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The Relationships of Closely Related Cossid Moth Species among Geographically Diverse with Respect to Indian Cossid Moth: (Lepidoptera: Cossidae)

Veto Khesoh [a], Melevolu Thisa [a], Roman Yakovlev [b], Kekuneil Henry Ltu [c], Zavei Hiese [c,d] and Prabhakar Maddela [a*]

[a] Department of Chemistry, Nagaland University, Lumami-798627, Nagaland, India.
[b] Altai State University, Pr. Lenina 61, Barnaul, 656049, Russia.
[c] Nagaland Science & Technology Council (NASTEC), Kohima-707004, Nagaland, India.
[d] Nagaland Institute of Science & Technology, Kohima, India.

Authors' contributions

This work was carried out in collaboration among all authors. Author VK wrote and design the manuscript. Authors VK and KHL collected the adult moth specimens (Chinocossus acronyctoides) from Kedima-Nagaland and Author ZH provided the facilities required for the entire accomplishment of the study. Author RY helped with the insect identification, Authors MP and MT reviewed, provided valuable feedback, editing and designing the manuscript. All authors read and approved the final manuscript.

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**Corresponding author: Email: maddelap.org@gmail.com;*

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ABSTRACT

This research article presents a molecular phylogenetic investigation focusing on closely related Cossid moth species within the Cossidae family from diverse geographical areas. The study aims to identify the species as well determine the evolutionary relationships among closely related Cossid moth species as many of them looks very similar and are difficult to distinguish only basing on morphological characters. We first amplified approximately 700 base pairs (bp) of the mitochondrial cytochrome c oxidase subunit-1 gene (MT-CO1), extracted from the thoracic region of the adult cossid moths which were collected from Kedima-Nagaland, India. Although, morphologically many Cossid species has been extensively studied, its evolutionary relationship to other members of the same genus, family or sub-families remains uncertain being a very diverse group. Therefore, this study provides the first description of the evolutionary relationship between the studied Cossid moth with respect to other geographically diverse Cossid species. To analyse the connections among the Cossid species, phylogenetic trees were constructed using data from an additional 58 Cossid species. The Maximum Likelihood and Bayesian analysis were used to construct the phylogenetic tree. In Both the techniques it demonstrated robust bootstrap support for the obtained phylogenetic relationships. Our study effectively identified the Cossid species and presented compelling evidence for the CO1 gene's efficacy in differentiating closely related species, as corroborated from our phylogenetic analysis. These results enhance our understanding of the evolutionary dynamics within the Cossidae family and offer valuable insights into the phylogenetic associations among closely related Cossid moth species. Here, we have also reported new MT-CO1 sequence of the studied Cossid species and have deposited in GenBank database (PP358253).

Keywords: Cossidae; COX1/MT-CO1/COI; cossids; phylogenetic tree.

1. INTRODUCTION

The order Lepidoptera consists of butterflies and moths. The family Cossidae consists of more than 700 species and 110 genera worldwide. Sometimes, the family Cossidae, due to its boring behaviour on wood and its reputation for being destructive during the larval phases, is considered as the carpenter moths [1,2,3]. They are also called goat moths because of the aromatic pungent smell in the larval stages [4,5,6]. However, the pungent smell disappears as they grow into adult moths. Anecdotal beliefs suggest that the pungent characteristic may function as a defence mechanism to protect the larvae from predators [5]. While many Cossid species are considered pest [7,8,9], the larvae of the investigating Speciesare known to be a venerated species. They serve as exotic food, particularly in the north-eastern part of India, Kohima-Nagaland. The moth larvae are also sold at high prices and are in high demand in the market. The local indigenous people also believe that the larvae have therapeutic benefits. A kilo of the larvae would cost around ₹10,000, thereby providing high socio-economic value to local sellers. Some other Cossidae species, like *Comadia redtenbacheri* (1848 by Carl Eduard Hammerschmidt) larvae, are also reported to be edible [10,11].

The life cycle of the identifying moth starts as an egg and the peak season for adult moths to oviposit starts from the month of April to June. The adult moth usually lays the egg on the bark of the Oak tree particularly *Quercus serrata* (1784 by Murry) and upon hatching, the young larvae slowly bore into the hard wood of the Oak tree. A female adult moth can lay up to 300-400 eggs. Thus, a single host tree can harbour a very high number of larvae, ranging from a few hundreds to even more than a thousand in a cluster. This Cossid larvae, upon maturation, metamorphoses into adult moths.

