Article

Novel Gene Rearrangements in the Mitochondrial Genomes of Cynipoid Wasps (Hymenoptera: Cynipoidea)

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Abstract: Cynipoidea is a medium-sized superfamily of Hymenoptera with diverse lifestyles. In this study, 16 mitochondrial genomes were newly sequenced, 11 of which were the first obtained mitochondrial genomes in the family Liopteridae and four subfamilies (Anacharitinae, Aspicerinae, Figitinae, and Parnipinae) of Figitidae. All of the newly sequenced mitogenomes have unique rearrangement types within Cynipoidea, whereas some gene patterns are conserved in several groups. nad5-nad4-nad4L-nad6-cytb was remotely inverted and two rRNA genes were translocated to nad3 downstream in Ibaliidae and three subfamilies (Anacharitinae, Eucoilinae, and Parnipinae within Figitidae); two rRNA genes in Aspicerinae, Figitinae, and Liopteridae were remotely inverted to the cytb-nad1 junction; rrnL-rrnS was translocated to the cytb-nad1 junction in Cynipidae. Phylogenetic inference suggested that Figitidae was a polyphyletic group, while the Ibaliidae nested deep within Cynipoidea and was a sister-group to the Figitidae. These results will improve our understanding of the gene rearrangement of the mitogenomes and the phylogenetic relationships in the Cynipoidea.

Keywords: base composition; codon usage; evolutionary rate; gene rearrangements; phylogeny

1. Introduction

Cynipoidea is a medium-sized superfamily of Hymenoptera, including around 223 genera with 3200 species described worldwide [1,2]. It includes five generally accepted extant families, Austrocynipidae, Cynipidae, Figitidae, Ibaliidae, and Liopteridae [2,3]. Cynipoid wasps exhibit a wide range of lifestyles [1,4,5]. The most well-known members are phytophagous and easily observed as gall-formers, while the majority of the species are small parasitoids or hyperparasitoids. Although previous molecular and/or morphological studies contributed to elucidating the family or subfamily relationships within Cynipoidea [1,6,7], the phylogenetic relationships within the Cynipoidea are still unclear and need further study, especially the phylogenetic relationships within the family Figitidae and Cynipidae.

The typical insect mitochondrial genome is a circular molecule that is 14–19 kb in size and encodes 37 genes, including 13 protein-coding genes (PCGs), two ribosomal RNA (rRNA) genes, and 22 transfer RNA (tRNA) genes [8]. Mitogenomes have been widely used for phylogenetics, though with the limitation of a relatively high evolutionary rate and the presence of base composition bias [8–10]. Most mitogenomes of insects are highly conserved, possessing the ancestral mitogenome arrangement; however, hymenopteran mitogenomes...
show extremely high rates of genome rearrangements [11–14]. tRNA rearrangements are widespread in Hymenoptera, with at least one tRNA rearrangement found in every sequenced hymenopteran species. Gene rearrangements are usually confined to specific lineages, which can help with phylogenetic reconstruction at lower taxonomic levels, such as the subfamily level in Braconidae [13]. At present, the complete or partial mitogenomes of only seven cynipoid species are available in GenBank (https://www.ncbi.nlm.nih.gov/; accessed on 20 January 2022). The synapomorphic mitochondrial gene rearrangement characters and their phylogenetic utility could not be fully assessed due to limited taxon sampling in the early studies.

In this study, 16 mitogenomes of Cynipoidea were newly sequenced by next generation sequencing (NGS), and 11 of them were the first obtained mitogenomes in the family Liopteridae and four subfamilies (Anacharitinae, Aspicerinae, Figitinae, and Parnipinae) of Figitidae. The obtained information from the study will also facilitate future phylogenetic research of Cynipoidea. Furthermore, we analyzed the main features of the newly generated mitogenomes and those of other Cynipoidea species. We also analyzed gene rearrangement patterns. Finally, the phylogeny of Cynipoidea was reconstructed by combining the available mitogenomes.

