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Information from the mitochondrial genomes of two egg parasitoids, *Gonatocerus* sp. and *Telenomus* sp., reveals a controversial phylogenetic relationship between Mymaridae and Scelionidae

Zhao-Can Shen, Lei Chen, Long Chen, Yuan-Xi Li*

Department of Entomology, Nanjing Agricultural University, Nanjing 210095, China

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ABSTRACT

The taxonomic status and phylogenetic affinities of Mymaridae and Scelionidae are controversial, based on similarities between these families in the characteristics of adults, larvae, and eggs. In this study, we sequenced the mitochondrial (mt) genomes of representatives from these two families and found that the derived secondary structure of tRNA-Arg was the same in each family due to the absence of the D-stem. The segment of "*cox1 trnL*$_2$ *cox2 trnK trnD atp8 atp6 cox3*" in *Gonatocerus* sp. (Mymaridae) is conserved and distinct from those of four other species of Chalcidoidea but similar to that in Proctotrupoidea and Platygastroidea. However, phylogenetic analysis indicated that *Gonatocerus* sp. was sister group to other species of Chalcidoidea. Comparisons based on complete gene orders may be more useful in a phylogenetic and systematic context, as different branches may exhibit partially homoplastic gene orders.

1. Introduction

The Mymaridae (Apocrita: Chalcidoidea) and Scelionidae (Apocrita: Platygastroidea), consisting of approximately 1530 and 3308 species, respectively, are two diverse families of parasitic Hymenoptera with a worldwide distribution [1,2]. Mymaridae are egg parasitoids of other insects, including Cicadelloidea, Delphacidae, Miridae, and Coleoptera, while Scelionidae are egg parasitoids of insects in Orthoptera, Hemiptera, Neuroptera and Lepidoptera. The hosts of both families include important agricultural and forest pests, and these two families are therefore of great significance in the biological control [3].

These families have often been thought to have a close phylogenetic relationship as their adults and eggs share many common characteristics [4,5]; nevertheless, the taxonomic and phylogenetic relationships between Mymaridae and Scelionidae are controversial. The Mymaridae was initially considered a genus of Proctotrupoidea by Curtis and was later erected as a family within Chalcidoidea [6,7]. However, Kozlov et al. [8] thought that Mymaridae had a common origin with Serphitidae and should be considered part of Proctotrupoidea. Furthermore, Rasnitsyn [4] proposed that both Mymaridae and Serphitidae were derived from within Scelionidae and transferred all 3 families to his newly established superfamily, Diaprioidea. Contrary to these opinions, Gibson [5], after an analysis of adult and egg characteristics, found that the relationship between Mymaridae and Chalcidoidea was closer than that with Scelionidae. The phylogenetics of Chalcidoidea is still controversial, although Mymaridae has been accepted by most taxonomists as a family within Chalcidoidea. Rasnitsyn proposed Proctotrupoidea (which mainly refers to the current Platygastroidea) as the sister group of Chalcidoidea, primarily based on structural similarities between the larvae of Mymaridae and Scelionidae [9,10]. In contrast, an analysis of molecular markers (18S, 28S, COI, and EF-1α) supported the sister group relationship between Mymaridae and other species of Chalcidoidea, with Chalcidoidea as a sister group of Mymarommatoidea [11] or Diaprioidea [12].

The mitochondrial (mt) genome has been considered an ideal molecular marker for species identification and for phylogenetic or evolutionary studies because of its features of rare recombination, maternal inheritance, conserved gene components, and high AT content [13,14]. In addition, variation in the physical characteristics of the mt genome, such as gene content, genome size, gene rearrangements, and RNA secondary structures can also provide useful phylogenetic information [13,15–17]. Poor representation amongst Hymenoptera lineages, however, restricts the evolutionary utility of the mt genome, especially in the Proctotrupomorpha (encompassing the superfamilies Proctotrupoidea, Cynipoidea, Diaprioidea, Mymarommatoidea, Platygastroidea, and Chalcidoidea). Only 12 mt genomes from Proctotrupomorpha have been reported to date, and no mt genome sequences are available from the Mymaridae Therefore, the main purpose of the

* Corresponding author.
E-mail address: yxli@njau.edu.cn (Y.-X. Li).

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Table 1
List of published mitochondrial genomes from Proctotrupomorpha and out-groups used in the phylogenetic analyses.

