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Multigene analysis of *Leucoma wiltshirei* Collenette, 1938 using combined mitochondrial and nuclear DNA sequences (Lepidoptera: Erebidae)

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Abstract

The study focuses on molecular identification of *Leucoma wiltshirei* Collenette, 1938, a significant pest in Iranian oak stands. It employs DNA sequencing of mitochondrial and nuclear gene fragments for accurate identification. The 12S rRNA gene region is highlighted for its suitability in identifying *Leucoma* Hübner, [1822] species. The analysis revealed genetic characteristics and diversity within the *Leucoma wiltshirei* gene fragments, contributing valuable information for species identification and understanding their evolutionary relationships.
Keywords: Lepidoptera, Erebidae, *Leucoma*, genetic identification, mtDNA, nDNA, Oak pest, phylogenetic analysis, taxonomic uncertainties, Zagros forests, Iran.

Análisis multigénico de *Leucoma wiltshirei* Collenette, 1938 utilizando secuencias combinadas de ADN mitocondrial y nuclear (Lepidoptera: Erebidae)

Resumen

El estudio se centra en la identificación molecular de *Leucoma wiltshirei* Collenette, 1938, una plaga importante en los robledales iraníes. Se emplea la secuenciación del ADN de fragmentos de genes mitocondriales y nucleares para una identificación precisa. Se destaca la región del gen 12S rRNA por su idoneidad para identificar especies de *Leucoma* Hübner, [1822]. El análisis reveló características genéticas y diversidad dentro de los fragmentos génicos de *Leucoma wiltshirei*, aportando información valiosa para la identificación de especies y la comprensión de sus relaciones evolutivas.
Palabras clave: Lepidoptera, Erebidae, *Leucoma*, identificación genética, ADNmt, ADNn, plaga del roble, análisis filogenético, incertidumbres taxonómicas, bosques de Zagros, Irán.

Introduction

Insect pests can indeed pose significant challenges to oak forests, and their interactions with trees are a complex aspect of forest ecosystems (Ferrenberg, 2016). While insects are indeed integral to ecosystems and play various roles (Haack & Byler, 1993), certain pest species can negatively impact forest biological balance due to climate-triggered insect defoliators as mentioned in Abdi (2019) and the abundance of natural enemies (Nealis, 1991). Exactly, caterpillars and other leaf-feeding insects can pose a significant threat to oak trees (Evans, 1987). Zagros forests of Iran are classified into two distinct parts located in northern and southern Zagros. In the latter, *Quercus brantii* Lindl. dominates the forest ecosystem (Sagheb-Talebi et al. 2014). The defoliation caused by Lepidoptera pests can have significant implications for the overall health and vitality of

the oak ecosystems (Kulman, 1971). Indeed, *Leucoma wiltshirei* Collenette, 1938 is recognized as a key pest that can cause substantial damage to oak trees in the Zagros forests (Sadeghi et al. 2009). The historical description of *L. wiltshirei* based on a holotype collected by the pioneer lepidopterologist Edward Parr Wiltshire in 1935 from Rawandiz in the Kurdistan region of Iraq, is significant. This early documentation is a key reference in the systematics, distribution, bionomy and population dynamics as well as of control measures of *L. wiltshirei,* a destructive pest in Iranian oak stands (Abai, 1980; Abai, 1981). Despite this degree of importance of the pest, there is a need for accurate and early detection. Recording the identity DNA sequences of the pest, brings several advantages for both forest pest control managers and researchers involved in systematic study and taxonomy. Identifying pests using genetic sequences is a valuable and increasingly common approach in modern biological research (Hebert et al. 2003). Genetic data such as DNA sequencing can provide a more precise and comprehensive understanding of the pests' taxonomy and their evolutionary relationships (Hosseini-Chegeni & Tavakoli, 2023; Karthika et al. 2016). This study focusing on the molecular identification of *L. wiltshirei* and the phylogenetic analysis using mitochondrial and nuclear DNA gene sequences. The study's focus is praised as a commendable and comprehensive approach to address the challenges posed by *L. wiltshirei* in Iranian oak stands.

