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Comparative analysis of seven mitochondrial genomes of *Phymatostetha* (Hemiptera: Cercopidae) and phylogenetic implications

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ABSTRACT

In this study, we present seven mitochondrial genomes (mitogenomes) of *Phymatostetha*. Each mitogenome contains the entire set of 37 genes, which arranged in the same order as the putative ancestral pattern of insects. The nucleotide composition of *Phymatostetha* mitogenomes is biased toward A/T, with rRNAs and PCG12 (i.e. the first and second codon positions of PCGs) exhibit the highest and lowest A + T content, respectively. Relative synonymous codon usage of PCGs also show that degenerate codons are biased to use more A/T than G/C. All tRNAs exhibit typical clover-leaf structure, with the exception of *trnS1*. Additionally, unpaired nucleotides are detected in *trnS1* anticodon stem and *trnR* acceptor stem. Phylogenetic relationships, based on the dataset of 13 PCGs, 22tRNAs, and two rRNAs, are analyzed using both the Bayesian and maximum likelihood methods. Our results clearly revealed the systematic status of *Phymatostetha* species and robustly supported the monophyly of this genus, in which *Phymatostetha semele* is sister to other *Phymatostetha* species. It was demonstrated that mitogenome was an effective molecular marker to adequately resolve phylogeny at low taxonomic levels.

1. Introduction

The typical insect mitochondrial genome (mitogenome) is a circular molecule with relatively stable gene organization. It varies from 15 to 18 kb and contains 13 protein-coding genes (PCGs), 22 transfer RNA genes (tRNAs), two ribosomal RNA genes (rRNAs), and a control region [1–3]. Owing to unique characters such as small size, strict orthologous genes, low rate of recombination, and fast evolution rate [4,5], mitogenome has been extensively used as a molecular marker for population genetics, phylogeny, and evolution at different taxonomic levels [6–9]. Furthermore, genome-level features, including nucleotide composition, gene rearrangement, and structural genomic characters, have also been widely used for comparative and evolutionary genomics, and phylogenetic analyses [2,10–13].

Phymatostetha (Hemiptera: Cercopidae) is a small but brightly colored Old World tropical genus, which includes appropriately 30 species [14,15]. Like other spittlebugs, they present the nymphal habit of covering themselves with copious spittle masses, which are produced through continuously ingesting the xylem sap [16,17]. Spittle mass has been considered as an effective barrier against desiccation, predation, parasitism, and damaging solar radiation [16,18,19]. In addition, many spittlebug nymphs tend to aggregate in one spittle mass and cause more serious economic damage to their host plants [20]. Some species of *Phymatostetha* are reported as the pests of banana [21,22]. Therefore, it is significant to accurately clarify the taxonomic status and phylogenetic relationships of *Phymatostetha* species. However, only some scattered taxonomic studies and limited molecular data are available [14,15,23]. Additionally, many species are also difficult to be identified because of insufficient diagnostic characters. Even less is known about the phylogenetic relationship within *Phymatostetha*, especially among closely related species. Therefore, detailed molecular data are required for the comparative and evolutionary genomics, and phylogenetic analyses of the genus *Phymatostetha*.

To date, only one complete mitogenome of *Phymatostetha* is available in GenBank (Accession number MG878381), which is quite limited and restricts our understanding about the taxonomic status, genome-level characters, and phylogenetic relationships of this genus. In this study, we sequenced seven other mitogenomes of *Phymatostetha* species, presented comprehensive comparative genomic analyses, and explored the phylogenetic relationships within *Phymatostetha*.

2. Materials and methods

2.1. Sample and DNA extraction

Seven adults of *Phymatostetha* species were collected (Table 1). Voucher specimens were preserved in absolute ethyl alcohol and stored at −80 °C freezer in Institute of Zoology, Chinese Academy of Sciences.

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Table 1
Summary of mitogenomes used in this study.

