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Diastolembia Thailandensis, A Remarkable New Genus and Species of Embiids (Embioptera: Embiidae) from Thailand

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Abstract: This research aimed at the morphological and molecular characterization of specimens collected in Thailand to extend the available information on webspinners (Embioptera). The purpose of this article is to describe *Diastolembia thailandensis* **n. gen.**, **n. sp.** (Embiidae) from Tak and Chiang Mai provinces. Alate males of this new species are distinguished by having a large lobe of basal segment of left cercus, expansion as angular and echinulate at the apex. Hind basitarsus is short with a single papilla. Meanwhile, apterous females are without distinctive characters. This discovery brings a total of 89 species under 24 genera worldwide for this insect family. Additionally, part of the mtDNA-COI gene of a peculiar new taxon and other Thai webspinner species was reconstructed for a phylogenetic position using the Maximum Likelihood method.

Keywords: *Diastolembia thailandensis*, Embiidae, Embioptera, new genus and species, Thailand.

泰國松香，來自泰國的一個顯著的恩比茲屬和新種（昆蟲綱：蟲科）

摘要：這項研究旨在對在泰國收集的標本進行形態學和分子表徵，以擴展有關網絡旋轉器(膜翅目) 的可用信息。本文的目的是描述泰國松香新一代，新物種 (蟲科) 來自達克省和清邁省。這種新物種的有翼雄性的特徵是左側尾毛的基部有一個大的裂片，在頂端擴張成有角和纓狀。後足底短，有一個乳頭。同時，多才多藝的女性沒有鮮明的個性。這一發現為這個昆蟲家族帶來了全世界 24 屬下的 89 種物種。此外，使用最大似然法重建了一個特殊的新分類群和其他泰國網絡旋轉器物種的公噸底部-手錶基因的一部分，用於系統發育位置。

关键词：泰國松香，蟲科，蟲翅目，新屬和種，泰國。

1. Introduction

Commonly called web spinners, insects in the order Embioptera (also known as Embiodea and Embiidina) are not as well-known as other taxa because of their secretive nature and mostly tropical and subtropical distribution. Furthermore, defining and naming species is challenging because of the lack of good characters in adult females, who are juvenile in form and lacking in family-defining traits [1]. However, adult females and their offspring are the most common stages collected in the field. Adult males exhibit useful characteristics for taxonomy but generally do not live long after reaching maturity and are hard to find—therein lies another challenge for taxonomists who must establish laboratory cultures to discover these males. Despite these difficulties, recent studies centered in SE Asia, especially Thailand, have yielded descriptions of new species and demonstrated that targeted collecting could contribute to our knowledge of this unique group. For example, based on a phylogenetic analysis that included specimens collected in Thailand, even a new

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Family—Ptilocerembiidae—was erected in 2012 by Miller et al. [2].

In addition to taxonomic work, research on embiopteran silk—the proteins, genes, glands, and spinning behavior [1, 3–5]—continues to mount, revealing intriguing aspects of their biology. However, work remains to be done to resolve questions about phylogeny despite recent analyses based on molecular data and morphology [2]. Authors of those reports lamented the lack of material for resolving questions such as re-defining the original family name for the order, the Embiidae (Burmesiter, 1839). At this point, Embiidae is polyphyletic in the most recent analysis [2] and is currently split into three groups ("Embiidae 1, 2, and 3"). Intending to add to an emerging understanding of Thai embiopterans, the purpose of this report is twofold: (1) to describe a new genus and species based on morphological and molecular characters and (2) to apply DNA barcoding to 20 embiopteran specimens collected from diverse habitats in Thailand to test whether that method can be used to identify them correctly. We chose to analyze the cytochrome c oxidase I (COI) gene because of its demonstrated usefulness in revealing relationships amongst closely related species in several other insect groups (e.g., beetles, butterflies, and flies) [6–8]. Given the difficulties in determining species identity, as mentioned above, DNA barcoding may provide a reliable technique when one has only adult female embiopterans at hand.

