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The Partial Nucleotide Sequences of the Mitochondrial Genes, *COI* and *16s rRNA*, of Fireflies in the Genera *Pygoluciola*, *Trisinuata*, and *Medeopteryx* (Coleoptera: Lampyridae)

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Abstract

Fireflies in the genera *Pygoluciola*, *Medeopteryx*, and *Trisinuata* distribute throughout lower northern Thailand. Five morphospecies of these fireflies were collected and nucleotide sequences were analyzed. We sequenced the partial of two mitochondrial genes; *Cytochrome c oxidase subunit I* (*COI*) and *16s rRNA*, and the phylogenetic trees were constructed and compared with the firefly nucleotide sequences in the NCBI database. The *COI* gene was amplified using LCO1490 and HCO2198 primers while specific primers of LR-J-13020a and LR-N-13398a were used to analyze amplicons of *16s rRNA*. The results showed the average GC-contents in the nucleotide of five firefly morphospecies were found to average 30.28% and 22.15% in (*COI*) and *16s rRNA*, respectively. The variable site in *COI* nucleotide sequences was 399 of the total 520 nucleotides (76.73%) whereas 91 of the total 418 nucleotides (21.77%) were found in *16s rRNA* nucleotide sequences. Construction of the phylogenetic trees of those five firefly morphospecies with reference accession samples indicated five clades when analyzed by *COI* nucleotide sequences and two clades were revealed in *16s rRNA* gene. According to these results, *COI* nucleotides are one of the most effective methods for differentiating between and within firefly species. The addition of the *16s rRNA* gene analysis showed an increase in the precision of firefly identification. Those five firefly morphospecies were separated using both the *COI* and *16s rRNA* nucleotide sequences.

Keywords**:** *Cytochrome c oxidase subunit I (COI); 16s rRNA; firefly; Pygoluciola; Medeopteryx; Trisinuata*

1. Introduction

Since molecular analysis has been established as a viable approach for researching the phylogenetics of animal taxa, including insects, it has been employed to clarify the phylogenetic relationship between and within species. DNA sequences from any species serve as species barcodes, providing comprehensible and reliable identification. Mitochondrial genomes (mito-genomes) are circular double-stranded molecules that range in size from 14 to 39 kb. The insect mitochondrial DNA is a small size (14-20 kb) and closed-circular molecule, which encodes 37 genes, including 13 protein-coding genes, two ribosomal RNA genes, and 22 tRNA genes (Boore, 1999). Hence, they have been utilized to explore the evolutionary relationships among and within animal species, including insects, mitochondrial genomes have become prominent tools for investigating molecular evolution, population genetics, and phylogenetic analysis of metazoans (Clary, & Wolstenholme, 1985; Boore, 1999; Cameron, 2013). There are several mitogenome studies in some inset taxa, but some are still under investigations, this included fireflies in the order Coleoptera. Recently, the complete or near-

complete mitogenome of 73 insect species have been reported (Cameron, & Whiting, 2008), such as *Phthonandria atrilineata* (Geometridae) of the order Lepidoptera (Yang et al., 2009).

Many mitochondrial genes, including the *16S* and *CO1* genes, are frequently used to establish phylogenetic trees in insects, particularly the *CO1* gene, which is genetically conserved in almost all insect groups and therefore a very useful marker to identify insect species. This gene successfully revealed the correct identification of 200 closely related lepidopteran species (Hebert et al., 2003), for example, *Astraptes fulgerator*, (Hebert et al., 2004), the species in the genera *Asiopodabrus, Chrysochroa*, and *Denticollinae* (Han et al., 2012; 2016; Kang et al., 2012). Based on the molecular phylogeny of the mitochondrial genomes and nuclear ribosomal DNA, Martin et al. (2017) proposed that the genus *Lamprigera* should be transferred from the Lampyrinae clade to the Lampyridae *incertae sedis*, even though its morphology differs from the Luciolinae, particularly its flashing behavior. Furthermore, the evolutionary relationship between several Chinese and Japanese fireflies in the Lampyridae taxon has been studied utilizing the luciferase gene and mitochondrial *16s rRNA* gene (Suzuki, 1997; Dong et al., 2008; Li et al., 2006).

Fireflies belong to the family Lampyridae, a member of the most abundant insect species, Coleopteran. The complete mitochondrial genomes of 28 species in five subfamilies of the Lampyrids have been reported (Ge et al., 2022). To enlighten the evolution and relationships within Lampyridae, the mitochondrial genome sequences are necessary. Recently, several reports published mitochondrial genome of fireflies, for example, *Asymmetricata circumdata* (Luan, & Fu, 2016), *Luciola lateralis* (Maeda et al., 2017), *Pteroptyx maipo* (Fan, & Fu, 2017), *Abscondita anceyi* (Hu, & Fu, 2018a), *Luciola curtithorax* (Hu, & Fu, 2018b), *Curtos fulvocapitalis* (Li et al., 2022).

Fireflies in the genera *Pygoluciola*, *Medeopteryx*, and *Trisinuata* are found in some areas of the lower northern Thailand. Not only they are sympatric in some areas, but they possess ambiguous characteristics. In our study, we sequenced the partial nucleotide sequence of two mitochondrial genes, *COI* and *16s rRNA* of two undescribed species of *Pygoluciola*, one species of *Medeopteryx*, and two species of *Trisinuata*. The nucleotide sequences of these genes were aligned, phylogenetic trees were constructed and were compared with some other species of fireflies.

2. Objectives

To identify the five fireflies (*Pygoluciola* sp.1, *Pygoluciola* sp.2, *Trisinuata* sp.1, *Trisinuata* sp.2 and *Medeopteryx* sp.) in the genera *Pygoluciola*, *Medeopteryx*, and *Trisinuata* based on morphological study, and *COI* and *16s rRNA* gene nucleotides.