Family	Species	Size (bp)	Accession no.
Cercopidae	*Phymatostetha huangshanensis*	17,785	MG878381
Cercopidae	*Phymatostetha semele*	14,569	MK123949
Cercopidae	*Phymatostetha signifera*	14,579	MK123950
Cercopidae	*Phymatostetha stalii*	14,574	MK123951
Cercopidae	*Phymatostetha punctata* 1	14,591	MK127916
Cercopidae	*Phymatostetha punctata* 2	14,591	MK127917
Cercopidae	*Phymatostetha* sp.1	14,548	MK127918
Cercopidae	*Phymatostetha* sp.2	14,584	MK127919
Cercopidae	*Cosmoscarta bispecularis*	15,426	KP064511
Cercopidae	*Callitettix versicolor*	15,374	NC_020031
Cercopidae	*Abidama producta*	15,277	NC_015799
Cercopidae	*Paphnutius ruficeps*	14,841	NC_021100

For each *Phymatostetha* species, genomic DNA was extracted from legs of a single adult using the DNeasy Blood & Tissue kit (Qiagen), following the manufacturer's protocols.

2.2. PCR amplification and sequencing

The mitogenomes of *Phymatostetha* species were amplified by a set of primers that designed from the mitogenomic sequence of *Phymatostetha huangshanensis* [23]. The amplification conditions and sequencing strategies were also conducted as described previously.

2.3. Bioinformatic analysis

The newly sequenced *Phymatostetha* mitogenomes were submitted to GenBank (Accession numbers: MK123949-51, MK127916-19). Sequence assembly, annotation, and alignment were described in our previous study [23]. Nucleotide composition and relative synonymous codon usage (RSCU) were calculated in MEGA 6.05 [24]. Composition skew was analyzed with the following formulas: AT skew = [A − T] / [A + T] and GC skew = [G − C] / [G + C] [25]. The comparable gene identity map was generated by CGView Comparison Tool [26], with the query length of 100 bp in BLAST search. The tRNAs secondary structures were predicted by Mitos WebServer [27], with Mito genetic code of invertebrate.

2.4. Phylogeny analysis

Phylogenetic analyses were performed on the dataset of 13 PCGs, 22 tRNA, and two rRNAs from twelve complete or nearly complete mitogenomes of Cercopidae, in which seven mitogenomes of *Phymatostetha* were newly sequenced (Table 1). Nucleotide alignments