Species	Family	Superfamily	Accession number	Reference
Gonatocerus sp.	Mymaridae	Chalcidoidea	MF776883	Present study
Telenomus sp.	Scelionidae	Platygastroidea	MF776884	Present study
Nasonia girault	Pteromalidae	Chalcidoidea	NW_001815691	[27]
Philotrypesis pilosa	Agaonidae	Chalcidoidea	JF808723	[28]
Megaphragma amalphitanum	Trichogrammatidae	Chalcidoidea	KT373787	[29]
Trichogramma dendrolimi	Trichogrammatidae	Chalcidoidea	KU836507	Chen et al., unpublished
Trissolcus basalis	Scelionidae	Platygastroidea	JN903532	[30]
Idris sp.	Scelionidae	Platygastroidea	KF696670	[31]
Ceratobaeus sp.	Scelionidae	Platygastroidea	KF696669	[31]
Leptopilina boulardi	Figitidae	Cynipoidea	KU665622	[32]
Ibalia leucospoides	Ibaliidae	Cynipoidea	KJ814197	[33]
Vanhornia eucnemidarum	Vanhorniidae	Proctotrupoidea	NC_008323	[34]
Pelecinus polyturator	Pelecinidae	Proctotrupoidea	KM104167	[33]
Monomachus antipodalis	Monomachidae	Diaprioidea	KM104168	[33]
Pristaulacus compressus	Aulacidae	Evanioidea	NC-022849	[35]
Gasteruption sp.	Gasteruptiidae	Evanioidea	KJ619460	[36]

present study was to increase our understanding of the phylogeny of Proctotrupomorpha by clarifying the phylogenetic relationships of the Mymaridae and Scelionidae, through mt genome sequences. In the present study, we sequenced the mt genomes of *Gonatocerus* sp. (Mymaridae) and *Telenomus* sp. (Scelionidae) and conducted phylogenetic analyses using these new mt genomes along with those previously published from Proctotrupomorpha.

2. Materials and methods

2.1. Sample collection and DNA extraction

Specimens of *Gonatocerus* sp. and *Telenomus* sp. were collected from a rice field of Jiangsu Province, China, and both specimens were stored in 100% ethanol at −20 °C prior to DNA extraction. DNA was extracted from each individual with a Wizard® Genomic DNA Purification Kit (Promega (Beijing) Biotech Co., Ltd. Beijing, China) according to the manufacturer's protocol and stored at −20 °C.

2.2. Mt. genome amplification

Short fragments of *cox1* and *cob* were amplified and sequenced with a range of universal insect mt primers (*cox1*: C1-J-1718/C1-N-2191; *cob*: CB-J-10933/CB-N-11367) [18]. From the initial *cox1* (518 bp) and *cob* (470 bp) sequence, taxon-specific primers (*cox1* to *cob* and *cob* to *cox1*) were designed to obtain the remaining mt genome via long PCR (Table S1). PCR was performed with a proofreading Tks Gflex DNA Polymerase (Takara Bio Inc., Japan), which checks each nucleotide during DNA synthesis and excises mismatched nucleotides in the 3′ to 5′ direction. The 50-μl PCR reaction mixture contained 1 μl of Tks Gflex DNA polymerase (1.25 U/μl), 25 μl of 2 × Gflex PCR Buffer (Mg^{2+}, dNTP plus), 1 μl of each primer (20 μM), 3 μl of DNA (approximately 400 ng), and 19 μl of water. The cycling conditions were 94 °C for 1 min and 35 cycles of 98 °C for 10 s, 55 or 60 °C for 15 s and 68 °C for 10 min. The PCR products were used for Illumina sequencing.

