The first complete mitochondrial genome from the genus *Paramenexenus* (Phasmatodea: Lonchodidae) and a phylogenetic analysis of Lonchodidae

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Abstract: Lonchodidae is the family with the largest number of genera in Phasmatodea. Due to the lack of complete mitochondrial genome data for most genera, it is not possible to determine the phylogenetic relationships between different genera in Lonchodidae. This study reports the first complete mitochondrial genome sequence in the genus *Paramenexenus*, represented by the species *P. congnatus*. The mitochondrial genome of *P. congnatus* is 17,315 bp in length and contains 13 protein-coding genes, 2 ribosomal RNA genes, 22 transfer RNA genes, and one non-coding control region. The evolutionary rates of the 13 PCGs in Lonchodidae, the *atp8* and *nad2* genes exhibit higher rates of evolution, while the *cox1* and *cox2* gene have lower rates of evolution. We conducted phylogenetic analyses using maximum likelihood and Bayesian inference methods, with 20 species from 13 genera as ingroups and one species of Phylliidae as outgroups. The phylogenetic trees show that Lonchodidae was mainly divided into two clades, the subfamily Necrosciinae from the monophyletic group, and the genus *Paramenexenus* belonged to Necrosciinae.

Key words: Necrosciinae; mitochondrial genome; phylogeny; stick insect

齿臀蛸属首个完整线粒体全基因组描述及长角棒蛸科系统发育分析（蛸目：长角棒蛸科）

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摘要：长角棒蛸科是蛸目属级阶元数量最多的 1 个科。目前，由于缺乏该科大部分属的线粒体全基因组，因而无法明确各属间的系统发育关系。本研究以拟长瓣齿臀蛸为代表，报道了齿臀蛸属第 1 个线粒体全基因组序列。拟长瓣齿臀蛸的线粒体基因组全长为 17,315 bp，它包含 13 个蛋白质编码基因、2 个核糖体 RNA 基因、22 个转运 RNA 基因和 1 个非编码控制区。本文使用长角棒蛸科中 13 属 20 种为内群，选取叶蛸科 1 种为外群，分别使用最大似然法和贝叶斯法构建长角棒蛸科的系统发育关系。系统发育树显示长角棒蛸科主要分为 2 支，长角枝蛸亚科为单系群，齿臀蛸属属于长角枝蛸亚科。

关键词：长角枝蛸亚科；线粒体基因组；系统发育；竹节虫

Introduction

Stick insects inhabit moist forests, where they conceal themselves on or in close proximity

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to host plants during the daytime and actively forage at night. They play a crucial role as phytophagous insects within forest ecosystems (Blüthgen *et al.* 2006). Lonchodidae is the largest family within Phasmatodea, comprised of two subfamilies (Necrosciinae and Lonchodinae) and encompassing a total of 161 genera. In China, the Lonchodinae consists of 5 genera and 58 species, while Necrosciinae includes 38 genera and 172 species (Brock *et al.* 2025).

Paramenexenus is a genus of the Necrosciinae, exhibiting both tropical and subtropical distributions. It encompasses a total of six species worldwide, with three endemic to Sri Lanka, two to Vietnam and one to China. Specifically, *Paramenexenus congnatus* predominantly inhabits Guangxi and Guangdong (Chen & He 2008). In controlled rearing conditions, we observed that *P. congnatus* primarily feeds on *Michelia figo* and *Rosa chinensis*.

In recent years, there has been a surge in studies focusing on complete mitochondrial genomes and phylogenetic analyses. Notably, the successful sequencing of the complete mitochondrial genomes of numerous stick insect species has been achieved (Kômoto *et al*. 2011; Zhou *et al*. 2017; Song *et al.* 2020; Dong *et al*. 2021; Xu *et al*. 2021; Zhang & Guo 2022; Yuan *et al.* 2023; Clarke *et al*. 2024). According to data obtained from the National Center for Biotechnology Information (NCBI), a complete mitochondrial genome is available for a total of 41 stick insect species worldwide. Among these, Lonchodidae comprises 19 species.