Studies of evolutionary relationships within important groups of Lepidoptera has been extensively conducted, initially focusing on morphology and later incorporating DNA data as a valuable tool for species identification [12]. In the DNA barcoding system, the mitochondrial gene (MT-CO1) stands out for its remarkable effectiveness in differentiating species and studying their phylogenetic relationships [13,14,15]. There by increasing the use of DNA fragments as a genetic marker from the mitochondrial DNA has become a productive approach for phylogenetic studies as well as studying closely-related species [16,17,18]. The fact that MT-CO1 has A-T (Adenosine-Thymine) rich regions, which are important components responsible for transcription and replication, as well as having a high nucleotide substitution rate, it makes a suitable marker for phylogenetic studies. Although its controversial for choosing

CO1 as the standard barcode example, for some plant and fungal species [19,20], but it is effective and efficient for studying insect like for Lepidopteran species and a common technique employed by many researchers to study about them [21,22,23].

Our study not only aims to determines the species but to establish a phylogenetic relationship with other closely related Cossid species, this helps in easy identification. If two species are closely related, they are more likely to share similar traits or characteristics, such as anatomy, physiology, behaviour, and ecological roles [24]. Our hypothesis that the investigating species is a closely related Cossid moth and that it might be one of the sister species of Cossidae groups. So, with the help of phylogenetic studies we not only able to understand the evolutionary relationships between these Cossid groups but can further determine how these Cossid species are related to each other and how they have evolved over time. From this study we will also be able to see the evolutionary relationships of these species which will be shown classified according to the hierarchical groups based on their shared ancestry, allowing for more accurate classification and naming of species. CO1 is known to be highly efficient for discerning between vertebrate and invertebrate species [22] and exhibits superior phylogenetic resolution comparing to other mitochondrial genes [25,26].

Here we use MT-CO1 gene sequence as well as morphological analyses to identify the collected species and assess its phylogenetic relationships to other Cossid moths.

2.MATERIALS AND METHODS

2.1 Insect Sample Collection

The adult Cossid moth species were collected from the field site Kedima-Kohima, Nagaland-India. The GPS position for latitude was 25° 33’33” N and longitude was 94°10’50” E.

2.2 Molecular Procedure

2.2.1 DNA extraction, Polymerase chain reaction (PCR) amplification, cloning and sequencing

For extracting DNA QIAGEN DNeasy kit was used to extract the DNA from the thoracic region of adult moth. Firstly, the adult specimens were collected from Kedima-Nagaland for molecular analysis. Then, three specimens of the adult moth were chilled in -20 °C and was wiped clean with ethanol 70% to avoid contamination. The adult moths were then decapitated in the thoracic region and fresh tissue was taken out. DNeasy blood and tissue kit was used by following the manufacturers protocol. The extracted DNA was amplified by PCR using the forward primer Lepf1 5'-TTCAACCAATCATAAAGATATTGG-3' and reverse primer LepR1 5'-TAAACTTCTGGATGTCCAAAAAATCA-3' as used by Vergara et al. [27] (Table 1). Then the resultant DNA extract was PCR-amplified. The amplification process was 5µl qiagen master mix hotstar, 0.5 µl forward primer, 0.5 µl reverse primer, water 2 µl and finally DNA template 2 µl making a total of 10 µl. The PCR temperature cycling follows at 94 °C for 15 min followed by denaturation at 94 °C for 30 cycles, annealing at 45-55 °C for 40 s and extension at 72 °C for 1 min and final extension at 72 °C for 5 min. The amplified fragment was separated in 2% agarose gel and shown in Fig. 1. Upon amplification the final product was sent to sequencing facility for direct sequencing. The DNA sequences obtained herein were compared (using the algorithm BLASTn) with those available in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm. nih.gov).