2.3. Mt genome sequencing, annotation and bioinformatic analysis

For Illumina sequencing of the PCR products, Single-indexed Illumina library was prepared with the TruSeq Nano DNA LT Prep Kit (Illumina, San Diego, CA, USA) following the manufacturer's instruction. In brief, at least 1 μg (8–10 kb) of the PCR products were sheared to desired size (~400 bp) by Covaris M220 (Covaris, Inc., Woburn, MA, USA) (Covaris settings: duty factor 20%, displayed power 50 W, cycles 500, duration 55 S, temperature 20 °C). Blunt ends were generated with End Repair Mix (Illumina-supplied consumables). After adding an 'A' base to the 3' end of the blunt phosphorylated DNA fragment, a 60 bp adapter index was ligated to each end of the fragment. The ligation products (~520 bp) were purified through gel-electrophoresis, and were amplified to generate the library. The qualified library was run on the Illumina HiSeq X Ten platform in PE150 mode (150 bp × 2). Raw sequencing data was generated by the Illumina base calling software CASAVAv1.8.2 (http://support.illumina.com/sequencing/sequencing_software/casava.ilmn), and sequences containing adaptors or primers identified by SeqPrep [19] (https://github.com/jstjohn/SeqPrep). Sickle (https://github.com/najoshi/sickle) was used for read trimming (default parameters) to obtain clean data for this study.

Illumina sequence reads obtained for the *Gonatocerus* sp. and *Telenomus* sp. were assembled into contigs using Geneious 9.1.4 [20] with the partial *cox1* sequence and *cob* sequences as reference fragments. The specific parameters were as follows: minimum overlap, 140, minimum overlap identity, 98%; and maximum mismatches per read, 3%. Transfer RNA genes were identified using the MITOS WebServer [21] and ARWEN [22], or these genes were found manually based on anticodon and secondary structures. Protein-coding genes (PCGs) were identified for open reading frame (ORF) searches in Geneious using the invertebrate mt genetic code and BLASTp searches conducted in NCBI [23]. rRNA genes were identified in MITOS and compared with those published from related species. Tandem repeats in the AT-rich region were predicted using Tandem Repeats Finder (http://tandem.bu.edu/trf/trf.html). Nucleotide sequence data for *Gonatocerus* sp. and *Telenomus* sp. have been deposited in GenBank under accession numbers MF776883 and MF776884.

Nucleotide composition, codon usage, and relative synonymous codon usage (RSCU) values as well as nucleotide substitution statistics were determined using MEGA6 [24]. GC and AT asymmetries were calculated following the formulas of Hassanin et al. [25]. The AT and GC skews were measured for the major (J) strand of each genome. The formulae used were AT skew = (A - T)/(A + T) and GC skew = (G - C)/(G + C) [26].

2.4. Phylogenetic analysis

The present study analyzed a total of 16 taxa from six superfamilies, including five Chalcidoidea, four Platygastroidea, two Cynipoidea, one Diaprioidea, and two Proctotrupoidea taxa as well as two out-groups from the Evanioidea (Table 1). Nucleotide sequences from each PCG and rRNA genes were aligned by MAFFT [37]. Large gaps and ambiguous sites were deleted manually after alignment. The data matrix combining all codons of the protein-coding genes and two rRNA genes (PCGs + rrnL + rrnS) were analyzed using two phylogenetic approaches: Bayesian inference (BI) and maximum likelihood (ML). To standardize the partitioning strategy as recommended for phylogenetic analyses using mitochondrial genome [15], PartitionFinder v2.1.1 [38]

Table 2
Characteristics of the two mitochondrial genomes sequenced in this study.

	Gonatocerus sp.				*Telenomus* sp.			
Gene	Length	AT (%)	AT skew	GC skew	Length	AT (%)	AT skew	GC skew
All genes	15,554	87.5	−0.9823	−0.0005	16,923	84.6	0.0378	−0.2338
13-PCG	11,142	85.9	−0.1212	0.0638	11,136	81.4	−0.1032	−0.0323
atp6	681	84.9	−0.0967	−0.1258	660	79.4	−0.0353	−0.2330
atp8	160	97.5	−0.1025	0.0000	168	85.1	0.0341	−0.1216
cox1	1539	78.4	−0.1454	0.1111	1527	74.5	−0.0631	−0.0781
cox2	666	82.9	−0.0797	0.0526	681	78.6	0.0166	−0.2744
cox3	786	82.4	−0.1359	0.1477	804	77.0	−0.0793	−0.1043
cytb	1140	82.5	−0.1308	0.0230	1140	77.6	−0.0593	−0.1839
nd1	945	84.8	−0.0909	0.0980	912	82.7	−0.2551	0.2529
nd2	1041	92.7	−0.1068	−0.1781	1005	87.4	−0.0275	−0.4173
nd3	354	89	−0.1753	0.1818	345	83.8	0.0239	0.1912
nd4	1318	88.3	−0.1008	0.1111	1350	83.9	−0.2372	0.3548
nd4L	258	90.3	−0.0986	0.1959	282	89.4	−0.1342	0.7944
nd5	1672	88	−0.1500	0.1000	1668	83.3	−0.1741	0.3054
nd6	582	92.3	−0.1138	0.0649	594	88.7	−0.0214	−0.4867

