# Using the Cytochrome *c* Oxidase Subunit I Gene as a Molecular Tool to Identify Firefly Larva Species

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## ABSTRACT

The larval stage of the firefly is the longest period of its life cycle that may take a year or more to complete. Collected larval samples need to be reared until they are adult and then they can be identified. The *COI* gene is a molecular marker widely used in identifying insect species. According to heredity, genetic inheritance from parents can pass to the offspring. We aimed to identify firefly species of collected larvae by comparing the *COI* gene sequence with known adult species, *Pyrocoelia praetexta*. Collected larva samples were divided into two groups, the first group was reared into adults in the laboratory and were morphologically identified. The second group with six larvae was used to analyze the *COI* gene sequences along with three adult *P. praetexta* samples. The sequences of the gene were aligned together with the *COI* gene of other three firefly species. The phylogenetic tree was constructed with the Maximum Likelihood method and showed the relationship among these observed firefly samples. Reared larval samples were identified as *P. praetexta* when they reached the adult stage. All collected larva samples and adults of *P. praetexta* were categorised in the same clade with the 99-100% branch support value and lower evolutionary divergence, indicating that they were the same species. Therefore, the *COI* gene could be a useful genetic marker to identify firefly species from larval samples. The results supported the idea that firefly samples could be identified in the larval stage using an appropriate genetic marker.

*Keywords*: *Firefly, Pyrocoelia praetexta, Cytochrome c oxidase subunit I, COI gene*

## INTRODUCTION

The life cycle of fireflies involves a complete metamorphosis consisting of egg, larval, pupal, and adult stages. Adult fireflies of all species live on land, but in the first three life stages, different species can be either aquatic, semi-aquatic, or terrestrial, depending on the species. Several previous studies have reported that the larval stage of many firefly species constitutes the longest period of their life cycle. For example, a study on terrestrial firefly *Photuris fulvipes* in Brazil showed that its egg stage lasted an average of 19 days, while its larval stage lasted an average of 282 days (Rosa, 2007). The life cycle of the aquatic firefly *Luciola ficta* was investigated in Taiwan by (Ho et al., 2010), who found that females spent about 337.1±31.2 days in the larval stage, while males spent about 307.6±34.1 days. The larval period accounted for about 84% of *Luciola ficta*'s life span. Another aquatic firefly, *Luciola substriata* was observed in Hubei Province, China, by (Fu et al., 2012). Larvae of this species overwintered in ponds and lakes from September to April/May. After that, they went through their pupal and adult stages during the summer, at which time mating and oviposition occurred. The larval stage of *L. substriata*, too, was the longest stage in its life cycle.

Although there are thousands of known firefly species worldwide, there has never been a way to distinguish between these different species while they are still in the larval stage. Therefore, to accomplish specific species identification from larvae, researchers have been imperative to raise collected larvae to their adult stage, at which point the species can be identified by morphology. There are two problems with that current workaround. The first issue is that the duration of the larval stage, as just mentioned, is quite long. The second issue is that firefly larvae are notoriously and burdensome to rear to adulthood successfully. The reasons for low survival rates in the laboratory are not known.

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Molecular analysis is one method currently used to address taxonomical uncertainties in many sorts of organisms. The Cytochrome *c* oxidase subunit I *(COI*) gene is one of the most widely used molecular markers in the study of animals because of its unique properties and high reliability. This gene is genetically conserved in almost all insect groups and a pervasive marker for insect studies (Hebert et al., 2003). Several controversial studies on insect identification, especially those with complicated morphological characters, have been cleared by using molecular approaches. This technique has been conducted to clarify the ambiguous morphological characters of dry museum specimens of a skipper, *Astraptes fulgerator* (Lepidoptera: Hesperiidae). The sequences of the *COI* gene of these specimens revealed that the *Astraptes fulgerator* was a species complex (Hebert et al., 2004). The method is also valuable to detect cryptic and/or pseudo-cryptic species with little morphological differentiation, e.g., *Asiopodabrus* in Cantharidae (Kang et al., 2012); *Chrysochroa* in Buprestidae and *Denticollinae* in Elateridae (Han et al., 2012; Han et al., 2016). In firefly and luminous insect studies, the origin and evolution of bioluminescence were explored using molecular markers. Also, their taxonomic statuses have been investigated by combined morphological characters and molecular markers (Suzuki, 1997; Branham & Wenzel, 2001; Branham & Wenzel, 2003; Li et al., 2006; Sagegami-Oba et al., 2007). Han (2019) using the DNA barcoding analysis on *COI* gene sequences to examine the species statuses of the firefly subgenus *Hotaria sensu lato*. This subgenus was previously identified as a member of the genus *Luciola*, based on morphological data which consisted of four morphospecies: *Luciola (Hotaria) parvula*, *L.* (*H.*) *unmunsana*, *L*. (*H*.) *papariensis*, and *L*. (*H*.) *tsushimana*. The results for *COI* gene sequence analysis clarified that the two types of *L*. (*H*.) *parvula* from Japan could be separated as distinct species. The other three morphospecies *L*. (*H*.) *unmunsana*, *L.* (*H*.) *papariensis*, and *L*. (*H.*) *tsushimana*) with unclear morphological characters, are assigned as distinguished and implicate groups by molecular analysis.

