Using *COI* Gene Sequence for Species Identification of Webspinners (Embioptera) in Thailand
การใช้ลำดับเบสของยีน *COI* สำหรับระบุชนิดของแมลงปั่นใย (Embioptera) ในประเทศไทย

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Abstract

The webspinners (Embioptera) is a smallish cosmopolitan order of an estimated 2,000 species, of which roughly 400 have been named. The most distinct autapomorphy is the silk spinning apparatus located in the front legs of all individuals. Approximately 90 genera have been grouped into 13 families. However, taxonomic knowledge of these insects is poorly developed in Thailand. Therefore, Thai embiids were selected for molecular phylogeny based on mitochondrial gene cytochrome c oxidase I (*COI*) in this study. The phylogenetic construction using the unweighted pair group method with arithmetic average (UPGMA) could separate all samples into two main clusters, the outgroup (order Mantodea) and the nine embiids which could be further subdivided into two groups. Group 1 represented the family Oligotomidae (*Aposthonia borneensis*, *A. ceylonica*, *Lobosembia* mandibulata, *A. problita* and *Eosembia auripecta*), while Group 2 represented the family Embiidae (*Oedembia* sp.) and Ptilocembiidae (*Ptilocerembia* spp.). Nucleotide diversity among embiid species was 0.226±0.016. The results indicated that *COI* mtDNA is a suitable method for rapid species identification.

Keywords: Webspinners, mitochondrial DNA, DNA sequence

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บทคัดย่อ

แมลงปั่นใยจัดอยู่ในอันดับ Embioptera ซึ่งเป็นอันดับเล็กๆ อันดับหนึ่งโดยทราบชนิดแล้วประมาณ 400 ชนิด จากการประมาณจำนวนชนิดทั้งหมดถึง 2,000 ชนิด แมลงปั่นใยมีลักษณะเฉพาะพิเศษโดยที่ขาคู่หน้าสามารถผลิตเส้นใยได้ ทั้งนี้ประมาณ 90 สกุลได้ถูกจัดไว้ใน 13 วงศ์ แต่อย่างไรก็ตาม ความรู้ทางอนุกรมวิธานของแมลงชนิดนี้ยังมีอยู่อย่างจำกัดในประเทศไทย ดังนั้นจึงมีการนำตัวอย่างแมลงปั่นใยในประเทศไทยมาศึกษาทางด้านความสัมพันธ์เชิงวิวัฒนาการโดยทำการศึกษายีน *COI* ในไมโทครอนเดีย ผลการศึกษา แผนภูมิพันธุกรรมโดยวิธี UPGMA สามารถแบ่งตัวอย่างตัวอย่างทั้งหมดได้เป็น 2 กลุ่มใหญ่ เป็น outgroup ในอันดับ Mantodea และกลุ่มแมลงปั่นใย 9 ชนิด ซึ่งสามารถแบ่งย่อยได้เป็น 2 กลุ่ม โดยที่กลุ่ม 1 อยู่ในวงศ์ Oligotomidae (*Aposthonia borneensis*, *A. ceylonica*, *Lobosembia mandibulata*, *A. problita* and *Eosembia auripecta*) ขณะที่กลุ่มที่ 2 มีสมาชิกจากวงศ์ Embiidae (*Oedembia* sp.) และ Ptilocembiidae (*Ptilocerembia* spp.) ความแปรผันของลำดับนิวคลีโอไทด์ระหว่างชนิดโดยรวมเท่ากับ 0.226±0.016 ผลการศึกษาในครั้งนี้แสดงให้เห็นว่าการใช้ยีน *COI* เป็นวิธีการที่มีความเหมาะสมสำหรับการจัดจำแนกชนิดที่รวดเร็ว

คำสำคัญ: แมลงปั่นใย, ไมโทคอนเดรียดีเอ็นเอน, ลำดับเบสของดีเอ็นเอ

Introduction

Insects belonging to invertebrates (Phylum Arthropoda) are the earth's most varied organism (Daly *et al*., 1998). Nearly three-quarters of all described animal species are insects (Borror *et al.,* 1989; Daly *et al*., 1998; Gullan and Cranstan, 2004). Their numbers far exceed all other terrestrial animal species, and are found in almost every terrestrial habitat on the earth's surface. They have diversified to fill almost every environmental niche imaginable (Putman, 1983; Gullan and Cranstan, 1994), making them one of the most vital components of most terrestrial ecosystems. Like other insects, the webspinner is a relatively small insect order (Embioptera), comprising of about 400 known species in 13 families, whose members mainly appear in the tropical and subtropical realms (Ross, 2000, 2007). They are very interesting insects with unique, social behaviors and ecology including some primitive morphological characters and evolutionary biology. Ross (2007) stated that Thailand had a particularly rich and varied embiid fauna and many unknown species are awaiting the species identification.