was used to simultaneously choose partitioning schemes and substitution models for the data matrix, and the results are shown in Table S2. The BI analyses were performed with MrBayes 3.2.2 [39], and were run for 10 million generations, with tree sampling every 1000 generations and a burn-in of 25% trees. ML analyses were performed using the GTRGAMMAI model in raxmlGUI1.3 [40]. Clade support was assessed using a nonparametric bootstrap with 1000 replicates. The phylogenetic trees were viewed and edited in FigTree v1.4.0 [41].

3. Results and discussion

3.1. General features of mt genomes

The complete mt genome of *Gonatocerus* sp. was 15,554 bp sequenced with an average coverage of 5460 (Table 2). 36 genes—including 13 PCGs, 21 *trn* genes and two *rrn* genes—as well as one AT-rich region were found in this genome (Table S3). We could not identify tRNA-Leu^{CUN} (*trnL1*), perhaps because the secondary structure of the gene was atypical [42]. Alternatively, this *trn* might have been lost because the codons (CUU, CUA, CUC and CUG) transferred by *trnL1* were missing in *Gonatocerus* sp. (Table S4). A nearly complete mt genome of *Telenomus* sp., with a length of 16,923 bp, was sequenced with an average coverage of 25,360. The assembly contained 37 genes as well as one AT-rich region (Table 2 and Table S3). We were unable to completely assemble part of the non-coding region between *trnY* and *trnQ*. The inability to assemble this region may have been due to the presence of short tandem repeats [30,43]. We also attempted to sequence this region using conventional Sanger methods testing four primer pairs; however, this approximately 1000 bp fragment could not be sequenced. AT content of the two genomes was 84.60% and 87.5%, for *Gonatocerus* sp. and *Telenomus* sp. respectively (Table 2), a strong AT bias as reported in other hymenopterans [37]. Each of genome exhibited negative GC skew; AT skew of *Gonatocerus* sp. was also negative, whereas that *Telenomus* sp. had positive AT skew.

3.2. Protein-coding genes

Ten of the 13 PCGs were located on the majority strand in *Gonatocerus* sp., while nine of them showed this location in *Telenomus* sp. (Table S3). In the mt genome of *Gonatocerus* sp., PCGs accounted for 71.63% (11,142 bp total) of the whole genome, whereas in *Telenomus* sp., the corresponding percentage was 69.8% (11,136 bp) of the obtained region. The overall AT content of the 13 PCGs was 85.9% in *Gonatocerus* sp., ranging from 78.4% (*cox1*) to 97.5% (*atp8*), and 81.4% in *Telenomus* sp., ranging from 74.5% (*cox1*) to 89.4% (*nd4L*) (Table 2).

The start codons of all PCGs are “ATN” in these genomes. In the *Gonatocerus* sp. mt genome, ten genes stop with TAA; three use the incomplete stop codon “T”, which is common in invertebrate mt genomes [44,45]. However, in *Telenomus* sp., all the PCGs stop with TAA, except for *nd2*, which stops with TAG (Table S3). The RSCU values in the mt genomes of *Gonatocerus* sp. and *Telenomus* sp. also reflect a significant bias toward A and T nucleotides which is commonly found in other species of Hymenoptera (Table S4) [46]. The codons (CUU, CUA, CUC and CUG) transferred by *trnL1* were lost in the mt genomes of *Gonatocerus* sp. (Table S4), and were not found in other species.

3.3. Transfer RNA and noncoding regions

Most tRNA genes in both genomes have a typical cloverleaf structure, except for tRNA-Ser^{AGN} (*trnS1*) and tRNA-Arg (*trnR*), in which the D-stem is absent (Fig. 1 and Fig. S1). A missing D-stem has been reported for *trnS1* in many insect species [34,47,48] but *trnR* missing D-stems has only previously been found in three species of Scelionidae. A missing D-stem has been proposed to be a shared, derived characteristic of the family Scelionidae [31]. The missing D-stem in *trnR* from *Gonatocerus* sp. may indicate a close relationship with Scelionidae [13].

