The first complete mitochondrial genome of subfamily Eustrotiinae (Lepidoptera: Noctuidae): genome description and its phylogenetic implications

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Abstract: The Noctuidae is the largest family within the order Lepidoptera. Many species of moths in Noctuidae are known to be significant pests for crops. However, there is currently a lack of available mitochondrial genome sequences for the subfamily Eustrotiinae, a clade within the Noctuidae. In this study, we report the first complete mitochondrial genome from the subfamily Eustrotiinae, represented by the species of *Maliattha signifera*. The mitochondrial genome of *M. signifera* is 15,430 bp in length and contains 13 protein-coding genes, two ribosomal RNA genes, 22 transfer RNA genes, and one non-coding control region. We conducted phylogenetic analyses using maximum likelihood and Bayesian inference methods, with 60 exemplars of Noctuidae as ingroups and two species of Euteliidae as outgroups. The phylogenetic trees show that the subfamilies Plusiinae, Bagisarinae, Heliothinae, Cuculliinae, Noctuinae, Ipimorphinae, and Eustrotiinae form the monophyletic groups, while the subfamilies Hadeninae, Xyleninae, Acronictinae, and Amphipyrinae are found to be non-monophyletic. The aim of this study is to explore the phylogenetic relationship among subfamilies within Noctuidae and clarify the phylogenetic placement of *M. signifera* through analysis of mitochondrial genome data.

Key words: Noctuoidea; *Maliattha signifera*; mitochondrial genome; gene rearrangement; phylogeny

文夜蛾亚科第一个完整的线粒体基因组描述及系统发育分析（鳞翅目：夜蛾科）

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摘要：夜蛾科是鳞翅目中物种数量最多的科。夜蛾科的一些蛾类昆虫是作物上的重要害虫。然而，目前缺乏夜蛾科中文夜蛾亚科的线粒体基因组序列。本研究以标瑙夜蛾为代表，报道了文夜蛾亚科第 1 个线粒体全基因组序列。标瑙夜蛾的线粒体基因组全长为 15,430 bp，包含 13 个蛋白质编码基因、2 个核糖体 RNA 基因和 22 个转运 RNA 基因以及 1 个非编码控制区。本文使用夜蛾科中的 60 个物种为内群研究对象，并选取 2 个尾夜蛾科昆虫作为外群，分别使用最大似然法和贝叶斯法构建夜蛾科的系统发育关系。系统发育树显示，金翅夜蛾亚科、青夜蛾亚科、实夜蛾亚科、冬夜蛾亚科、夜蛾亚科、Ipimorphinae 和文夜蛾亚科为单系群，而盗夜蛾亚科、木夜蛾亚科、剑纹夜蛾亚科和杂夜蛾亚科为非单系群。本研究旨在通过对线粒体基因组数据的分析，研究夜蛾科中各亚科之间的系统发育关系，并阐明标瑙夜蛾的系统发育地位。

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关键词：夜蛾总科；标瑙夜蛾；线粒体基因组；基因重排；系统发育

Introduction

Over the past few years, there has been a significant advancement in high-throughput sequencing technology which has led to the widespread use of mitochondrial genomes in insect systematic studies. This is primarily due to the numerous advantages associated with these genomes. For instance, they have a relatively small gene content and high copy numbers in each cell, making them easy to sequence and assemble. Additionally, they have a relatively conservative organizational structure and exhibit maternal inheritance. Moreover, mitochondrial genomes in insects have a fast evolution rate, making them useful for evolutionary studies (Cameron 2014; Simon *et al*. 1994, 2006).

In insects, the mitochondrial genome is typically a circular molecule that encodes a total of 37 genes. These genes include 13 protein-coding genes (PCGs), two ribosomal RNA (rRNA) genes, and 22 transfer RNA (tRNA) genes (Cameron 2014). In addition to coding genes, there is a non-coding control region in the mitochondrial genome, known as the A+T-rich region. This region plays a crucial role in the replication and transcription of the mitochondrial genome (Boore 1999).

The noctuid moth family (Noctuidae) is one of the largest families within the order Lepidoptera, and it comprises a diverse range of species (Heppner 1991). Many genera within this family, such as *Spodoptera* and *Helicoverpa*, are notorious for their economically destructive feeding behavior. Species like *Spodoptera frugiperda* and *Helicoverpa armigera* cause significant crop losses, amounting to millions of pounds annually (Karim 2000; Montezano *et al*. 2018). Despite their economic importance, research on the phylogeny of Noctuidae has been limited, hindering a comprehensive understanding of its evolutionary relationships. Therefore, characterizing mitochondrial genomes from additional species within this family would greatly increase our understanding of genomic evolution and contribute to phylogenetic analyses.

Currently, there are no existing mitochondrial genome sequences available for Eustrotiinae, a subfamily within the Noctuidae. In this study, we successfully obtained the complete mitochondrial genome of *Maliattha signifera*, which represents the first species within the subfamily Eustrotiinae. We conducted sequencing, assembling, annotation, and analysis of the mitochondrial genome of *M. signifera*, examining its characteristics and determining its phylogenetic position within the Noctuidae family. Additionally, we compared it with all other accessible mitochondrial genome sequences within the Noctuidae to analyze subfamily-level phylogenetic relationships and elucidate the phylogenetic placement of *M. signifera*.

