The mitochondrial genome of *Megascolia azurea* (Christ) (Hymenoptera: Scoliidae) with its phylogenetic implications

Zhen LIU①, Chun CHEN, Shiqi CHEN

Key Laboratory of Zoology in Hunan Higher Education, Hunan University of Arts and Science, Changde, Hunan 415000, China

Abstract: We sequenced and annotated the mitochondrial genome of *Megascolia azurea* (Christ, 1791), the only representative of the genus *Megascolia* Betrem, 1964 in the family Scoliidae in China. It is a larval parasitoid of the rhinoceros beetles *Xylotrupes gideon* (Linnaeus, 1767) and *Oryctes rhinoceros* (Linnaeus, 1758) (Coleoptera). The mitogenome is 16,335 bp in length and possesses 38 mitochondrial genes. However, unlike most animal mitochondrial genomes, it contains one extra *trnM* gene. Gene rearrangement shows an inversion event involving protein-coding and tRNA genes. The protein-coding genes in this sequence and another 8 species from the Aculeata were used for phylogenetic analysis by maximum likelihood (ML) analysis and Bayesian inference (BI) analyses using *Cotesia vestalis* from the family Braconidae as the outgroup. The topology of the resulted phylogenetic tree demonstrated that *M. azurea* is sister to *Megacampsomeris prismatic*, a species also belonging to family Scoliidae. Scoliidae was recovered as a sister to the clade of (Apidae + (Chrysididae + (Tiphiidae + Mutillidae) + (Formicidae + Vespidae))), followed by Pompilidae which was located at a basal position.

Key words: mitochondrial genome; phylogeny; scoliid wasp

天蓝巨土蜂的线粒体基因组及其系统发育含义（膜翅目：土蜂科）

刘珍①，陈淳，陈诗琦

湖南文理学院，动物学湖南省高等教育重点实验室， 湖南 常德 415000

摘要：天蓝巨土蜂 *Megascolia azurea* (Christ, 1791)是中国土蜂科巨土蜂属 *Megascolia* Betrem, 1964 的唯一代表，是鞘翅目姬独角仙 *Xylotrupes gideon* (Linnaeus, 1767) 和椰蛀犀金龟 *Oryctes rhinoceros* (Linnaeus, 1758)的幼虫寄生蜂。我们测定了天蓝巨土蜂的线粒体基因组，其长度为 16,335 bp 且拥有 38 个线粒体基因。与其他动物线粒体相比，该线粒体基因包含一个额外的 *trnM* 基因。基因重排涉及蛋白编码基因和 tRNA 基因的倒置。通过最大似然法和贝叶斯推理分析两种方法以菜蛾盘绒茧蜂为外群对天蓝巨土蜂和其他 8 种针尾部昆虫的线粒体蛋白编码基因进行了系统发育分析，所获得的系统树拓扑结构表明，土蜂科是（蜜蜂科 +（青蜂科 +（沟土蜂科 + 蚁蜂科）+（蚁科 + 胡蜂科））） 的姐妹群，而蛛蜂科与以上构成姐妹群并位于系统发育树的基部。

关键词：线粒体基因组；系统发育；土蜂

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① Corresponding author, E-mail: qingniao8.27@163.com

Introduction

Scoliidae is a family distributed worldwide, with about 560 recognized species in 43 genera belonging to two subfamilies: Proscoliinae and Scoliinae (Osten 2005). Species of Scoliidae are larval parasitoids of scarabaeoid beetles and pollinators of various plants (Liu *et al*. 2021a). *Megascolia azurea* (Christ, 1791) is among the largest in body size and widely distributed in the Oriental Region and probably bivoltine in Hong Kong (Liu *et al*. 2021b; Christopher & Christopher 2021). *Xylotrupes gideon* (Linnaeus, 1767) and *Oryctes rhinoceros* (Linnaeus, 1758) (Coleoptera) were reported as hosts of this species (Betrem 1928).