The AT-rich region in the mt genomes of *Gonatocerus* sp. is 509 bp long and contains two tandem repeats: one consists of two 51 bp units, and the other of two 21 bp units (Fig. S2). Altogether, 409 bp of short noncoding regions were identified in *Gonatocerus* sp., with the largest one consisting of 122 bp between *nd1* and *rrnL*. The long noncoding region between *nd1* and *rrnL* may be related to the loss of *trnL1*, which is located between *nd1* and *rrnL* in the ancestral mt genome. The AT-rich region is 999 bp in length in *Telenomus* sp. and included one 51-bp-long tandem repeat and two 19-bp-long polyT sections, which were difficult to sequence (Fig. S2). We could not obtain part of the region between *trnY* and *trnQ*, and we were therefore unable to determine whether it was simply a large non-coding region or a duplicated AT-rich region as reported for many species [33,49–51]. The size of the other short noncoding regions in *Telenomus* sp. was 480 bp in total, ranging from 1 to 143 bp.

3.4. Genome organization

In *Gonatocerus* sp., most PCGs are located in ancestral positions for insects, with only *nad5* inverted compared with the organization of the ancestral pancrustacean (Fig. 2). The relative positions of tRNA, however, were highly variable, with seven tRNAs in derived positions: *trnA* and *trnM* inverted and moved to the *rrnL-nad2* junction; *trnE*, *trnV* and *trnQ* also moved from their original positions; and *trnF* and *trnI* inverted without transposition. Rearrangement events mainly occurred at the *nd3-nd5* and *rrnL-rrnS* junctions and around the AT-rich region. All of

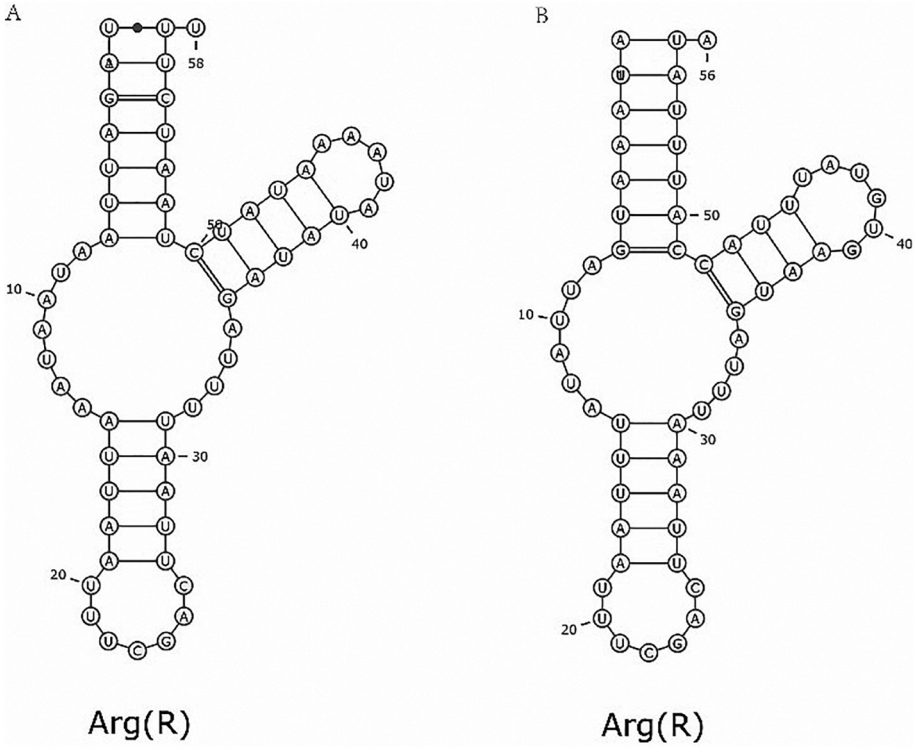

Fig. 1. Predicted secondary structures of tRNA-Arg in *Gonatocerus* sp. (A) and *Telenomus* sp. (B).

those junctions are "hot spots" where gene rearrangement has been noted to occur frequently in hymenopterans [33,52–54]. The gene order of *Telenomus* sp. is nearly identical to that of *Trissolcus basalis,* in which 11 tRNAs have changed position compared to the ancestral pancrustacean. In both genomes, *trnV* has moved out of the *rrnL-rrnS* junction, and a derived placement of *trnV* might be a shared derived character for Platygastroidea and Chalcidoidea [31].