Methods

Sample collection and identification

Leucoma adults were collected in northern Khuzestan province, located in the zone of southern Zagros. These samples captured during the outbreak occurred during the spring and summer of 2022 in the region. Dead specimens were transferred to the laboratory for morphological identification, which included examining wing venation and genitalia, in accordance with traditional methods used in lepidopteran taxonomy (Abai, 1980), and followed by molecular assays.

Table 1. Primer details and PCR conditions used in this study.

Gene	Primer sequence (5′→3′)	Product size* (bp)	Touchdown temperature profile
D-18S rRNA	F: GAG GGA GCC TGA GAA ACG G R: ACC TTG TTA CGA CTT TTA CTT CCT CTA	1465-8	
U-18S rRNA	F: GAG GGA GCC TGA GAA ACG G R: ACC TTG TTA CGA CTT TTA CTT CCT CTA	1465-8	95 °C—4 min; 94 °C—1 min, 60–50 °C—30 s
Ef1α	F: CCC GTT TCG AGG AAA TCA A R: GCA GCA TCA CCA GAT TTG AT	706	(annealing at 60 °C with 1°C decrease per cycle
12S rRNA	F: TTA ATA ACT AAT TTT GTG CCA GC R: GAC GGG CAA TAT GTA CAT	560, 594	until 50 °C), 72 °C—90 s [× 10]; 94 °C—1 min, 50
Cytb	F: CAT ATT GGR CGA RGA ATT TAT TAT G R: GCA ATW ACT CCY CCT AAT TTA TTA G	590	°C—30 s, 72 °C—90 s [× 30]; 72 °C—10 min
COI	C1-J-1718**: GGA GGA TTT GGA AAT TGA TTA G C1-N-2776***: GGA TAA TCA GAA TAT CGT CGA G	1106	

* Product sizes were calculated according to sequences of different *Leucoma* species deposited in GenBank,
** Simon et al. (1994), *** Hedin & Maddison (2001)

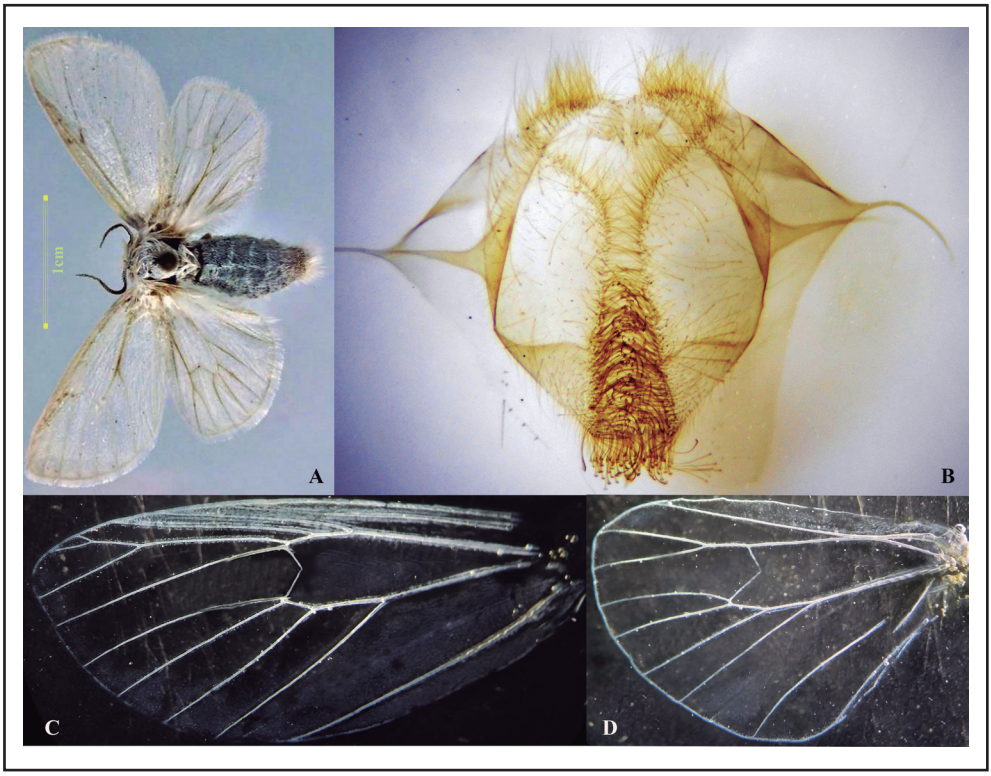
DNA extraction, amplification and sequencing