3. Materials and methods

3.1 Sample collections

We surveyed paddy fields, orchards, agricultural areas, parks, and natural habitats in lower northern Thailand. Adult fireflies were collected and maintained separately in plastic boxes before being transferred to a laboratory. The samples were anesthetic in a refrigerator for 30 minutes and they were then divided into two groups. The first group was preserved in 70% ethanol for identification, while the second in 95% ethanol for molecular analysis. Adult firefly identifications were performed using the following identification guides:

- Systematics and phylogenetics of Indo-Pacific Luciolinae fireflies (Coleoptera: Lampyridae) and the description of new Genera (Ballantyne, & Lambkin, 2013).
- Taxonomy and species distribution of fireflies (Coleoptera: Lampyridae) in the North of Thailand (Nak-eiam, 2015).

For molecular analysis, total DNA was extracted from 50-100 mg of adult firefly thorax, antennae, and legs using the BioFact™ Genomic DNA Prep Kit (Biofactory) per the manufacturer's instructions. The extracted DNA was loaded in a 1% TBE agarose gel with 1X SYBR® Safe DNA gel stain (Invitrogen, USA) and observed under UV light using Gel documentation to quantify the quantity of DNA (Bio-Rad, USA).

3.2 DNA amplification

Using the Polymerase Chain Reaction (PCR) and two gene-specific primers (*COI* and *16s rRNA*) (Table 1), 25 ng of firefly DNA was amplified. The PCR master mix contained 25 µL of 10X buffer, 50 mM $MgCl_2$, 10 mM dNTPs, 10 µM forward and reverse primers, and Taq DNA polymerase (Invitrogen, USA). The PCR amplification was carried out in a thermal cycler with the following profile: started at 94°C for 3 minutes, amplified by 35 cycles of 94°C for 45 seconds, annealing for 30 seconds at a temperature specific to each marker as indicated in Table 1, and 72°C for 1 minute, followed by 7 minutes at 72°C for a final extension.

Table 1 Gene-specific primer sequences for *COI*, and *16s rRNA*

Genes	Primer sequences (5'-3')	Annealing temperature (°C)	Expected size (bp)	References
COI	LCO1490: GGTCAACAAATCATAAAGATATTGG HCO2198: TAAACTTCAGGGTGACCAAAAAATCA	47.5	658-708 bp	Vrijenhoek et al. (1994)
16s rRNA	LR-J-13020a_F: ACGCTGTTATCCCCAAGGTA LR-N-13398a_R: CGCCTGTTTAACAAAAACAT	54	400-470 bp.	Stanger-Hall et al. (2007)

The PCR products were analyzed on 1.5 % TBE agarose gel electrophoresis containing 1xSYBR® Safe DNA gel stain (Invitrogen, USA).

3.3 Nucleotide sequencing and analysis

Per the manufacturer's instructions, PCR products of each gene were purified using the BioFact™ Gel & PCR purification System (Biofactory). The nucleotide sequence length analysis revealed that the *COI* gene sequence is approximately 658-708 bp, while the 16S rRNA gene is approximately 400-470 bp. The purified DNA was then eluted with 30 µL of elution buffer, and the purified PCR products were sequenced by Bionics Corporation (Korea). Phylogenetic trees were constructed by the ClustalW program and Maximum Likelihood and Bootstrap 1000 methods in MEGA X utilizing nucleotide sequence data of *COI* and *16s rRNA* genes (Kumar et al., 2018; Urtgam et al., 2023). The pairwise nucleotide sequences of each sample were performed with *COI* and *16s rRNA* sequences of six firefly species. *Asymmetricata circumdata* was employed as an outgroup, along with nucleotide sequences from several additional firefly species obtained from GenBank (NCBI).

4. Results

Five morphospecies of fireflies; i.e., two morphospecies of *Pygoluciola*, two morphospecies of *Trisinuata*, and one morphospecies of *Medeopteryx* were collected from the regions of lower northern Thailand. The partial nucleotide sequences of two mitochondrial genes were sequenced and analyzed. The phylogenetic trees were then constructed using the Neighbor-joining method, the Tamura-Nei model with Gamma distribution, and 1000 Bootstrap replicates based on partial nucleotide sequences of *Cytochrome c oxidase* subunit I (*COI*) and *16s rRNA* genes. *Pygoluciola* sp.1, *Pygoluciola* sp.2, *Trisinuata* sp.1, *Trisinuata* sp.2, and *Medeopteryx* sp., nucleotide sequences were examined and compared to firefly nucleotide sequences in the NCBI database.

4.1 Sequence alignment of molecular marker genes

The nucleotide sequences of *Cytochrome c oxidase subunit I* (*COI*) and *16s rRNA* genes in *Pygoluciola* sp.1, *Pygoluciola* sp.2, *Trisinuata* sp.1, *Trisinuata* sp.2, *Medeopteryx* sp. were aligned with *Asymmetricata circumdata* using ClustalW in MEGA11. The alignment file was constructed with T-Coffee program (Notredame et al., 2000). GC-contents in nucleotide of five fireflies; *Pygoluciola* sp.1, *Pygoluciola* sp.2, *Trisinuata* sp.1, *Trisinuata* sp.2, *Medeopteryx* sp. were found to average 30.28% in *Cytochrome c oxidase subunit I* (*COI*) and 22.15% in *16s rRNA*, respectively (Table 2).

The variable site was found in *Cytochrome c oxidase subunit I* (*COI*) nucleotide sequence (Figure 1) as 399 of the total 520 nucleotides (76.73%) whereas 91 of the total 418 nucleotides (21.77%) were found in *16s rRNA* nucleotide sequence (Figure 2). This investigation revealed that *Cytochrome c oxidase subunit I* is one of the most variable genes in insect molecular markers.

Table 2 Nucleotide contents in *Cytochrome c oxidase subunit I* (*COI*) and *16s rRNA* gene of five firefly species.