In general, both sexes of adult insects are required for species identification. For webspinners, only remarkable morphological characteristics including wings and genitalia (terminalia) of adult males have been widely examined. Whereas, all females and immature have no distinguish characteristics for determination (Ross, 2007). In Thailand, taxonomic study based on major morphology has widely been observed (Ross, 2007; Poolprasert and Edgerly, 2011; Poolprasert et. al., 2011a, b; Poolprasert, 2012; Poolprasert, 2014; Poolprasert and Edgerly, 2014). Resulting in relationship within and between groups of webspinner is still poorly known. To clarify the evolutionary relationship of these Thai webspinners, the molecular technique (DNA barcoding) has been initially adopted so far (Miller et al., 2012).

Nowadays, molecular technique based on DNA sequence can be applied for the taxonomic identification of known and unknown specimens (McKenzie et al., 2003). Even though several different DNA based methods are potential available for many purposes such as quarantine and forensic examinations, one general applied method used approach known as DNA barcoding has been widely applied as methods to efficiently describe biodiversity (Armstrong and Ball, 2005). This technique uses DNA fragments gained from specific genes such as the cytochrome c oxidae I (*COI*) gene, a fragment of about 500-800 base pairs from mitochondrial DNA. *COI* has been extensively used in several animal species (Hebert et al., 2003a, 2003b). It is more conserved and very appropriate for species identification since its sequence has a low variability (less than 1-2%), even for the closely-related species its value is less than 1%. In addition, *COI* gene is one of the most common to be deliberated in implying the relationships among closely-related species in numerous insect groups (butterflies, flies and beetles), as an individual gene or its combination with other genes (Hebert et al., 2003a; 2003b; 2010). The knowledge about phylogenetic relationships of Thai embiids is still unclear. In this study, therefore, we primarily attempted to sequence *COI* gene in order to indentify to species level especially from female specimens using DNA barcode and to evaluate former phylogenetic hypotheses regarding nine embiid species belonging to main three families including Oligotomidae, Embiidae and Ptilocerembiidae found in Thailand.

Materials and Methods

1) Embiids sample collection

A total of nine embiids were gathered from main collection of Pibulsongkran Rajbhat University, Phitsanulok province, Thailand. All female specimens preserved in 95% alcohol were used for molecular technique.

2) DNA extraction, Amplification and Sequencing

Total genomic DNA was extracted from a single leg of webspinners using DNeasy® Blood & Tissues kit (Qiagen, Germantown, MD, USA). The protein-coding mitochondrial *COI* gene for molecular analysis was used in this study. The primers used for the polymerase chain reaction (PCR) amplification were LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer et al., 1994). Each PCR reaction was performed using a final volume of 20 μl containing 4 μl of 5x PCR enhancer, 2 μl of 10x HF reaction buffer, 0.4 μl 10 mM dNTP mix, 0.3 μl of each primer (10 μmol/L), 0.3 μl of Long and high fidelity DNA polymerase (0.75 U) (Biotechrabbit, Germany), 10.7 μl of nuclease free water and at least 2 ng of genomic DNA template.

The cycling program include an initial activation step of 3 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 48 °C for 1 min and extension at 72 °C for 1 min and a final extension step of 5 min at 72 °C. The amplification products were analyzed by electrophoresis in 1% agarose gel in 1x TAE buffer. A single band of PCR products were purified using the GenUP PCR/Gel Cleanup Kit (Biotechrabbit, Germany) as described by manufacture's instruction and direct sequences by Macrogen, Inc (http://www.macrogen.com).