Material and methods

Sample collection and DNA extraction

Adult specimens of *M. signifera* were collected on Yaoshan Mountain, Henan Province, China, at the geospatial coordinates N33°47′21″, E112°19′40″, on July 2022. These

specimens were preserved in 95% ethanol and stored at -80 °C. Adult specimens were deposited in the Entomological Museum of Henan Agricultural University. The total DNA was extracted from the thoracic muscles using the TIANamp Genomic DNA Kit (TIANGEN 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 *M. signifera* was sequenced using high-throughput sequencing (MGI2000 platform, BGI Genomics Co., Ltd, Wuhan, China). A total of 2 gigabytes of raw sequencing data was generated. Quality control was performed using NGS QC Toolkit v2.3.3 (Patel & Jain 2012) with its default settings. To assemble the mitogenome, the GetOrganelle v1.7.5.2 (Jin *et al*. 2020) assembler was utilized with the animal_mt database (-F animal_mt) to identify, filter, and assemble the reads specifically associated with our target.

Mitogenome annotation and analyses

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 CS6. To generate the circular mitochondrial genome map of *M. signifera* (Fig. 1), we employed the OrganellarGenomeDRAW webserver (Greiner & Lehwark 2019). Line maps depicting the gene order for mitochondrial genomes within the Noctuidae family were drawn using PhyloSuite (Zhang *et al*. 2020). The newly sequenced mitogenome of *M. signifera* has been submitted to GenBank and assigned the accession number OQ111926.

The MEGA X (Kumar *et al*. 2018) was utilized to calculate the nucleotide composition and relative synonymous codon usage (RSCU) of the mitochondrial genome sequences. The AT and GC-skew values were determined using the formula described in Reyes *et al*. (1998). Additionally, we employed the tool CREx (http://pacosy.informatik.uni-leipzig.de/crex/form) (Bernt *et al*. 2007) to identify the most parsimonious explanation for the gene arrangement observed in Noctuidae.

Nucleotide diversity (*Pi*) analyses were performed for the 13 PCGs in Noctuidae species, and a sliding window analysis (with a 200 bp sliding window and 20 bp step size) was conducted using DnaSP v.5.0 (Librado & Rozas 2009). 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 Noctuidae mitochondrial genomes.

Phylogenetic analysis

As ingroups, we included 60 mitochondrial genomes from eleven subfamilies of Noctuidae. Two species from Euteliidae were selected as outgroups for 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. Poorly aligned sites of the 13 PCGs genes

were removed using trimAI (Capella-Gutiérrez *et al*. 2009) with the "automed1" selection parameter settings as described above. Individual gene alignments were then concatenated using FASconCAT-G_v1.04 (Kück & Longo 2014). Two datasets were compiled for phylogenetic analysis: 1) PCG_aa: amino acid sequences of the 13 PCGs; and 2) PCG_nt: nucleotide sequences of the 13 PCGs.

Phylogenetic analyses were conducted using Maximum Likelihood (ML) and Bayesian Inference (BI) approaches. ML analyses were performed using IQ-TREE 2.2.2 (Nguyen *et al*. 2015), and branch support (BS) values were computed using ultrafast bootstrap (Minh *et al*. 2013) with 10,000 replicates.