However, the genus *Paramenexenus* lacks available mitochondrial genome sequences. In this study, we used second-generation sequencing (NGS) to obtain the complete mitochondrial genome of *P. congnatus*. By integrating these data with existing complete mitochondrial genomes of Lonchodidae, we constructed a phylogenetic tree using Bayesian Inference (BI) and Maximum Likelihood (ML) methods. The primary objectives were to discuss the taxonomic status of *Paramenexenus* and elucidate the affinities among Necrosciinae's genera while providing a research foundation and molecular data for studying the subfamily Necrosciinae as well as establishing groundwork for future phylogenetic investigations of higher orders within Lonchodidae.

Material and methods

Sample collection and DNA extraction

The specimens of *P. congnatus* were collected on Rentou Mountain, Guangxi, China, at the geospatial coordinates N21.698041°, E107.849648°, in October 2022. All specimens were preserved in 95% ethanol and stored at -30°C. Adult specimens were deposited in the Insect Collection of the Southwest Forestry University. All specimens were identified by the corresponding author before DNA extraction. The total DNA was extracted from the thoracic and leg muscles using the Sangon Genomic DNA Kit (SANGON BIOTECH CO., LTD), following the manufacturer's instructions. The quality and concentration of the total DNA were assessed using a NanoDrop 2000 spectrophotometer and 1.5% agarose gel electrophoresis, respectively.

Mitogenome sequencing and assembly

The isolated DNA of *P. congnatus* was sequenced by the next-generation sequence method on the Illumina NovaSeq platform (Personalbio Technology Co., Ltd, Shanghai, China). The second-generation sequencing data was assembled using A5-miseq v20150522 (Coil & Jospin 2015) and SPAdesv3.9.0 (Bankevich *et al.* 2012). Collinearity analysis was performed using Mummer v3.1 (Kurtz *et al.* 2004) to determine the positional relationship between fragment overlap clusters and fill in any missing sequences between these clusters. The final mitochondrial sequence was obtained after correction with Pilon v1.18 (Walker *et al.* 2014).

Genome annotation and sequence analysis

We utilized the default settings of the MITOS Web Server (Bernt *et al.* 2013) to identify and determine the boundaries of each gene within the mitochondrial genome. Additionally, we analyzed the folding of tRNA secondary structures using this server. In order to accurately predict tRNAs, we selected Mito/Chloroplast as the search source and utilized the genetic code specific to invertebrate mitochondrial genomes. Visual illustrations of the secondary structures of tRNA genes were created by using Adobe Illustrator 2020. To generate the circular mitochondrial genome map of *P. congnatus* (Fig. 1), we used the OGDRAW webserver (Greiner *et al.* 2019). The newly-sequenced mitogenome of *P. congnatus* was submitted to GenBank and assigned the accession number PQ613292.