Phylogenetic relationships among major lineages of the Aculeata have been studied over the years (Branstetter *et al*. 2017; Brothers 1975, 1993; Pilgrim *et al*. 2008; Wilson *et al*. 2013; Zheng *et al*. 2021). Mitochondrial genomes are one of the most highly studied genomic systems for molecular evolution and phylogenetic inference (Cameron 2014). Mitochondrial genomes have been extensively sequenced in the Hymenoptera and provide useful genetic markers for phylogenetic inferences (Dowton *et al*. 2009a, b; Tang *et al*. 2018; Zheng *et al*. 2018; Zheng *et al*. 2021). However, compared to other groups of the Hymenoptera, there is a lack of representative mitochondrial genomes for several major lineages of the Aculeata, such as the Scoliidae and Tiphiidae (Tang *et al*. 2018). There is only one identified scoliid species, namely *Megacampsomeris prismatica* (*Campsomeris prismatica*)(unverified) sequenced in NCBI (https://www.ncbi.nlm.nih.gov/)previously.

In this study, we determined the mitochondrial genome of *M. azurea* by next-generation sequencing. A thorough description of its genome features and analysis of phylogenetic relationships within the major Aculeata lineages are also provided to illuminate evolutionary patterns across the stinging wasps.

Material and methods

Collection, Identification and DNA extraction

Materials were all collected by hand netting in Taiyang Mountain (29.17° N, 111.71°E), Changde, China in August 2020. The collected specimens were stored in 100% ethanol and stored at −80°C before DNA extraction.

Genomic DNA was extracted from the legs of single specimens using a Ezup Column Animal Genomic DNA Purification Kit (Sangon Biotech, China) following the manufacturer's protocols. Extracted genomic DNA were qualified by NanoPhotometer® (IMPLEN, CA, USA) and Qubit 3.0 (Invitrogen, Life Technologies, Carlsbad, CA, USA) and a Nanodrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Voucher specimens are deposited in the Laboratory of Hunan University of Arts and Science, Changde, China.

Genome sequencing, assembly, annotation and analysis

The extracted genomic DNA of the specimen was sheared into fragments of approximately 350 bp in length using an Ultrasonic Processor Covaris S220 (Covaris, Inc. MS, USA). High-throughput sequencing libraries were constructed using the Illumina TruSeq

DNA PCR-Free HT Kit and sequenced using an Illumina Novaseq6000 with the strategy of producing 150 bp paired-ends by the Annoroad Gene Tech. (Beijing) Co., Ltd. Quality of raw sequencing reads was checked by FastQC version 0.11.3 (Andrews 2010), and low-quality reads and sites were filtered by Trimmomatic version 3.2.57 (Bolger *et al*. 2014).

The target mitochondrial reads were filtered out using BLAST (BLASTn with E value: 1×10^{-5}) against a reference data set containing Braconidae mitochondrial genomes via the FastqExtract script (Crampton Platt *et al*. 2015). The mitochondrial genome of *M. azurea* was assembled by IDBA_UD version 1.1.3 (Peng *et al*. 2012) and SPAdes version 3.15.2 (Bankevich *et al*. 2012) with default parameters.

Annotation of the assembled genome was performed by using MITOS Web Server (Bernt *et al*. 2013). Start and stop codons of protein-coding genes (PCGs) were manually adjusted in Geneious Prime v11 by referencing the published mitogenomes of Microgastrinae. The online tRNAscan-SE service (http://lowelab.ucsc.edu/tRNAscan-SE/) and XRNA tools were used to confirm locations of transfer RNA (tRNA) genes. Characteristics of mitochondrial genomes of sequenced species in Scoliidae were analyzed, including gene arrangements and base compositions and codon usages of PCGs according to methods mentioned in Yuan *et al*. (2022). Gene rearrangements were analyzed by comparison with the putative ancestral type of *Drosophila melanogaster*.