2. Materials and Methods

2.1. Taxa Sampling, Specimen Identification

All samples were collected by handpicking from several ecosystem types ranging from highly degraded anthropogenic places to native forests in lower northern (Tak province) and northern (Chiang Mai province) Thailand (Fig. 1). Morphological traits were examined and photographed with a handheld digital microscope (AM-413T-FVW Dino-Lite Pro White) and the DinoCapture Program for measurement. The head, genitalia, and tarsi of males and the sternite patterns of females were illustrated with a stereomicroscope with a drawing tube attachment. All photographic plates were generated with Adobe Photoshop CS6. Afterward, specimens were morphologically identified to the genus and species levels using the primary published literature of Szumik [9] and LucaÑas and Lit [10] before molecular identification. All specimens have been deposited as voucher specimens in the collection of Pibulsongkram Rajabat University (PSRU), Phitsanulok, and the Chulalongkorn University Museum of Natural History (CUMNH), Bangkok, Thailand. Some samples preserved in 95% ethyl alcohol were further investigated regarding molecular identification with mitochondrial COI barcoding.

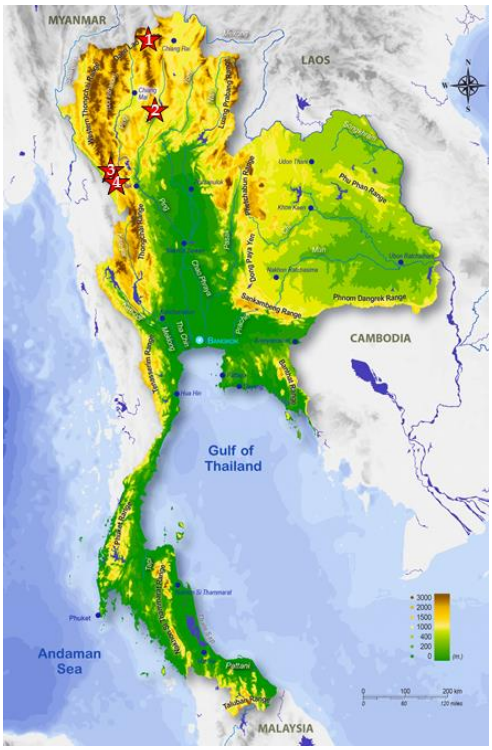

Fig. 1 Collecting localities of *Diastolembia thailandensis* **n. gen., n. sp.** (filled stars), 1) Fang and 2) Sanpatong districts, Chiang Mai province, 3) Mae Ramat and 4) Mae Sod districts, Tak province Thailand

2.2. DNA Extraction, PCR, Amplification, Sequencing, and Alignment

For confirmation of identification at the molecular level, DNA was isolated from somatic tissues (leg, thorax) of embiopteran samples using the DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD, US, catalog #69504), followed the manufacturer's protocols. The partial gene sequence of mitochondrial cytochrome oxidase I (COI) was PCR amplified using forward primer (LCO1490) with DNA sequence 5'-GGT CAA CAAATC ATAAAG ATA TTG G-3' and reverse primer (HCO2198) with the DNA sequence of 5'-TAAACT TCA GGG TGA CCAAAAAAT CA-3' [6]. Each PCR reaction was conducted using a final volume of 20 µl containing 4 µl of 5x PCR Enhancer, 2 µl of 10x HF Reaction Buffer, 0.4 µl of 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 GmbH, Germany), 10.7 µl of nuclear-free water and at least 2 ng of the DNA template. The cycling program comprised an initial activation step of 2 min at 94 °C, followed by 33 cycles of 30 s at 92 °C (denaturation), annealing temperature at 50 °C for 45s, extension at 72 °C for 1 min and a final extension of 8 min at 72 °C. The amplification product was quantified by 1% agarose gel electrophoresis for fragment size in 1x TAE buffer. According to the manufacturer's instructions, the PCR amplified products with the expected length were column purified using the GenUP PCR/Gel Cleanup Kit (biotechrabbit, Germany). Afterward, the purified product was directly sequenced

with forward and reverse primers by Macrogen, Inc. (http://www.macrogen.com).