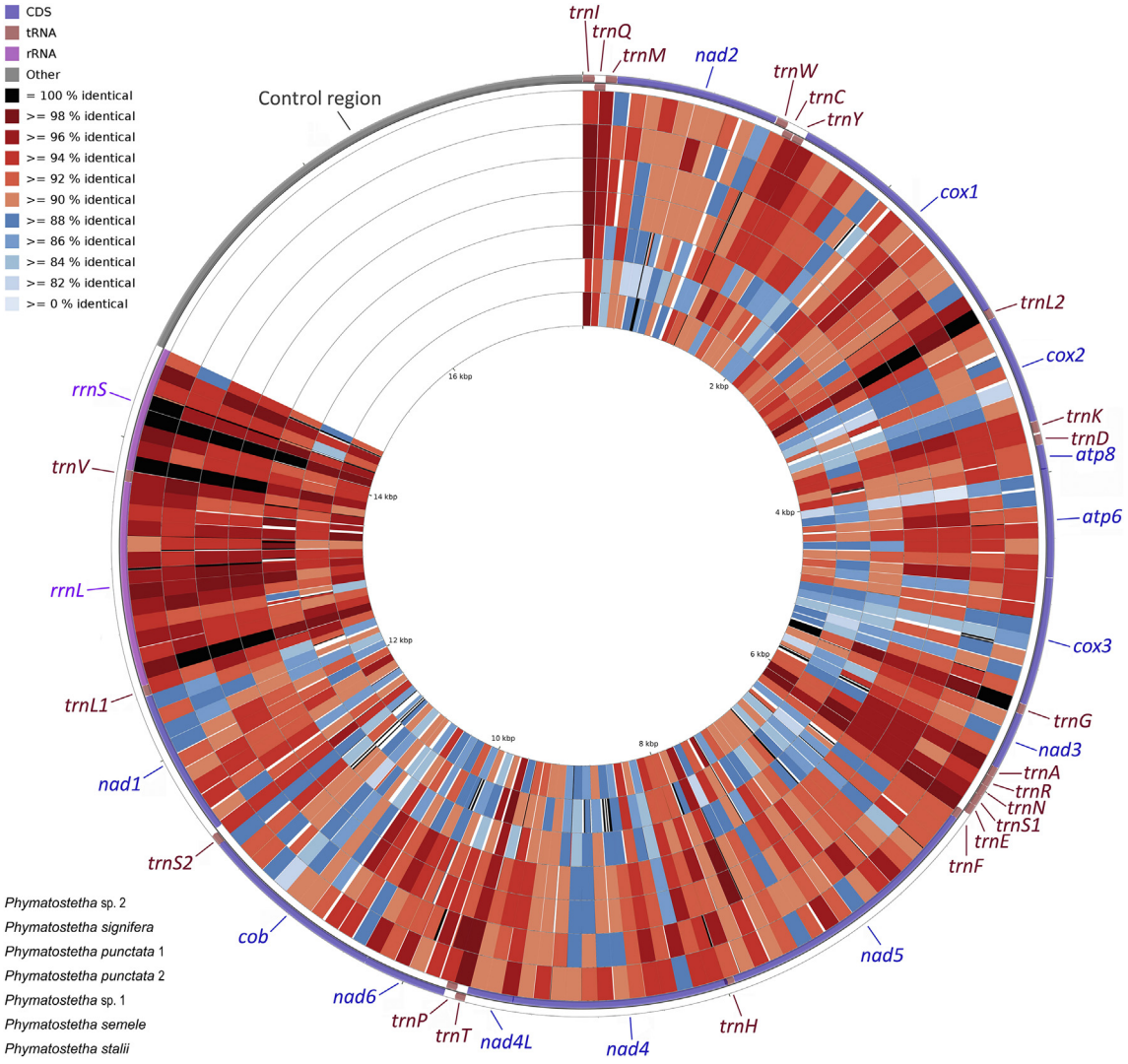

Fig. 1. Circular map of the seven *Phymatostetha* mitogenomes. Different color presents the sequence identity of BLAST searches. The mitogenome that most similar to the reference mitogenome (*P. huangshanensis*) is placed closest to the outer edge of the map. The species from outside to inside are as follows, respectively: *Phymatostetha* sp.2, *P. signifera*, *P. punctata* 1, *P. punctata* 2, *Phymatostetha* sp.1, *P. semele*, and *P. stalii*.

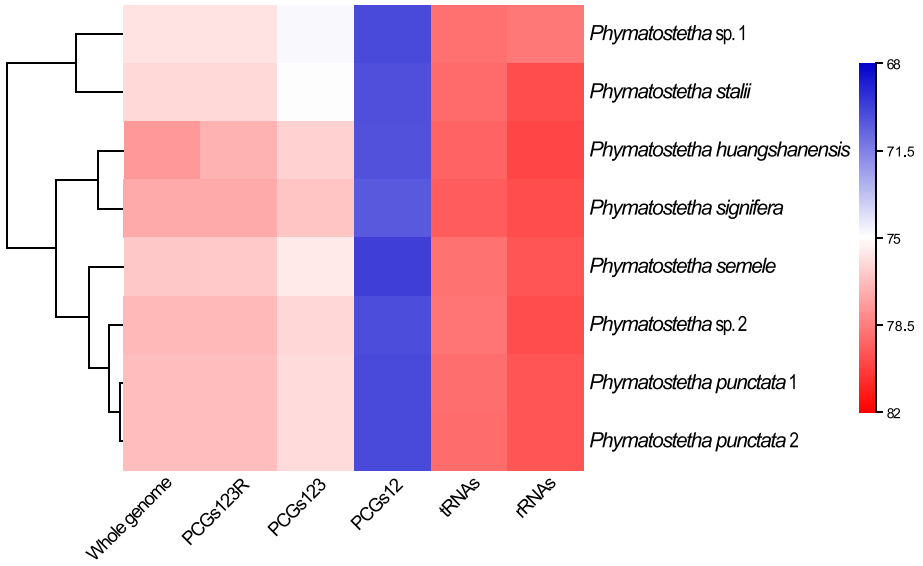

Fig. 2. Base composition of various datasets among *Phymatostetha* mitogenomes. The y-axis indicates hierarchical clustering of *Phymatostetha* species based on the A + T content. PCGs12, indicates the first and second codon positions of PCGs; PCGs123, indicates all codon positions of PCGs; PCGs123R, indicates PCGs123, tRNAs, and rRNAs.