The survey of firefly diversity in the northern region of Thailand reported various un-identified species (Nak-eiam, 2015). To complete the taxonomic status of firefly species, researchers need to explore insight into morphological characters, biology such as life cycle, and ecology such as habitat preferences. The study of firefly biology, both in larval and adult stages, can be done in field and laboratory conditions. In laboratory conditions, the longer periods of larva development may be the intricate periods. Because these larvae may confront the obstruction of unusual development or even die during this period (Fu & Meyer-Rochow, 2013). Hence, rearing fireflies from the larval stage to the adult stage is a time-consuming protocol. Besides, for those sympatric species, collections of fireflies may be ambiguous, especially in larval stages. The larval stages in some closely related firefly species seem to be similar. Hence, it is difficult to tell them apart. For example, larvae of *Photuris fulvipes* are reciprocal to other *Photuris* sp. therefore, it is possible to miss-identify the species (LaBella & Lloyd, 2013). These complications may cause difficulties for researchers and may lead to an unsuccessful study. The inconvenience lies in the proper identification of different species. Especially for the identification of new species. As we know that, the inheritance that passes from parents to offspring is consistent over generations. Hence, the tracing of genetic information gives insight into the inherited background of each species. Molecular markers have been used to clarify ambiguous traces; therefore, the *COI* gene is widely used to investigate genetic traits. *Pyrocoelia praetexta* is one of the common terrestrial firefly species which distribute throughout the lowland area of the lower northern Thailand. This species resides in agricultural areas and natural habitats. In this study, we investigate the possibility of using an analysis of the *COI* gene of firefly samples to confirm the identity of those larvae with an adult of known species, such as *P. praetexta*.

## MATERIALS AND METHODS

### Sample collections and identification

We surveyed and collected larvae of fireflies that were expected to be Pyrocoelia praetexta from a field in Phitsanulok province, Thailand. The larvae were kept in a plastic box and transferred to the laboratory. Flying adults from the same location were also collected by an insect sweeping net and then were preserved in a vial with 70% ethanol. The adult samples were identified using identification guides as follows,

- 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).

After identification, the adults were preserved individually in a vial with 95% ethanol for molecular study along with the larvae. Larva samples were divided into two groups. The first group consisted of four larvae that were reared in the insectary room until they reached the adult stage and were identified to a specific level using the above references. The second group of six larvae was preserved individually in a vial with 95% ethanol for further study. For the molecular analysis, three adult and six larvae firefly samples were employed.

**DNA extraction**

Total DNA was extracted from 50-100 mg of muscle tissue of both adult and larva firefly samples using BioFact™ Genomic DNA Prep Kit (BIOFACT, Daejeon, Korea) according to the manufacturer's instructions. The extracted DNA was analyzed in 1% agarose gel electrophoresis containing 1xSYBR® Safe DNA gel stain (Invitrogen, USA) and kept at -20°C for further analysis.