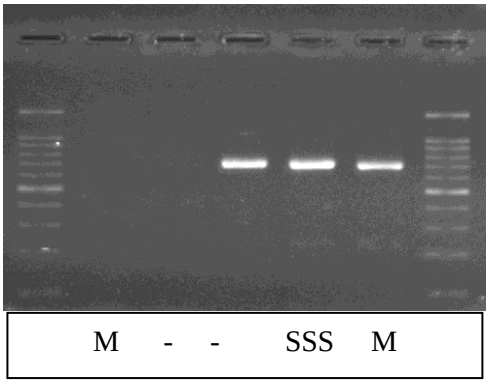

Fig. 1. Gel picture used for mitochondrial sequencing (M= Marker, S= Sample, - = Null)

2.3 Phylogenetic tree construction of the Cossid species

A total of 60 species (Table 2), 58 selected Cossid species, an out-group and the studied species were subsequently aligned for the construction of phylogenetic tree. The phylogeny construction was performed by employing software’s like RAxMLGUI1.5b2 [28] and MEGA X [29] for Maximum likelihood (ML) studies and Mr Bayes 3.2.7 [30] for Bayesian analysis. Two independent runs of four chains of 300,000 Metropolis-coupled Markov chain Monte Carlo

(MCMC) generations were run until the deviation of split frequencies was smaller than value 0.01, with sample trees taken every 1000 generations. The consensus tree was constructed with a burn-in of 25%. Both the ML and Bayesian analyses were run with models conforming to GTR+I+G, with model parameters estimated by the respective program. ML analyses were bootstrapped pseudo replicated 1000 times.

Table 1. Primer pairs used to amplify the mitochondrial gene CO1.

Sl no.	Primer Name	Primer sequence (5'-3')	Amplicon region
1	LepF1	ATTCAACCAATCATAAAGATATTGG	CO1
2	LepR1	AAACTTCTGGATGTCCAAAAAATCA	CO1

Table 2. Cossidae species with their Accession numbers used in this study. The studied species is highlighted in bold.

Sl no.	Species Names	Accession number/Genbank	Locality
1	*Acossus centrensis*	GU092174.1	Canada
2	*Acossus populi*	KM554595.1	Canada
3	*Archaeoses magicosema*	HQ951835.1	Australia
4	*Archaeoses pentasema*	GU828800.1	Australia
5	*Archaeoses polygrapha*	HQ951823.1	Australia
6	*Chalcidica hyphinoe*	AB983491.1	Indonesia
7	*Chinocossus acronyctoides*	PP358253	Nagaland
8	*Comadia_henrici*	HM426788.1	USA
9	*Comadia redtenbacheri*	JN673376	Mexico
10	*Comadia redtenbacheri*	JN673377	Mexico
11	*Cossus afghanistanus*	MF596151.1	Afghanistan
12	*Cossus cossus*	GU828604.1	Europe
13	*Cossus cosssus*	HM376793.1	Germany
14	*Cossus cossus*	HM870975.1	Finland
15	*Cossus cossus*	HM914074.1	Italy
16	*Cossus cossus*	JF860045.1	Italy
17	*Cossus cossus*	KX040085.1	Germany
18	*Cossus cossus*	KX045460	Romania
19	*Cossus cossus*	KX070766.1	Germany
20	*Duomitus ceramica*	HQ952090.1	Australia
21	*Duomitus ceramica*	KX928975.1	Thailand
22	*Endoxyla coscinophanes*	HQ952055.1	Australia
23	*Endoxyla coscinophanes*	HQ952057.1	Australia
24	*Endoxyla duponchelii*	HQ952052.1	Australia
25	*Endoxyla didymoplaca*	HQ951959.1	Australia
26	*Endoxyla epicycla*	HQ952068.1	Australia
27	*Eogystia hippophaecolus1*	KC791455.1	China
28	*Eogystia hippophaecolus2*	KC791446.1	China
29	*Givira brunneoguttata*	KF491763.1	Chile
30	*Givira ethela*	KF492379.1	USA
31	*Givira lotta*	JF847156.1	USA
32	*Givira modisma_*	JQ553818.1	Costa Rica
33	*Givira mucida*	GU828543.1	North America
34	*Givira mucida*	KF492383.1	USA, Arizona
35	*Givira tristani*	JQ550596.1	Costa Rica
36	*Hermophyllon anceps*	AB983485.1	Indonesia
37	*Indarbela obliquifasciata*	GU828829.1	Thailand
38	*Kerzhnerocossus tannuolus*	MF071456.1	Russia
39	*Metarbelinae sp.*	GU828771.1	Africa
40	*Morpheis sp.*	JQ559047.1	Costa Rica
41	*Morpheis xylotribus*	JN287262.1	French Guiana
42	*Panauquarlesi*	HM377294.1	Indonesia
43	*Phragmataecia castaneae*	HM874160.1	Italy
44	*Phragmataecia castaneae*	HQ968493.1	Liechtenstein
45	*Phragmataecia castaneae*	KX071724.1	Germany
46	*Prionoxystus macmurtrei*	GU087518.1	North America
47	*Prionoxystus robiniae*	GU090139.1	North America