Genomic DNA (gDNA) of a representative whole female was extracted using the ionic detergent cetyltrimethylammonium bromide (CTAB; Merck, Darmstadt, Germany) and a chloroform-isoamyl alcohol mixture according to Doyle & Doyle (1987). DNA sample was tested to amplify six partial gene fragments of the moth specimen by polymerase chain reaction (PCR) using the primers (Table 1) designated for the first time in this study except primer *COI* gene. For designing of the primers, GenBank sequences of different *Leucoma* species were aligned using GeneDoc® v. 2.7 (Nicholas et al. 1997) and the desired conserved loci were selected by visual observation. Selected fragments were analyzed using Oligoanalyzer v. 3.1 (www.eu.idtdna.com/analyzer/applications/oligoanalyzer) (Integrated DNA Technologies, Iowa, USA). The specificity of the candidate primers for our PCR was tested in NCBI primer BLAST database (www.ncbi.nlm.nih.gov/tools/primer-blast) in order to finding oligos specific to our PCR template. The various target genes consist of mitochondrial genomic: cytochrome oxidase subunit I (*COI*), cytochrome b (*CytB*), small subunit (SSU) ribosomal RNA (rRNA) of the mitochondrial ribosome (12S rRNA) and nuclear genomic: upward (U-18S rRNA) and downward (D-18S rRNA) small subunit ribosomal RNA, elongation factor 1-alpha (*Ef1α*). Finally, the oligonucleotide primers were synthesized by the SinaClon company (Tehran, Iran). PCR reactions were performed in 30 µl mixture consisting of 10.5 µl double-distilled water, 15.5 µl Master Mix RED® (Ampliqon, Odense, Denmark), 1 µl from each 10 pM primers, 2 µl gDNA template (50–100 ng/µl) in a thermocycler, BioRad MyCycler® (Applied Biosystems, Waltham, MA, USA). PCR products were visualized by electrophoresis on 1% agarose gel stained with SYBR® safe DNA gel stain (Invitrogen, Burlington, USA), and finally submitted to a third-party service provider for Sanger sequencing. Sequencing was done on Applied bioSyestems-ABI, 3130XL in the Codon Genetic Group®-Iran, using the same primers as in PCR as two directional. The ABI output sequences were edited manually using FinchTV® (Qt Company, Espoo, Finland). All sequences were submitted to GenBank via BankIt® and accession numbers were assigned.