Species	Nucleotide contents in five fireflies (%)							
	COI				*16s rDNA*			
	T	C	A	G	T	C	A	G
Pygoluciola sp.1	32.11	15.46	37.26	15.17	32.29	14.94	44.34	8.43
Pygoluciola sp.2	32.11	15.46	37.26	15.17	33.49	13.73	43.86	8.92
Trisinuata sp.1	31.22	16.05	38.59	14.14	34.95	13.11	43.45	8.50
Trisinuata sp.2	31.22	15.76	38.29	14.73	33.33	13.38	44.77	8.52
Medeopteryx sp.	32.11	15.61	38.44	13.84	34.98	12.81	43.84	8.37
Avg.	31.75	15.67	37.97	14.61	33.80	13.60	44.05	8.55

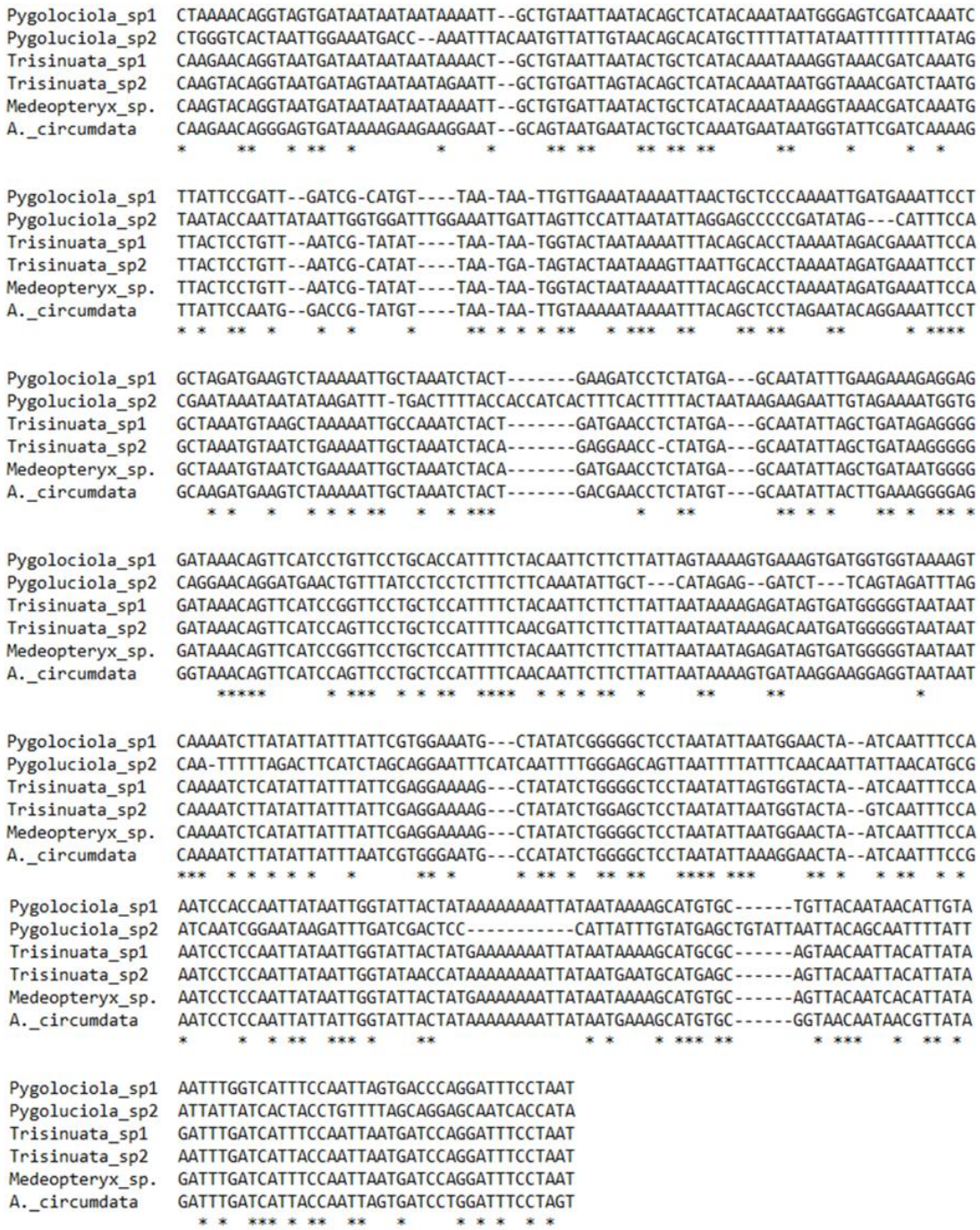

Figure 1 Sequence alignment of *COI* gene in *Pygoluciola* sp.1, *Pygoluciola* sp.2, *Trisinuata* sp.1, *Trisinuata* sp.2, *Medeopteryx* sp. and *Asymmetricata circumdata*.