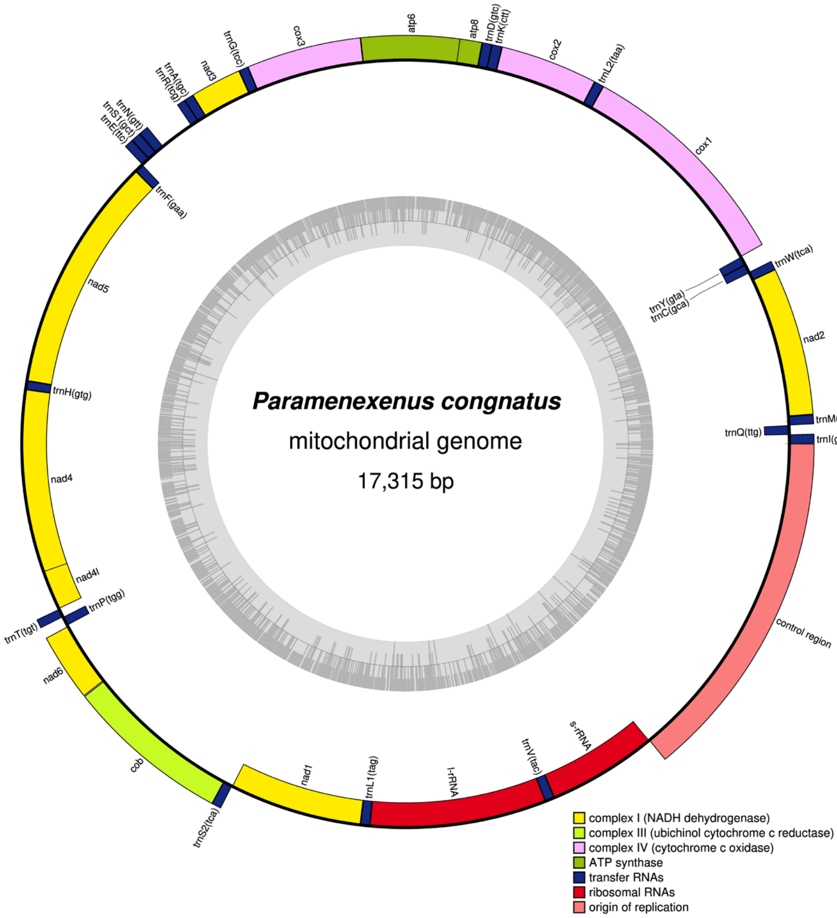

Figure 1. Circular map of the mitochondrial genome of *P. congnatus.*

The nucleotide composition and relative synonymous codon usage (RSCU) of the mitochondrial genome sequences was calculated via the Rscript function. Base composition and RSCU values (relative synonymous codon usage) were analyzed using PhyloSuite 1.2.2 (Zhang *et al.* 2020). The AT and GC-skew values were determined using the formula described in Reyes *et al.* (1998).

Nucleotide diversity (Pi) analyses were performed for the 13 PCGs in Lonchodidae species, and a sliding window analysis (with a 200 bp sliding window and 20 bp step size) was conducted using DnaSP v.6.0 (Rozas *et al*. 2017). Moreover, the software was utilized to calculate the non-synonymous substitution rates (Ka), synonymous substitution rates (Ks), and ratios of non-synonymous to synonymous substitution (Ka/Ks) for each of the concatenated 13 PCGs in Lonchodidae mitochondrial genomes

Phylogenetic analysis

The mitochondrial whole genome of *P. congnatus* and 19 other Lonchodidae taxa were selected as ingroups for this paper (Table 1). A species from Phylliidae was selected as the outgroup for conducting the phylogenetic analyses. Nucleotide and amino acid sequences for the 13 PCGs were aligned using MAFFT v.7 (Katoch & Standley 2013) with the iterative refinement method of E-INS-i and the invertebrate mitochondrial genetic codon table.

Table 1. List of species used to construct the phylogenetic tree

Taxon	Family	Subfamily	Species	GenBank No.	Length
Ingroups	Lonchodidae	Lonchodinae	*Carausius sp.*	OQ682524.1	16198
			Eurycantha calcarata	MW915467.1	16280
			Megalophasma granulatum	KY124331.1	15275
			Phraortes illepidus	AB477460.1	16456
			Phraortes lii	ON493672.1	16456
			Phraortes lianzhouensis	OQ682528.1	16690
			Phraortes sp.	AB477464.1	16867
			Stheneboea repudiosa	OQ682531.1	16376
		Necrosciinae	*Calvisia medogensis*	KY124330.1	16107
			Lopaphus sphalerus	OQ682526.1	17761
			Lopaphus albopunctatus	OQ682525.1	15011
			Micadina phluctainoides	AB477466.1	16507
			Micadina brachyptera	MT025192.1	15879
			Marmessoidea bispina	OQ682527.1	16630
			Neohirasea stephanus	OL405132.1	18021
			Neohirasea japonica	AB477469.1	15305
			Sosibia gibba	OM257176.1	15907
			Sosibia ovata	OM257177.1	16142
			Sipyloidea sipylus	AB477470.1	17001
			Paramenexenus congnatus	PQ613292	17315
outgroup	Phylliidae	Phylliinae	*Pulchriphyllium bioculatum*	OQ682529.1	16868