of each PCG were aligned separately with codon-based multiple alignments using MUSCLE that implemented within MEGA 6.05. The tRNA and rRNA genes were aligned individually using MAFFT 7.310 with the Q-INS-i algorithm [28]. Additionally, Gblock 0.91b [29] was conducted to eliminate poorly aligned sequences. The best partitioning schemes and evolutionary models (Table S1) were recommended by PartitionFinder 2.1.1 [30] with Bayesian Information Criterion (BIC). The dataset was pre-defined by both gene types and codon positions.

Phylogenetic inferences were conducted under the criteria of maximum likelihood (ML) and Bayesian inference (BI). Analyses were rooted in two Callitettixini species according to the result of previous phylogenetic study [23]. The maximum likelihood (ML) analysis was conducted using IQ-TREE 1.6.5 [31]. Branch support was performed with 1000 replicates of ultrafast likelihood bootstrap. Bayesian inference (BI) was carried out using MrBayes 3.2.6 [32] through the CIPRES Science gateway [33]. Two independent runs (each with three hot chains and one cold chain) were performed simultaneously for 10,000,000 generations, with sampling every 1000 generations and a relative burn-in of 25%. The convergence of the two independent runs was assessed by average standard deviation (SD) of split frequencies (<0.01).

3. Results and discussion

3.1. Genome structure

This study sequenced seven mitogenomes of *Phymatostetha*, with the length ranged from 14,548 bp in *Phymatostetha* sp.1 to 14,591 bp in *Phymatostetha punctata* (Table 1). Because of complicated secondary structure and high A + T content, the control regions were failed to be amplified. Each mitogenome contained the typical set of 37 genes,

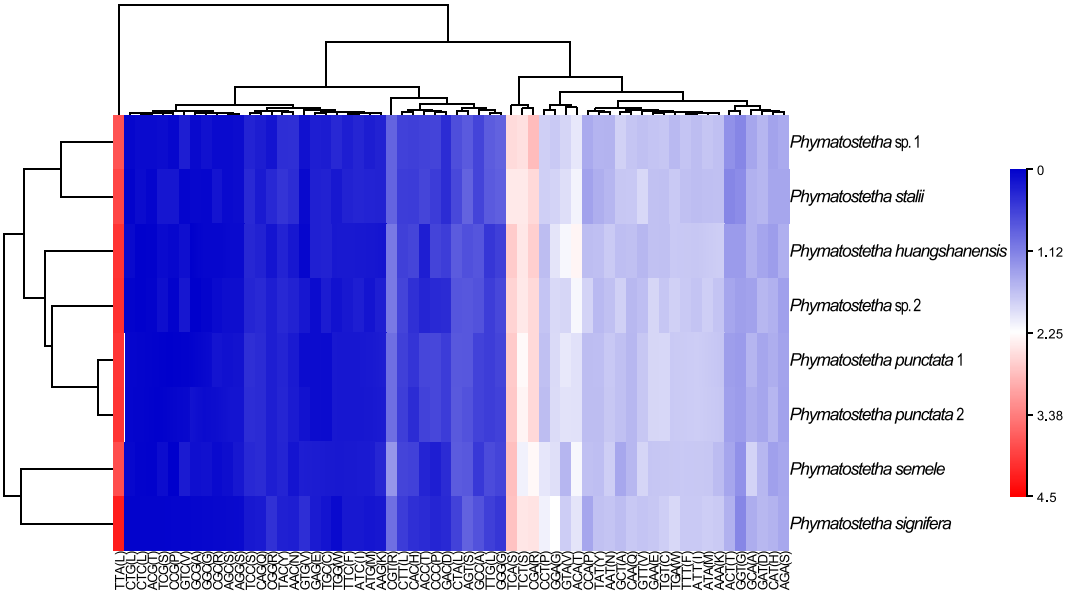

Fig. 3. Relative synonymous codon usage of PCGs in *Phymatostetha* mitogenomes. The x-axis and y-axis exhibit hierarchical clustering of codon frequencies and *Phymatostetha* species, respectively.