3) Alignment of sequences and Phylogenetic analysis

Similarity search for each sequence was perfprmed using BLAST (https://www.ncbi.nlm.nih.gov/). Partial *COI* sequences were initially aligned in MEGA6 v6.06 (Tamura et al., 2013) using ClustalW (1.6) with the default settings (Gap Opening Penalty = 15, Gap Extension Penalty = 6.66 in both pairwise and multiple alignments). All *COI* sequences were finally trimmed to 630 base pairs. Then phylogeny

construction was performed using Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) tree building method. Genetic distances were computed using Kimura's 2-parameter (K2P) test (Kimura, 1980). The statistical confidence of a particular clade in all the tree building methods was evaluated by using bootstrap test with 1000 replications. Moreover, overall AT bias and nucleotide diversity (**TT**±SD.) were computed using DNAsp v5.10.01 program (Librado and Rozas, 2009).

Result and Discussion

The DNA sequence of the mitochondrial 5'*COI* gene region was analyzed in order to identify the species based on female materials and to infer phylogenetic relationship of the webspinners (order Embioptera). DNA fragments containing 630 base pairs of *COI* gene were obtained from nine embiid species belonging to three families (Oligotomidae, Embiidae and Ptilocerembiidae). When performing a BLAST search, It was found that all female specimens tested could provide species marches of more than 96% of sequence similarity. The average nucleotide composition proportions for the embiid sequences were detected to be A = 30.00%, T = 31.20%, G = 13.40% and C = 25.40% (Table 1). This finding was conformed to the results of the *COI* gene region of other insect groups exhibiting a bias towards adenine and thymine (Karthika et al., 2016). In general, the base composition of the *COI* fragment varied among the species but it was commonly demonstrated with an overall AT bias of 67.27 and GC of 32.73 (Karthika et al., 2016). In the same way, the A+T and G+C contents for this result were 61.20% and 38.80%, respectively (Table 1). In this regard, interspecific variation in the base composition in *COI* was relatively low for the total nucleotides (0.226±0.016). The genetic divergence for the coccinellid species ranged from 0.085 (*Ptilocerembia rossii* and *Pt. catherinae*) to 0.422 (*Oedembia* sp. and *Lobosembia mandibulata*) (Table 2).

Table 1 DNA composition in various species of webspinners containing nucleotides with pyrimidine (Thymine (T) and Cytosine (C)) and purine (Adenine (A) and Guanine (G)) bases.

	Nucleotide composition (%)			
Species	**T**	**C**	**A**	**G**
Ptilocerembia rossii	27.70	30.50	28.80	13.00
Ptilocerembia catherinae	26.80	31.70	27.30	14.20
Ptilocerembia roepkei	28.20	30.50	29.00	12.30
***Oedembia* sp.**	25.50	30.00	29.80	14.70
Aposthonia borneensis	35.00	18.50	33.80	12.70
Aphosthonia prolita	34.30	19.80	32.30	13.50
Aphosthonia ceylonica	34.30	21.70	29.50	14.50
Eosembia auripecta	34.00	23.30	29.80	12.80
Lobosembia mandibulata	34.80	22.30	29.80	13.00
Average	**31.20**	**25.40**	**30.00**	**13.40**

Table 2 Genetic distance (K2P) between embiid species obtained from *COI* gene sequence analysis.

Scientific name	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
(1) Ptilocerembia rossii									
(2) Ptilocerembia catherinae	0.085								
(3) Ptilocerembia roepkei	0.251	0.236							
(4) Oedembia sp.	0.360	0.345	0.363						
(5) Aposthonia borneensis	0.389	0.365	0.369	0.371					
(6) Aphosthonia prolita	0.346	0.323	0.397	0.397	0.142				
(7) Aphosthonia ceylonica	0.362	0.370	0.369	0.391	0.278	0.269			
(8) Eosembia auripecta	0.368	0.367	0.359	0.363	0.297	0.268	0.237		
(9) Lobosembia mandibulata	0.377	0.360	0.359	0.422	0.236	0.291	0.330	0.320	

A phylogenetic tree of nine species (five genera) belonging to three families (Oligotomidae, Embiidae and Ptilocerembiida) under the order Embioptera and relevant outgroup (order Mantodea) was constructed based on *COI* sequence using