Mitochondrial genomes of 10 species from Hymenoptera were chosen to construct the phylogenetic relationships of Aculeata. *Cotesia vestalis* from Braconidae was chosen as the outgroup. The PCGs were aligned using G-INS-i algorithms implemented in MAFFT v7.407 (Katoh & Standley 2013). The best partition schemes of substitution models for the matrix were searched in PartitionFinder v2.1.12 with model selection = BIC and Branch lengths = unlinked between different subsets (Lanfear *et al*. 2012). MrBayes v3.2.7a (Ronquist & Huelsenbeck 2003) and IQ-TREE v1.6.12 (Nguyen *et al*. 2015) were used to reconstruct the phylogenetic tree, respectively, based on nucleotide (NU) sequences of PCGs.

Results

Genome structure and organization

A total of 26 Gb filtered clean data were produced in this sequencing. The mitochondrial genome of *M. azurea* is 16,335 bp in length (GenBank accession OP661167), containing 13 protein-coding genes (PCGs), 23 transfer RNA genes (tRNAs), and two ribosomal RNA (rRNA) genes. However, unlike most animal mitochondrial genomes, it contains one extra *trnM* gene that can be detected using either the IDBA or SPAdes assemblers.

The overlapping nucleotides from seven adjacent genes in the mitochondrial genome of *M. azurea* were discovered to be up to 43 bp in total, as the maximum length of overlap was 25 bp in length, locating at one junction (*rrnL-trnL1*). The total intergenic nucleotides were 660 bp in length and dispersed between 27 adjacent genes, with the longest gene spacer, 127 bp in length dispersing between *trnH* and *trnN*. Neither overlapping nor space nucleotides were found between the other 6 adjacent genes. The majority strand (J-strand) encoded seven PCGs (*atp6*, *atp8*, *cob*, *cox3*, *nad3*, *nad5*, and *nad6*) and ten tRNAs (*trnS2*, *trnT*, *trnH*, *trnN*, *trnR*, *trnA*, *trnG*, *trnD*, *trnC* and *trnY*) while the remaining genes were encoded on the minority strand (N-strand).

Table 1. Codon usage in the mitochondrial genome of *Megascolia azurea*

Codon	Number	RSCU	Amino acid	Codon	Number	RSCU
UUU	451	1.72	F	UUC	73	0.28
UUA	366	3.77	L	UUG	79	0.81
CUU	59	0.61		CUC	17	0.17
CUA	50	0.51		CUG	12	0.12
AUU	451	1.78	I	AUC	55	0.22
AUA	362	1.72		AUG	58	0.28
GUU	54	1.76	V	GUC	4	0.13
GUA	57	1.85		GUG	8	0.26
UCU	75	1.62	S	UCC	23	0.50
UCA	64	1.38		UCG	8	0.17
CCU	44	2.59	P	CCC	7	0.41
CCA	16	0.94		CCG	1	0.06
ACU	52	1.66	T	ACC	18	0.58
ACA	48	1.54		ACG	7	0.22
GCU	17	1.70	A	GCC	3	0.30
GCA	19	1.90		GCG	1	0.10
UAU	391	1.83	Y	UAC	37	0.17
UAA	443	1.74	*	UAG	65	0.26
CAU	54	1.89	H	CAC	3	0.11
CAA	63	1.64	Q	CAG	14	0.36
AAU	488	1.85	N	AAC	40	0.15
AAA	438	1.77	K	AAG	57	0.23
GAU	82	1.78	D	GAC	10	0.22
GAA	103	1.72	E	GAG	17	0.28
UGU	51	1.52	C	UGC	16	0.48
UGA	77	1.62	W	UGG	18	0.38
CGU	13	1.53	R	CGC	1	0.12
CGA	19	2.24		CGG	1	0.12
AGU	62	1.34	S	AGC	22	0.48
AGA	82	1.77		AGG	35	0.75
GGU	19	1.13	G	GGC	1	0.06
GGA	41	2.45		GGG	6	0.36

Abbreviation: RSCU, relative synonymous codon usage.