Phylogenetic analysis of the set of linked nucleotide sequences was performed using Bayesian inference (BI) and maximum likelihood (ML) methods. The optimal partitioning scheme and the corresponding nucleotide substitution model for each dataset were calculated in Phylosuite v1.2.2 by the PartitionFinder2 tool. The BI analysis was conducted using

MrBayes tool and a selected four independent Markov chains were run for 4000000 metropolis-coupled Markov chain Monte Carlo (MCMC) generations, sampling trees every 100 generations. The first 25% of samples were discarded as burn-in, and the remaining trees were used to calculate posterior probabilities (PP) in a 50% majority rule consensus tree. The ML analysis using the IQ-tree tool and choose best-fit model presented by PartitionFinder2 v2.1, and node confidence was assessed with 1,000 bootstrap replications.

Results and discussion

Genome features

The complete mitochondrial genome of *P. congnatus* is 17315 bp in length. It is a single circular DNA molecule containing of 13 protein coding genes (PCGs), 22 tRNAs, 2 rRNAs, and a control region (Fig. 1). The gene content and arrangement of these 37 genes are consistent with the published gene arrangement observed in other Lonchodidae stick insects. Among these, there are a total of 23 genes located on the minority strand (N-strand), including 9 PCGs (*nad2*, *nad3*, *nad6*, *cob*, *cox1*, *cox2*, *cox3*, *atp6*, *atp8*), as well as 14 tRNA genes (*trnI*, *trnM*, *trnW*, *trnL2*, *trnK*, *trnD*, *trnG*, *trnA*, *trnR*, *trnN*, *trnS1*, *trnE*, *trnT*, *trnS2*). The remaining 14 genes (*nad5*, *nad4*, *nad4L*, *nad1*, *trnQ*, *trnC*, *trnY*, *trnF*, *trnH*, *trnP*, *trnL1*, *trnV*, *l-rRNA*, *s-rRNA*) are encoded on the majority strand (J-strand), including 4 PCGs, 8 tRNAs and 2 rRNAs.

Furthermore, 15 gene overlaps are found in the mitochondrial genome, ranging from 1 to 8 bp in length. 6 small non-coding intergenic spacers are found in the mitochondrial genome, ranging from 1 to 249 bp in length. The largest non-coding intergenic spacer (control region) is 2435 bp in length and situated between the *s-rRNA* and *trnI*. Among the 13 PCGs, eleven PCGs have the commonly-observed ATN start codon, while *nad4L* and *nad1* have the atypical GTG and TTG codon. Furthermore, eight PCGs have the typical stop codon TAA or TAG, while *cox1*, *cox2* and *nad1* have incomplete stop codons with a single T residue, and *cox3* and *nad5* have incomplete stop codons with TA residue (Table 2).

Table 2. Annotation of the mitochondrial genome of *P. congnatus*

Gene	Coding strand	Position	Gene Length (bp)	Initiation codon	Stop codon	Intergenic nucleotide
trnI	N	1-67	67			
trnQ	J	65-133	69			-3
trnM	N	133-198	66			-1
nad2	N	199-1,215	1017	ATT	TAA	0
trnW	N	1,214-1,278	65			-2
trnC	J	1,271-1,336	66			-8
trnY	J	1,337-1,401	65			0
cox1	N	1,403-2,941	1534	ATG	T--	1
trnL2	N	2,937-3,001	65			-5
cox2	N	3,002-3,671	670	ATA	T--	0
trnK	N	3,672-3,741	70			0
trnD	N	3,741-3,807	67			-1
atp8	N	3,808-3,963	156	ATT	TAA	0

Table 2 (continued)