Phylogenetic analysis

The nucleotide sequences were compared to taxa available in GenBank using BLASTn analyses (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the correct species or homologous. We employed a focused approach by limiting BLASTn analysis to *Leucoma* genus, to obtain more specific and relevant results. Subsequently, all sequences were submitted to GenBank via BankIt® and accession numbers were assigned. Sequences were aligned using SeaView v. 5.0.4 (PRABI-Doua, Lyon, France) (Gouy et al. 2010), and genetic differences (distance matrix) among the *Leucoma* sequences were calculated using Maximum Composite Likelihood in the MEGA11 software (Tamura et al. 2021). A merged alignment sheet created that includes sequences from different genes 18S rRNA (upward and downward regions), *Ef1α*, 12S rRNA, *Cytb* and *COI* sequences belonging to lepidopteran taxa. The taxa include six genera and seven species of superfamily Noctuoidea, with *L. wiltshirei* (this study) and other species like *L. salicis* (Linnaeus, 1758), *Lymantria dispar* (Linnaeus,1758), *Euproctis similis* (Fuessly, 1775), *Catocala fraxini* (Linnaeus, 1758) (Erebidae) as in-group, and *Spodoptera exigua* (Hübner, [1808]) and *Lithophane socia* (Hufnagel, 1766) (Noctuidae) as outgroup. Finally, sequences from the present study and the GenBank nucleotide data were analyzed to construct a combined phylogenetic tree by MEGA11. Before that, a Partition Homogeneity Test (PHT) or Incongruence Length Difference (ILD) with heuristic search was performed under one thousand replicates between 18S rRNA (upward and downward), *Ef1α*, 12S rRNA, *Cytb* and *COI* sequences by PAUP v. 4.0 (Swofford, 2002). Phylogenetic relationships between taxa were inferred using the Neighbor Joining (NJ) method including in- and out-group taxa. The clades were arranged and labelled based on bootstrap support value, the genetic distance computed among the taxa and comparison to the outgroups. Moreover, for the clade *Leucoma* different support values containing 50% majority rule consensus, Clade Credibility and Posterior Probability were calculated using PAUP, MrBayes v. 3.2.7a (Huelsenbeck and Ronquist, 2001), BEAST v. 2.5 (Bouckaert et al., 2019) software, respectively. Jalview v. 2.11.3.2 software (Waterhouse et al. 2009) was used to determine the variable and conserved sites of six partial gene sequences of *Leucoma* and related taxa. The conserved, variable sites (parsim-informative sites and singletons) among the sequences in this study were determined using MEGA11.

Figure 1. *Leucoma wiltshirei*. **A.** female dorsal view. **B.** her genitalia. **C.** forewing D. hindwing (mounted on microscopic slide).



Results

Morphological identification: In total, 10 moth samples of the study were identified as female *Leucoma wiltshirei* based on the wing venation and genitalia characteristics (Figure 1). All these samples were collected from *Quercus brantii*, which is noted as the sole host of this particular pest in southern Zagros.

Two directional sanger sequencing: After sequence editing, the number of nucleotides for different genes in the study are as follows: D-18S rRNA: 488 bp, U-18S rRNA: 455 bp, *Ef1α*: 693 bp, 12S rRNA: 561 bp, *Cytb*: 572 bp, *COI*: 914 bp. Distance matrix (differences) show genetic similarity to *L. salicis* between one to 16% and among different *Leucoma* sequences available in GenBank (Table 2). Accession numbers for the sequences are including OQ596329.1 (*Ef1a*), OQ596330.1 (*Cytb*), OQ599898.1 (12S rRNA), OQ586447.1 (D-18S rRNA), OQ586442.1 (U-18S rRNA), OP221142.1 (*COI*).Phylogenetic analysis of the combined data set: A Partition Homogeneity Test (PHT) generated a p value of 0.47. Considering the incongruence threshold of $p = 0.05$ (Cunningham, 1997), suggesting that the datasets for D-18S rRNA, U-18S rRNA, *Ef1α*, 12S rRNA, *Cytb*, and *COI* were not substantially incongruent and could be combined for analysis. The combined phylogenetic tree is typically built based on genetic data, and the process involves comparing genetic sequences to estimate the evolutionary distances between different species (Figure 2). The combined phylogenetic tree showed evolutionary distances between *Leucoma wiltshirei* and nearest taxa, with 11% similarity to *Lymantria dispar* and 14% to *Euproctis similis*. The phylogenetic tree revealed distinct clades, with the family Noctuidae (*Spodoptera exigua* and *Lithophane socia*) forming a more distinct evolutionary lineage. The phylogeny of the various Lepidoptera genera is rooted in clades of selected out-group genera. Visual analysis using Jalview software indicated that the 12S gene region exhibited the greatest diversity

among the studied taxon sequences (Figure 3), making it suitable for identifying *Leucoma* species. Other gene fragments, such as *COI*, *Cytb*, and *Ef1α*, followed in terms of diversity. D-18S rRNA and U-18S rRNA genetic regions showed the lowest diversity, suggesting their conservation across studied species but may not be suitable for species-specific diagnosis. A total of 2877 conserved sites, 819 variable sites, and 426 parsimony-informative sites were identified using MEGA11. These sites provided insights into functional regions, genetic diversity, and evolutionary relationships in the studied sequence alignment or dataset.