most of which were coded on the J-strand (9 PCGs and 13 tRNAs), whereas the other genes (4 PCGs, 9 tRNAs, and two rRNAs) were located on the N-strand (Fig. 1). All the sequenced *Phymatostetha* mitogenomes exhibited identical gene arrangement, which was also consistent with the putative ancestral pattern of insects [7,10].

To better visualize the nucleotide identity in mitogenomes of *Phymatostetha*, the comparable circular map was presented (Fig. 1). Among different genes, *rrnL*, *rrnS*, and some tRNA genes (e.g. *trnL2*, *trnG*, and *trnT*) had much higher sequence conservation. Within PCGs, cytochrome oxidase (*cox*) genes were more conserved with *cox1* showed the highest similarity, whereas NADH dehydrogenase (*nad*) genes were more variable with *nad1* presented the maximal variation.

3.2. Nucleotide composition and codon usage

The nucleotide composition of *Phymatostetha* mitogenomes was biased toward A/T, with rRNA genes exhibited the highest A + T content (Fig. 2; Table S2). However, the dataset of PCG12 (i.e. the first and second codon positions of PCGs) presented the lowest A + T content. The biased usage of A/T could also be reflected in codon frequencies. Relative synonymous codon usage of *Phymatostetha* mitogenomes revealed that degenerate codons were tend to use more A/T than G/C (Fig. 3). For example, the third codon positions of the four most prevalent codons in *Phymatostetha*, including TTA, TCA, TCT, and CGA, were all composed of A/T. Conversely, GC-rich codons were seldom used in *Phymatostetha* species, such as CTG, CTC, and ACG. AT-skew and GC-skew presented similar patterns in all *Phymatostetha* mitogenomes, with positive AT-skew (from 0.115 to 0.166) and negative GC-skew (from −0.219 to −0.129) for the J-strand.

3.3. tRNA and rRNA genes

All 22 tRNAs typical of insect mitogenomes could be predicted in the seven *Phymatostetha* mitogenomes (Fig. 4). Most tRNAs were folded into canonical clover-leaf structure except for *trnS1*, with its dihydrouracil (DHU) arm formed a simple loop. Furthermore, *trnS1* anticodon stem in all *Phymatostetha* mitogenomes presented an unpaired nucleotide. Similar pattern could be found in the acceptor stem of *trnR*.