With the increasing number of mitochondrial genomes sequenced, gene rearrangements are quite common in Hymenoptera [55]. However, there are some conserved regions where genes rearrangement seldom occurred in rearranged mt genomes. For example, the positions of *nad5* to *cob* were conserved in Proctotrupomorpha and this segment was inverted only in *Ibalia leucospoides* (Fig. 3). Furthermore, closely related species often share identical unchanged gene orders, and partially homoplastic gene orders can provide important molecular information in a phylogeny [13,56,57]. The pattern of "*cox1 trnL*$_2$*cox2 trnD trnK atp8 atp6 cox3 trnG nad3*" was conserved across the four species of Platygastroidea, and an inverted segment "*-cox3 -atp6 -atp8 -trnD trnK -cox2 -trnL2 -cox1*" was shared by the four species of Chalcidoidea (*Trichogramma dendrolimi*, *Megaphragma amalphitanum*, *Philotrypesis pilosa* and *Nasonia giraulti*) [58]. However, the "*cox1 trnL*$_2$*cox2 trnK trnD atp8 atp6 cox3*" segment in *Gonatocerus* sp. was found to be conserved in this analysis, and was distinct from the sequences of the other four species of Chalcidoidea, but similar to those of

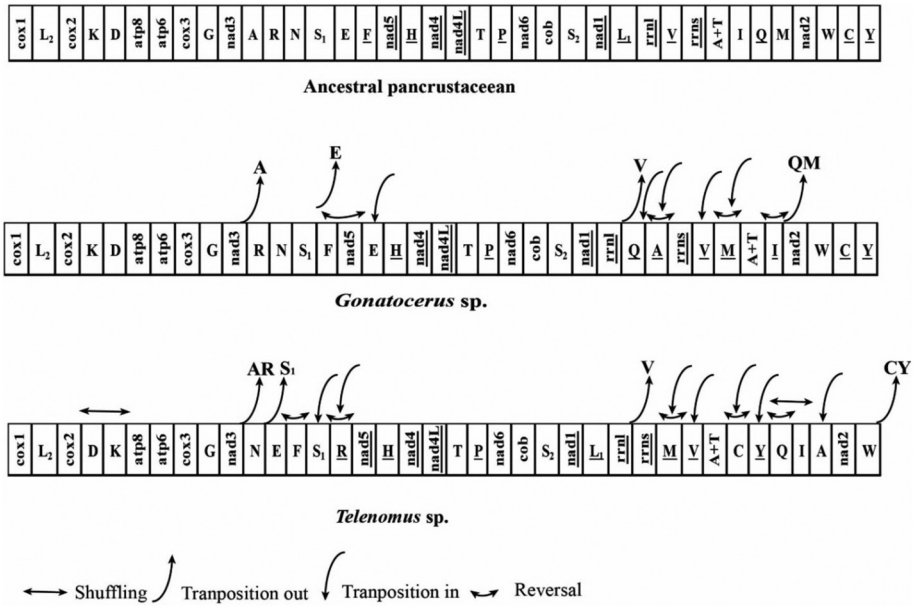

Fig. 2. Mitochondrial genome organization of *Gonatocerus* sp. and *Telenomus* sp. compared with that of the ancestral pancrustacean. The trn genes are indicated by single letter amino acid codes; L1, L2, S1, and S2 denote tRNA-Leu (CUN), tRNA-Leu (UUR), tRNA-Ser (AGN), and tRNASer (UCN), respectively. Genes are transcribed from left to right, except those indicated by underlining. Gene movements relative to the ancestral organization are indicated with arrows.