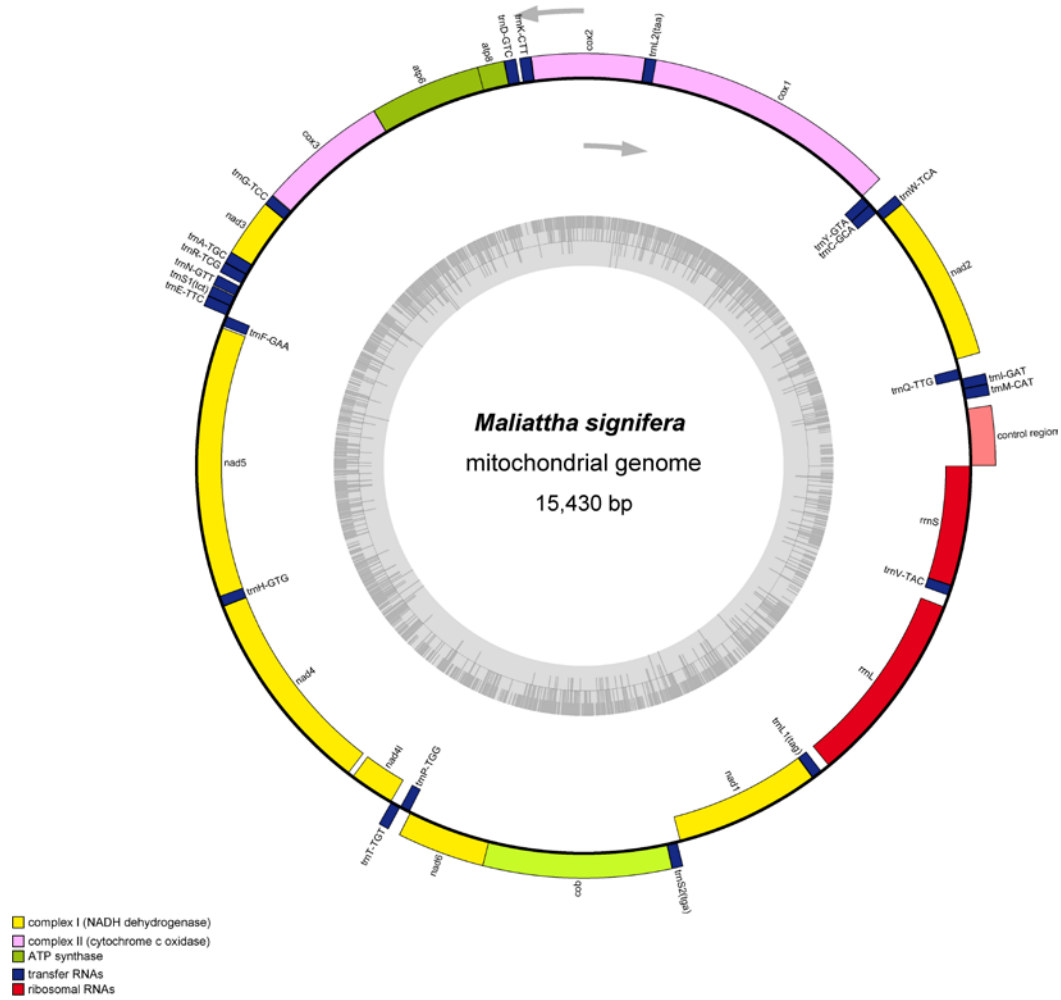

Figure 1. Circular map of the mitochondrial genome of *M. signifera*. The outer circle shows the gene map of *M. signifera*, with the genes outside the map encoded on the heavy strand (H-strand), whereas genes on the inside of the map are encoded on the light strand (L-strand). Genes are represented by different colour blocks.

The Bayesian phylogenetic analysis was conducted using MrBayes v3.2 software (Ronquist *et al*. 2012). The analysis utilized the Markov chain Monte Carlo (MCMC) method, running for a total of 10^7 generations. Every 1,000 generations, a sample was collected, with

the initial 25% of trees discarded as burn-in. Ultimately a phylogenetic tree was generated as the final result.

Results and discussion

Mitochondrial genome organisation and nucleotide composition

The complete mitogenome of *M. signifera* spans a length of 15,430 bp and contains the typical gene set of insect mitochondrial genomes, including two rRNAs, 22 tRNAs, 13 PCGs and a non-coding sequence (Table 1). In the mitochondrial genome of *M. signifera*, the H strand (heavy strand) contains a total of 23 genes. Among these genes, 9 are responsible for encoding proteins, while the remaining 14 genes are involved in the synthesis of transfer RNA molecules. On the other hand, the L strand (light strand) harbors 14 genes, including 4 protein-coding genes, 8 transfer RNA genes, and 2 ribosomal RNA genes. Furthermore, a control region of 362 base pairs in length is situated between the *rrnS* and *trnM* genes, playing a crucial role in regulating gene transcription.

Table 1. Annotation of the mitochondrial genome of *M. signifera*

Gene	Gene length	Start position	Stop position	Start codon	Stop codon	Coding strand
trnM	68	423	490			H
trnI	65	491	555			H
trnQ	69	621	553			L
nad2	1008	682	1689	ATT	TAA	H
trnW	67	1688	1754			H
trnC	68	1814	1747			L
trnY	66	1880	1815			L
cox1	1539	1887	3425	CGA	TAA	H
trnL2	67	3421	3487			H
cox2	685	3488	4172	ATG	T	H
trnK	71	4173	4243			H
trnD	72	4263	4334			H
atp8	165	4335	4499	ATC	TAA	H
atp6	678	4493	5170	ATG	TAA	H
cox3	789	5170	5958	ATG	TAA	H
trnG	65	5961	6025			H
nad3	354	6026	6379	ATT	TAG	H
trnA	71	6378	6448			H
trnR	65	6450	6514			H
trnN	66	6536	6601			H
trnS1	66	6606	6671			H
trnE	66	6672	6737			H

Continured Table 1.

Gene	Gene length	Start position	Stop position	Start codon	Stop codon	Coding strand
trnF	68	6808	6741			L
nad5	1743	8561	6819	ATT	TAA	L
trnH	69	8627	8559			L
nad4	1339	9966	8628	ATG	T	L
nad4l	291	10292	10002	ATG	TAA	L
trnT	65	10360	10296			H
trnP	65	10425	10361			L
nad6	534	10433	10966	ATA	TAA	H
cob	1146	10960	12105	ATG	TAA	H
trnS2	68	12104	12171			H
nad1	939	13129	12191	ATT	TAA	L
trnL1	68	13198	13131			L
rrnL	1259	14515	13257			L
trnV	67	14658	14592			L
rrnS	772	15430	14659			L
Control region	362	1	362			Non-coding sequence

Note: H: Heavy strand; L: Light strand; “T” indicates the incomplete stop codon.

PCGs and codon usage

Twelve out of the thirteen PCGs in the genome have the commonly observed ATN start codon, while *cox1* stands out with the atypical CGA codon. This CGA codon, which is present in the majority of mitochondrial genome sequences from other Lepidoptera species, is quite intriguing (Kim *et al*. 2010, 2018; Park *et al*. 2016; Jeong *et al*. 2021, 2022). These findings provide valuable insights for further exploration into the evolution and genetic variations of these species. Ten of the PCGs in the genome end with the typical stop codons TAA or TAG, while *cox2* and *nad4* have incomplete stop codons with a single thymine (T) residue.

The four PCGs on the L strand have a total length of 4,311 bp. They exhibit an AT content of 81.5% and a GC content of 18.5%. The AT skew is -0.193, and the GC skew is 0.326. The eight transfer RNA genes on the L strand have a total length of 540 bp. They have an AT content of 82.6% and a GC content of 17.4%. The AT skew is 0.027, and the GC skew is 0.383. The two ribosomal RNA genes on the L strand have a total length of 2,031 bp. They have an AT content of 84.9% and a GC content of 15.1%. The AT skew is 0.003, and the GC skew is 0.325. The nine PCGs on the H strand have a total length of 6,897 bp. They have an AT content of 78.5% and a GC content of 21.5%. The AT skew is -0.115, and the GC skew is -0.114. The fourteen transfer RNA genes on the H strand have a total length of 942 bp. They have an AT content of 81.4% and a GC content of 18.6%. The AT skew is 0.048, and the GC skew is 0.029 (Table 2).