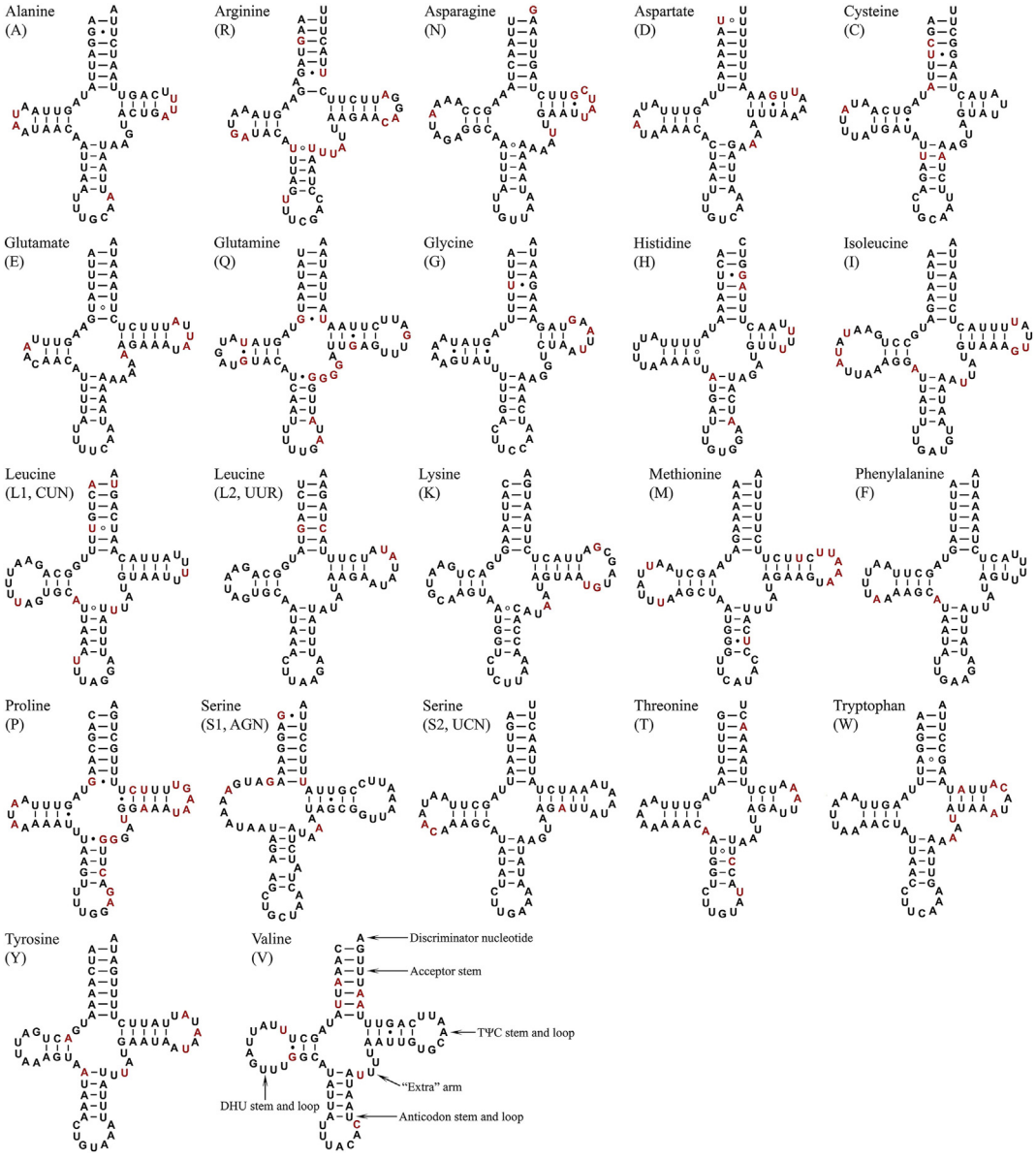

Fig. 4. Secondary structures of 22 tRNAs identified in the mitogenome of *P. semele*. The conserved and variable sites among *Phymatostetha* species are indicated with black and red, respectively.