A relatively high A+T content in the mitochondrial genome is common in Hymenoptera (Oliveira *et al.* 2008). The mitochondrial genome of *M. azurea* contains a significant bias toward A/T in nucleotide composition by 85%, which ranges from 78.4% (*cox1*) to 91.9%

(*nad2*) with 42.7% of A, 8.1% of G, 42.3% of T, and 6.9% of C (Fig. 1). This high A+T content is consistent with the other scoliids (Zheng *et al*. 2021), showing Scoliidae is among the highest in A+T content in the Aculeata.

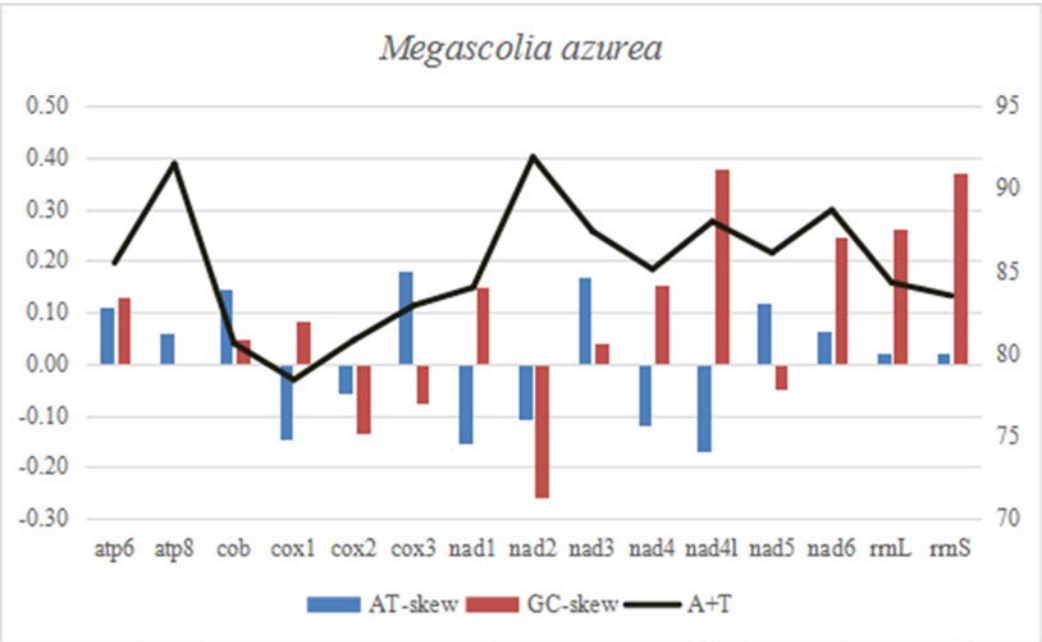

Figure 1. Base composition of PCGs and two rRNAs in the mitochondrial genome of *Megascolia azurea*.

The values of the GC skew in most genes were positive, except four genes (−0.14 in *cox2*, −0.08 in *cox3*, −0.26 in *nad2* and −0.05 in *nad5*) and the value of the AT skew were also mostly positive, except for six genes (−0.15 in *cox1* and *nad1*, −0.06 in *cox2*, and −0.11 in *nad2*, −0.12 in *nad4* and −0.17 in *nad4l*) (Fig. 1).

The RSCUs in the mitochondrial genomes of *M. azurea* (Table 1) show an overwhelming preference toward the usage of A and T, especially at the third position, owing to genetic code degeneracies, which to some extent explains the high bias of A+T content. The total number of codons in the *M. azurea* mitochondrial genome is 5328. Among the available codons, there are five most widely used amino acids with their corresponding codons in the following order, Leu-UUA, Pro-CCU, Gly-GGA, Arg-CGA, and Ala-GCA, respectively.