Figure 2. NJ bootstrap consensus combined tree (MEGA11) of Superfam.: Noctuoidea inferred from the molecular data set (3734 bp + gaps) including six partial gene sequences of *Leucoma* and related taxa as in-group (Fam.: Erebidae): 1: 488-bp D-18S rRNA, 2: 455 bp U-18S rRNA, 3: 693-bp Ef1α, 4: 561-bp 12S rRNA, 5: 572-bp Cytb, 6: 914-bp COI. Bootstrap values of *Leucoma* clade are in bold included NJ (MEGA11), NJ 50% majority rule consensus tree (PAUP), MP 50% majority rule consensus tree (PAUP), BI clade 10 credibility value (MrBayes), BI posterior probability value (BEAST), respectively. The taxon sequenced in the present study is highlighted in bold. Spodoptera exigua and 12 Lithophane socia (Fam.: Noctuidae) are included as out-group. BI: Bayesian Inference, D: 13 Downstream, MP: Maximum Parsimony, NJ: Neighbor-Joining, U: Upstream).

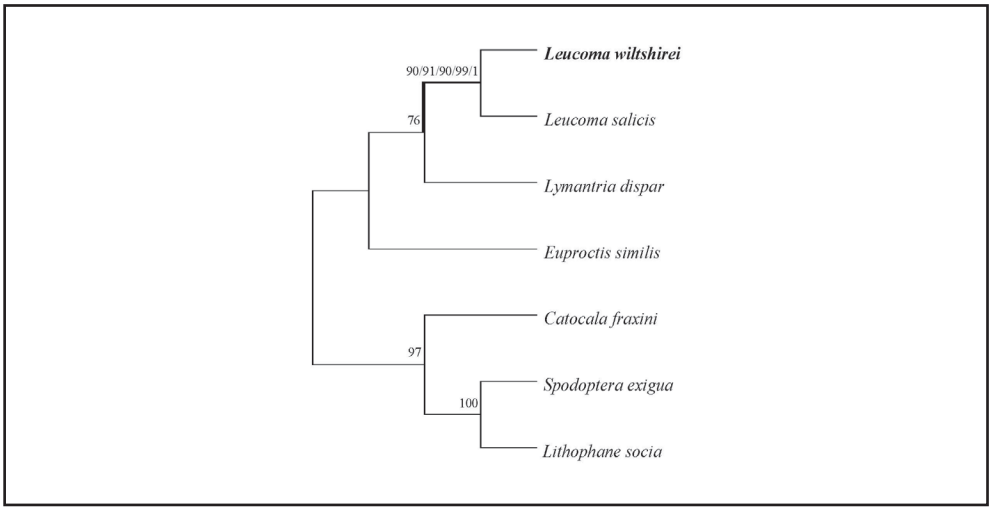


Figure 3. Variable and conserved sites of six partial gene sequences of *Leucoma* and related taxa mentioned in figure 2. Tiny color rods embedded in the dark part representing genetic variability.