2. Materials and Methods

2.1. Sample Identification and DNA Extraction

All 16 newly sequenced samples were identified based on the morphology of adults according to the taxonomic literature (Table S1). All specimens were initially preserved in 100% ethanol and then stored at 4 °C before DNA extraction. Whole genomic DNA was non-destructively extracted from every sample using the DNeasy tissue kit (Qiagen, Hilden, Germany), modified from previous studies [15,16]. Voucher specimens were deposited in the Institute of Insect Sciences, Zhejiang University (Voucher specimen numbers: ZJUH_20220001- ZJUH_20220016, Table S1).

2.2. Next-Generation Sequencing and Assembly

All libraries were constructed using the VAHTS® Universal DNA Library Prep Kit. Whole-genome data were generated on the Illumina NovaSeq platform (Illumina, San Diego, CA, USA) with a PE150 strategy (2 × 150 base, paired-end reads).

More than 2 GB of raw data of each sample was obtained. The raw reads were checked by FastQC v0.11.9 [17], with adapter contamination trimmed by Trimmomatic [18]. The target mitochondrial reads were filtered out using BLAST v2.9.0+ (BLASTn, E-value cutoff 1 × 10−5) against a reference dataset of published Cynipoidea mitogenomes [19]. The mitochondrial reads were assembled by SPAdes v3.0 [20] and IDBA v1.1.3 [21] with default parameters, respectively. Two assemblies were then integrated with GENEIOUS v2020.0.5 (Biomatters Ltd., San Diego, CA, USA).

2.3. Mitochondrial Genome Annotation and Analysis

Assembled contigs were initially annotated using the MITOS web server (http://mitos.bioinf.uni-leipzig.de/index.py; accessed on 15 June 2021) [22]. The start and stop positions of 13 PCGs were adjusted manually and corrected by aligning published data of Cynipoidea species in GenBank. The putative tRNA genes were confirmed by the tRNAscan-SE search server with their homologs from related species [23]. The obtained mitogenomes were submitted to GenBank (Accession number: OM677820-OM677835, Table 1).

The nucleotide composition of all components and the relative synonymous codon usage (RSCU) of PCGs were estimated using MEGA 11.0 [24]. The base composition values (AT and GC-skews) were calculated using the following formulas: AT-skew = (A – T)/(A + T) and GC-skew = (G – C)/(G + C) [25]. The numbers of the synonymous substitutions (Ks) and non-synonymous substitutions (Ka), and the ratios of Ka/Ks for each PCG were calculated in the DnaSP 6.0 [26]. The gene rearrangement of all protein-coding genes, all tRNAs,
and two rRNAs in the 20 cynipoid mitogenomes were analyzed by comparison with the ancestral mitogenomes and with each other.

Table 1. Information of mitochondrial genomes used in phylogenetic analysis.

| Family      | Subfamily          | Species                      | Accession Number |
|-------------|--------------------|------------------------------|------------------|
| Figitidae   | Anacharitinae      | Anacharis sp.                | OM677820 *       |
| Figitidae   | Anacharitinae      | Aegilips sp.                 | OM677821 *       |
| Figitidae   | Aspicerinae        | Melanips sp.                 | OM677822 *       |
| Figitidae   | Aspicerinae        | Prosaspicera validispina     | OM677823 *       |
| Figitidae   | Aspicerinae        | Pujadella villari            | OM677824 *       |
| Figitidae   | Parnipinae         | Parnips nigripes             | OM677835 *       |
| Figitidae   | Eucollinae         | Gastraspis sp.               | MG923497         |
| Figitidae   | Eucollinae         | Endecaneris sp.              | OM677825 *       |
| Figitidae   | Eucollinae         | Ganaspini sp.                | OM677826 *       |
| Figitidae   | Eucollinae         | Trybiographa sp.             | OM677827 *       |
| Figitidae   | Figitinae          | Figites sp. 1                | OM677828 *       |
| Figitidae   | Figitinae          | Figites sp. 2                | OM677829 *       |
| Ibaliidae   |                    | Ibalia leucospoides          | KJ814197         |
| Ibaliidae   |                    | Ibalia sp.                   | OM677830 *       |
| Liopteridae |                    | Paramblynotus sp.            | OM677831 *       |
| Liopteridae |                    | Oberthuerella shirkeyi       | OM677832 *       |
| Liopteridae |                    | Tessmannella kiplungi        | OM677833 *       |
| Cynipidae   |                    | Trichagalma acutissinae      | MN928529         |
| Cynipidae   |                    | Syngergus sp.                | MG923514         |
| Cynipidae   |                    | Saphonicrus sp.              | OM677834 *       |
| outgroup    |                    | Platygaster sp.              | MG923507         |
| Platygastridae |                  | Trissolcus basalis          | JN903532         |