Sl no.	Species Names	Accession number/Genbank	Locality
48	*Prionoxystus robiniae*	GU090140.1	North America
49	*Rhyacionia buoliana (out-group)*	KT132354.1	Canada
50	*Skeletophyllon tempestua*	HQ952072.1	Australia
51	*Streltzoviella insularis*	JN673375	Japan
52	*Trismelasmos maculatus*	AB983486.1	Indonesia
53	*Trismelasmos sp.*	HQ952048.1	Australia
54	*Trismelasmos tectorius*	HQ952075.1	Australia
55	*Trismelasmos tectorius*	HQ952074.1	Australia
56	*Xyleutes persona*	HQ952093.1	Australia
57	*Yakudza vicarius*	KC791470.1	China
58	*Zeuzera coffeae*	JN287265.1	China
59	*Zeuzera multistrigata*	JN287263.1	Japan
60	*Zeuzera pyrina*	JF854514.1	Hungary

3. RESULTS

3.1 Insect Species Identification

The investigating species was identified as *Chinocossus acronyctoides* which was an earlier described species by Moore in 1879 [31]. This was confirmed from morphological and genitalia analysis and comparing with from earlier described data. The fore wings are greyish-brown, grey more prominent at the apex with reticulate black patterns. In both the male and female moth have the same antennae exhibit homogeneity, featuring a uniform and uncomplicated structure which are dark-brown; abdomen greyish brown, paler beneath with pale bands above (Fig. 2). The moth pictured were also cross referred with the paper [32] and was further confirmed by expert Roman V Yakovlev.

3.2 Phylogenetic Analysis of the Cossid species

CO1 sequence obtained from the studied species was used to draw the phylogenetic tree with respect to the other species of Cossidae family, it showed statistically well resolved phylogram. The analysis was inferred from 661bp of the nucleotide fragment. The phylogenetic analysis was conducted using maximum likelihood with 1000 bootstrap replicates to assess the robustness of the inferred phylogenetic relationships. The resultant phylogram showed well bootstrap support for Maximum Likelihood (ML) as well for Bayesian posterior probability when it was analyzed with three different methods, Mr Bayes and Randomized Axelerated Maximum Likelihood (RAXML) and MEGA X. The analysis showed six separate statistically well supported clades (Fig. 3). All the analyzed species fall into their own respective clades. *Chinocossus acronyctoides* felled in to the Cossinae family clade, the proposed subfamily of Cossidae forming a monophyletic clade with the other Cossinae species revealing that *Chinocossus acronyctoides* is the sister species of the subfamily, Cossinae. If insect species have dissimilar CO1 sequences it is quite improbable that two insect species are closely related [33]. So, from our study findings it supports our hypothesis suggesting that our identified species is a sister species from the Cossidae family and indeed a sister species of Cossinae subfamily group. This also highlights the accuracy and a good approach for selecting CO1 gene as a genetic marker and a desired method for identifying species especially for this Cossidae groups.

3.3 Partial CO1 Region

Three CO1 sequences of *Chinocossus acronyctoides* were obtained and they were all about 700 bp in length. There are no CO1 sequence data of *Chinocossus acronyctoides* available in GenBank. So, we have provided the first CO1 sequence from *Chinocossus* genus particularly for *Chinocossus acronyctoides.* The present CO1 sequences of *Chinocossus acronyctoides* was deposited in the GenBank database (http://www.ncbi.nlm. nih.gov). The GenBank accession number is PP358253. When the obtained CO1 gene sequence of *Chinocossus acronyctoides* was blast search at NCBI blast Cossid species *Yakudza vicarious* match the best with 91.60% identical to the new *Chinocossus acronyctoides* sequence. Other species like *Cossus cossus, Eogystia hippophaecolus, Kerzhnerocossus tannuolus* and *Streltzoviella insularis* matched 91.16%, 91.16%, 90.65% and 90.48% respectively, which suggest that *Chinocossus acronyctoides* is aclosely related to the other Cossinae species from diverse geographical regions Table 2. The highlighted words in bold (Fig. 3) next to the phylogram, in respect to each colour clade represents the sub-family name.