**Polymerase chain reaction**

Cytochrome *c* oxidase subunit I (*COI*) gene was amplified using Polymerase chain reaction (Mullis et al.,1986). Twenty ng of total DNA was used with *Taq* DNA polymerase (Invitrogen, USA) according to the manufacturers' instructions. The PCR master mix containing 25 µl of 10x *Taq* buffer, 3 mM $MgCl_2$, 200 µM of each dNTP, 200 nM of each specific primer for *COI* gene (*COI*-F; 5'-GGAGCTCCTGACATAGCATTCCC-3' and *COI*-R; 5'-CCCGGTAAAATTAAAATATAAACTTC-3') (Simon et al., 1994) was carried out in T100™ Thermal Cycler (Bio-Rad, USA) with optimal condition following; heated at 94°C for 3 min and amplified by 30 cycles of 94°C for 45 seconds, 47.5°C for 30 seconds and 72°C for 1 min followed by incubating at 72°C for 7 min for a final extension (Urtgam & Jongjitvimol, 2020). The PCR products were analyzed on 1.5% agarose gel electrophoresis containing 1xSYBR® Safe DNA gel stain (Invitrogen, USA) and visualised under the UV light with Bio-Rad Gel Documentary (Bio-Rad, USA).

**Nucleotide analysis**

PCR product was purified using BioFact™ Gel & PCR purification System (BIOFACT, Daejeon, Korea) according to the manufacturers' instructions. The purified PCR product was sequenced via Sanger DNA sequencing by Bionics Company (Korea). The 9 nucleotide sequences of *P. praetexta* were aligned together with 3 nucleotide sequences of *Pyrocoelia rufa* (AF277831.1), *Luciola curtithorax* (NC038225.1) and *Luciola lateralis* (AF360951.1) with ClustalW and the phylogenetic tree was constructed using the Maximum Likelihood method and Tamura-Nei model with Gamma distribution together with 1,000 replicates of Bootstrap in MEGA X (Kumar et al., 2018).

## RESULTS AND DISCUSSION

**Sample collections and identification**

Out of four larvae collected for rearing to adulthood, only two survived to reach adulthood. They were compared with the collected adult samples and were identified using the identification references mentioned above (Nak-eiam, 2015; Ballantyne & Lambkin, 2013). All samples were identified, based on morphological characteristics as *Pyrocoelia praetexta*. The brief distinguished characters of adults are dark brown head with paler brown antennae, long serrate antennae, pronotum is semicircular shaped and is orange yellow, elytra is dark brown with narrowly yellowish orange line surrounding the elytron except for the base and the length is as long as total body length, compose of eight tergal segments which almost are very dark brown, posterior margin of tergite 8 (T8) is shortly rounded *trisinuate*, ventral abdomen comprises of seven visible segments, light organs is in the middle of ventrite 6 (V6) and ventrite 7 (V7) as transversely oblong band with rounded apices. This result confirmed that the larvae and the adult samples were the same species (Figure 1).

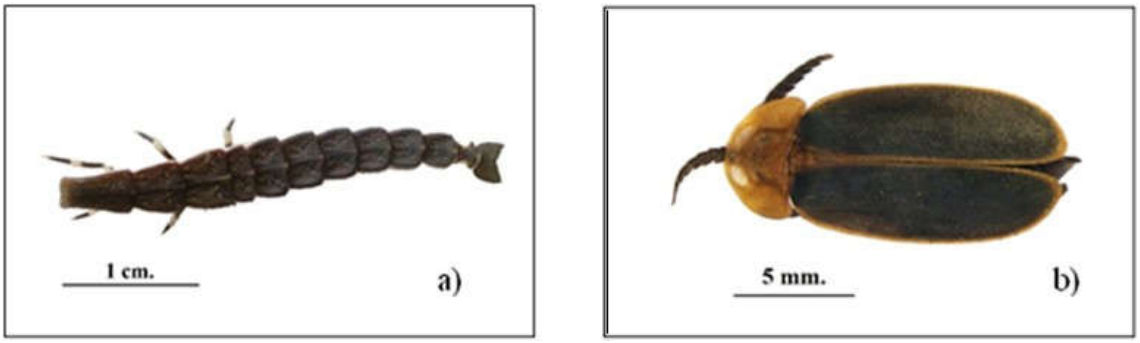


Figure 1 Samples collected a) larval stage, b) adult stage.