2.3. Alignment of Sequences and Phylogenetic Analysis

A similarity search for each sequence was verified using BLAST (https://www.ncbi.nlm.nih.gov/). Partial COI sequences were initially aligned in MEGA7 [11] 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 468 base pairs. The sequences obtained after removing the primers used for PCR amplification were deposited to NCBI-Genbank BankIt (https://www.ncbi.nlm.nih.gov/BankIt/) under accession numbers (MH187261- MH187280). The statistical confidence of a particular clade in all the tree-building methods was evaluated by using the maximum likelihood (ML) method based on the Kimura 2-parameter model with 1000 bootstrap replicates.

3. Results and Discussion

3.1. Morphological Study and Systematics

Order: Embioptera, Shipley 1904

Family: Embiidae Burmeister, 1836, type genus: *Embia* Latreille, 1925.

Genus *Diastolembia* **n. gen.**

Type species**:** *Diastolembia thailandensis* **n. sp.**, by present designation (monotypic).

Diagnosis. The alate male of *Diastolembia* is usually distinguished from other genera in the family Embiidae as follows: The submentum is subsquare to square. The lobe of the basal segment of the left cercus (LC_1) is generally large, expansion as angular or globose, and echinulate at the apex. Medial sclerite (MS) is narrow and short, posterior margin becoming membranous. Right hemitergite (10R) is very long, longer than left hemitergite (10L). The terminal with the right hemitergite (10RP) process is very short, obtuse where the process of the left hemitergite (10LP) is very long, tapered caudally. Hind basitarsus short with one papilla. Apterous female without distinctive characters except for only one basitarsal papilla.

Etymology. Diastole- (from the Greek διαστολή = dilation), about its basal segment of left cercus (LC_1) is generally dilated, large and lobed and -embia (from the Greek εμβια = living), referring to webspinners that spend most of their lives inside their silk galleries.

Diastolembia thailandensis **n. sp.** (Figs 2–4)

***Material examined*.** Holotype; 1♂ (CUMNH), Thailand, Tak province, Mae Ramat district, dry evergreen forest, 16°58.331'N, 098°32.213'E, 213 m, 04. IV. 2012, leg. P. Poolprasert. 2 ♂♂ paratypes, 3♀♀ female paratypes; 1♀ (CUMNH), same data as holotype. 2♂♂, 3♀♀ (CUMNH) Mae Sod district, hill evergreen forest, 16°45.837'N 098°54.533'E, 788 m, 26. VI. 2011, leg. P. Poolprasert. 1♂, 3♀♀ (CUMNH), Chiang Mai province, Sanpatong district, hill evergreen forest, 18°32.608'N, 098°31.521'E, 1237 m, 02. III. 2008, leg. J.S. Edgerly and P. Poolprasert; 1♂ (CUMNH), Fang district, mixed deciduous forest, 20°04.499'N 099°14.616'E, 615 m, 31. III. 2008, leg. J.S. Edgerly and P. Poolprasert. **Distribution**. This species is known from northern Thailand (Chiang Mai and Tak provinces)

Etymology. The species name Diastolembia thailandensis (Lat.), referring to Thailand, the country of origin.

Description. Alate male (n = 3), mean (range): Head width x length 1.6 (1.5-1.8) x 1.9 (1.8-2.1) mm, body length 14.2 (13.8-15.2) mm, width 2.1 (2.0-2.3) mm, forewing length 11.3 (11.2-11.4) mm, hindwing length 10.3 (10.1-10.8) mm.

Head: Capsule as broad as long, blackish. Eyes entirely dark, large prominent subreniform. Submentum quadrate, darkish. Antennae darkish throughout, 20 antennomeres.

Thorax: Yellowish to orange throughout. Wings with MA forked, blackish with hyaline inter-ventral lines. All legs were darkish. The hind leg was with only one basitarsal papilla.