Gene	Coding strand	Position	Gene Length (bp)	Initiation codon	Stop codon	Intergenic nucleotide
atp6	N	3,957-4,634	678	ATG	TAA	-7
cox3	N	4,634-5,422	788	ATG	TA-	-1
trnG	N	5,422-5,485	64			-1
nad3	N	5,486-5,839	354	ATT	TAG	0
trnA	N	5,838-5,903	66			-2
trnR	N	5903-5963	61			-1
trnN	N	6213-6281	69			249
trnS1	N	6282-6349	68			0
trnE	N	6350-6413	64			0
trnF	J	6412-6478	67			-2
nad5	J	6479-8202	1724	ATT	TA-	0
trnH	J	8203-8266	64			0
nad4	J	8266-9597	1332	ATG	TAA	-1
nad4L	J	9591-9881	291	GTG	TAA	-7
trnT	N	9884-9947	64			2
trnP	J	9948-10015	68			0
nad6	N	10023-10490	468	ATA	TAA	7
cytb	N	10494-11627	1134	ATG	TAG	3
trnS2	N	11626-11693	68			-2
nad1	J	11695-12660	966	TTG	T(AA)	1
trnL1	J	12661-12729	69			0
rrnL	J	12730-14013	1284			0
trnV	J	14014-14082	69			0
rrnS	J	14083-14880	798			0
CR	N	14881-17315	2435			0

Nucleotide composition and codon usage

Table 3. Nucleotide composition and skewness of *P. congnatus* mitochondrial genome

Region	A%	T%	G%	C%	A+T%	G+C%	AT skew	GC skew
Whole genome	44.42	32.47	9.71	13.40	76.89	23.11	0.155	-0.160
nad2	45.23	34.61	7.18	12.98	79.84	20.16	0.133	-0.288
cox1	33.96	35.98	14.41	15.65	69.94	30.06	-0.029	-0.041
cox2	41.49	33.28	11.19	14.03	74.77	25.22	0.110	-0.113
atp8	48.08	37.18	5.13	9.62	85.26	14.75	0.128	-0.304
atp6	39.68	35.40	9.88	15.04	75.08	24.92	0.057	-0.207
cox3	35.79	35.28	13.58	15.36	71.07	28.94	0.007	-0.062
nad3	40.11	36.72	7.91	15.25	76.83	23.16	0.044	-0.317
nad5	29.18	50.46	12.59	7.77	79.64	20.36	-0.267	0.237
nad4	26.88	51.80	13.14	8.18	78.68	21.32	-0.317	0.233
nad4l	25.09	56.01	12.71	6.19	81.10	18.90	-0.381	0.345
nad6	43.59	39.53	5.13	11.75	83.12	16.88	0.049	-0.392
cytb	36.51	36.77	11.64	15.08	73.28	26.72	-0.004	-0.129
nad1	26.09	51.97	13.56	8.39	78.06	21.95	-0.332	0.236
rrnL	30.14	48.91	13.55	7.40	79.05	20.95	-0.237	0.294
rrnS	33.21	43.86	14.29	8.65	77.07	22.94	-0.138	0.246

The *P. congnatus* mitogenome exhibited a significantly higher A+T content with a relative content of 76.89%. The base A accounted for 44.42 %, T for 32.47 %, G for 9.71 %, and C for 13.4 %. The lowest A+T content was observed in cox1 at 69.94%, while the highest was found in atp8 at 85.26%. The AT-skew and GC-skew values for the *P. congnatus* whole genome were calculated as 0.155 and -0.16 respectively (Table 3).

The Relative Synonymous Codon Usage (RSCU) in the mitochondrial genome of *P. congnatus* is shown in Figure 2. Differences in nucleotide composition are reflected not only in AT and GC offsets but also in codon usage. Leu2 (UUA), Arg (CGA), Ser2 (UCA) and Thr (ACA) were observed to be the most frequently used codons. The amino acid compositions were mostly A or U, indicating the strong AT bias in the whole mitochondrial genome. However, the codons Gln (CAG), Arg (CGC) and Arg (CGG) were missing.