Protein coding genes (PCGs)

The total length of 13 encoded PCGs is 11,011 bp in *M. azurea*, comprising 67% of the entire mitochondrial genome length. The size of the PCGs in the *M. azurea* mitochondrial genome is similar to the other corresponding ortholog (MH748671, 10,699 bp) in NCBI. The overall AT content of PCGs is 85.4%. The gene with the highest A + T content is *nad2* with 91.9%, followed by *atp8* with 91.5%.

All PCGs in the *M. azurea* mitochondrial genome initiated with the typical start codon ATN (seven with ATA, four with ATT, and two with ATG) as is the case of other Hymenoptera insects (Cameron *et al*. 2008; Castro & Dowton 2005; Cha *et al*. 2007; Crozier & Crozier 1993; Oliveira *et al*. 2008; Wei *et al*. 2009; Yuan *et al*. 2021). Approximately all

PCGs in the *M. azurea* mitochondrial genome terminate with a complete stop codon TAA or TAG. Only one PCG (*nad4*) used the incomplete stop codon T, which has been commonly reported in other invertebrates (Ojala *et al*. 1981).

tRNA and rRNA genes

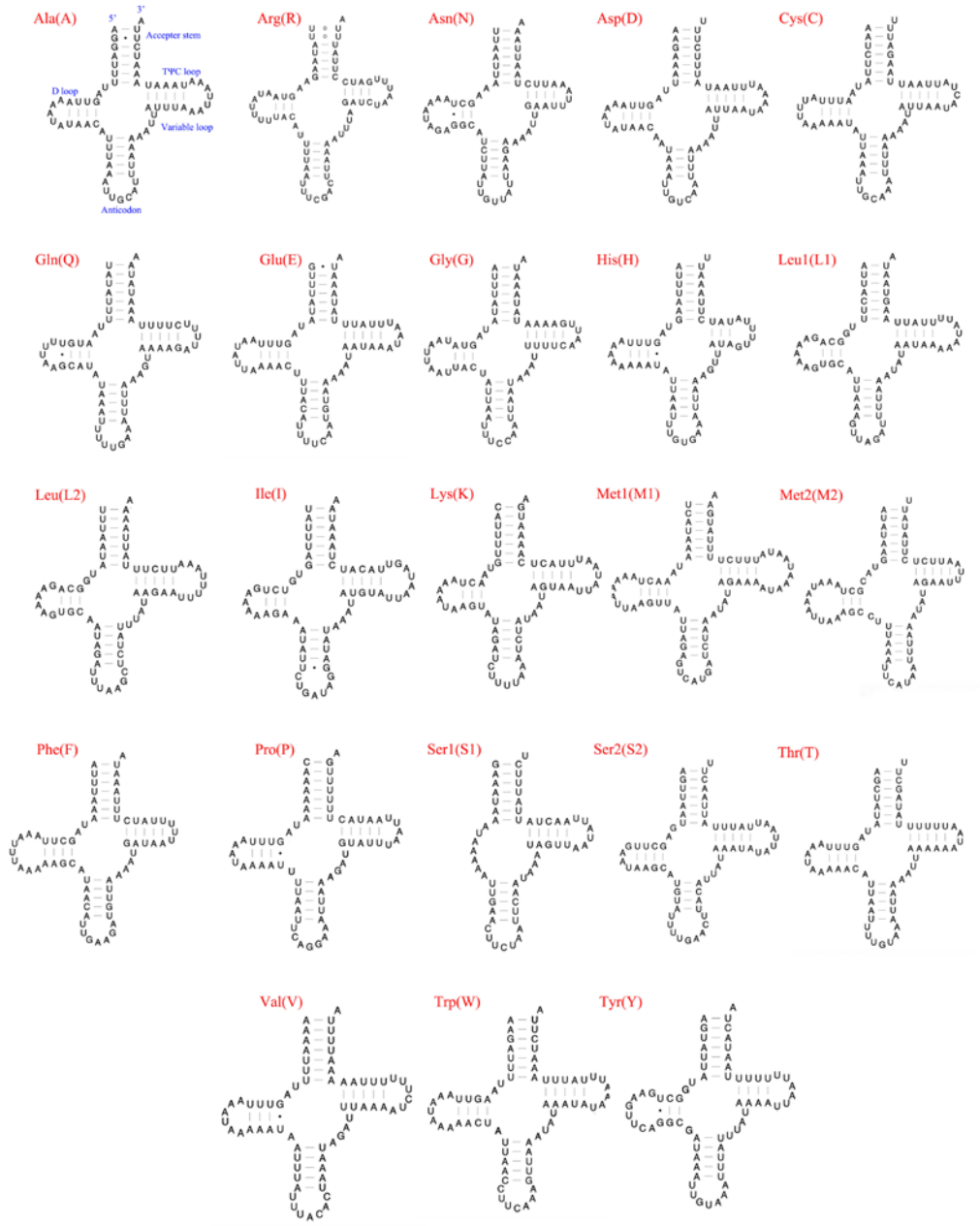

Figure 2. Predicted secondary structures for the 23 typical tRNA genes of *Megascolia azurea* mitochondrial genome. Base-pairing is indicated as follows: Watson-Crick pairs by lines, wobble GU pairs by dots and other non-canonical pairs by circles, tRNA, transfer RNA.