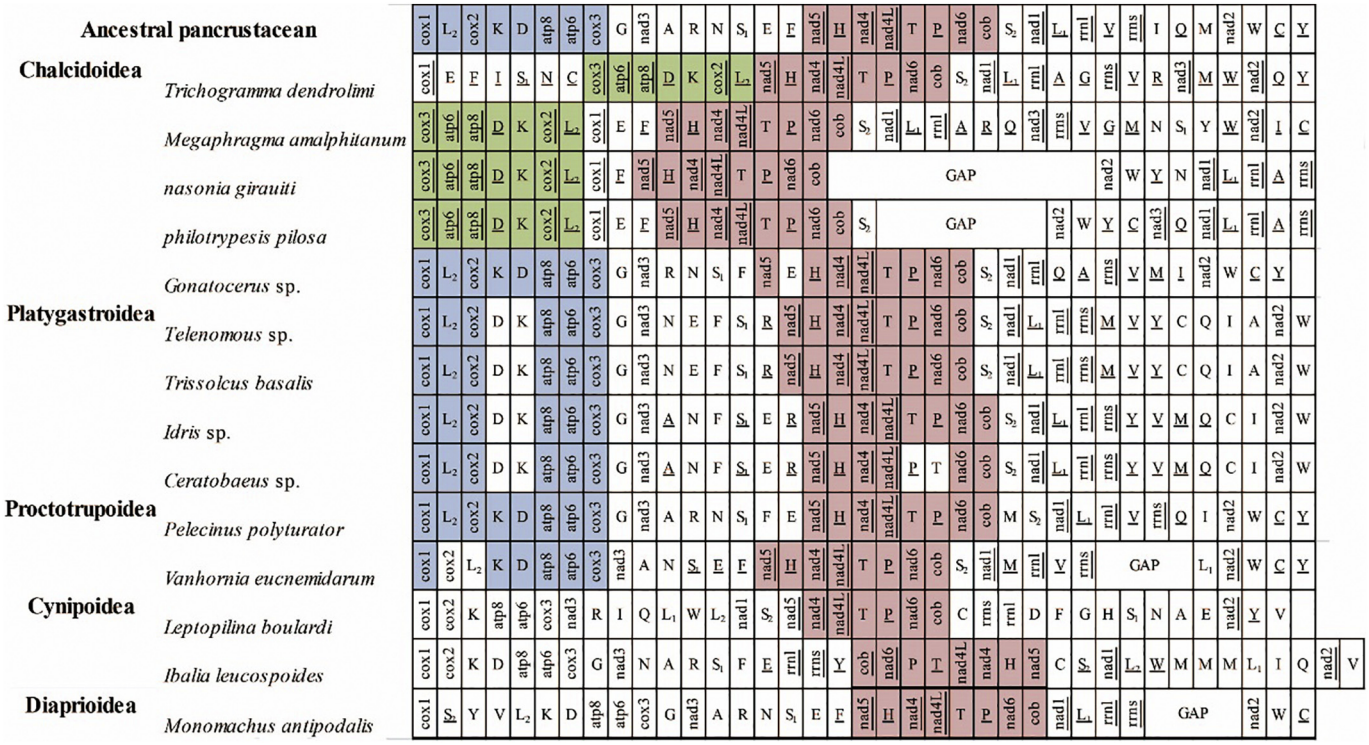

Fig. 3. Gene orders of the currently sequenced Proctotrupomorpha mitochondrial genomes. The trn genes are indicated by single letter amino acid codes; L1, L2, S1, and S2 denote tRNA-Leu (CUN), tRNA-Leu (UUR), tRNA-Ser (AGN), and tRNASer (UCN), respectively. Genes are transcribed from left to right, except those indicated by underlining. Partially homoplastic gene orders are shown in the same color.

