Saudi Journal of Biological Sciences 28 (2021) 7253–7256

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Identifying two moth species (Lepidoptera: Ditrysia) from Saudi Arabia using mitochondrial *16S rRNA* sequences

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ARTICLE INFO

Article history:
Received 20 June 2021
Revised 28 June 2021
Accepted 10 August 2021
Available online 19 August 2021

Keyword:
Lepidoptera
Ditrysia
Noctuoidea
Pyraloidea
16s rRNA

ABSTRACT

The mitochondrial genetic markers are considered useful tools for discrimination between more closely related lepidopteran taxa. Therefore, the present study aimed to investigate the role of mitochondrial (mt) *16 s rRNA* gene in the determination of the taxonomic position for two moth species within Ditrysia clade. Maximum likelihood analysis has indicated a well-supported dendrogram based on the Tamura-Nei model for the recovered lepidopterans. The mt *16 s rRNA* query sequences from 24 species within seven families were analyzed. This analysis and bootstrap confidence revealed two major clades representing Glossata suborder within Lepidoptera, with a close relationship of Noctuoidea + (Pyraloidea (Hesperioidea + Papilionoidea)). The subfamily Heliothinae forming a sister group with Risobinae (Noctinae + Hadeninae). In addition, there is a clear observation about the close relation between Phycitinae + Galleriinae within Pyraloidea and Cyrestinae + Limenitidinae within Papilionoidea. The present study supported that the *Helicoverpa* and *Meroptera* species are the first accounts of these genera inhabiting Saudi Arabia.

1. Introduction

Lepidoptera Linnaeus, 1758 is one of the four largest insect orders that containing about 165,000 species (Regier et al., 2009). It served as important model systems for studies of genetics, physiology, development, and many aspects of biological research (Roe et al., 2009). Glossata Fabricius, 1775 is a suborder within Lepidoptera that included two nested subclades Heteroneura and Eulepidoptera (Mitter et al., 2017). Heteroneura is a natural group in the Lepidopteran insects that comprises over 99% of all butterflies and moths. The most securely established large subgroup of Lepidoptera is the clade Ditrysia Borner, 1925, which contains 29 superfamilies and 98% of lepidopterans (van Nieukerken et al., 2011). Noctuoidea is the largest speciose superfamily within Ditrysia that followed by Pyraloidea and Geometroidea (Zahiri et al., 2011). The Heliothinae is a subfamily within the Noctuidae family that encompasses 381 species within 28 genera (Pogue, 2013). The genus *Helicoverpa* Hardwick, 1965 is a group of 18 polyphagous species belonged to Heliothinae (Mitter et al., 1993). Phycitinae Zeller, 1839 is a subfamily within the Pyralidae family that includes 530 species (Neunzig, 2003). The *Meroptera* genus was erected by Grote, 1882 and includes six species.

Due to the lack of enough morphological identification criteria for insect species, molecular diagnostic tools have been recommended to distinguish among diverse species (Lewter and Szalanski, 2007). Different genetic markers have been performed in insect phylogeny, including mitochondrial and nuclear DNA genes (Riccieri et al., 2020). Molecular data using ribosomal RNA (rRNA) genes has provided valuable insights for understanding genetic diversity and improving classifications for highly diverse insect groups (Zahiri et al., 2011). Recently, the mitochondrial *16 s rRNA* gene was considered a useful identification tool for insect relationships at different taxonomy levels (Dellaporta et al., 2006). To date, there are no available data about molecular phylogenetic analysis of Lepidoptera in Saudi Arabia. Therefore, the goal of this study is to determine the utility of the mitochondrial *16 s rRNA* gene for the identification of some lepidopteran species within Heliothinae and Phycitinae.

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2021.08.030
1319-562X/

2. Materials and methods

2.1. DNA extraction and PCR amplification

Ethanol preserved samples were used to extract genomic DNA using QIAamp® DNA Mini Kit (QIAGEN, Germany). The purity and concentration of DNA were estimated using NanoDrop™ 2000/2000c Spectrophotometer V1.0 User manual (Thermo Fisher Scientific, Wilmington, DE 19810, USA). The mitochondrial (mt) *16S rRNA* gene was targeted and amplified through polymerase chain reaction (PCR) using the following primers, 16sRNA-FW 5′-CGC TGT TAT CCC TAA GGT AA-3′ and 16sRNA-RV 5′-CTG GTA TGA AAG GTT TGA CG −3′, as mentioned by Li et al. (2010). Each PCR mixture of 20 μl volume and consisted of 4 μl 5 × FIREPol® Master Mix, 2 μl of genomic DNA, 0.6 μl of each primer, and nuclease-free water for a final reaction volume of 20 μl. PCR conditions used the following profile: 95 °C for 5 min and 35 cycles of 95 °C for 15 s, 48 °C for the 30 s, 72 °C for 1 min, and 72 °C for 5 min. Reactions were conducted using Thermal cycler PCR (Veriti® 96-Well Thermal Cycler, Applied Biosystems). The amplified PCR products were confirmed by 1.5% agarose gel electrophoresis in 1 × TAE buffer and purified before the sequencing reaction using QIAquick® PCR purification Kit (QIAGEN, Germany).