The entire 22 ancestral tRNAs commonly existing in the animal mitochondrial genome were successfully detected in the mitochondrial genome of *M. azurea*. But unlike other animal mitochondrial genomes, there was an extra *trnM* detected in the mitochondrial genome of *M. azurea*. These tRNA genes were scattered throughout the mitochondrial genome. Generally, the lengths of tRNAs range from 59 (*trnS1*) to 71 bp (*trnM2*). All tRNA genes in the mitochondrial genome of *M. azurea* have typical clover-leaf secondary structures except *trnS1* is lacking the D loop (Fig. 2). The DHU-stem and TΨC-stem of the mitochondrial tRNA in *M. azurea* varied in length. The size of its anticodon loop of all tRNAs was 7 bp, except for that in *trnS2* (9 bp). The size of the amino-acid acceptor stem of all tRNAs was 7 bp. Such variations are normal in other insect mitochondrial tRNAs (Sheffield *et al*. 2008; Wei *et al*. 2010; Peng *et al*. 2017). A total of 11 mismatched base pairs were detected with 9 G-U pairs and 2 U-U pairs.

Gene rearrangements

Gene rearrangements were evident compared with the ancestral type of *Drosophila melanogaster*. The rearrangements include protein-coding genes and tRNA genes. Inverse transposition occurred in gene clusters *trnI* to *trnK*, *trnH*, *trnS1* and *trnE*. Transposition occurred in *trnF* and local inversion occurred in *nad5* (Fig. 3). The rearrangements were similar to two unnamed mitogenomes of Scoliidae in Zheng *et al*. (2021) except an extra *trnM* gene and shows the rapid evolution of this group compared to other lineages of Hymenoptera.

Figure 3. Gene rearrangement in the mitochondrial genome of *Megascolia azurea*.