Abdomen: Brown throughout, paler ventrally, tenth abdominal tergite diagonally cleft leftward to its base. Terminalia with hemitergites separated basally by a plate of MS. 10L smaller and shorter than 10R. 10LP medium long, broad basally, then evenly tapered to apex. 10RP very short, blunt distally. EP sclerotized. HP dull blackish brown. LPPT slender, sclerotized, slightly arced leftward, fused to HP. LC_1 thick with a finely and densely echinulate, globose, subapical lobe.

Apterous female (n = 7, mean (range) ± SD): Head width × length 1.8 (1.7-2.1) ± 0.34 x 2.2 (2.0-2.4) ± 0.16 mm, body length 16.4 (14.7-17.3) ± 0.23 mm, width 2.2 (2.1-2.3) ± 0.72 mm.

Head: Capsule as broad as long, sides short, parallel broadly arcuated caudally, brown. Eyes are dark, smaller, and less kidney-shaped than in males. Submentum trapezoidal. Antennae are entirely brown without white tip except for few basal segments paler, 18 antennomeres.

Thorax: Purplish brown throughout, paler at sides and ventrally. All legs concolorous with thorax except for femorotibial joints pale in color. Hind leg has only one basitarsal papilla.

Abdomen: Concolorous thorax with cream stripe lateral plate. Cerci are brownish. Sternite 8 medially inset into the plate

Remarks. Colonies of this species commonly occur on tree trunks in natural forests. The insects feed on foliose lichens (Fig. 2C). The silk is often camouflaged with pulverized bark or fecal pellets, resembling silk of many species of the family Ptilocerembiidae. Galleries

can cover a wide area of the bark surface. Development requires one year, with adults appearing between April and July.