**Nucleotide contained in Cytochrome *c* oxidase subunit I gene of *Pyrocoelia praetexta***

The Cytochrome *c* oxidase subunit I (*COI*) gene was amplified in six larvae and three adults of *Pyrocoelia praetexta* with specific primer following by nucleotide analysis via the standard sanger sequencing method with both of forward and reverse primer. The nucleotide of *COI* form both forward and reverse sequences were combined in MEGA X. The nine amplicons of *COI* of *P. praetexta* are in the range of 405-491 bp correlated with the nucleotide size of *COI* in *P. rufa* (403 bp) (Li et al, 2003). The nine *COI* sequences of *P. praetexta* were aligned, and the nucleotide contained therein were characterized using ClustalW in MEGA X. The aligned sequence was finally trimmed to 405 bp (Figure 2) with GC contained as 33.54, 32.35 and 33.14% in the larva, adult, and average of both larva and adult, respectively. (Table 1).

The data revealed that the ratio of A:T:C:G of Cytochrome *c* oxidase subunit I in *P. praetexta* is about 2:2:1:1. Note that the ratio of base composition in *COI* gene reported in this study is consistent with those of mitochondrial genomes in the other species e.g., *Pygoluciola qingyu*, *Abscondita terminalis*, *Emeia pseudosauteri* (Liua & Fu, 2020). Moreover, most of the base substitution in the *COI* gene of *P. praetexta* was point mutation, and transition mutation occurs more frequently than transversion mutation (Figure 2). Transition mutation could generally be found due to tautomeric shift mutation during the evolution of UV induction mutation and give raise to the evolution of living things.