Fig. 2. A. Adult male moth, B. Adult female moth and C. Genitalia of an adult moth

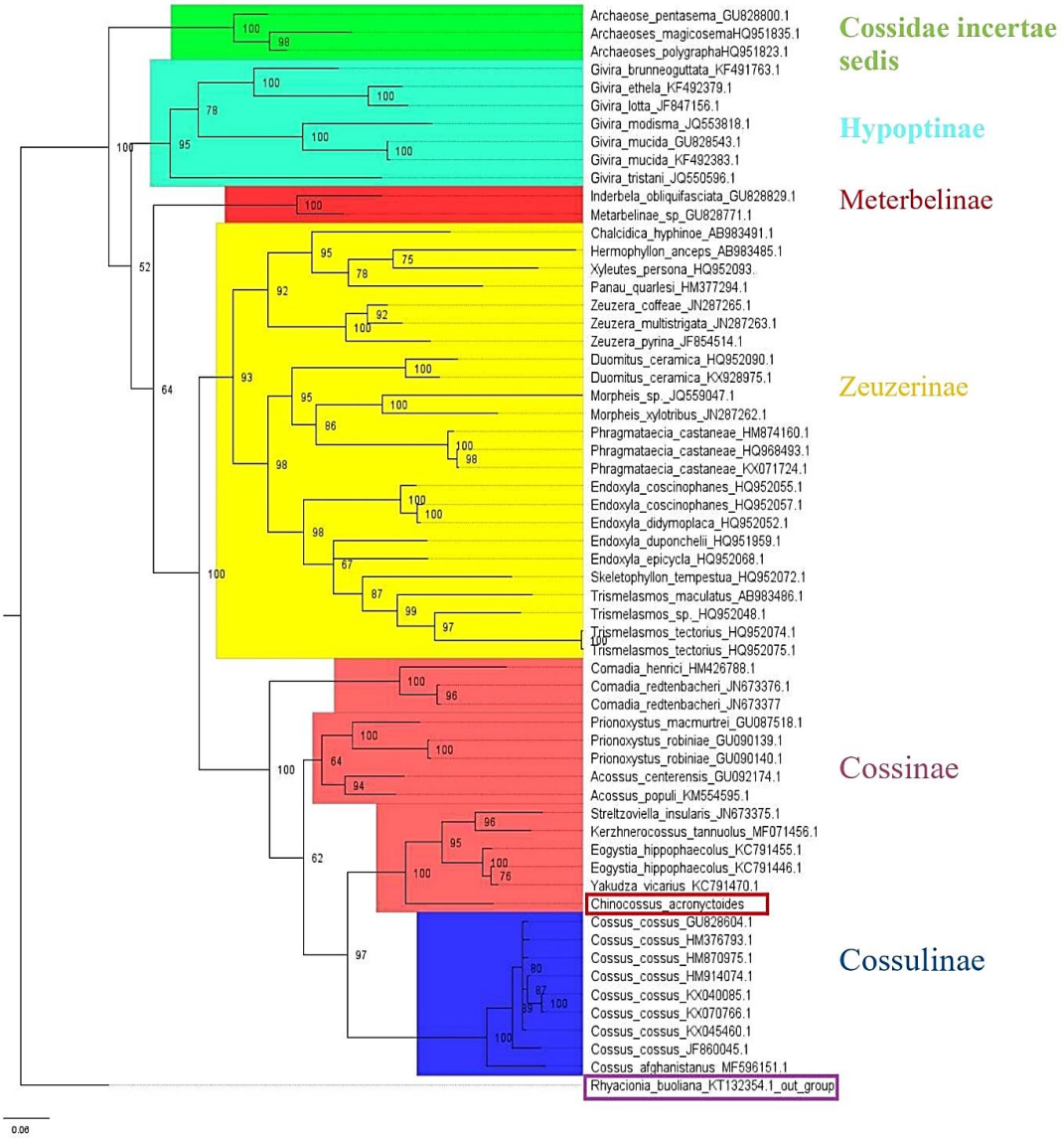

Fig. 3. Phylogenetic tree construction of the 62 species from a 661 nucleotide fragments of available COI sequences with an out-group *Rhyacioniabuoliana*(Purple). The numbers on the nodes shows the ML bootstrap or Bayesian posterior probability. The study species *Chinocossusacronyctoides* (Red) showing good resolution in all the methods. Scale bar is 0.06 substitutions per position