Table 2. Nucleotide composition and skewness of the *M. signifera* mitochondrial genome

Feature	Size (bp)	AT (%)	AT-skew	GC-skew
PCGs-H	6,897	78.5	-0.115	-0.114
PCGs-L	4,311	81.5	-0.193	0.326
rrnL-L	1,259	85.2	0.005	0.290
rrnS-L	772	84.6	0.002	0.378
rRNAs-L	2,031	84.9	0.003	0.325
tRNAs-H	942	81.4	0.048	0.029
tRNAs-L	540	82.6	0.027	0.383
Whole genome	15,430	81.2	0.007	-0.202

Note: H: Heavy strand; L: Light strand.

Analysis of nucleotide diversity and evolutionary rate in the family Noctuidae

The nucleotide diversity and evolutionary rates of the 13 PCGs in Noctuidae are shown in Table 3. The nucleotide diversity ranges from 0.091 (*cox1*) to 0.170 (*nad6*). The *nad6* gene (nucleotide diversity index, *Pi* = 0.170) exhibits the highest nucleotide diversity among all the PCGs, followed by the *atp8* gene (*Pi* = 0.137), *cob* gene (*Pi* = 0.118), and *nad2* gene (*Pi* = 0.113). In contrast, the *cox1* gene (*Pi* = 0.091), *cox2* gene (*Pi* = 0.093), and *nad4* gene (*Pi* = 0.093) have relatively lower nucleotide diversity indices and are considered conservative genes.

Table 3. Nucleotide diversity and evolution rate analyses of the 13 PCGs in the mitochondrial genome of Noctuidae

Gene	Nucleotide diversity	*Ka*	*Ks*	*Ka/Ks*
atp6	0.109	0.043	0.332	0.130
atp8	0.137	0.108	0.273	0.396
cox1	0.091	0.017	0.319	0.053
cox2	0.093	0.032	0.304	0.105
cox3	0.105	0.033	0.357	0.092
cob	0.118	0.040	0.372	0.108
nad1	0.109	0.066	0.249	0.265
nad2	0.113	0.065	0.296	0.220
nad3	0.112	0.048	0.352	0.136
nad4	0.093	0.057	0.219	0.260
nad4l	0.095	0.062	0.215	0.288
nad5	0.102	0.064	0.230	0.278
nad6	0.170	0.119	0.350	0.340

Regarding the evolutionary rates of the 13 PCGs in Noctuidae, the *atp8* gene (0.396), *nad6* gene (0.340), and *nad4l* gene (0.288) exhibit higher rates of evolution, while the *cox1* gene (0.053) and *cox3* gene (0.092) have lower rates of evolution.

The Relative Synonymous Codon Usage (RSCU) values for the mitochondrial genome of *M. signifera* were computed and are presented in Fig. 2.

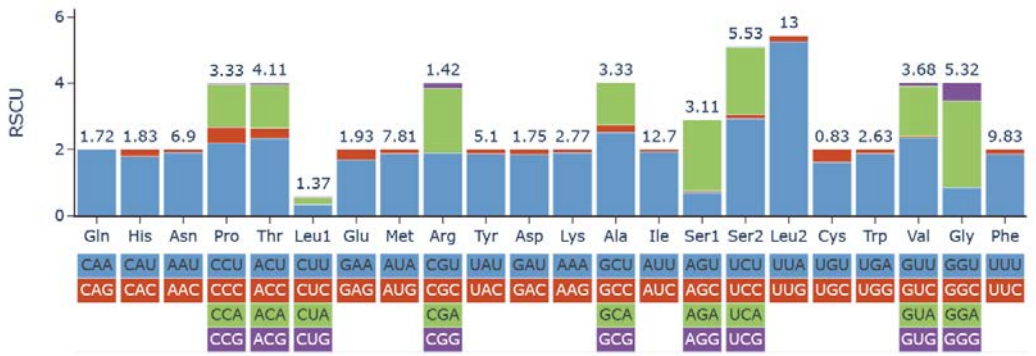

Figure 2. Codon usage of the mitochondrial genome of *M. signifera*. RSCU: relative synonymous codon usage. Codon families are indicated on the X axis and frequency of RSCU on the Y axis.

Gene rearrangement

The gene order of *M. signifera* (Eustrotiinae) differs considerably from that of the putative insect species but it is comparatively similar to other gene orders of Noctuidae species. The main difference lies in the rearrangement of the tRNA cluster of *trnI–trnQ–trnM* to *trnM–trnI–trnQ*) (Fig. 3).

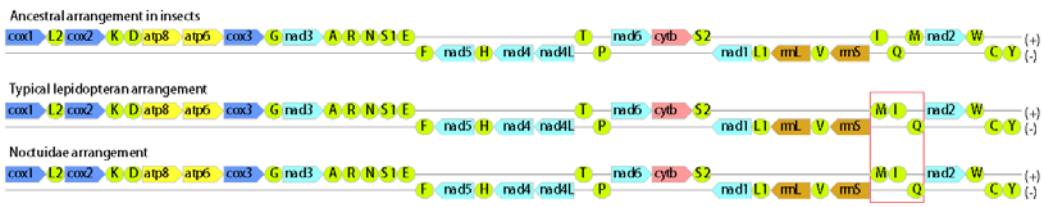

Figure 3. Linear arrangement of the mitochondrial genome of Noctuidae. Genes shown with "-" signs are located on the minor strand (L-strand), while others are located on the major strand (H-strand).

tRNA genes and rRNA genes

The lengths of tRNAs varied from 65 nucleotides (*trnI*, *trnG*, *trnR*, *trnT* and *trnP*) to 72 (*trnD*). The secondary structures of these tRNAs were depicted in Fig. 4. Out of the 22 tRNAs, only 21 could be folded into typical cloverleaf shapes. The tRNA genes *trnS1* lacked the Dihydorouridine (DHU) arm due to unmatched base pairs.