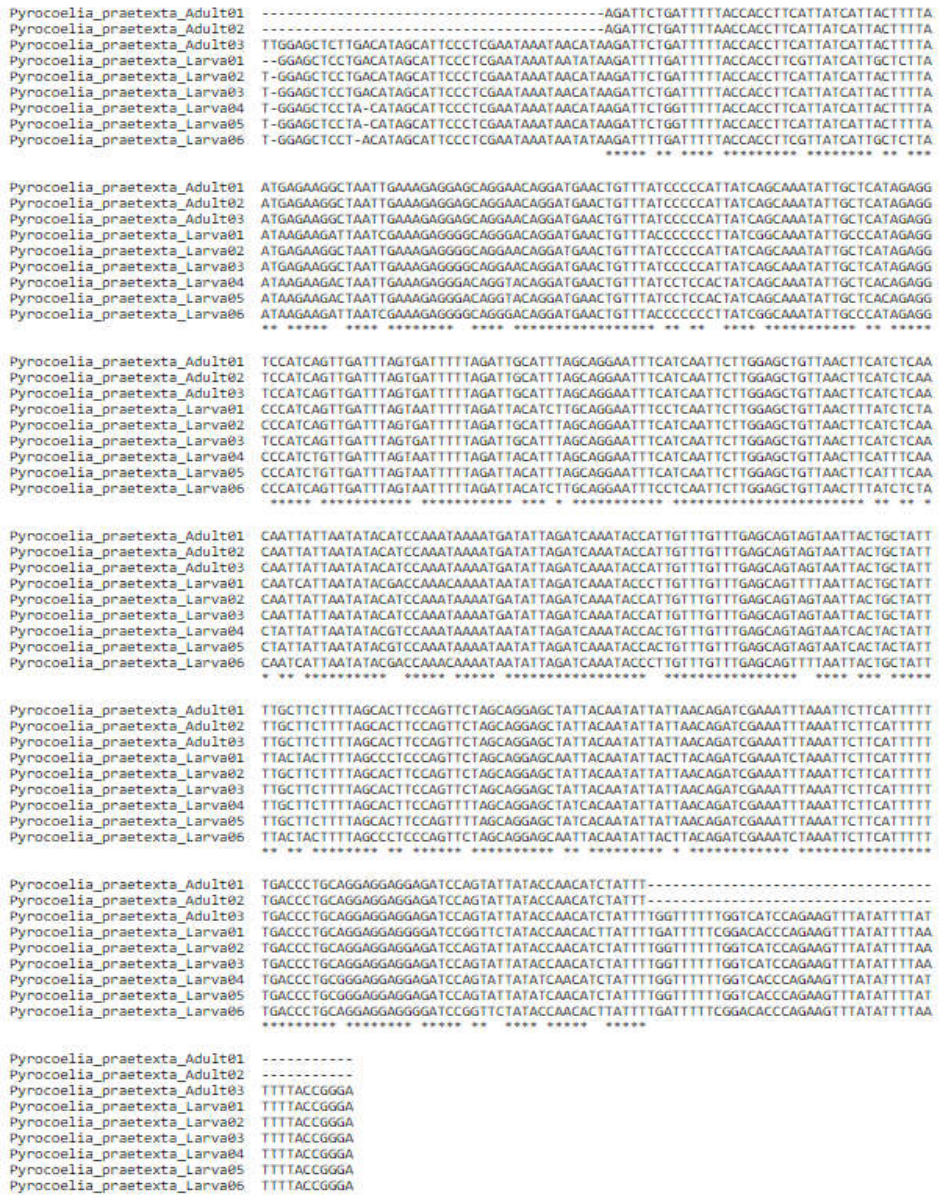


**Figure 2**. Multiple alignment of 9 Cytochrome *c* oxidase subunit I nucleotide sequences of Pyrocoelia praetexta using T-COFFEE, Version_11.00 (Di Tommaso et al., 2011). The symbol '*' indicates monomorphic nucleotides present in all the nine *Pyrocoelia praetexta COI* sequences.

**Phylogenetic tree analysis of Cytochrome *c* oxidase subunit I gene in *Pyrocoelia praetexta***

The sequences of the *COI* gene in larva and adult *P. praetexta* were aligned together with the *COI* gene in other fireflies. Three reference *COI* gene nucleotides of *Pyrocoelia rufa* (AF277831.1); *COI* of 403 bp, *Luciola curtithorax* (NC038225.1); mitochondrion complete genome of 16,882 bp, and *Luciola lateralis* (AF360951.1); *COI* of 403 bp, were retrieved from NCBI and were used as the database for phylogeny study. Finally, the aligned sequence was trimmed to 306 bp and the phylogenetic tree was constructed via the Maximum Likelihood method and Tamura-Nei model with Gamma distribution together with 1,000 replicates of Bootstrap were performed (Fig. 3). The phylogenetic tree showed that all those six larval and three adult samples of *P. praetexta* were categorized in the same group with 97-100% branch support value, indicating the closed relationship

among these samples. In addition, larvae of *P. praetexta* were separated into 3 clades with the first clade consisted of larvae No 02/03 together with adult *P. praetexta* (No 01/02/03). The second clade comprised of larvae No 04/05, and larvae No 01/06 were in the third clade. Even though, larvae No 01/04/05/06 were not categorized in the same clade i.e., larva No 02/03 together with adult *P. praetexta* (98%), larva No 04/05 (100%), and larva No 01/06 (99%). Even though, all *P. praetexta* was categorized in the same group with high branch support value as 97%. This result similar to those of the finding in 2 firefly species distributed in Sarawak and Peninsula Malaysia i.e., *Pteroptyx tener* and *Pteroptyx bearni* which was categorized into 2 clades within each species (Jusoh et al., 2014). Moreover, the similar result was also found in the Korean endemic firefly, *Luciola unmunsana*, which was categorised into 3 clades (Han et al., 2019), and also recorded in *Luciola lateralis* by Kim et al. (2001). These data indicated that structured variations in the DNA barcode could generally occur in fireflies, especially regarding with the geographical interference. However, the evolution distance confirmed that all the 9 samples of *P. praetexta* (6 larvae and 3 adult samples) are still assigned as the same species based on the DNA barcode of Cytochrome *c* oxidase subunit I gene with a very low value of the evolutionary divergence at 0.139 (Table 1). However, the inter-species evolution distances between *P. praetexta* and other species studied were high; i.e., *P. praetexta* and *P. rufa* (AF277831.1) (0.516), *Luciola curtithorax* (NC038225.1) (1.399), *Luciola lateralis* (AF360951.1) (2.129) and overall evolution distances was 0.666 (Table 1). The evolution distance of these 4 species shows that intra-specific divergence of those firefly samples observed in this study was lower than those inter-specific divergence (illustrating 4 to 12-fold) which are well correlated with the finding in genus *Luciola* (4 to 7-fold) (Han et al., 2019).

Therefore, the results from our study confirmed that the DNA barcode of *COI* gene could distinguish and separate the firefly species according to the literatures of animal identification based on DNA barcode of *COI* such as bird, insects, as well as firefly (Hebert et al., 2004; Foottit et al., 2008; Salokannel et al., 2012; Landvik et al., 2013; Jusoh et al., 2014). In this research, the DNA barcode of *COI* gene sequence could be used as a genetic marker to identify *P. praetexta* both in the larva and adult stages. This finding is consistent with the previous study exhibiting the successful identification of larva and female Lampyridae (Jusoh et al., 2014). However, the accuracy of identification for both larva and adult *P. praetexta* could be improved by employing DNA barcode of *COI* together with morphological study.