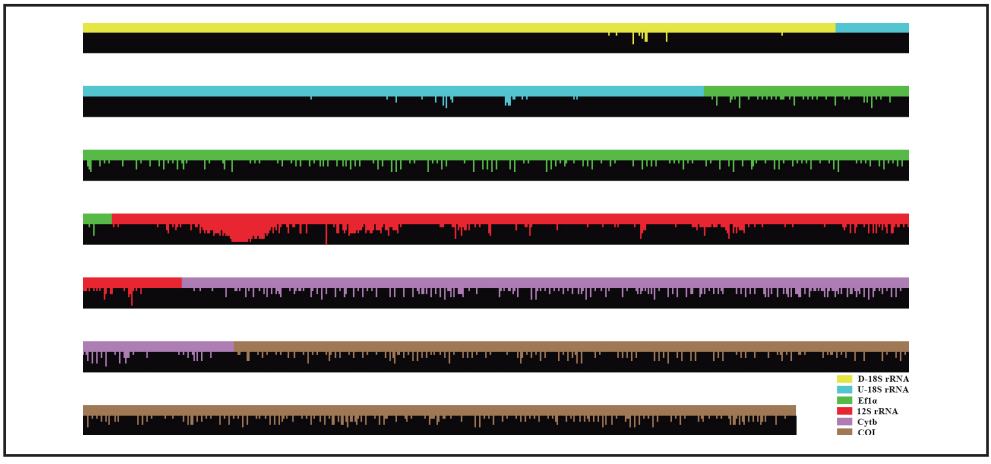


Table 2. Genetic distance between *Leucoma wiltshirei* and different *Leucoma* sequences available in GenBank based on Maximum Composite Likelihood (MCL) calculated by MEGA11.

Gene	*L. candida*	*L. chrysoscela*	*L. luteipes*	*L. melanoscela*	*L. nyingchiensis*	*L. parallela*	*L. salicis*	*L. sartus*	*L. sericea*
D-18S rRNA							1		
U-18S rRNA							2		
Ef1α		7					10		10
12S rRNA		7					8		
Cytb		15					15		
COI*	17	18	15	18	13	18	16	22	19

*Due to the limitations in the available COI sequence data, only 425 base pairs were calculated for comparison with the species (except *L. salicis*).

Discussion

This study confirms the identity of *Leucoma wiltshirei* through genetic analysis of mitochondrial and nuclear DNA. The 12S rRNA mitochondrial gene region exhibits the greatest diversity, making it suitable for identification. Other gene fragments, such as *COI*, *CytB*, and *Ef1α*, also show diversity. D-18S rRNA and U-18S rRNA genetic regions are more conserved and less suitable for species-specific diagnosis. Our study was devised and executed to resolve taxonomic uncertainties surrounding the identity, employing a multi-gene molecular analysis. Mitochondrial and nuclear ribosomal RNA genes have been more widely used to deduce relationships among various Lepidoptera taxa (Elameen et al. 2024; Ma et al. 2020; Wiemers et al. 2010; Zhao et al. 2019). Wahlberg et al. (2009) discovered a significant and well-supported discrepancy between the results obtained from mitochondrial DNA and those from nuclear DNA when estimating the species relationships within the genus *Polygonia* (Lepidoptera: Nymphalidae). Bertrand et al. (2014) demonstrate congruent patterns of nuclear and mitochondrial lineages for the *Urbanus* Hübner, [1807] complex (Lepidoptera: Hesperiidae), based on Bayesian phylogenetic trees constructed from *COI*, *Cytb*, and *Ef1α* sequences. Pazhenkova & Lukhtanov (2019) emphasized the importance of simultaneously analyzing nuclear and mitochondrial genes in *Brenthis* Hübner, [1819] (Lepidoptera: Nymphalidae) to assess their compatibility with morphological species. The key finding is that the specific nuclear genome is more specific in determining the borders of morphological species compared to the specific mitochondrial genome alone or the combination of mitochondrial and nuclear genes. DNA sequence data have provided new insights into the origins of the hawkmoth genus *Hyles* Hübner, [1819] (Lepidoptera: Sphingidae). The authors utilized a combination of mitochondrial sequences (e.g., *COI*) and nuclear sequences such as *Ef1α* (Hundsdoerfer et al. 2009). Zahiri et al. (2012) unveils the most robust phylogenetic hypothesis to date for the higher taxa within Erebidae, leveraging novel molecular data from eight independent gene regions including mitochondrial gene (*COI*) and seven nuclear genes (such as *EF-1α*). Mitochondrial and nuclear sequences were utilized to differentiate closely related species of the genera *Plebejus* (Vodolazhsky & Stradomsky, 2010) and *Polyommatus* Latreille, 1804 (Vodolazhsky et al. 2011) (Lepidoptera: Lycaenidae).