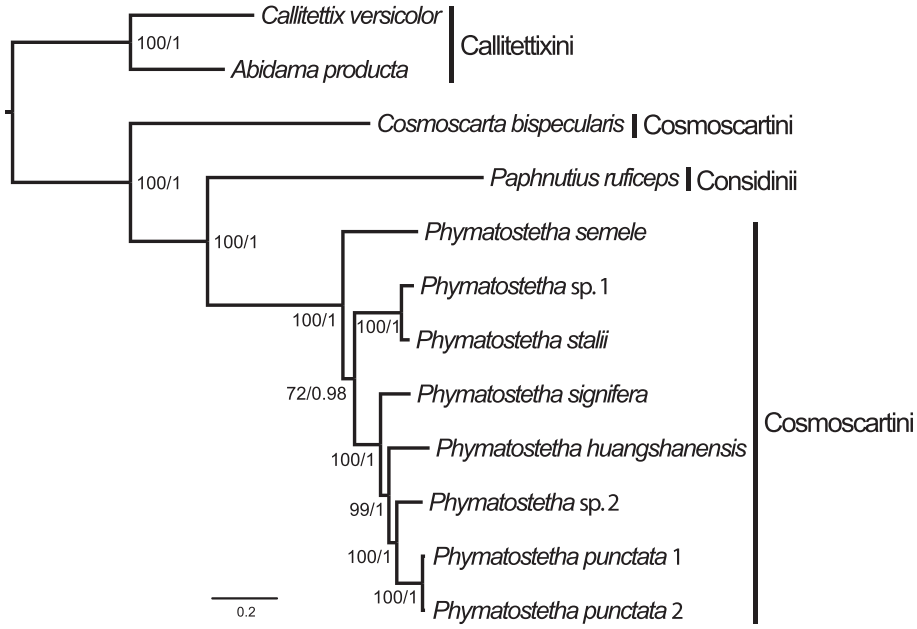

Fig. 5. Phylogenetic tree inferred from mitogenomes of *Phymatostetha*. Numbers at the nodes are Bayesian posterior probabilities (PP, left) and ML bootstrap (BS, right) values.

This was an unusual structure, but had also been proposed in some other hemipteran tRNA stems [34,35].

Except for the anticodon arm, nucleotide substitutions on tRNA stems were always less than the corresponding loops (Fig. 4). The length of acceptor stem (7 bp), anticodon stem (5 bp), and anticodon loop (7 bp) presented high conservation. Except for the DHU arm of *trnS1*, the stems of DHU and TΨC were 3–4 bp and 3–5 bp in length, respectively. However, the extra arm and loops of DHU and TΨC were more variable, with obvious nucleotide substitutions and length variation. Additionally, noncanonical match of G-U and mismatches of U-U, A-A, A-G, and A-C were scattered throughout tRNA stems. These unmatched base pairs had also been reported in other hemipterans [35,36]. They either represented unusual pairings [37] or could be restored through post-transcriptional editing processes [38].

As in other insect mitogenomes, *rrnL* and *rrnS* genes were encoded on N-strand and located at the conserved positions between *trnL1* and *trnV*, and between *trnV* and the control region, respectively (Fig. 1). Among the seven *Phymatostetha* mitogenomes, the length of *rrnL* varied from 1241 bp in *Phymatostetha* sp.1 to 1269 bp in *Phymatostetha stalii* and *P. huangshanensis*, whereas the largest and smallest *rrnS* genes were 771 bp in *Phymatostetha semele* and 783 bp in *P. punctata*, respectively. The A + T content of rRNAs ranged from 78.69% in *Phymatostetha* sp.1 to 80.09% in *P. huangshanensis*.

3.4. Phylogenetic analysis

Phylogenetic analyses were conducted on nucleotide sequences of 13 PCGs, 22 tRNA, and two rRNAs derived from twelve Cercopidae mitogenomes, in which seven *Phymatostetha* mitogenomes were newly sequenced. The BI and ML methods generated fully resolved trees with identical topology (Fig. 5). The monophyly of *Phymatostetha* was robustly supported (PP = 1.00; BP = 100), in which *P. semele* was sister to the remaining *Phymatostetha* species. Although *Phymatostetha* sp.1 was difficult to be identified to species with morphological methods, it was inferred has sister to *P. stalii*. In addition, the genus relationships of (*Cosmoscarta* + (*Phymatostetha* + *Paphnutius*)) were well supported (PP = 1.00; BP = 100), which were also consistent with previous phylogenetic study inferred from mitogenomes of Cicadomorpha [23]. Accordingly, the tribe Cosmoscartini was recovered as a non-monophyletic group with respect to the species of *Paphnutius ruficeps*, which was classified within the tribe Considinii. Therefore, more sampling might be needed to adequately resolve genus relationships within the tribe Cosmoscartini.

This study firstly presented the comparative genomic analyses and phylogenetic relationships within *Phymatostetha*, which would improve our understanding about mitochondrial differentiation and evolution of the genus. It was demonstrated that mitogenome was an effective molecular marker to adequately resolve phylogeny at low taxonomic levels. More mitogenomes at different taxonomic levels will also be useful to improve our understanding of mitogenomic evolution and phylogenetic relationships within Cercopidae.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2018.12.174.

Declarations of interest

The authors declare no conflict of interest.

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