Phylogenetic analyses

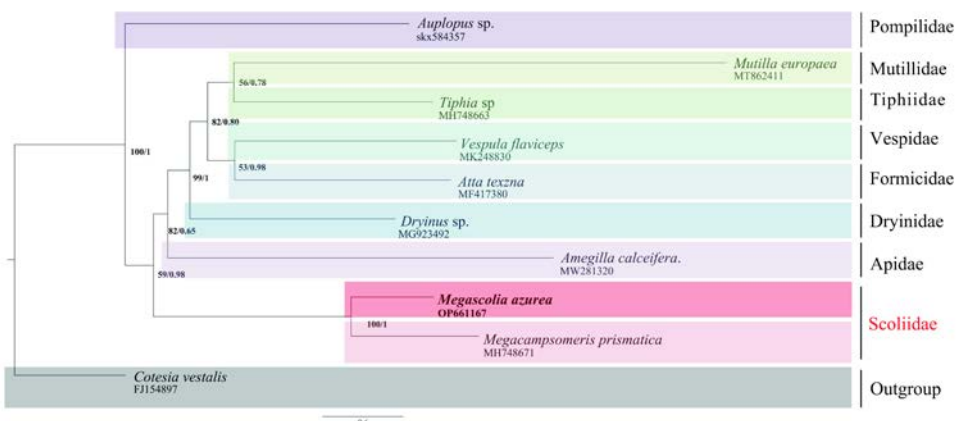

Figure 4. Phylogenetic tree of maximum likelihood (ML) methods and Bayesian inference (BI) using matrixes of 13 PCGs in mitochondrial genomes of 9 representative lineages in Aculeata and outgroup *Cotesia vestalis*. The support values of the corresponding nodes are shown above (left is bootstraps of ML, right is posterior probability of BI).

Table 2. Optimal partitioning scheme for mitogenome dataset determined by PartitionFinder

Subset	Subset Partitions	Sites	Best Model
1	c1p3, cbp3, c3p3, c2p3	1354	GTR+G
2	a6p3, n3p3, a8p3, n2p3, n6p3	958	GTR+G
3	n5p1, n4p1, n1p1, n4lp1, n3p1	1477	GTR+I+G
4	n6p1, n2p1, a8p1	622	GTR+I+G
5	n1p2, n5p2, n4p2	1273	GTR+I+G
6	cbp1, c2p1, c3p1, a6p1	1060	GTR+I+G
7	n4lp2, a8p2, n2p2, n6p2	704	GTR+I+G
8	n4lp3, n5p3, n4p3, n1p3	1355	GTR+G
9	n3p2, c2p2, cbp2, a6p2, c3p2	1182	GTR+I+G
10	c1p2	508	GTR+G
11	c1p1	508	GTR+I+G

We conducted phylogenetic analyses on the concatenated nucleotide sequences (PCG123) of *M. azurea* and other eight species from the Aculeata. *Cotesia vestalis* from Braconidae was chosen as the outgroup to validate the phylogenetic position of *M. azurea* within Aculeata. Optimal partitioning strategy and models were selected using PartitionFinder v2.1.12 (Table 2). The resulted 10 data matrices were subjected to BI and ML analyses. The BI and ML analyses generated the same topology (Fig. 4). Generally, the support values of the corresponding nodes in the phylogenetic tree of BI were relatively higher than in MI in this study, indicating that MrBayes performs more reliably than ML methods in mitochondrial phylogenomic analyses of this group, and corresponds with other results of Hymenoptera (Dowton *et al*. 2009a; Wei *et al*. 2014). In the present analyses, *M. azurea* is sister to *M. prismatic*, with a solid confidence value (bootstraps = 100, posterior probability = 1), consistent with the morphological characters. Pompilidae was always located at basal positions of the Aculeata in both analyses. The monophyly of the superfamily Pompiloidea were not recovered in both analyses, with *Mutilla europaea* (Mutillidae) clustering with Tiphiidae in another clade. The sister relationship between the Formicidae and Vespidae was recovered in both analyses with *Vespula flaviceps* clustering with *Atta texana*, as in other results based on nuclear genomic data (Branstetter *et al*. 2017; Johnson *et al*. 2013; Peters *et al*. 2017), mitochondrial data (Zheng *et al*. 2021) and molecular combining morphological data (Pilgrim *et al*. 2008). Chrysididae was sister to the group (Tiphiidae + Mutillidae) and (Formicidae + Vespidae), followed by Apidae and Scoliidae in our analyses. Mutillidae always had the longest branch length based on BI and ML analyses, which is congruent with a former study (Zheng *et al*. 2021). More mitogenomes sequences of more taxa will be needed to study the phylogenetic relationships within Scoliidae and other stinging wasps. Considerable uncertainty remains regarding higher-level relationships within Aculeata.

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