* Newly obtained in this study.

2.4. Phylogenetic Analysis

A total of 20 mitogenomes representing the superfamily Cynipoidea, including 16 newly obtained taxa, were used for phylogenetic analyses. Two species from Platygasteridae, Platygaster sp. and Trissolcus basalis were used as outgroups (Table 1). The PCGs were realigned using the G-INS-i algorithm implemented in MAFFT v7.464 [27]. Bayesian inference analysis (BI) was conducted with MrBayes v3.2.7a [28] using the most optimal partition schemes and best model schemes (Table S2) acquired by PartitionFinder v1.1.1 [29]. Four independent Markov chains were run for 100 million generations, with tree sampling occurring every 1000 generations and a burn-in of 25% of the trees. The stationarity of the run was assessed by Tracer v1.7. (ESS values > 200) [30]. Maximum likelihood (ML) analysis was performed with RAxML-HPC2 v8.2.12 [31] under the GTR+GAMMA model. A total of 200 runs for different individual partitions were conducted with 1000 bootstrap replicates.

3. Results and Discussion

3.1. General Features of Mitochondrial Genomes

We obtained 16 new mitogenomes from the taxa of Cynipoidea. The 37 typical genes were identified in each of the newly sequenced mitogenomes except for Trybiographa sp. (missing trnF), Figites sp. 1 (missing trnW, trnN, trnL1, trnS2, and trnV), Figites sp. 2 (missing trnL1), and Pujadella villari (missing trnQ) (Figure 1). The complete control region (CR) of all the species failed to be assembled, possibly due to low similarity between reference and high A and T contents, common in insect mitogenomes, especially in Hymenoptera [19].

The A + T content for the sequenced region of the mitogenomes in the Cynipoidea ranged from 79.36% (Trybiographa sp.) to 87.01% (Paramblynotus sp.) (Table 2). There was no significant difference in base composition among the species of Cynipoidea. Relative high A + T content in the mitogenomes is not unusual in Hymenoptera compared with other orders [32].
Figure 1. Mitogenomic architecture of the Cynipoidea referenced with the ancestral insect mitochondrial genome. An, Anacharitinae; Eu, Eucoilinae; Li, Liopteridae; Fi, Figitinae; As, Aspicerinae; Ib, Ibalia; Pa, Parnipinae; Cy, Cynipidae. Families are shown in different colors.

Table 2. Base composition of 16 mitochondrial genomes in Chalcidoidea.