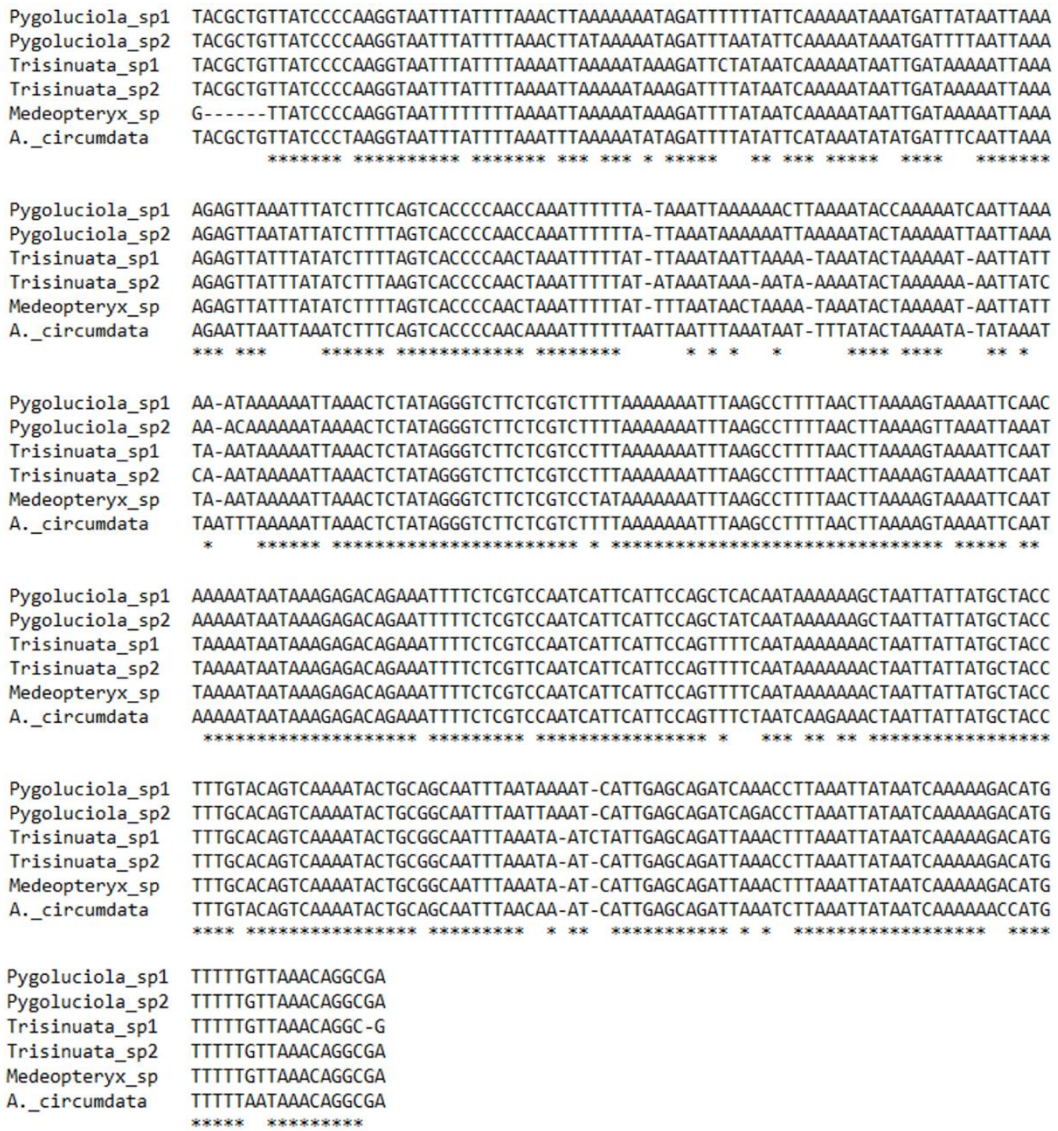

Figure 2 Sequence alignment of *16s rRNA* gene in *Pygoluciola* sp.1, *Pygoluciola* sp.2, *Trisinuata* sp.1, *Trisinuata* sp.2, *Medeopteryx* sp. and *Asymmetricata circumdata*.

4.2 *COI* gene

The *COI* gene was amplified using LCO1490 and HCO2198 primers, resulting in a PCR product aligned to 705 bp, which is consistent with a previous study. The LCO1490 and HCO2198 primers were designed to cover the nucleotide range of 1490-2198 bp in mitochondrial DNA with high efficiency for invertebrate identification (Vrijenhoek et al., 1994). Evolutionary divergence of firefly estimated with Kimura-two parameter (K2P) based on *COI* gene show a high variation with the gene from 32.4 ± 7.1 - 92.3 ± 6.4% with an overall variation of 42.4 ± 2.2% (Table 3). This data revealed that the variation of the *COI* gene was very high which could be used in the firefly species identification. The *COI* nucleotide sequences of all five firefly specimens were aligned with the 22 fireflies in the eight genera retrieved from the NCBI database as references (Figure 5). Clade (A) of the genus *Pteroptyx* with an 98% bootstrap value, consisted of 5 *Pteroptyx* spp. as well as *Inflata indica*, *Emarginoptyx trilucida*, *Medeopteryx* sp., and *Trisinuata* sp., was the first of the 3 primary clades established with tree branch support with a value greater than 50%. With a low tree branch support value, *Medeopteryx* sp., *Trisinuata* sp.1, and *Trisinuata* sp.2 were grouped with *Medeopteryx* sp. (OL891941.1). The second

clade (B) (76% bootstrap value) which could separate into 2 sub-clades of clade B-I, with a 76% bootstrap value, was formed of four *Abscondita* spp. and clade (B-II), with a 69% bootstrap value, was the genus *Pygoluciola*, which included five *Pygoluciola* spp.: *Pygoluciola* sp.1 XHF-2022a (OM201323.1), *Pygoluciola* sp.1 GL-2018 (MG200085.1), *Pygoluciola* sp.2 (OM201324.1), *Pygoluciola qingyu* (MG200086.1) and *Pygoluciola dunguna* (MT106241.1), as well as the two samples from this study: *Pygoluciola* sp.1 and *Pygoluciola* sp.2. whereas, *Luciola substriata* was separated into the third clade (C). These *COI* nucleotide sequences distinguished *Asymmetricata circumdata* from the others. The average genetic divergence (%K2P) in *COI* of these fireflies (5 fireflies and reference species) was 42.4 ± 2.2 %. The *COI* has been used as animal DNA barcoding approach, with the average genetic divergence of family, genus, and species of 11.16%, 6.84%, and 0.25%, respectively.