In our study, the phylogenetic tree analysis of superfamily Noctuoidea revealed that *Leucoma wiltshirei* shares a common ancestor with *L. salicis*. These two species, along with *Lymantria dispar* and *Euproctis similis*, form a cohesive clade within the Erebidae family, totaling four species. Interestingly, *Catocala fraxini*, another member of the Erebidae family, is positioned outside this clade and instead clusters with species from the out-group of the Noctuidae family, specifically *Spodoptera exigua* and *Lithophane socia*. This discovery indicates the presence of paraphyly within the Erebidae family, which is also mentioned in Zahiri et al. (2012) about the genus *Rivula* Guenée, [1845] and the Phaegopterina subtribe within the Erebidae Lepidoptera, discussing some paraphyletic taxa. However, it's claimed that the relationships among the four families within the Noctuoidea, such as Noctuidae and Erebidae, are not clear (Zahiri et al. 2023). The superfamily Noctuoidea is indeed a large taxonomic group within the order Lepidoptera. It comprises several families, one of which is the family Erebidae. As of last update, it included approximately 1,760 genera and 24,569 species, making it one of the largest families within the superfamily Noctuoidea (van Nieukerken et al. 2011).

In our multi-gene analysis, we employed five methods, namely Neighbor-Joining (NJ), NJ with 50% majority rule consensus, 50% majority rule consensus using Maximum Parsimony (MP), clade credibility value from Bayesian Inference (BI), and posterior probability value from BI, to evaluate the accuracy of the *Leucoma* clade. Various phylogenetic software tools were utilized for this assessment. The accuracy of this clade, which comprises two species, *L. wiltshirei* and *L. salicis*, ranged from 90% to 100%. Dai et al. (2012) utilized five distinct analysis methods-Maximum Likelihood (ML), Neighbor-Joining (NJ), "best close match" (BCM), Minimum Distance (MD), and a BP-based method-across both single-gene and multiple-gene analyses to support the monophyly of the moth genus *Dendrolimus* Germar, 1812 (Lepidoptera: Lasiocampidae). As well as the simultaneous use of mitochondrial and nuclear genomes for phylogenetic analysis has been employed to study butterflies of this genus. The results indicate that the interpretation of nuclear and mitochondrial data can differ, leading to varying conclusions about species differentiation (Kononov et al. 2016).

Our examination revealed 2877 conserved sites, 819 variable sites, and 426 parsimony-informative sites. These findings offer vital genetic insights necessary for evaluating the distribution and population dynamics of this significant pest species with global implications. Similar investigations aiming for polymorphism, genetic diversity, and genetic structure assessment have been conducted on various moth species worldwide, such as the study by Chen et al. (2013) analyzing *Lymantria dispar* (Lepidoptera: Erebidae) in China. Our analysis revealed genetic characteristics and diversity within the *Leucoma wiltshirei* gene fragments, contributing valuable information for species identification and understanding their evolutionary relationships.

Conclusion

This study confirms the identity of *Leucoma wiltshirei* through multi-gene molecular analysis, highlighting the 12S rRNA mitochondrial gene as the most diverse and suitable for identification. The phylogenetic analysis shows that *L. wiltshirei* and *L. salicis* form a cohesive clade within the Erebidae family, with significant paraphyly observed in the family. The findings provide essential genetic insights for species identification and understanding evolutionary relationships, with implications for managing this pest species globally.

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Conflit of Interest

The authors declare that there is no known financial interest or personal relationships that could have influenced the work presented in this article.

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