| Species                  | Whole Genome | Protein-Coding Genes |
|--------------------------|--------------|----------------------|
|                          | Length (bp)  | A + T (%)            | Length (bp)  | A + T (%) | AT-Skew | GC-Skew |
| Anacharis sp.            | 18,513       | 80.57                | 11,381       | 78.67     | −0.0560 | −0.1112 |
| Aegilips sp.             | 16,709       | 84.00                | 11,080       | 81.75     | −0.0832 | −0.0722 |
| Melanips sp.             | 16,103       | 85.52                | 11,168       | 83.67     | −0.1121 | 0.0323  |
| Pr. validispina          | 15,938       | 84.27                | 11,136       | 82.88     | −0.1075 | 0.0336  |
| Pu. villari              | 16,650       | 79.51                | 11,148       | 76.81     | −0.1241 | 0.0662  |
| Endecameris sp.          | 16,234       | 84.95                | 11,133       | 83.26     | −0.0946 | −0.0687 |
| Ganaspini sp.            | 17,078       | 82.62                | 11,183       | 80.81     | −0.0891 | −0.0596 |
| Trybiographa sp.         | 16,034       | 79.36                | 11,123       | 76.91     | −0.0878 | −0.1340 |
| Figites sp. 1            | 15,333       | 84.24                | 10,986       | 82.90     | −0.0930 | 0.0495  |
| Figites sp. 2            | 16,775       | 83.09                | 11,145       | 81.31     | −0.0984 | 0.0302  |
| Italia sp.               | 17,176       | 86.40                | 11,069       | 85.66     | −0.1175 | 0.0422  |
| Paramblynotus sp.        | 15,482       | 87.02                | 11,173       | 85.58     | −0.1127 | 0.0130  |
| O. sharkeyi              | 16,053       | 84.04                | 11,199       | 82.58     | −0.1060 | −0.0169 |
| Te. kiplingi             | 15,724       | 83.41                | 11,154       | 81.12     | −0.1021 | −0.0028 |
| Saphonecrus sp.          | 16,482       | 85.36                | 11,271       | 82.97     | −0.1049 | 0.0083  |
| Pa. nigripes             | 16,876       | 84.46                | 11,168       | 83.82     | −0.1114 | 0.0515  |

3.2. Base Composition, Codon Usage, and Evolutionary Rate

All 13 PCGs were identified in the newly generated mitogenomes, with sizes ranging from 10,986 bp (Figites sp. 1) to 11,381 bp (Anacharis sp.). The entire A + T content of all the PCGs ranged from 76.81% (Pu. villari) to 85.66% (Italia sp.) (Table 2). The AT-skew in all Cynipoidea mitogenomes was negative. The GC-skew in Anacharitinae, Eucoilinae, and Liopteridae was also negative, whereas Paramblynotus sp. in Liopteridae was positive (Table 2), which is an unusual feature of cynipoid mitogenomes. Two rRNA genes (rrnS and rrnL) were identified in all mitogenomes. The length of rrnS ranged from 792 bp (Aegilips sp.) to 879 bp (Endecameris sp.), and the size of rrnL ranged from 1231 bp (Ganaspini sp.) to 1464 bp (Pu. villari) (Table S3).

The relative synonymous codon usage (RSCU) values of all four subfamilies were plotted in Figure 2, and all possible synonymous codons of the 22 amino acids were present. A or T nucleotides were used with higher frequency in the third codon position than...
other nucleotides, and A was used more often than T. The four most frequently used codons—AUA (Met), AUU (Ile), UUA (Leu$^2$), and UUU (Phe)—were observed (Figure 2, Table S4). These results are consistent with published mitogenomes of other wasps [33,34].

Figure 2. Relative synonymous codon usage (RSCU) of 16 cynipoid mitogenomes. Codon families are provided on the X-axis along with the different combinations of synonymous codons that code for that amino acid. RSCU is defined on the Y-axis.

Ka (nonsynonymous substitutions) and Ks (synonymous substitutions) are used as indicators of selective pressure [35]. The evolutionary rates (Ka, Ks, and Ka/Ks) of PCGs vary considerably among genes (Figure 3, Table S5). In Cynipoidea, the Ka values of 13 PCGs ranged from 0.1177 (cox1) to 0.3506 (atp8), and Ks values ranged from 0.2566 (nad6) to 0.4494 (cox1). The average Ka/Ks ratios were estimated to investigate the evolutionary rates of Cynipoidea PCGs. Ratios ranged from 0.2619 (cox1) to 1.1850 (nad6). The genes nad2, atp8, and nad6 > 1, indicating that they evolved at a faster rate in Cynipoidea. This result was similar to that of the Apoidea [36] and Ichneumonidae [37] in Hymenoptera.