4.3 *16s rRNA* gene

The amplicons of *16s rRNA* were conducted with the specific primers of LR-J-13020a and LR-N-13398a which covered the mitochondrial DNA that correlated with the previous report of the North American fireflies in Stanger-Hall et al. (2007). The alignment of the *16s RNA* nucleotide sequence was constructed with 418 bp using *16s rRNA* nucleotide sequences of five firefly specimens together with the 18 fireflies in the nine genera of *Abscondita*, *Aquatica*, *Asymmetricata*, *Inflata*, *Luciola*, *Medeopteryx*, *Pygoluciola*, *Sclerotia*, and *Triangulara* retrieved from the NCBI database as a reference. Evolutionary divergence of firefly estimated with Kimura-two parameter (K2P) based on *16s rRNA* gene show a high variation with the gene from 9.0±0.5-10.8±0.8% with an overall variation of 9.8±0.2% (Table 3). This data revealed that the variation of the *16s rRNA* gene was high which could be used to improve the firefly species identification based on nucleotide sequencing. The phylogenetic tree was constructed with tree support branch values of 72% and 100%, two major clades were revealed (Figure 4). The first clade (clade A) comprises two groups of five firefly species. *Inflata indica* (NC 039700.1) and *Medeopteryx* sp. (OL891941.1) provided a 70% branch support value in the first group, whereas *Trisinuata* sp.1, *Trisinuata* sp.2, and *Medeopteryx* sp. provided a 56% branch support value for the second group. Based on the partial *16s rRNA* gene sequence, this clade confirmed a close genetic relationship between *Trisinuata* sp. and *Medeopteryx* sp. With a 72% tree support branch, *Pygoluciola* sp.1 and *Pygoluciola* sp.2 specimens were placed in the same clade (clade B) as the other five fireflies in the genus *Pygoluciola*. With a 51% bootstrap value, *Pygoluciola* sp. 2 was grouped with *Pygoluciola* sp. FM18 (MK292102.1) and *Pygoluciola* sp. 1 XHF-2022a (OM201323.1), whereas *Pygoluciola* sp. 1 may have separated from the ancestor at an earlier branch. The four firefly species in the genus *Abscondita* were strongly supported into a group (73% bootstrap value) whereas the genera *Aquatica*, *Luciola*, *Sclerotia*, and *Triangulara* were grouped with 93% tree branch support value. The *16s rRNA* gene variation in *Asymmetricata circumdata* (NC 032062.1) may differ greatly from those of other fireflies and showed that it separated off from the ancestor at an early stage. The classifications of fireflies based on *16s rRNA* nucleotides were validated by the phylogenetic tree constructed using the *16s rRNA* nucleotide sequence. Additionally, the phylogenetic tree based on the *16s rRNA* nucleotide sequence helped identify those two *Pygoluciola* species. It demonstrated the separation of *Pygoluciola* sp.1 and *Pygoluciola* sp.2, with a low branch support value of only 13%, and indicated that *Pygoluciola* sp.2 is closely related to those *Pygoluciola* species in the database.

Table 3 Evolutionary divergence between sequences estimated with Kimura-two parameter (K2P) based on *COI*, and *16s rRNA* gene.

Species	%K2P (mean±S.E.) of 5 fireflies (in this study) to the others based on	
	***COI* gene**	***16s rRNA* gene**
Pygoluciola sp.1	92.3 ± 6.4	9.9 ± 0.4
Pygoluciola sp.2	32.4 ± 7.1	9.0 ± 0.5
Trisinuata sp.1	87.9 ± 6.2	10.5 ± 0.8
Trisinuata sp.2	87.6 ± 6.4	10.0 ± 0.7
Medeopteryx sp.	85.8 ± 4.4	10.8 ± 0.8
Over all	**42.4 ± 2.2**	**9.8 ± 0.2**

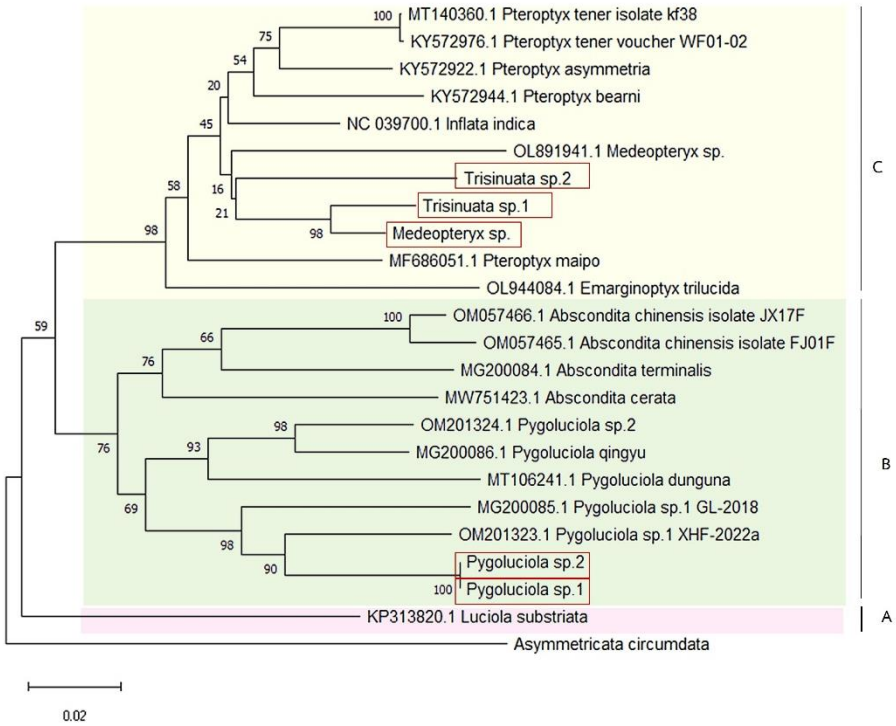

Figure 3 Phylogeny study of five fireflies; *Pygoluciola* sp.1, *Pygoluciola* sp.2, *Trisinuata* sp.1, *Trisinuata* sp.2 and *Medeopteryx* sp. analyzed by Cytochrome c oxidase subunit I (*COI*) gene. The tree was constructed by Maximum Likelihood method and Tamura-Nei model with Gamma distribution. The numbers shown on each node is the branch support value with 1000 replicates of the Bootstrap method (Kumar et al., 2018). The scale bar indicates 0.2 changes per nucleotide position.