The *rrnL* gene has a length of 1,259 base pairs, with an AT content of 85.2%. The AT skew is 0.005, while the GC skew is 0.290. The *rrnS* gene is 772 base pairs long, with an AT content of 84.6%. The AT skew is 0.002, while the GC skew is 0.378. These genetic characteristics provide valuable insights into the composition and structure of the *rrnL* and *rrnS* genes (Table 2).

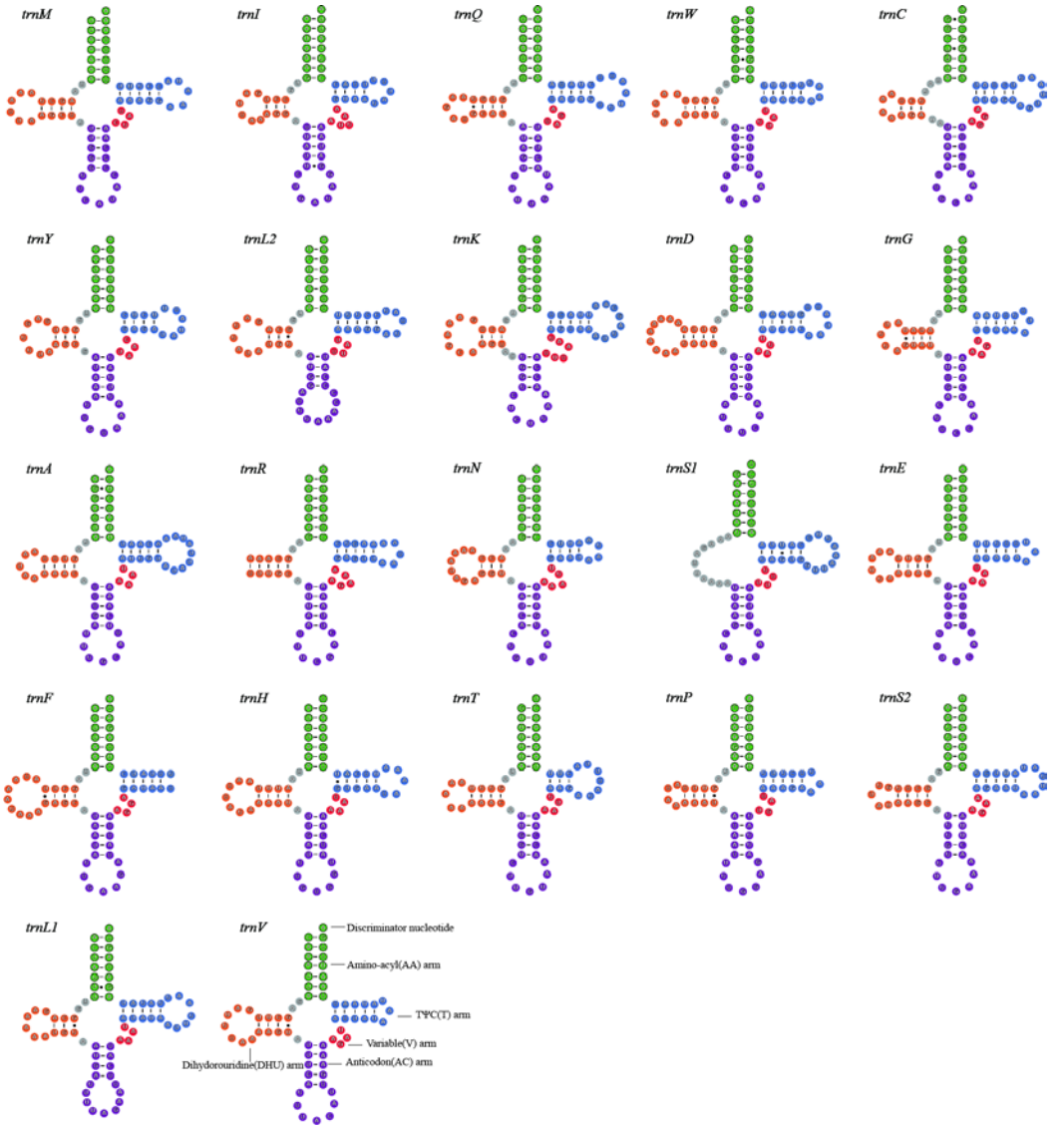

Figure 4. The secondary structures of tRNA genes inferred for the mitogenome of *M. signifera*. Watson-Crick pairs are indicated by lines, and wobble GU pairs are indicated by dots. The non-canonical pairs are not marked.

Phylogenetic analysis

Based on the analysis of nucleotide and amino acid datasets using two different phylogenetic methods, the phylogenetic trees were constructed (Figs 5–8). The results reveal that Plusiinae, Bagisarinae, Heliothinae, Cuculliinae, Noctuinae, Ipimorphinae, and Eustrotiinae formed monophyletic groups. However, the subfamilies Hadeninae, Xyleninae, Acronictinae, and Amphipyrinae, were found to be non-monophyletic.