2.2. Sequencing and phylogenetic analysis

Amplicons were sequenced with ABI 3130 Genetic Analyzer (Applied Biosystems®, USA) using BigDye™ Terminator Cycle Sequencing Kit (Perkin Elmer Inc., USA). The resulting sequences were linked to database searches using BLASTn to verify the species' identity. The sequences were aligned using ClustalX (Thompson et al., 1997) and corrected manually by BioEdit v5.89 (Hall, 1999). Phylogenetic analysis was performed by the Maximum Likelihood (ML) method with the Tamura-Nei model. Tree reliability was estimated by the bootstrap method under appropriate models for substitution with 1000 replicates using MEGA 7.0 (Kumar et al., 2016).

3. Results

A partial sequence of the mt *16S rRNA* amplicons revealed the presence of two lepidopterans species, one of them belonging to family Noctuidae (*Helicoverpa punctigera*) and the second one within family Pyralidae (*Meroptera pravello*) with an average size (GC content) of 221 (17.2%) and 221 (14%) bp, respectively. The sequenced data were registered in GenBank (accession numbers of gb| MK063902.1 for *H. punctigera* and gb| MK063894.1 for *M. pravello*). The current phylogeny based on the analysis of 24 related lepidopteran taxa was carried out using the maximum likelihood (ML) method (Fig. 1).

The present cladogram is constructed by members of the Lepidoptera order in the clade Ditrysia and represented by four superfamilies: Noctuoidea, Hesperioidea, Pyraloidea, and Papilionoidea (Fig. 1). The phylogenetic tree consisted of two clades, Clade I including taxa in two subclades belonging to the above-mentioned superfamilies with different families, as following: subclade (A) for Noctuoidea represented by two families of Noctuidae and Notodontidae, and subclade (B) includes Hesperioidea clustered members of Hesperiidae, Pyraloidea represented by the Crambidae family, and Papilionoidea includes family Papilionidae. Clade II is divided into two subclades that clustered the remaining members of the last two superfamilies: subclade (C) for Pyraloidea includes one family Pyralidae, and subclade (D) for Papilionoidea represented by Nymphalidae family.

Our phylogeny indicated a close relationship of Noctuoidea + (Pyraloidea (Hesperioidea + Papilionoidea)). Heliothinae forming a sister group with Risobinae (Noctinae + Hadeninae). Phycitinae and Galleriinae within Pyraloidea sisters to Cyrestinae and Limenitidinae within Papilionoidea. The ML tree showed a well-resolved distinct clade for the recovered species with other members of lepidopterans taxa especially those belonging to the family Noctuidae and deeply embedded within the genus *Helicoverpa* with a close relationship to the previously described *Helicoverpa* species especially to *H. punctigera* (gb| KF977797.1, MG437200.1, MG437201.1) in the sister taxon with a strong support value of 84. In addition, the other lepidopteran species formed a well distinct clade with members of Pyraloidea especially the family Pyralidae, and deeply embedded within the genus *Meroptera* with a close relationship to the previously described *M. pravello* (gb| MK063894.1) in the same taxon with a moderate support value of 54.

4. Discussion

The insect order Lepidoptera forms an important part of the natural environment (Kristensen et al., 2007). The morphological identification for different insect species using standard taxonomic features is considered a challenge due to little available information until now (Perera et al., 2015). A phylogenetic investigation based on DNA barcodes has succeeded in the species-level identification of Lepidopterans (Jinbo et al., 2011). Our phylogeny strongly supported the inter-relationship between the recovered species with other lepidopteran taxa at the generic and species level based on the analysis of the mt *16 s rRNA* gene, this agreed with and Liu et al. (2014) who suggested that mitochondrial DNA genes (*16S*, *COI*, *COII*, and *NDI*) represented as useful genetic markers for clarifying the relationship between different lepidopteran species.

Pelham (2008) expected the higher classification of butterflies that comprises three superfamilies, the Papilionoidea, Hesperioidea, and Hedyloidea, which are phylogenetically embedded within the moths; this data agreed with our results but only represented by the former two superfamilies. In addition, moths demonstrated in the current phylogeny with the remaining superfamilies observed in the ML tree with special reference to the presence of lepidopteran species recovered herein. Also, Papilionoidea demonstrated to be paraphyly in origin that forming a single clade together with Hesperioidea, this is consistent with the earlier phylogenetic analysis of Lepidoptera by Kristensen and Skalski (1999). The present study agreed with the hypothesis of Mitchell et al. (2006) about the construction of Noctuoidea in numerous families represented herein by only Noctuidae and Notodontidae. Our phylogeny demonstrated the monophyly of Noctuoid species, this data agreed with Zahiri et al. (2012), and Regier et al. (2013). In addition, Cho et al. (2008) and Arnemann et al. (2016) indicated that the mitochondrial genes are suitable for resolving *Helicoverpa* phylogeny with monophyly in origin which is supported by bootstrap values of greater than 70%, this is consistent with our results.