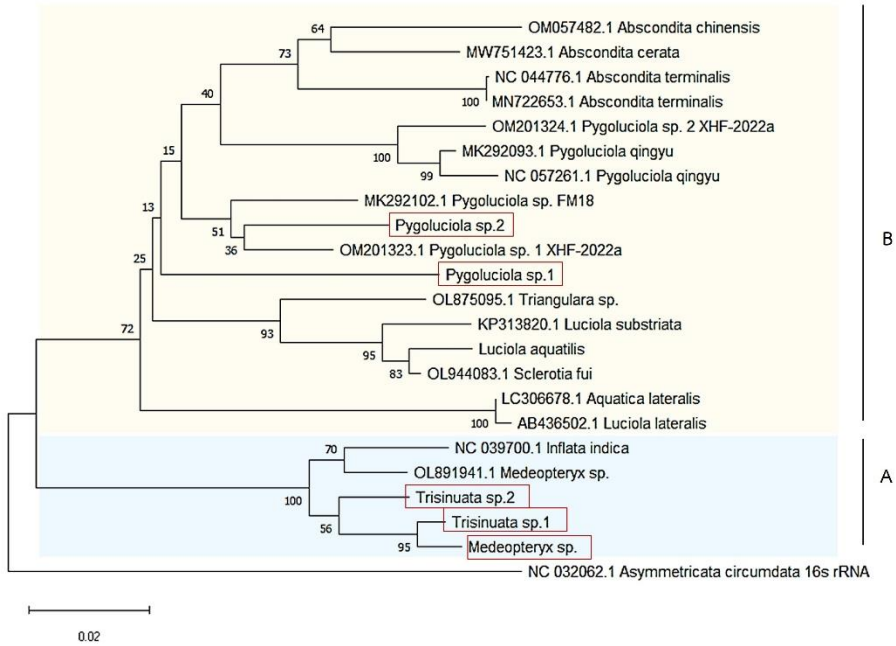

Figure 4 Phylogeny study of five fireflies; *Pygoluciola* sp.1, *Pygoluciola* sp.2, *Trisinuata* sp.1, *Trisinuata* sp.2 and *Medeopteryx* sp. analyzed by *16s RNA* gene. The tree was constructed by Maximum Likelihood method and Tamura-Nei model with Gamma distribution. The numbers shown on each node is branch support value by 1000 replicates of Bootstrap method (Kumar et al., 2018). The scale bar indicates 0.02 changes per nucleotide position.

5. Discussion

The average %GC content of the *COI* gene was 30.50% in these fireflies, which correlated with the %GC content of the *Pyrocoelia praetexta* (Wattanachaiyingcharoen, & Urtgam, 2022), and was comparable to the %GC content in the order Coleoptera (Hebert et al., 2003), and correlated with those of %GC content of mitochondrial genomes in the other fireflies (Liu, & Fu, 2020). Different levels of variation were identified in the %GC content of the mitochondrial genome across animal phyla, particularly in insects, which may indicate species separation (Clare et al., 2008). In insect taxon, the *COI* gene has been widely used to clarify those cryptic species, for example, Lepidopteran species (Hebert et al., 2003; 2004), some genera of Coleopterans (Han et al., 2012; 2016, Kang et al., 2012). Moreover, *COI* gene shows the ability to identify haplotypes in the *Vespa velutina* (Urtgam, & Jongjitvimol, 2020). In addition, nine cohabiting Taiwanese firefly species, including *Abscondita chinensis, Ab. cerata, Aquatica ficta, Lucila curtithorax, L. kagiana, L. filiformis, Curtos sauteri, C. costipennis,* and *Pyrocoelia praetexta,* could all be identified with high resolution using the *COI* (Goh et al., 2022). A number of firefly genera, including *Pteroptyx*, *Colophotia*, *Poluninius*, and *Pyrocoelia*, could also be recognized based on morphological characteristics and the *COI* gene. Moreover, species identification was successful for male-female at the adult and larva stages of *Pyrocoelia analis* based on different morphological characteristics together with molecular maker *COI* (Jusoh et al., 2014).

According to these findings, *COI* nucleotides are one of the most effective tools of categorizing the firefly's genera, and of differentiating between and within firefly species. In addition, the *16s rRNA* gene assists in improving the categorize of *Trisinuata* spp. and *Medeopteryx* sp. with strong tree support value. At the same time, the *16s rRNA* gene was thought to be a trustworthy DNA marker for identifying numerous animal taxa in both vertebrates and invertebrates, including the hydrozoan (Stanger-Hall et al., 2007; Mitani et al., 2009; Zheng et al., 2014; Elbrecht et al., 2016; Saad, 2019). The additional use of *16s rRNA* as DNA barcoding in hydrozoans may result in more accurate hydrozoan identification than using *COI* solely (Zheng et al., 2014). The *16s rRNA* gene has been shown to be extremely effective in increasing the precision of interspecies identification in Madagascan frogs and may aid in enhancing species identification in larval stages (Vences et al., 2005). Additionally, *16s rRNA* increased the amplification in some insects that are less elaborate when employing the *COI* gene, helping to lessen primer bias in animals including insect identification (Elbrecht et al., 2016). When it comes to identifying the Lampyridae in North America and demonstrating their shared resemblance to specimens from Europe, Asia, and tropical America, mitochondrial DNA, such as *16S* and *COI*, are highly effective (Stanger-Hall et al., 2007). Furthermore, the combination of *COI* and *16s RNA* gene can enhance the efficacy to distinguish firefly species together with the morphological study.