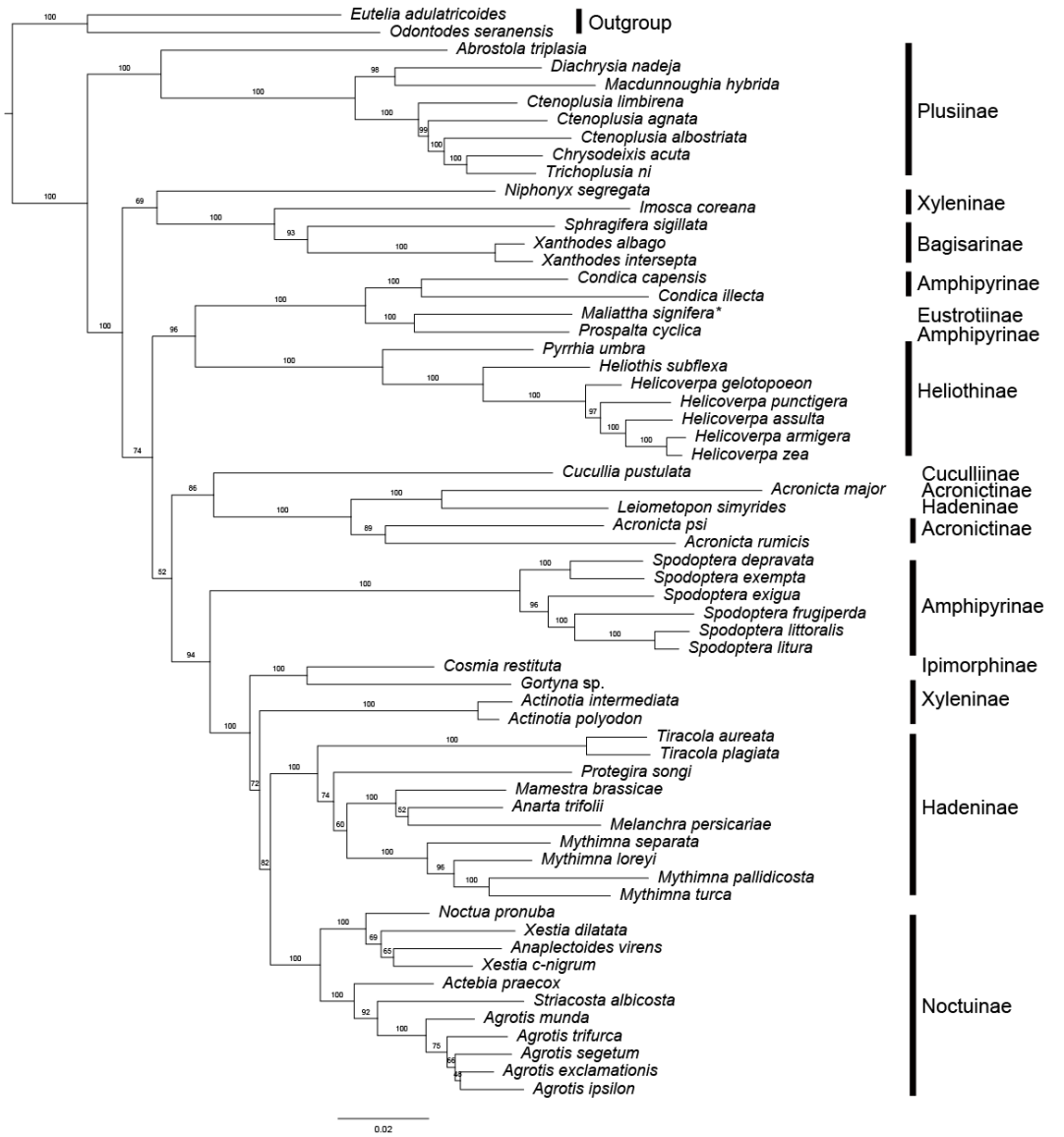

Figure 5. Phylogenetic tree from the amino acid sequences of 13 PCGs constructed based on maximum likelihood method.

This result was consistent with the findings of Xue *et al*. (2019), who employed 13 PCGs from 20 species of Noctuidae to construct the phylogeny. Their study indicated that Heliothinae and Plusiinae formed monophyletic groups, while Amphipyrinae were non-monophyletic (Xue *et al*. 2019). Similarly, Chen *et al*. (2022) utilized 13 protein-coding genes from Noctuoidea, encompassing 25 species of Noctuidae, to construct the phylogeny and also observed Heliothinae and Plusiinae as monophyletic (Chen *et al*. 2022). In addition, Mitchell *et al*. utilized two protein‐coding nuclear genes supporting Hadeninae as non-monophyletic (Mitchell *et al*. 2006). However, there are disparities with Xue *et al*. (2019) who identified Hadeninae and Acronictinae as monophyletic groups, and with Chen *et al*.

(2022) who found Hadeninae, and Amphipyrinae to be monophyletic (Xue *et al*. 2019; Chen *et al*. 2022). These inconsistencies may be attributed to the limited mitochondrial genomic data used by Xue *et al*. (2019) and Chen *et al*. (2022) which could account for the divergent outcomes when compared to our study.