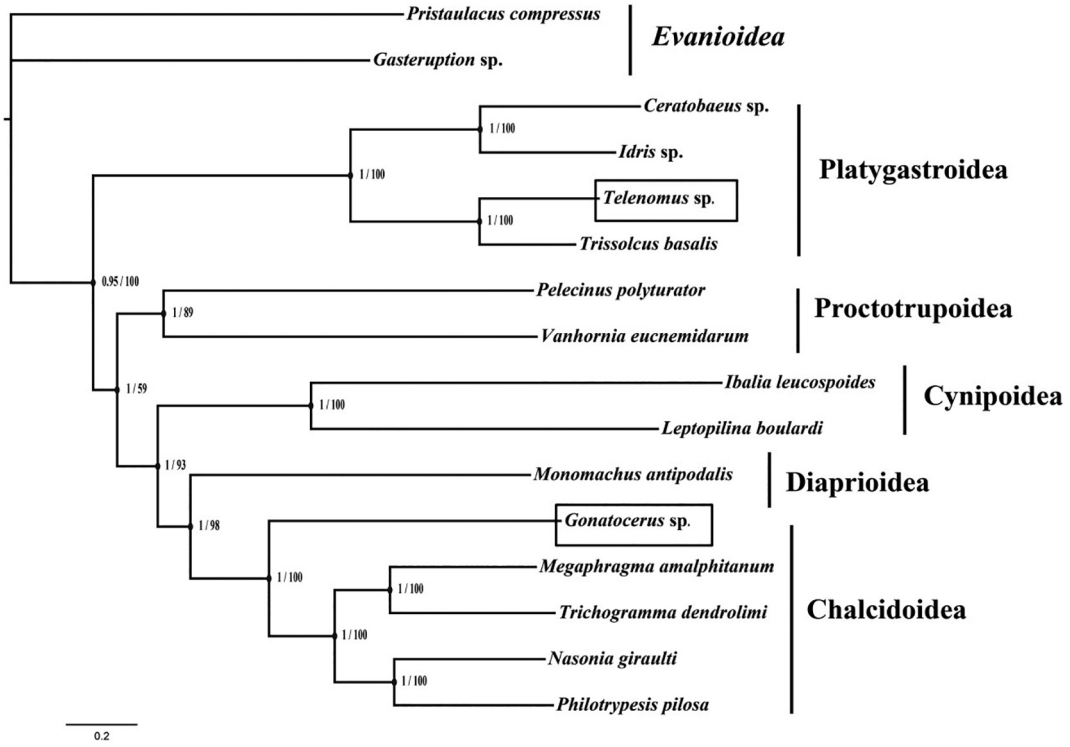

Fig. 4. Proctotrupomorpha phylogeny based on nucleotide sequences of mitochondrial protein-coding genes and rRNA genes. Bayesian posterior probabilities and bootstrap values (1000 replicates) are shown sequentially, separated by "/" near the respective nodes. The newly sequenced species are highlighted with frames. The scale bar indicates the number of changes per site.

Proctotrupoidea and Platygastroidea (Fig. 3).

3.5. Phylogenetic analyses

The topologies of the trees generated using the two phylogenetic approaches were identical, with strong support at most nodes (Posterior probabilities > 95% and bootstrap values > 70%) (Fig. 4) [59,60]. The two strategies support the monophyly of Chalcidoidea as well as a sister group relationship between *Gonatocerus* sp. and the remaining Chalcidoidea. These results are in agreement with those of Munro et al. [12] and Heraty et al. [10]. Diaprioidea (*Monomachus antipodalis*) was supported as the sister group of Chalcidoidea, and this result was identical to that of previous analyses [33,34,61]. However, the placement of Cynipoidea, varied between previous analyses, suggesting Cynipoidea as a sister group to Platygastroidea or Diaprioidea [61–63]. Our results indicated that Cynipoidea is sister to Diaprioidea plus Chalcidoidea, in agreement with the results of Mao et al. [31]. The Platygastroidea were well supported as monophyletic and as sister to the remaining Proctotrupomorpha; additionally, the relationship between Mymaridae and Chalcidoidea was shown to be obviously closer than that between Mymaridae and Scelionidae.

As a tool for examining phylogenetics, the mt gene order and the secondary structures of tRNA can resolve some contentious evolutionary questions [13,16,56]. However, the gene order of *cox2* to *cox3* in *Gonatocerus* sp. was conserved at specific positions, which were different from those of the reported species of Chalcidoidea and similar to other species in Proctotrupoidea and Platygastroidea. Furthermore, one derived tRNA secondary structure of *Gonatocerus* sp. was similar to that of Platygastroidea. These results differ from those based on phylogenetic analysis. This pattern could occur because Hymenopterans exhibit a high frequency of gene rearrangement, as the gene order of each family differs significantly from that of others (Fig. 3). For example, differences in rearrangement exist between species of Trichogrammatidae (*Megaphragma amalphitanum* and *Trichogramma dendrolimi*) (Fig. 3). Furthermore, although partially homoplastic gene orders can afford important molecular information in a phylogenetic and systematic context, comparisons based on complete gene orders are more useful as different branches may share the same rearrangement [64]. Additionally, the limited numbers of representatives from each family may also affect the analysis, especially for Diaprioidea, from which only one mt genome is available. Hence, the density of taxon sampling needs to be increased in future studies.

Competing interests

The authors declare that they have no competing interests.

Author contributions

Z.C.S. and Y.X.L. designed and conducted the experiment. Z.C.S. and C.L. performed sampling in the rice field. Z.C.S. and L.C. performed computational analysis. Z.C.S. and Y.X.L. wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2018.06.009.

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