6. Conclusion

According to the results, the nucleotide sequencing of the *COI* gene is capable of distinguishing different species of fireflies. Furthermore, comparable outcomes were obtained from the nucleotide sequencing of the *16s rRNA* gene. Combinations of the *COI* gene with the *16s rRNA* gene are effective in separating the five firefly species that were collected from lower northern Thailand (*Pygoluciola* sp.1, *Pygoluciola* sp.2, *Trisinuata* sp.1, *Trisinuata* sp.2 and *Medeopteryx* sp.). Consequently, analyzing the *COI* gene's nucleotide sequence in conjunction with the *16s rRNA* gene will improve the precision of firefly categorization.

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8. Ethics Approval

This research was approved for the Ethics of Use Animals for Scientific Work from Naresuan University (Approvement No. 64 01 004).

9. References

Ballantyne, L. A., & Lambkin, C. L. (2013). Systematics and phylogenetics of Indo-Pacific Luciolinae fireflies (Coleoptera: Lampyridae) and the description of new

genera. *Zootaxa*, *3653*(1), 1-162. https://doi.org/10.11646/zootaxa.3653.1.1

Boore, J. L. (1999). Animal mitochondrial genomes. *Nucleic Acids Research*, *27*(8), 1767-1780. https://doi.org/10.1093/nar/27.8.1767

Cameron, S. L. (2013). Insect mitochondrial genomics: Implications for evolution and phylogeny. *Annual Review of Entomology*, *59*, 95-117. https://doi.org/10.1146/annurev-ento-011613-162007

Cameron, S. L., & Whiting, M. F. (2008). The complete mitochondrial genome of the tobacco hornworm, *Manduca sexta* (Insecta: Lepidoptera: Sphingidae), and an examination of mitochondrial gene variability within butterflies and moths. *Gene*, *408*(1-2), 112-123. https://doi.org/10.1016/j.gene.2007.10.023

Clare, E. L., Kerr, K. C., von Königslöw, T. E., Wilson, J. J., & Hebert, P. D. (2008). Diagnosing mitochondrial DNA diversity: applications of a sentinel gene approach. *Journal of Molecular Evolution*, *66*, 362-367. https://doi.org/10.1007/s00239-008-9088-2

Clary, D. O., & Wolstenholme, D. R. (1985). The mitochondrial DNA molecule of Drosophila yakuba: nucleotide sequence, gene organization, and genetic code. *Journal of Molecular Evolution*, *22*, 252-271. https://doi.org/10.1007/BF02099755

Dong, P. X., Hou, Q. B., Li, X. Y., & Liang, X. C. (2008). Cloning, expression and sequence analysis of a luciferase gene from the Chinese firefly *Pyrocoelia pygidialis*. *Zoological Research*, *29*(5), 477-484. https://doi.org/10.3724/SP.J.1141.2008.05477

Elbrecht, V., Taberlet, P., Dejean, T., Valentini, A., Usseglio-Polatera, P., Beisel, J. N., ... & Leese, F. (2016). Testing the potential of a ribosomal 16S marker for DNA metabarcoding of insects. *PeerJ*, *4*, Article e1966. https://doi.org/10.7717/peerj.1966

Fan, Y., & Fu, X. (2017). The complete mitochondrial genome of the firefly, *Pteroptyx maipo* (Coleoptera: Lampyridae). *Mitochondrial DNA Part B*, *2*(2), 795-796. https://doi.org/10.1080/23802359.2017.1398598.

Ge, X. Y., Liu, T., Kang, Y., Liu, H. Y., & Yang, Y. X. (2022). First complete mitochondrial genomes of Ototretinae (Coleoptera, Lampyridae) with evolutionary insights into the gene rearrangement. *Genomics*, *114*(2), Article 110305. https://doi.org/10.1016/j.ygeno.2022.110305

Goh, K. S., Wang, L. J., Ni, J. H., & Wang, T. Y. (2022). Luminescent characteristics and mitochondrial COI barcodes of nine cohabitated Taiwanese fireflies. *PeerJ*, *10*, Article e14195. https://doi.org/10.7717/peerj.14195

Han, T., Kang, T., Jeong, J., Lee, Y., Chung, H., Park, S., ... & Park, H. (2012). Pseudocryptic speciation of *Chrysochroa fulgidissima* (Coleoptera: Buprestidae) with two new species from Korea, China and Vietnam. *Zoological Journal of the Linnean Society*, *164*(1), 71-98. https://doi.org/10.1111/j.1096-3642.2011.00763.x

Han, T., Lee, W., Lee, S., Park, I. G., & Park, H. (2016). Reassessment of species diversity of the subfamily Denticollinae (Coleoptera: Elateridae) through DNA barcoding. *PLoS One*, *11*(2), Article e0148602. https://doi.org/10.1371/journal.pone.0148602

Hebert, P. D., Cywinska, A., Ball, S. L., & DeWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(1512), 313-321. https://doi.org/10.1098/rspb.2002.2218

Hebert, P. D., Penton, E. H., Burns, J. M., Janzen, D. H., & Hallwachs, W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences*, *101*(41), 14812-14817. https://doi.org/10.1073/pnas.040616610

Hu, J., & Fu, X. (2018a). The complete mitochondrial genome of the firefly, *Abscondita anceyi* (Olivier)(Coleoptera: Lampyridae). *Mitochondrial DNA Part B*, *3*(1), 442-443. https://doi.org/10.1080/23802359.2018.1456373

Hu, J., & Fu, X. (2018b). The complete mitochondrial genome of the firefly,

Luciola curtithorax (Coleoptera: Lampyridae). *Mitochondrial DNA Part B*, *3*(1), 378-379. https://doi.org/10.1080/23802359.2018.1437817