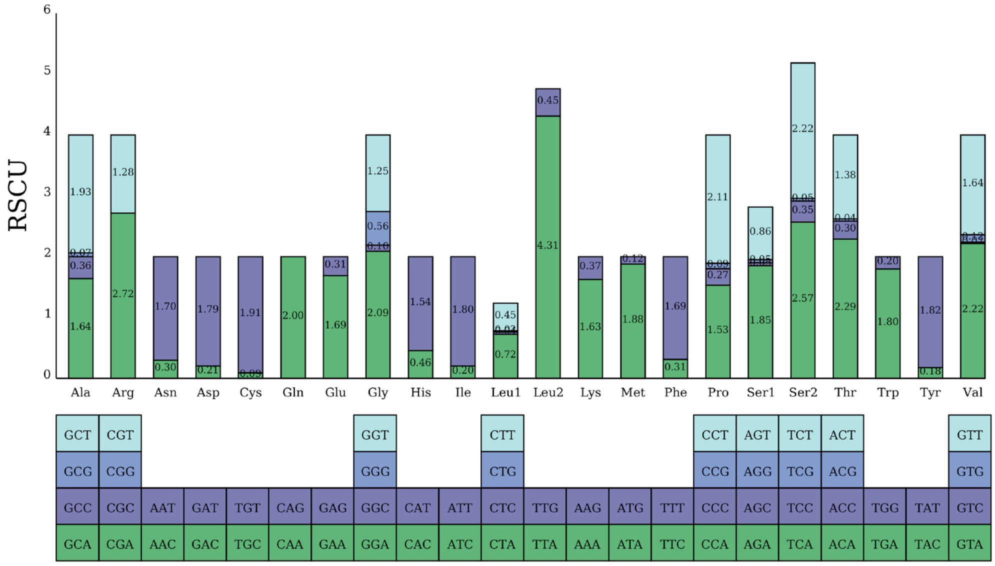

Figure 2. Codon usage of the mitochondrial genome of *P. congnatus*. RSCU: relative synonymous codon usage. Codon families are indicated on the X axis and frequency of RSCU on the Y axis.

Transfer RNA and ribosomal RNA genes

The mitochondrial genome of *P. congnatus* had 22 tRNA genes, as in other Phasmatodea mitogenomes. The lengths of tRNAs varied from 61 nucleotides (*trnR*) to 70 (*trnK*). The secondary structures of these tRNAs are depicted in Fig. 3 where 21 tRNAs have a typical cloverleaf structure and *trnS1* lacks a dihydrouridine (DHU) arm due to unmatched base pairs. The *rrnL* gene is predicted to be between *trnV* and *trnL1*. It is 1284 bp long with an A+T content of 79.05%. The *rrnS* gene has 798 bp in length and has 77.07% A+T content. The *rrnL* and *rrnS* genes are separated by trnV. For the two rRNAs, the AT-skews are negative and the GC-skews are positive.

Nucleotide diversity and evolutionary rate in Lonchodidae

The nucleotide diversity and evolutionary rates of the 13 PCGs in Lonchodidae are shown in Table 4. The nucleotide diversity ranges from 0.147 (*cox1*) to 0.300 (*atp8*). The *atp8* gene (nucleotide diversity index, Pi = 0.300) exhibits the highest nucleotide diversity among all the PCGs, followed by the *nad2* gene (Pi = 0.242), *nad6* gene (Pi = 0.236), *atp6* gene (Pi = 0.223), *nad5* gene (Pi = 0.213), *nad3* gene (Pi = 0.211), and *nad4* gene (Pi = 0.206). In contrast, the *cox1* gene (Pi = 0.147), *nad1* gene (Pi = 0.167), and *cox2* gene (Pi = 0.177) have relatively lower nucleotide diversity indices and are considered conservative genes. For the evolutionary rates of the 13 PCGs in Lonchodidae, the *atp8* gene (0.931), *nad6* gene (0.547) and *nad2* gene (0.528) exhibit higher rates of evolution, while the *cox1* gene (0.104) and *cox2* gene (0.220) have lower rates of evolution.

