR clean-up purification kit. If more than one band was present, the appropriately sized PCR product was cut off from the gel and purified using a biospin gel extraction kit. All fragments were sequenced in both directions, and some PCR products were sequenced by primer walking strategy. The identified sequences were assembled by seqman (DNASTAR 2001), BioEdit and Chromas 2.22, and then the complete mtgenome sequences of T. japonica and A. yunnanensis were manually checked. The coverage of each mtgenome was above two times.

Sequence analysis
Gene encoding proteins, rRNA and tRNA were identified according to their amino acid translation or secondary structure features, respectively. Individual gene sequences were compared with the available homologous sequences of Orthoptera species in GenBank. A total of 22 tRNA genes were identified using software tRNA Scan-SE 1.21 (http://lowelab.ucsc.edu/tRNAscan-SE) and their cloverleaf secondary structures and anticodon
sequences were identified using DNASIS (Ver.2.5, Hitachi Software Engineering).

**The reconstruction of phylogenetic trees**
In order to evaluate the phylogenetic relationships in *Orthoptera*, phylogenetic trees were established based on nucleotide/amino acid sequences of 13 protein-coding genes and whole mtgenome sequences of 37 *Orthoptera* species whose complete mtgenome sequences were available in GenBank by using two *Blattaria* species (*Periplaneta fuliginosa* and *Eupolyphaga sinensis*), two Isoptera species (*Reticulitermes flavipes* and *Coptotermes formosanus*) and one Mantodea specie (*Tamolanica tamolana*) as outgroup [6]. Mtgenome sequences were downloaded from GenBank (Table 2).

**Alignments and bayesian analyses**
The nucleotide and amino acid sequences were aligned by ClusterW in MEGA 4.0 with manual refinements [16]. One alignment was based on the complete mtDNA sequences, except for the highly variable ETAS (extended termination associated sequence) domain within regulation region, creating a sequence of 15,612 nt positions. The second alignment was based on the complete set of codons (except stop codons) creating a concatenated sequence of 10,989 nt positions (3663 amino acid positions) corresponding to the 13 protein-coding genes.

Bayesian analyses were performed by MRBAYES 3.1.2, with gaps treated as missing data [10]. The best fitting substitution model judged by Akaike information criterion (AIC) was determined by MrMODELTEST 2.3 [17]. For each BI analysis, two independent sets of monte carlo markov chains (MCMC) were run, each with one cold and three heated chains for $1 \times 10^6$ generations, and every 1000 generations were sampled. The burn-in parameter was estimated by plotting-lnL against the generation number using TRACER v1.4.1, and the retained trees were used to estimate the consensus tree and Bayesian posterior probabilities [18].

**Results**
**Genome organization and gene arrangement**
By sequencing and sequence assembly, a total of 37 genes were found in mtgenomes of *T. japonica* and *A.
Table 2: A total of 37 Orthoptera species were used in reconstruction of phylogenetic trees. Two Blattaria species, two Isoptera species and one Mantodea species were considered as outgroup.

| Taxa                | Species                        | Accession  |
|---------------------|--------------------------------|------------|
| Caelifera/Tetrigioidea | Tetrix japonica                | JQ272702   |
|                     | Alulatettix yunnanensis        | EF513373   |
|                     | Acridacircum cirolimbata       | JX835120   |
|                     | Acridiella versicolor          | JQ982750   |
| Caelifera/Acridoidea | Gryllus campestris             | EF513373   |
|                     | Orthoptera saundersi           | EF513373   |
|                     | Orthoptera arenicola           | EF513373   |
| Caelifera/Ensifera   | Ensifera versicolor            | EF513373   |
|                     | Ensifera apicata               | EF513373   |
|                     | Ensifera burchelli             | EF513373   |
|                     | Ensifera caniculari            | EF513373   |
|                     | Ensifera dubia                 | EF513373   |
|                     | Ensifera fuscata               | EF513373   |
|                     | Ensifera lineatipes            | EF513373   |
|                     | Ensifera monticola             | EF513373   |
|                     | Ensifera sexilimbata           | EF513373   |

The nucleotide composition of these two mitogenomes (T. japonica and A. yunnanensis) biased toward adenine and thymine (75.57% in T. japonica and 75.24% in A. yunnanensis). ATN was the preferred initiation codon of 13 protein-coding genes in T. japonica and A. yunnanensis, including 8 ATG, 3 ATA, 1 TAC and 1 ATT. TAA and TAG were considered to be the termination codons of these 13 protein-coding genes in T. japonica and A. yunnanensis, except one T of nad5 gene in A. yunnanensis (Table 3). Besides, the A+T-rich regions of the two mtgenomes were also located between small rRNA and tRNA genes, which were considered to be the termination codons of these 13 protein-coding genes in T. japonica and A. yunnanensis, except one T of nad5 gene in A. yunnanensis (Table 3). Besides, the A+T-rich regions of the two mtgenomes were also located between small rRNA and tRNA genes, which were considered to be the termination codons of these 13 protein-coding genes in T. japonica and A. yunnanensis, except one T of nad5 gene in A. yunnanensis (Table 3).