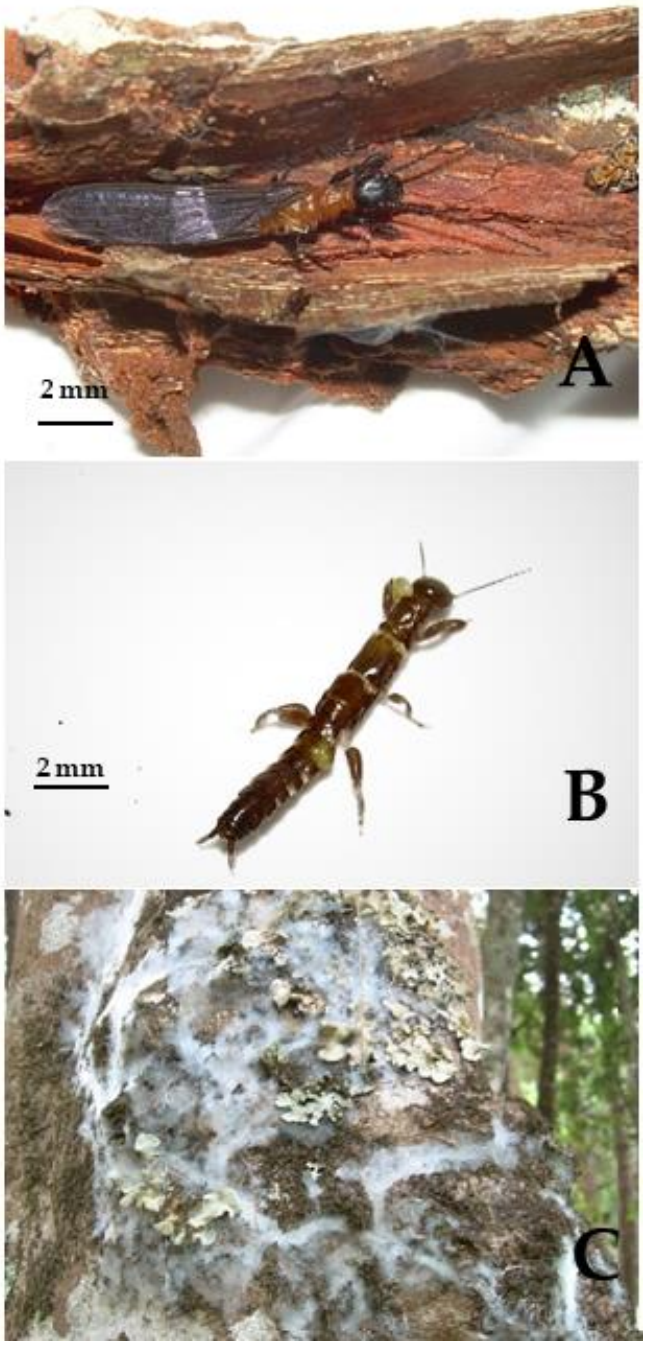

Fig. 2 *Diastolembia thailandensis* n. gen., n. sp. (A) male, (B) female with sclerogibbid parasite visible on the left side of prothorax, and (C) silk gallery

According to Ross [12], Embiopterans are relatively rich and diverse in SE Asia, especially Thailand. Ross produced a key to families and recognized an Oriental cluster for the order. He listed two species in the genus *Oedembia*, found in India, Nepal, Pakistan, and Myanmar. This genus is most closely related to *Parembia* Davis, 1939, treated in error as a subgenus of *Embia*. Later, it is considered *Oedembia*, a distinct genus because of its swollen submentum and more long left tergal process. A transverse dorsal fold on the basal segment of the left cercus lobe is absent in *Parembia*. There are also other distinctions. Upon close inspection, we found that the characters of our unknown embiopteran found in the current survey were not similar to those of the genera mentioned above. It differs by having a single basitarsal papilla.

On the other hand, some additional characters, such as the hypandrium (H) and its process (HP), are seen in the genus *Ptilocerembia* (Ptilocerembiidae). However, it can be distinguished by the medial sclerite (MS), narrower and longer than in *Ptilocerembia*. In addition, the basal segment of the left cercus (LC_1), normally dilated, large and lobed, is a unique character of this newly named species. For these reasons, we believe this embiopteran should be treated as the new genus and species as *Diastolembia thailandensis* **n. gen., n. sp.** (Figs 3 and 4)