Unweighted Pair-Group Method with Arithmetic Mean (UPGMA). Phylogenetic analysis demonstrated two main mt*COI* clades (Oligotomidae and Embiiodae + Ptilocerembiidae) (Figure 1). All species of family Oiligotomidae were monophyletic and closely related to each other, whereas *Oedembia* sp. (family Embiidae) was closely related to *Ptlilecerembia* group (family Ptilocerembiidae. *Oedembia* is a Southeast Asian group which, although well-supported as sister group to *Ptilocerembia*, shared no unambiguous morphological synapomorphies with that group. Given that Embiidae has experienced considerable historical change in taxon composition. In addition, *COI* discriminated well at the intergeneric and interspecific levels (Miller et al., 2012).

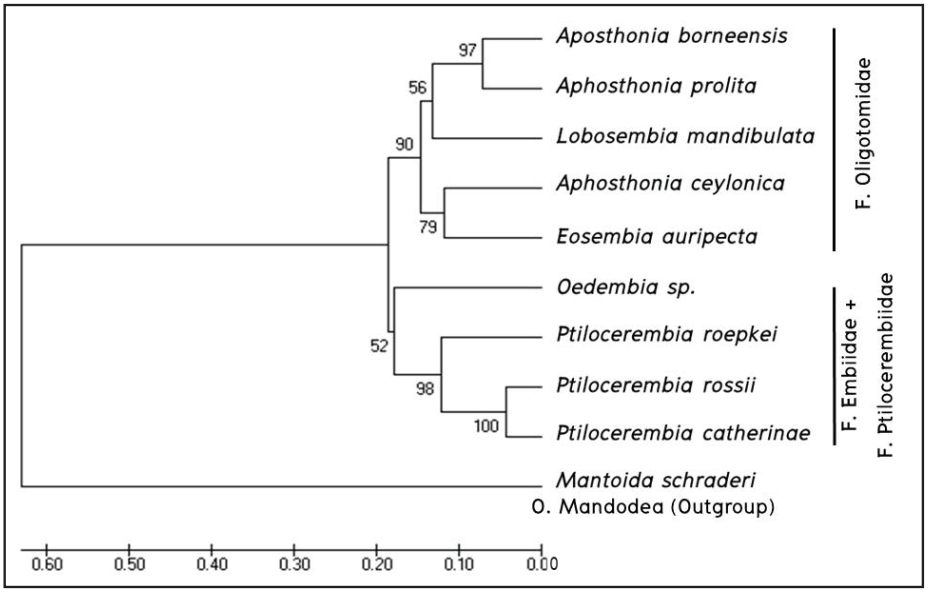

Figure 1. Phylogenetic relationships between families and species of embiids based on UPGMA of *COI* sequence.

This study demonstated that DNA sequencing was an effective tool for rapid, reliable species identification of Embioptrans. Other studies, focusing, for example on Lepidoptera (butterflies and moths), Hymenoptera (bees and wasps), Neuropterida (lacewings and antlions), Heteroptera (true bugs), Myriapoda (millipedes, centipedes, pauropods, and symphylans) and Orthoptera (grasshoppers and crickets) have obtained similar results, strongly suggesting the efficacy of DNA sequencing for all arthropods (Hebert et al., 2003a; 2003b; 2010).

Conclusion

Based on DNA sequencing, it was found that all Thai webspinners could be well classified into species level, suggesting *COI* mtDNA sequencing is an appropriated technique for rapid species identification and can be applied for other insect group. For phylogenetic relationship among nine Thai embiids, five species (*Aphostonia borneensis, A. ceylonica, A. problita, Eosembia auripecta* and *Lobosembia mandibulata*) belonging to family Oligotomidae was recovered as monophyletic, whereas, *Oedembia* sp. (family Embiidae) was still in the same group of Ptiocerembia (family Ptilocerembia). However, the tests of monophyly for this insect order (Embiotera) were relatively weak due to the small and unrepresentative taxon sampling that was available. The lack of strong support for interfamilial relationships demonstrated that this data alone was inadequate to clearly resolve relationships among embiid families. Future research which builds on this study should provide additional insight regarding the relationships among these enigmatic lineages and establish a foundation for a more natural and stable classification for this insect group.

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