Phylogenetic analyses

Based on 13 concatenated protein-coding nucleotide sequences, the topology of established phylogenetic tree was similar with the reconstructed tree based on the whole mtgenome sequences. Differently, Teleogryllus emma of Gryllidae was revealed to be basal to all other Orthoptera species in phylogenetic tree of protein-coding nucleotide sequences, which was conflicted with the monophyletic Gryllidae in phylogenetic tree of mtgenome (Fig. 1a, c). In phylogenetic tree based on amino acid, Thrinclus schrenkii was found to belong to Pamphagoidea among various species of Acridoidea, which was also not consistent with the monophyletism of Acridoidea (Fig. 1b). According to the 37 Orthoptera species, 13 concatenated protein-coding DNA sequences were suspected to be accurate and effective for phylogenetic reconstruction of Orthoptera species.

As shown in Fig. 1a, two Orthopteran suborders, Caelifera and Ensifera were both recovered as monophyletic
In Caelifera branch, Acridoidea, Pyrgomorphoidea and Tettigioidea were monophyletic groups. The phylogenetic relationships of these superfamilies were Acridoidea and Pamphagoidea > Pygromorphoidea > Pneumoroidia > Eumastacoida > Tettigioidea > Tridactyloidea. In Ensifera, a sister-group relationship between Tettigonioida and Rhaphidophoroidea was revealed.

### Discussion

According to our previous studies, the mtgenomes of *T. japonica* (15,128 bp) and *A. yunnanensis* (15,104 bp) were circular molecules (GenBank accession numbers: JQ340002 and JQ272702) [19, 20]. In this study, a total of 37 typical genes and a regulation region were found in the mtgenomes of *T. japonica* and *A. yunnanensis*, which

| Feature | Strand | Position | Initiation codon/Stop codon | Anticodon |
|---------|--------|----------|-----------------------------|-----------|
| trnI    | J      | 1–64     | ATG/TAA ATG/TAA             | GAT       |
| trnQ    | N      | 65–133   | ATG/TAA ATG/TAA             | TGG       |
| trnM    | J      | 134–201  | ATG/TAA ATG/TAA             | CAT       |
| nad2    | J      | 201–1202 | ATG/TAA ATG/TAA             | TCA       |
| trnW    | J      | 1201–1266| ATG/TAA ATG/TAA             | GCA       |
| trmC    | N      | 1259–1324| ATG/TAA ATG/TAA             | GTA       |
| trmY    | N      | 1325–1388| ATG/TAA ATG/TAA             | TAA       |
| COI     | J      | 1386–2924| ATG/TAA ATG/TAA             | TGG       |
| mlu(UUR)| J      | 2920–2983| ATG/TAA ATG/TAA             | GTT       |
| COII    | J      | 2984–3667| ATG/TAA ATG/TAA             | GCT       |
| trnD    | J      | 3666–3729| ATG/TAA ATG/TAA             | TCA       |
| trmK    | J      | 3730–3797| ATG/TAA ATG/TAA             | TCC       |
| atp8    | J      | 3802–3957| ATG/TAA ATG/TAA             | TGG       |
| atp6    | J      | 3951–4622| ATG/TAA ATG/TAA             | GTT       |
| COIII   | J      | 4625–5428| ATG/TAA ATG/TAA             | GCT       |
| tmG     | J      | 5412–5474| ATG/TAA ATG/TAA             | TCC       |
| nad3    | J      | 5472–5828| ATG/TAA ATG/TAA             | TGG       |
| tmA     | J      | 5827–5891| ATG/TAA ATG/TAA             | TGG       |
| tmN     | J      | 5950–6013| ATG/TAA ATG/TAA             | TGG       |
| tmS     | J      | 6013–6081| ATG/TAA ATG/TAA             | TGG       |
| tmE     | J      | 6081–6144| ATG/TAA ATG/TAA             | TGG       |
| tmF     | N      | 6143–6205| ATG/TAA ATG/TAA             | TGG       |
| nadS    | N      | 6207–7922| ATG/TAA ATG/TAA             | TGG       |
| tmH     | J      | 7926–7989| ATG/TAA ATG/TAA             | TGG       |
| nadD    | N      | 7989–9314| ATG/TAA ATG/TAA             | TGG       |
| nad4L   | N      | 9308–9598| ATG/TAA ATG/TAA             | TGG       |
| tmT     | J      | 9601–9666| ATG/TAA ATG/TAA             | TGG       |
| tmP     | N      | 9667–9730| ATG/TAA ATG/TAA             | TGG       |
| nad6    | J      | 9732–10,226| ATG/TAA ATG/TAA            | TGG       |
| cob     | J      | 10,226–11,362| ATG/TAA ATG/TAA         | TGG       |
| tmI(UCN)| J      | 11,361–11,428| ATG/TAA ATG/TAA     | TGG       |
| nad1    | N      | 11,441–12,385| ATG/TAA ATG/TAA   | TGG       |
| trmL    | N      | 12,380–12,442| ATG/TAA ATG/TAA | TGG       |
| 16S     | N      | 12,443–13,739| ATG/TAA ATG/TAA | TGG       |
| trnV    | N      | 13,741–13,811| ATG/TAA ATG/TAA | TGG       |
| 12S     | N      | 13,812–14,597| ATG/TAA ATG/TAA | TGG       |

*J* represents sense strand, *N* represents antisense strand.