The present phylogeny indicates that the Pyraloidea family split into two families of Pyralidae and Crambidae, this data consistent with Regier et al. (2009, 2012) and Heikkilä et al. (2015) who stated the paraphyly of Pyraloidea concerning the presence of Pyraustinae and this superfamily represented by two monophyletic families of Pyralidae and Crambidae that forming a sister group with each other. In addition, the Crambidae and Pyralidae considered being microlepidoptera that more closely related to other families of macromoths including Noctuidae and Notodontidae, this agreed with Heikkilä et al. (2015). The present study indicated a sister group within Pyraloidea of Galleriinae + Phycitinae, confirming

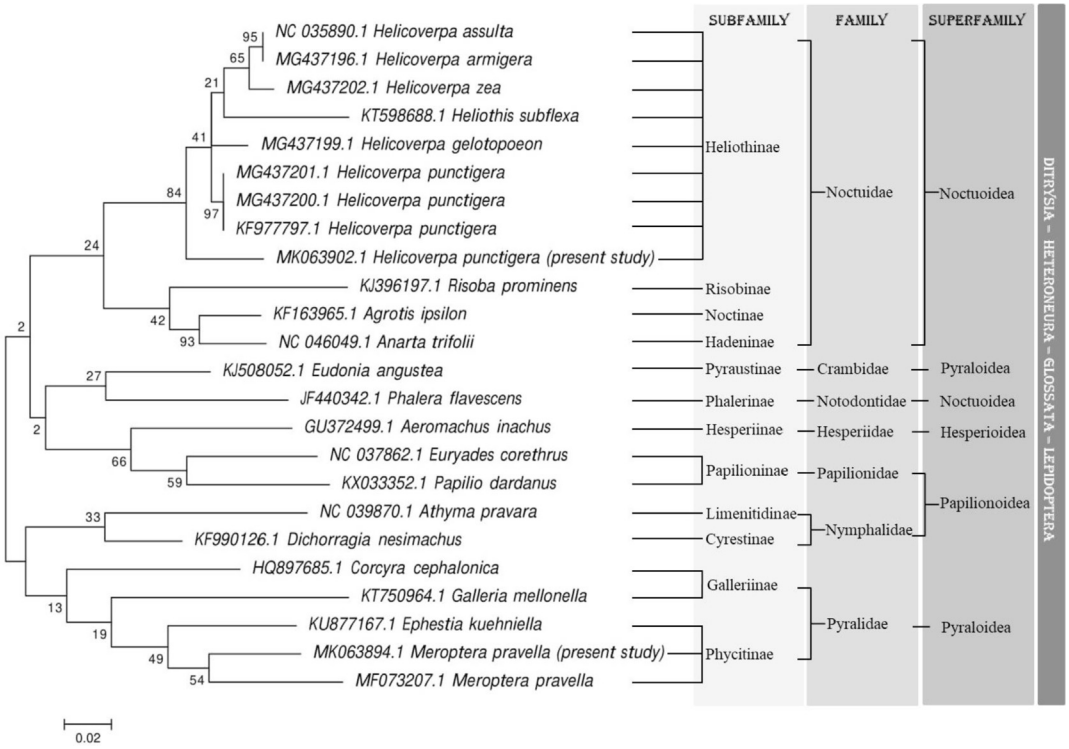

Fig. 1. Maximum likelihood analysis based on the Tamura-Nei model for the recovered lepidopterans. The tree with the highest log likelihood (−1393.38) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

the findings of Regier et al. (2012) stated that the relationships between Galleriinae + (Phycitinae + (Epipaschiinae + Pyralinae). In addition, our phylogeny demonstrates the basal position of *Meroptera pravella* within subfamily Phycitinae of the Pyralidae family, this agreed with Munroe and Solis (1998). Therefore, Sequence alignments between the current lepidopteran species and other taxa from GenBank showed a high percentage of identity between these species with previously sequenced *Helicoverpa* and *Meroptera* species within the Noctuidae and Pyralidae families, respectively.

5. Conclusion

Our data highlighted the need for higher taxonomic levels for different lepidopterans include a combination of morphology and phylogeny results to reach the specific level for each taxon.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was supported by the Researchers Supporting Project number (RSP-2021/99), King Saud University, Riyadh, Saudi Arabia.

Data Availability All data generated or analyzed during this study are included in this published article.

Animal Research (Ethics) Not applicable.

Consent to Participate (Ethics) Being the corresponding author to this manuscript, I state that all authors agree to its submission, and the co-authors have authorized the Corresponding author.

Consent for Publish (Ethics) Being the corresponding author to this manuscript, I state that all authors agree to its submission, and the co-authors have authorized the Corresponding author.

Plant Reproducibility Not applicable.

Clinical Trials Registration Not applicable.

Author Contribution All authors participate equally.

Funding

Authors extend their appreciation to Researchers Supporting Project (RSP-2021/99) King Saud University, Riyadh, Saudi Arabia.

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