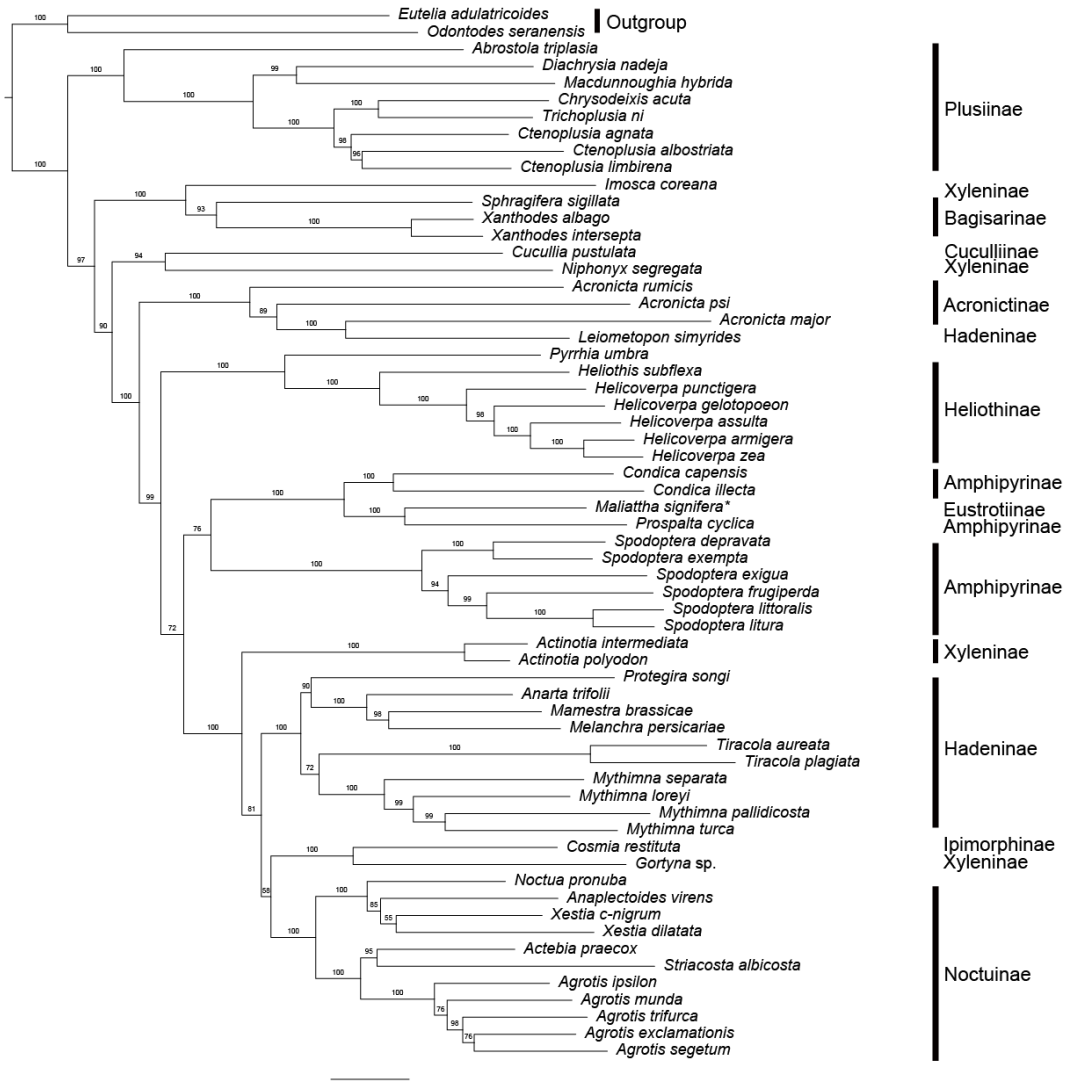

Figure 6. Phylogenetic tree from the nucleotide sequences of 13 PCGs constructed based on maximum likelihood method.