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Table 3 Annotation of the mitochondrial genomes in *Tetrix japonica* (Tj) and *Alulatettix yunnanensis* (Ay)
exhibited similar gene order and orientation with other Orthopteran insects. The conserved mtgenome structure in divergent insects identified their close genetic relationships [10]. In addition, the main nucleotide composition of these two mtgenomes was revealed to be adenine and thymine (75.57% of T. japonica and 75.24% of A. yunnanensis). Although the nucleotide composition was slightly lower than that found in some other Orthoptera insects (Locusta migratoria 75.3%, Oxya chinensis 75.9% and Acrida willemsi 76.2%), it was still corresponded well to the normal range of insect mtgenomes from 69.2% to 84.9% [10]. These data should be useful for developing mtgenome genetic markers for species identification of Orthoptera insects.

In mtgenomes of T. japonica and A. yunnanensis, 22 tRNA genes were identified in the same relative genomic positions as observed in other Orthoptera insects. The typical cloverleaf secondary structures and anticodons of these tRNAs were also similar to those found in other metazoan animals. As the only major non-coding region in insect mtgenome, the regulation region (A+T rich region) biased on A+T nucleotides were evolved under a strong directional mutation pressure [21]. It has been reported the A+T rich region was varied greatly in insects, from 70 bp in Ruspilia dubia to 4601 bp in Drosophila melanogaster [22, 23]. In this study, A+T rich regions in 531 bp length with 82.67% A+T and 460 bp length with 80.87% A+T located between small rRNA and tRNA^the were revealed in T. japonica and A. yunnanensis, respectively. This region may limit its use for both inter- and intra-specific analyses in evolutionary studies.

In phylogenetic analyses, a similar topology of the established phylogenetic trees based on the whole mtgenome sequences and concatenated protein-coding nucleotide sequences were revealed. However, Teleogryllus emma of Gryllidae basal to all other Orthoptera species based on nucleotide sequences was conflict with the monophyletic Gryllidae based on mtgenome sequences. This phenomenon may be explained by that the mitochondrial non-protein-coding sequences of Orthoptera species, such as tRNA genes with nucleotide conservation were different from protein-coding sequences with relatively fast evolutionary rate, thereby disturbing phylogenetic reconstruction [24]. In addition, the phylogenetic tree based on amino acid showed that Thrinchus schrenkii of Pamphagoidea was nested within Acridoidea, which was conflicted with the monophyletism of Acridoidea. As amino acid sequences were usually conserved due to invisible synonymous substitutions in amino acid level, nucleotide sequences may be more reliable for phylogenetic reconstruction of closely related Acridoidea species [25]. These results of phylogenetic trees in 37 Orthopteran species indicated that the best way for phylogenetic reconstruction of Orthoptera was based on the concatenated protein-coding nucleotide sequences, but not the amino acid sequences and entire mtgenomes. As shown in phylogenetic trees based on concatenated protein-coding nucleotide sequences, two Orthopteran suborders, Caelifera and Ensifera, were both recovered as monophyletic groups, which were consisted with previous studies of morphological and molecular data [5]. The phylogenetic relationships of the superfamilies in Caelifera also supported previous results of Flook and Rowell [13]. Besides, a sister group relationship between Tettigonioidae and Rhaphidophoroidea was revealed in Ensifera, which was also consist with the results presented by

![Fig. 1 Phylogenetic tree established by concatenated protein-coding DNA sequences (N = 13) a, concatenated amino acids b, and whole mtgenome sequences c of 37 Orthopteran species and 5 outgroups (two Blattaria species, two Isoptera specie and one Mantodea specie). The red underline is the species position of Alulatettix yunnanensis and Tettix japonica.](image-url)
Fenn et al. [5] and Zhou et al. [26], The assumption that Gryllidae was basal to all other Ensifera received strong supports. In conclusion, T. japonica and A. yunnanensis, together with other Orthoptera species, exhibited the same mitochondrial genome organization. The concatenated nucleotide sequences of 13 protein genes were suitable markers for reconstruction of phylogenetic relationship in orthopteroid species. The relationships of Tridactyloidea as sister group of Tettigidea in Caelifera and Rhaphidophoroidea as sister group of Tettigonioida in Ensifera were identified. However, this study was still limited by insufficient species, and their phylogenetic relationships were not accurately identified. Further researches on mtgenome data and morphological characters were still needed to reveal the relationships of Orthoptera species.

Additional files
Additional file 1: Figure S1. Predicated secondary structure of the 22 tRNA genes of Tettix japonica.
Additional file 2: Figure S2. Predicated secondary structure of the 22 tRNA genes of Alulatettix yunnanensis.

Authors' contributions
YS and DL carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. YS and DL carried out the immunoassays. YS and BX participated in the sequence alignment. DL and GJ participated in the design of the study and performed the statistical analysis. BX and GJ conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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