4. DISCUSSION AND CONCLUSION

The main focus of the study was to identify and distinguish the studied species as well to bring out a phylogenetic relationship amongst closely related cossid species. Our identifying species *Chinocossus acronyctoides* is a Cossid moth which belongs to the sub-family Cossinae. Till date there are five different species recorded from this genus [34] and no studies on their phylogeny have been properly described with respect to other Cossidae species. The distribution of this genus ranges from, the *Chinocossus greeni* Sri Lanka and China [35], *Chinocossus hunanensis* China [36], *Chinocossus marcopoloi* China-Yunnan [37], *Chinocossus vjet* Vietnam [38], and *Chinocossus acronyctoides* which has a broader distribution into many countries like India, Pakistan, China (Yunnan, Hunan, Anhui, Jiangxi, Guangxi), Vietnam and Philippines [38,39]. Cossid species are very diverse and difficult to differentiate as they look very similar and many of them can be identified mistakenly for the other species just by basing on appearance. But with the knowledge of phylogenetic tree and the inclusion of genetic markers this problem can be overcome. According to the phylogenetic tree constructed in this study *Chinocossus acronyctoides*, *Yakudza vicarious* then followed by *Eogystia hippophaecolus, Kerzhnerocossus tannuolus* and *Streltzoviella insularis*, appears to be more closely related species with a recent common ancestor. Their genetic sequences exhibit a high degree of similarity, approximately 90%, and form a distinct monophyletic clade, indicating a shared evolutionary lineage. In our phylogenetic analysis of 59 closely related species we estimated a substitution rate of 0.06 substitutions per site indicating that on average, 6substitution occur per 100 nucleotide sites. In all the techniques that we used to draw the phylogenetic tree, showed powerful bootstrapped support.

Using phylogenetic analysis which not only helps to identify but also helps in naming species by accurately discriminating and classify them basing on their shared ancestry. We not only identified our species but explored the phylogenetic relationships of *Chinocossus acronyctoides* with respect to other closely related Cossid moth species from diverse geographical region. The fact that these moth species are very diverse and so the status of their monophyly is often in dispute. Presently in our study *Archaeose sp.* is one of those sub-family where their monophyly is still in dispute. Similarly, as in the case of most other genera of moths, the systematic classification of this Cossidae family is still debatable [5], especially on the monophyly and the relationship within the family due to the fact that the Cossidae family is very varied and the unavailability of CO1 sequences for required species makes it even more difficult to study phylogenetically. Here we have provided a phylogenetic relationship of *Chinocossus acronyctoides* with respect to other Cossidae species and *Archaeoses sp.* which is in dispute with its monophyly [5]. The newly obtained CO1 sequence for *Chinocossus acronyctoides* was deposited in GenBank, which will serve as a valuable asset for future research on the biodiversity and evolutionary relationships of Cossinae moths or Cossid moths in general. This sequence represents the first step towards elucidating the evolutionary connections between *Chinocossus acronyctoides* and other members of the Cossidae family. The fact that this research brings about the phylogenetic relationship of *Chinocossus acronyctoides* with respect to the other species of Cossidae from diverse region, it not only revolutionizes our understanding of the evolutionary history and intricate interconnections within the Cossidae family, but also brings forth critical insights into the complex and dynamic evolutionary pathways of closely related Cossid moth species. Certainly, due to the unavailability of more *Chinocossus acronyctoides* CO1 sequences from other region (China, Vietnam) or other species from the same genus has made our study a little limited but by unravelling these hidden evolutionary connections from our analysis, our study sets a new paradigm for future research in Cossid moth phylogenetics, contributing a broader spectrum on biodiversity, conservation, and evolutionary biology.

Overall, the study findings advance the area of evolutionary biology and lay the groundwork for future investigations into the biodiversity and evolution of Cossid moths.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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