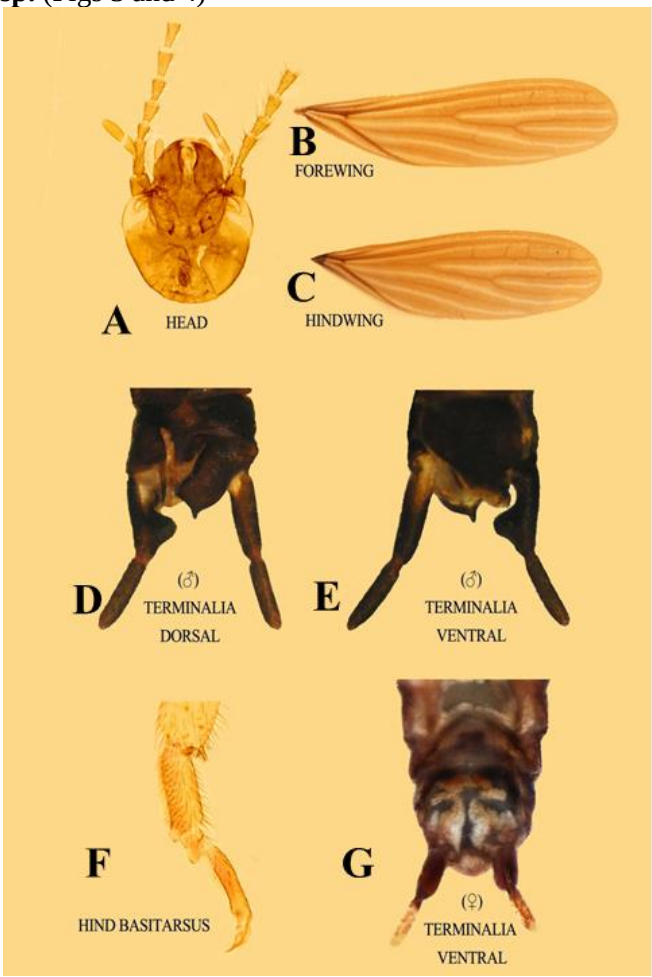

Fig. 3 Diastolembia thailandensis n. gen., n. sp. (A) Head of male. (B) Forewing of male. (C) Hind wing of male. (D) Teminalia (dorsal) of male. (E) Teminalia (ventral) of male. (F) Hind tarsus of male. (G) Sternites of female. MA = anterior media

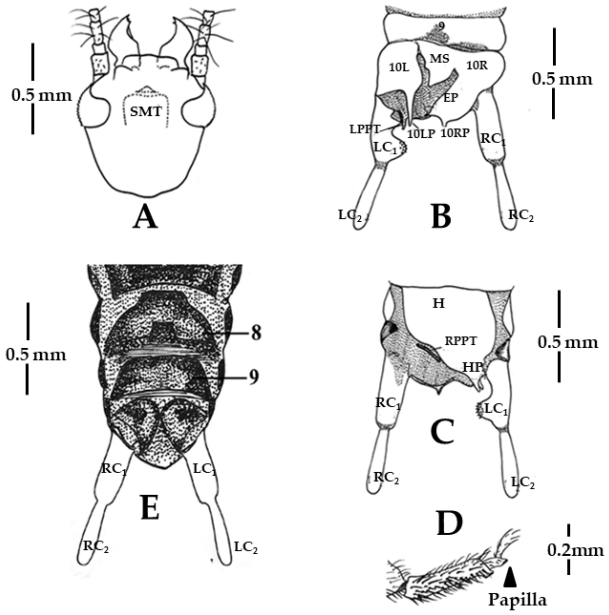

Fig. 4 Illustration of Diastolembia thailandensis n. gen., n. sp. (A) Head of male. (B) Terminalia (dorsal) of male. (C) Terminalia (ventral) of male. (D) Hind basitarsus of male. (E) Sternites of female. Abbreviations: 8 (T8, S8) = eighth abdominal tergite/sternite; 9 (T9, S9) = ninth abdominal tergite/stergite; 10L and 10R = hemitergites of the tenth segment; 10LP and 10RP = left and right tergal processes; EP = epiproct (segment 11); H = hypandrium (sternite 9); HP = hypandrium process; LPPT = left paraproct; LC_1 and LC_2 = first and second segments of left cercus; left cercus basipodite (LCB); left cercus-basipodite process (LCBP); RC_1 and RC_2 = first and second segments of right cercus; SMT = submentum

3.2. DNA Barcoding Analysis

A total of 20 new sequences were generated and deposited in GenBank (Table 1). The alignment contained approximately 28 taxa belonging to three families. The sequence of the 5' region of COI was analyzed to identify species and infer phylogenetic relationships. DNA fragments containing 468 base pairs were gathered from the 20 samples that belonged to three main families: Oligotomidae, Embiidae, and Ptilocerembiidae. The transition/transversion rate ratios were k1 = 141.402 (purines) and k2 = 1.89 (pyrimidines). The overall transition/transversion bias was R = 23.05, where

R = [A*G*k1 + T*C*k2]/[(A+G)*(T+C)].

The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated, leaving 468 positions in the final dataset. The Maximum Likelihood (ML) method was used for phylogenetic analysis with MEGA7 [11], and bootstrap analysis provided support values for the branches.

In order to further test the confidence of branching orders, bootstrapping using 1,000 replicates values was done.