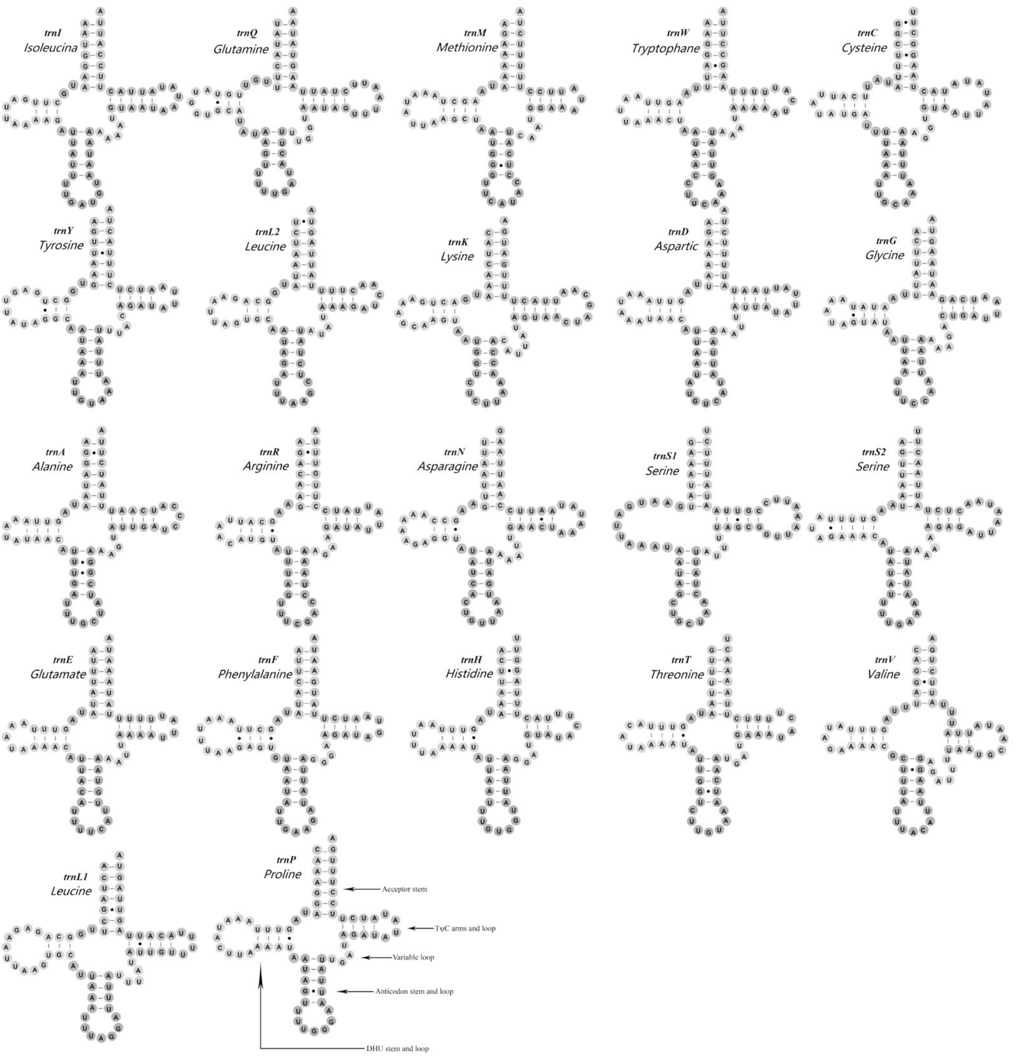

Figure 3. The secondary structures of tRNA genes inferred for the mitogenome of *P. congnatus*. Watson-Crick pairs are indicated by lines, wobble GU pairs are indicated by dots. The non-canonical pairs are not marked.