3.3. Gene Rearrangements

Compared with the putative ancestral mitogenome of insects, the mitogenomes of all the Cynipoidea in this study are extremely variable, and all PCGs, tRNAs, and rRNAs had various degrees of rearrangement (Figure 2).
Concerning the phylogenetic relationships of Cynipoidea, all analyses based on the PCGs matrix and two inference methods (BI and ML) generated congruent results with high nodal supports (Figure 4). The results indicate that the Figitidae is a polyphylectic group, consistent with the results based on UCEs [4]. The family Cynipidae is recovered as monophyletic by our data, as most previous studies assumed [1], but this contradicts the result of Blaimer et al. [4]. Adding more members to this family may help to show whether it is monophyletic or not. Ibbaliidae was formerly thought to be an early-branching cynipoid in most studies [6,7]. However, Blaimer et al. proposed that Ibbaliidae nested far inside Cynipoidea and was a sister-group to Figitidae [4]. Our research likewise came up with a similar result.
Based on UCEs, all analyses based on that types of all newly sequenced mitogenomes in this study have unique rearrangement types within Cynipoidea. The gene rearrangements in Cynipoidea are randomly distributed, though there are gene patterns conserved in several groups, for instance, nad5-nad4-nad4L-nad6-cytb was remotely inverted and two rRNA genes were translocated to nad3 downstream in Ibalidae, and three subfamilies Anacharitinae, Eucoilinae, and Parnipinae within Figitidae; two rRNA genes in Aspicerinae, Figitinae, and Liopteridae were remotely inverted to the cytb-nad1 junction; rrmL-rrnS was translocated to the cytb-nad1 junction in Cynipoidea.

**Figure 4.** Phylogenetic analyses of Cynipoidea based on nucleotide datasets of 13 PCGs. The scale bar corresponds to the estimated number of substitutions per site. Numbers separated by a slash on the node are posterior probability (PP) and bootstrap value (BV).

**4. Conclusions**

In this study, 16 Cynipoidea mitogenomes were newly obtained using the next-generation sequencing method, in which the mitogenomes of the family Liopteridae and four subfamilies Anacharitinae, Aspicerinae, Figitinae, and Parnipinae of Figitidae were first reported. The mitogenomes of all the Cypoidea in this research are highly variable. All of the newly sequenced mitogenomes in this study have unique rearrangement types within Cynipoidea. The gene rearrangements in Cynipoidea are randomly distributed, though there are gene patterns conserved in several groups, for instance, nad5-nad4-nad4L-nad6-cytb was remotely inverted and two rRNA genes were translocated to nad3 downstream in Ibalidae, and three subfamilies Anacharitinae, Eucoilinae, and Parnipinae within Figitidae; two rRNA genes in Aspicerinae, Figitinae, and Liopteridae were remotely inverted to the cytb-nad1 junction; rrmL-rrnS was translocated to the cytb-nad1 junction in Cynipoidea. The BI and ML analysis showed consistent topology and indicated that Figitidae was a polyphyletic group and Ibalidae nested far inside Cynipoidea and was a sister-group to Figitidae. Nevertheless, these results provide valuable information for understanding the evolution of Cynipoidea. Denser taxon sampling provides more accurate and comprehensive information for the further analysis of gene arrangement and the evolutionary history of Cynipoidea.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes13050914/s1, Table S1: Collection information of Cynipoidea species in this study; Table S2: The best schemes of partition and substation models selected in nucleotide dataset; Table S3: Length of tRNA and rRNA genes in the Cynipoidea mitogenomes; Table S4: Relative synonymous codon usage in 16 Cynipoidea mitogenomes; Table S5: Synonymous and nonsynonymous substitutional analysis of 13 protein coding genes.
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