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

Species	Genbank Acc No.	Nucleotide composition (%)			
		T	C	A	G
Oligotoma humbertiana	MH187261	26.92	28.85	30.98	13.25
Eosembia sp.	MH187262	33.33	24.02	29.25	13.40
Oligotoma nigra	MH187263	33.33	24.02	29.25	13.40
Diastolembia thailandensis **n.gen.,n.sp.**	MH187264	33.33	24.02	29.25	13.40
D. thailandensis **n. gen., n. sp.**	MH187265	33.33	24.02	29.25	13.40
D. thailandensis **n. gen., n. sp.**	MH187266	33.33	24.02	29.25	13.40
D. thailandensis **n. gen., n. sp.**	MH187267	33.33	24.02	29.25	13.40
Ptilocerembia thaidina	MH187268	33.33	24.02	29.25	13.40
P. catherinae	MH187269	33.33	24.02	29.25	13.40
P. rossi	MH187270	35.26	20.73	31.41	12.61
Aposthonia borneensis.	MH187271	26.28	33.76	27.99	11.97
Eosembia auripecta	MH187272	25.43	33.76	28.85	11.97
E. auripecta	MH187273	25.43	33.97	28.63	11.97
E. auripecta	MH187274	27.35	32.48	26.92	13.25
E. auripecta	MH187275	26.50	32.26	27.99	13.25
E. auripecta	MH187276	26.92	31.62	28.42	13.03
E. auripecta	MH187277	26.92	31.62	28.42	13.03
E. auripecta	MH187278	27.99	28.42	30.56	13.03
E. auripecta	MH187279	29.70	26.50	31.20	12.61
O. saundersii	MH187280	25.64	30.13	31.62	12.61
Average		30.23	27.40	29.34	13.03

Bootstrap values were appointed to each tree internal node and the values >80 are shown in Fig. 5.

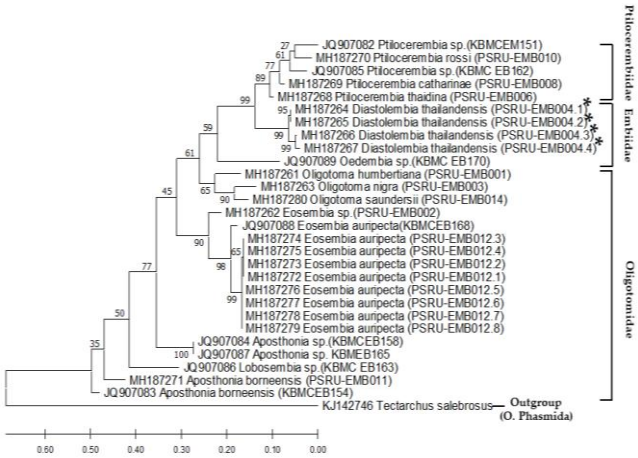

Fig. 5 Maximum Likelihood (ML) tree for *COI* barcodes of 28 Thai embiopteran specimens (three families), showing topology and bootstrap values. Numbers after current species names are voucher numbers for sequences determined by this study (see Table 1), * = new species of embiopteran (Embiidae). *Tectarchus salebrosus* (Order Phsamida), accession no. KJ142746 was used as an outgroup

A phylogenetic tree of Thai embiopterans belonging to Embiidae, Oligotomidae, and Ptilocerembiidae)

under Embioptera was constructed based on partial order sequences of mtDNA COI gene using the Maximum Likelihood (ML) method. The bootstrap values are shown at the branching points. The evolutionary history was inferred by using the ML method based on the Kimura 2-parameter model. The tree with the highest log likelihood (-3879.8884) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with a superior log-likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 28 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 468 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [11]. The evidence presented supports the monophyly of each embiopteran species among these three families (Fig. 5), which is previously defined by numerous morphological, behavioral, and molecular characters [2, 13]. Surprisingly, the family Oligotomidae is depicted as paraphyletic in this tree, suggesting that a thorough phylogenetic status within the Oligotomidae will be further needed to provide a more natural classification.