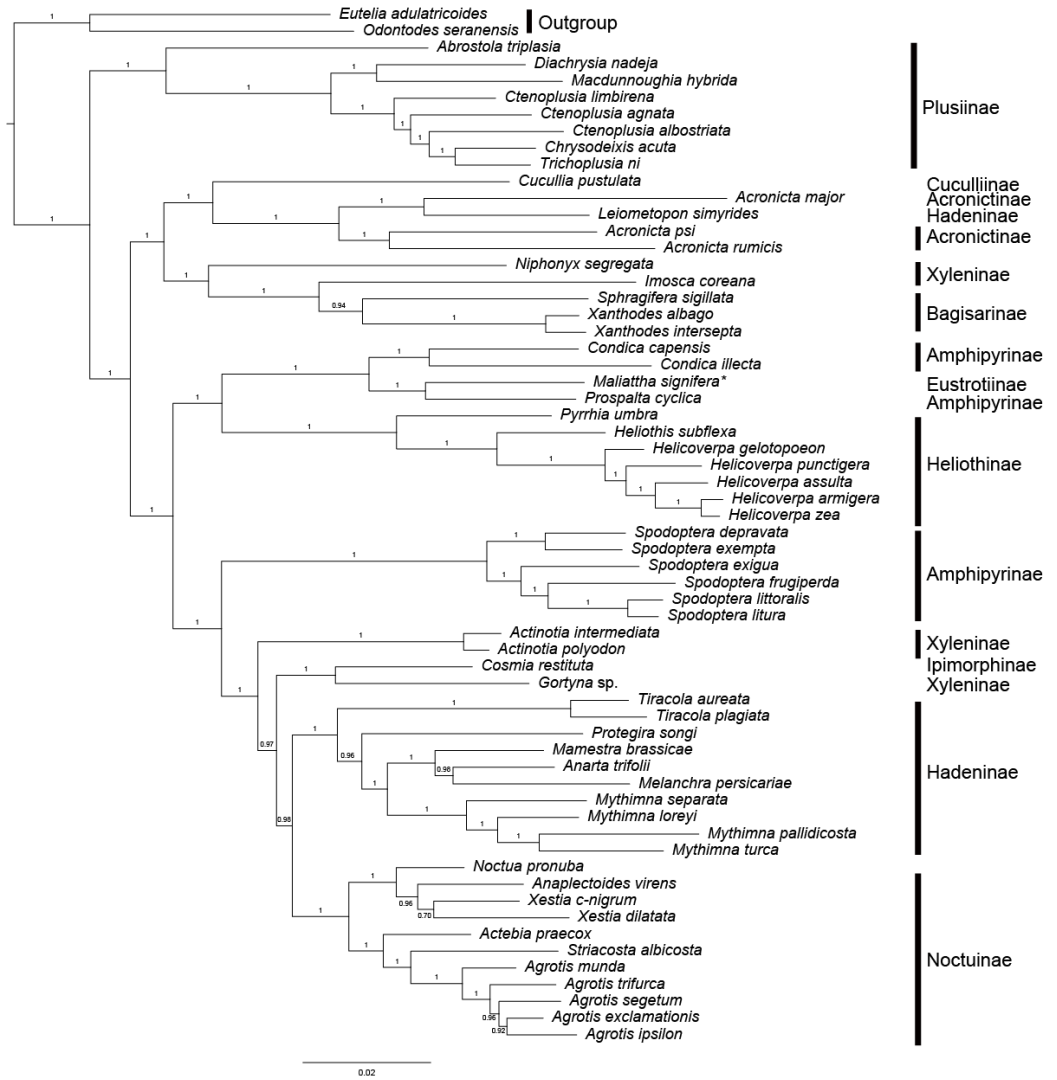

Figure 7. Phylogenetic tree from the amino acid sequences of 13 PCGs constructed based on Bayesian inference method.

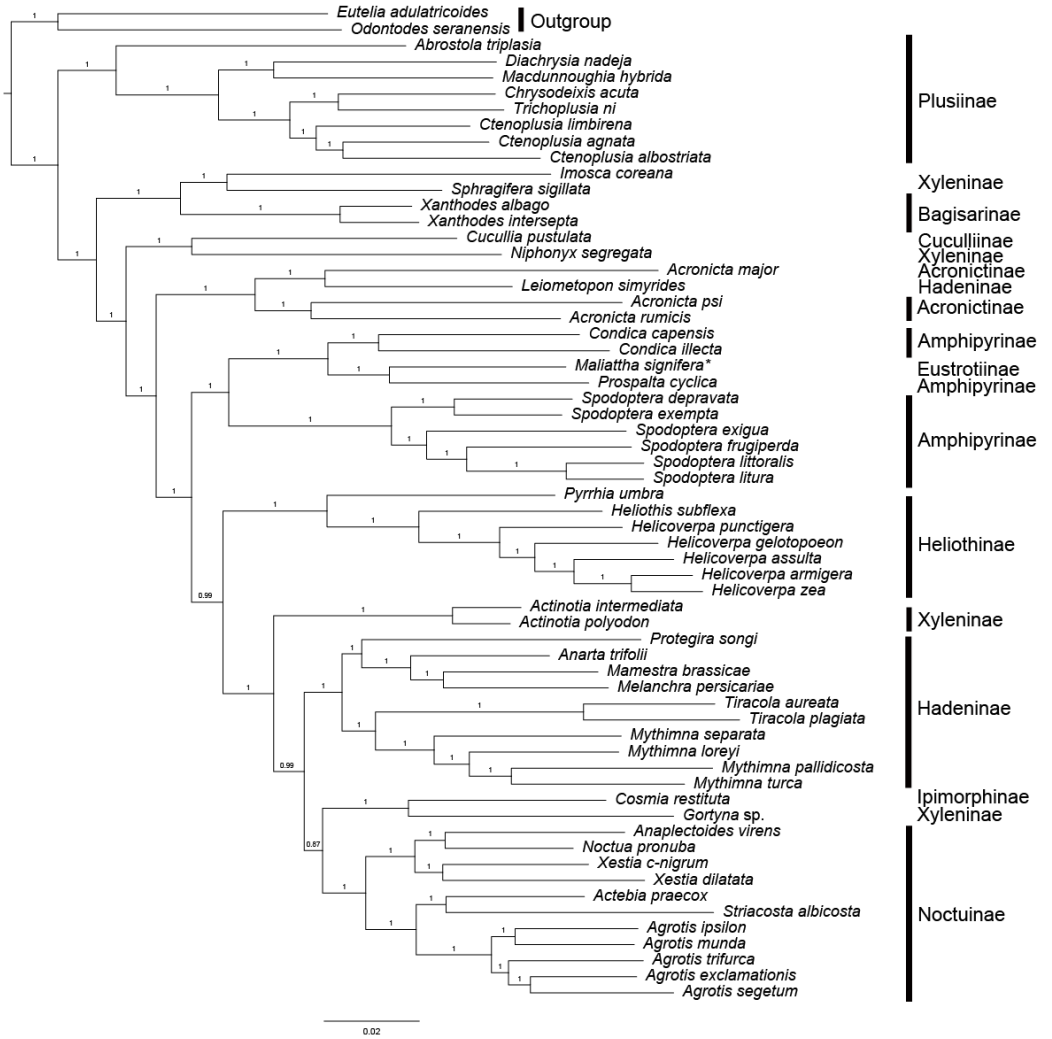

Figure 8. Phylogenetic tree from the nucleotide sequences of 13 PCGs constructed based on Bayesian inference method.

Conclusions

This study employed high-throughput sequencing technology to successfully assemble the complete mitochondrial genome sequence of *M. signifera*, marking the first comprehensive mitochondrial genome sequence within Eustrotiinae. The researchers conducted detailed annotation and analysis of the mitochondrial genome structure and other features of *M. signifera*. They also calculated the nucleotide diversity and evolutionary rates of the 13 PCGs in Noctuidae. Furthermore, by utilizing the mitochondrial genome data of all publicly available Noctuidae species (60) from the NCBI database as the ingroup and two species from Euteliidae as the outgroup, we constructed nucleotide and amino acid datasets. We then employed maximum likelihood and Bayesian methods to establish the phylogenetic

relationships within Noctuidae. This study provides valuable mitochondrial genomic data for further investigations into the internal phylogenetic relationships of Noctuidae.

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The mitogenome sequence newly generated in this study was deposited in GenBank with the accession number OQ111926.

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