Table 4. Nucleotide diversity and evolution rate analysis of the 13 PCGs in the mitochondrial genome of Lonchodidae

Gene	Nucleotide diversity	Ka	Ks	Ka/Ks
nad2	0.242	0.206	0.390	0.528
cox1	0.147	0.050	0.478	0.104
cox2	0.177	0.101	0.459	0.220
atp8	0.300	0.296	0.318	0.931
atp6	0.223	0.161	0.446	0.361
cox3	0.195	0.120	0.467	0.257
nad3	0.211	0.155	0.440	0.352
nad5	0.213	0.174	0.357	0.488
nad4	0.206	0.163	0.361	0.451
nad4L	0.196	0.148	0.374	0.397
nad6	0.236	0.203	0.371	0.547
cytb	0.195	0.116	0.479	0.242
nad1	0.167	0.118	0.345	0.342

Phylogenetic analyses

We used concatenated amino acid and nucleotide sequences of the 13 PCGs from 2 subfamilies in the family Lonchodidae, with Phylliidae as the outgroup (Fig. 4). The ML tree shows that two subfamilies (Lonchodinae and Necroscinae) of Lonchodidae clustered on different branches and Necroscinae was monophyletic. The *P. congnatus* belongs to the subfamily Necrosciinae, the generic relationships of Necrosciinae are *Paramenexenus* + [(*Sipyloidea* + (*Marmessoide* + (*Calvisia* + *Sosibia*)))] + [(*Lopaphus* + *Neohirasea* + *Micadina*)]. In the BI tree, the relationships are consistent with the outcome of the ML analysis.

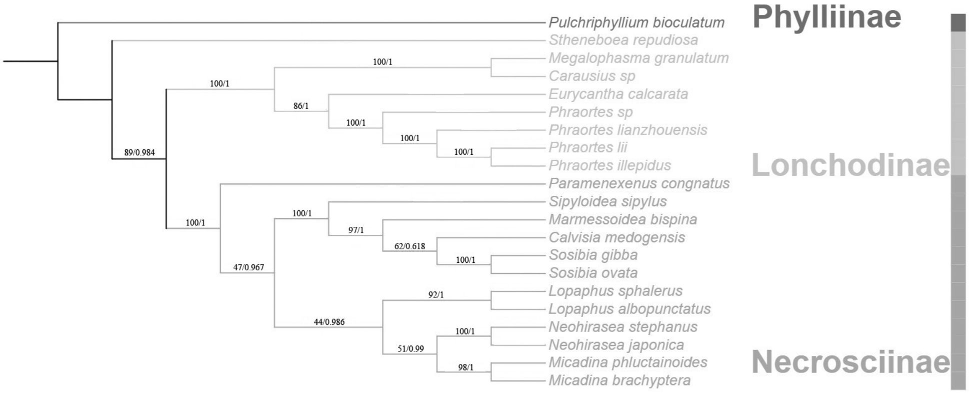

Figure 4. Phylogenetic tree from the nucleotide sequences of 13 PCGs constructed based on Maximum Likelihood (ML) and Bayesian Inference method (BI). Numbers at the branches represent bootstrap support (%) and Bayesian probabilities.

Conclusions and discussion

The mitochondrial genome of *P. congnatus* was successfully sequenced for the first time. The sequencing results revealed a length of 17,315 bp and include 13 protein-coding genes, 2 ribosomal RNA, 22 transfer RNA and one control region. Consistent with most Phasmida species, the total AT content was significantly higher than GC content. Furthermore, the absence of the DHU arm in trnS1 and the presence of a typical cloverleaf model in the remaining tRNA molecules are consistent with other insects' characteristics. Previous studies have reported gene rearrangement in species *Dajaca napolovi* and *Orthomeria smaragdinum* from the Aschiphasmatinae of Aschiphasmatidae, and *Megalophasma granulatum* from the Lonchodinae of Lonchodidae (Li *et al.* 2022), but no gene rearrangement was observed in *P. congnatus*.

Phylogenetic analysis results and morphological classification feature all support that *Paramenexenus* belongs to the subfamily Necrosciinae. Necrosciinae encompasses a total of 113 genera worldwide, out of which 8 genera possess complete mitochondrial genome data. Due to limited availability of molecular data for other genera in the Necrosciinae, we were unable to conclusively discuss their relationship with other genera within the Lonchodidae. Future research endeavors should prioritize the acquisition of additional molecular data from Lonchodidae in order to augment our comprehension of both phylogenetic relationships and morphological evolution not only within *Paramenexenus* but also across Phasmatodea as a whole.

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