Jusoh, W. F., Hashim, N. R., Sääksjärvi, I. E., Adam, N. A., & Wahlberg, N. (2014). Species delineation of Malaysian mangrove fireflies (Coleoptera: Lampyridae) using DNA barcodes. *The Coleopterists Bulletin*, *68*(4), 703-711. http://www.jstor.org/stable/24773405

Kang, T. H., Han, T. M., Okushima, Y., & Park, H. C. (2012). Integrative taxonomy of *Asiopodabrus fragiliformis* (Kang and Kim, 2000)(Coleoptera: Cantharidae) and its related species. *Zootaxa*, *3259*(1), 1-33. https://doi.org/10.11646/ZOOTAXA.3259.1.1

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular biology and evolution*, *35*(6), Article 1547. https://doi.org/10.1093/molbev/msy096

Li, W., Liu, Q., & Fu, X. (2022). The complete mitochondrial genome of the firefly *Curtos fulvocapitalis* (Coleoptera: Lampyridae). *Mitochondrial DNA Part B*, *7*(1), 1-3. https://doi.org/10.1080/23802359.2021.1958080

Li, X. Y., YANG, S., & Liang, X. C. (2006). Phylogenetic relationship of the firefly, *Diaphanes pectinealis* (Insecta, Coleoptera, Lampyridae) based on DNA sequence and gene structure of luciferase. *Zoological Research*, *27*(4), 367-374.

Liu, Q., & Fu, X. (2020). The genetic variations in the mitochondrial genomes of three Luciolinae fireflies. *Mitochondrial DNA Part B*, *5*(3), 3210-3214. https://doi.org/10.1080/23802359.2020.1806126

Luan, X., & Fu, X. (2016). The complete mitochondrial genome of the firefly, *Asymmetricata circumdata* (Motschulsky)(Coleoptera: Lampyridae). *Mitochondrial DNA Part B*, *1*(1), 553-555. https://doi.org/10.1080/23802359.2016.1199000

Maeda, J., Kato, D. I., Arima, K., Ito, Y., Toyoda, A., & Noguchi, H. (2017). The complete mitochondrial genome sequence and phylogenetic analysis of Luciola lateralis, one of the most famous firefly in Japan (Coleoptera: Lampyridae). *Mitochondrial DNA Part B*, *2*(2), 546-547. https://doi.org/10.1080/23802359.2017.1365640

Martin, G. J., Branham, M. A., Whiting, M. F., & Bybee, S. M. (2017). Total evidence phylogeny and the evolution of adult bioluminescence in fireflies (Coleoptera: Lampyridae). *Molecular phylogenetics and evolution*, *107*, 564-575. https://doi.org/10.1016/j.ympev.2016.12.017

Mitani, T., Akane, A., Tokiyasu, T., Yoshimura, S., Okii, Y., & Yoshida, M. (2009). Identification of animal species using the partial sequences in the mitochondrial 16S rRNA gene. *Legal medicine*, *11*, S449-S450. https://doi.org/10.1016/j.legalmed.2009.02.002

Nak-Eiam, S. (2015). *Taxonomy and species distribution of fireflies (Coleoptera: Lampyridae) in the north of Thailand* [Doctoral dissertation]. Department of Biology, Naresuan University, Phitsanulok.

Notredame, C., Higgins, D. G., & Heringa, J. (2000). T-Coffee: A novel method for fast and accurate multiple sequence alignment. *Journal of molecular biology*, *302*(1), 205-217. https://doi.org/10.1006/jmbi.2000.4042

Saad, Y. M. (2019). Analysis of 16S mitochondrial ribosomal DNA sequence variations and phylogenetic relations among some Serranidae fishes. *South African Journal of Animal Science*, *49*(1), 80-89. https://doi.org/10.4314/sajas.v49i1.10

Stanger-Hall, K. F., Lloyd, J. E., & Hillis, D. M. (2007). Phylogeny of North American fireflies (Coleoptera: Lampyridae): implications for the evolution of light signals. *Molecular phylogenetics and evolution*, *45*(1), 33-49. https://doi.org/10.1016/j.ympev.2007.05.013

Urtgam, S., & Jongjitvimol, T. (2020). Genetic evolution of Asian predatory wasp, *Vespa velutina*, in northern of Thailand based on cytochrome oxidase subunit I DNA barcoding. NU. *International Journal of Science*, *17*(2), 101-113.

Urtgam, S., Sumpradit, T., & Thurnkul, N. (2023). Isolation, Identification, and Application of Pigment-Producing Actinobacteria from Stingless Bee Hives for Handicraft Production. *Journal of Current Science and Technology, 13*(3), 564-573. https://doi.org/10.59796/jcst.V13N3.2023.749

Vences, M., Thomas, M., Van der Meijden, A., Chiari, Y., & Vieites, D. R. (2005). Comparative performance of the *16S rRNA* gene in DNA barcoding of amphibians. *Frontiers in zoology*, *2*(1), 1-12. https://doi.org/10.1186/1742-9994-2-5

Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol*, *3*(5), 294-299.

Wattanachaiyingcharoen, W., & Urtgam, S. (2022). Using the cytochrome c oxidase Subunit I gene as a molecular tool to identify firefly larva species. *NU. International Journal of Science*, *19*(1), 9-17.

Yang, L., Wei, Z. J., Hong, G. Y., Jiang, S. T., & Wen, L. P. (2009). The complete nucleotide sequence of the mitochondrial genome of *Phthonandria atrilineata* (Lepidoptera: Geometridae). *Molecular biology reports*, *36*, 1441-1449. https://doi.org/10.1007/s11033-008-9334-0

Zheng, L., He, J., Lin, Y., Cao, W., & Zhang, W. (2014). 16S rRNA is a better choice than COI for DNA barcoding hydrozoans in the coastal waters of China. *Acta Oceanologica Sinica*, *33*, 55-76. https://doi.org/10.1007/s13131-014-0415-8