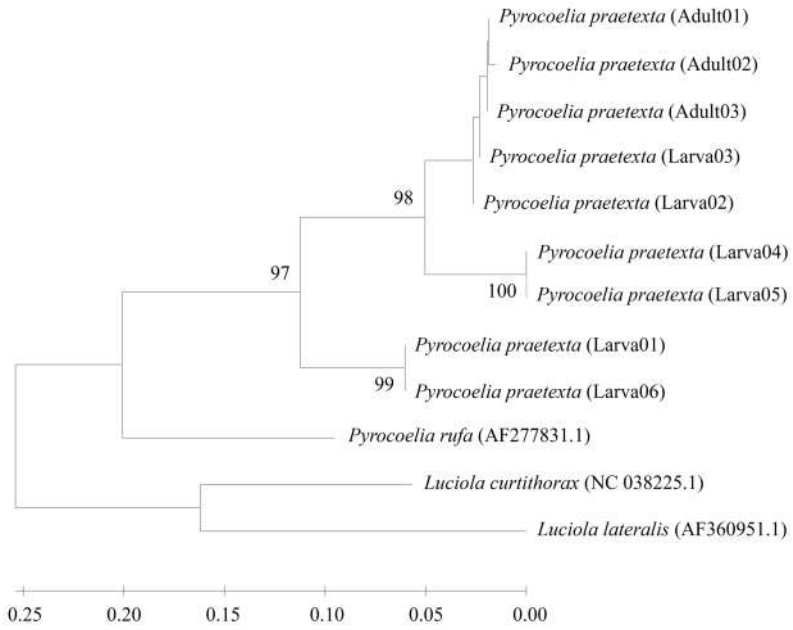


Figure 3 Phylogeny study of *Pyrocoelia praetexta* analyzed by Cytochrome *c* oxidase subunit I (*COI*) gene. The tree was constructed by Maximum Likelihood method and Tamura-Nei model with Gamma distribution. The numbers shown on each node only higher than 95% branch support value by 1,000 replicates of Bootstrap method (Kumar et al., 2018).

**Table 1.** Estimates of evolutionary divergence between Cytochrome *c* oxidase subunit I sequences.

| **Species/Species** | **Average** |
|---|---|
| *Pyrocoelia praetexta* (**within species**) | 0.139 |
| *Pyrocoelia praetexta / Pyrocoelia rufa* (AF277831.1) | 0.516 |
| *Pyrocoelia praetexta / Luciola curtithorax* (NC038225.1) | 1.399 |
| *Pyrocoelia praetexta / Luciola lateralis* (AF360951.1) | 2.129 |
| *Pyrocoelia rufa* (AF277831.1) */ Luciola curtithorax* (NC038225.1) | 0.840 |
| *Pyrocoelia rufa* (AF277831.1)*/Luciola lateralis* (AF360951.1) | 1.160 |
| *Luciola curtithorax* (NC038225.1)*/Luciola lateralis* (AF360951.1) | 0.582 |
| **Overall** | 0.666 |

## CONCLUSIONS

Our study suggested that the *COI* gene was able to distinguish *P. praetexta* from other firefly species. Within the *Pyrocoelia* clade, all larval and adult samples of *P. praetexta* were grouped into the same clade with 99-100% branch support value. This *P. praetexta* was obviously separated from another *Pyrocoelia* species, and two *Luciola* species. The results of the phylogenetic tree and the branch support value including the evolutionary divergence value indicated that all samples of *Pyrocoelia praetexta*, both larvae and adults, were categorized in the same species. This was also

supported by the morphological study of reared and collected adults. Therefore, the *COI* gene can be a significant genetic marker to identify the larval stage of other firefly species. This finding will be useful for the biological and ecological study of those ambiguous characters or those of sympatric species fireflies, as the researchers can be able to collect the samples at larval stages and use the *COI* gene nucleotide analysis compare with the known adult species to confirm the species directly. The appropriate genetic marker could be an effective tool to ensure that the collected larval firefly samples are precise species for further research.

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### Conflict of Interest Statement

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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