Based on this analysis and in corroboration with the morphological details described above, *Diastolembia thailandensis* **n. gen., n. sp.** (family “Embiidae 3” of Miller et al. [2]) appears closely related to the *Ptilocerembia* group in the family Ptilocerembiidae and could be considered as a sister group of *Ptilocerembia* in this time. At the time of the Miller et al. [2] analysis, we only had the name *Oedembia* as a possibility, based on the descriptions and dichotomous keys of the embiopterans of SE Asia by Ross [12]. Specimens, collected by us and previously designated as *Oedembia* sp. in Miller et al. [2], are now herein recognized as the newly named genus *Diastolembia* **n. gen.**

Diastolembia thailandensis **n. gen., n. sp.** is observed only in a Thai group which, although well-supported as sister group to *Ptilocerembia*, shared no unambiguous morphological synapomorphies with those species. Morphologically, Ptilocerembiidae can be distinguished from "Embiidae" by the left cercomeres that are fused, sometimes incompletely with a suture visible between cercomeres and basal cercomere without echinulations. Meanwhile, the left cercomeres are not fused for the new species, and basal cercomere is clavate or with distinct medial lobe bearing small echinulations in Embiidae. Embiidae has the most problematic definition in Embioptera because many of the diagnostic traits have similar corresponding traits in other taxa, and the group is not monophyletic. As previously defined, Embiidae has alate males with vein MA forked, the left basal cercomere apically clavate or with a medial process and bearing echinulations, and tenth abdominal tergite (T10) entirely divided medially [2].

This study explored that DNA sequencing was an effective yardstick for rapid, reliable species identification of embiopterans. Other investigations, 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 [6–8]. However, the use of the mitochondrial genome (mtDNA) and the nuclear genome (nuDNA) as the DNA barcode for species discrimination and phylogenetic reconstruction should be considered initially.

4. Limitations and Conclusion

4.1. Limitations

1) Some specimens are hard to find from the surveys. Alate males are very important to use for species identification. They are quite hard to find or collect from the surveys. Some field-collected specimens, therefore, needed to be reared until they metamorphosed into adults.

2) Most museum materials yielded low-quality DNA, limiting their generalized use in phylogeny. So, live or fresh specimens are required for molecular study. Besides, the next-generation sequencing methods should be initially applied for the systematics of Thai webspinners.

3) Regarding DNA barcode, the mtDNA COI gene is commonly applied for species identifications. However, a single gene is not suitable to reconstruct the species relationships; instead, it could reconstruct the COI gene relationships. The present analysis is acceptable, but adding more genes is highly recommended.

4.2 Conclusion

Previously unidentified *Diastolembia* species was discovered from surveys in the natural forests from western to northern parts of Thailand from 2008 to 2018. Therefore, we added a new genus (*Diastolembia*) from Thailand, formally announcing their occurrence in that region by naming the species *Diastemata thailandensis* **n. gen., n. sp.** Morphologically, the alate male can be readily distinguished from others by its head is broad as long. the antennae (20 antennomeres) are abruptly white distally, the anterior branch of the media (MA) is branched in the forewing and hindwing,

the left tergal process (10LP) is medium long, broad basally then evenly tapered to apex, (LC_1) is generally dilated, large and lobed, the hind basitarsus has only one papilla. Whereas apterous females can be recognized by the antennae (18 antennomeres) are entirely brown without white tip except for few basal segments that are relatively paler. The hind basitarsus has only one ventral papilla. In addition, results suggested that COI was a good DNA barcoding for embiopterans. However, a combination with other DNA fragments would increase identification efficiency.

This discovery supports the known zoogeography of the family "Embiidae 3" to the Oriental realm. This genus increases the total number of genera of webspinners known worldwide to 24 with 89 species. A single species found here is described and illustrated based on morphological traits and barcoding data. Also, new remarkable fauna and other Thai embiopterans were tested for phylogenetic status. Phylogenetic analysis based on the Maximum Likelihood method resolved the species following morphological traits and close relationship between sister species. Given the problematic state of the original family for the order, the Embiidae, we suggest that a concerted effort be made to analyze species from throughout its range. Now the California Academy of Sciences in San Francisco, USA has acquired the extensive collection of Edward S. Ross and made this available to researchers, progress is possible.

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