

# Molecular identification of some immature Lepidoptera causing *Quercus* L., defoliation in Lorestan province, western Iran (Insecta: Lepidoptera)

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## Abstract

*Quercus* L., dominate almost 40% of Iran's forest area. The second destructive group of insects is defoliator Lepidoptera distributed in the Zagros forests of Iran. We assessed Lepidoptera communities in Zagros forests ecosystems in Lorestan province, western Iran during a ca. 2-yr period that coincided with defoliation outbreaks experienced by this area. A number of 500 lepidopteran larva feeding *Quercus* leaves handy were collected. The phylogenetic relationship of Lepidoptera was analysed using BEAST software based on the Bayesian Inference method. In total, 14 lepidopteran taxa were identified based on DNA sequences of their immature stages. Six genera and eight species were identified using BLASTn. Here we developed a COI barcoding-based approach to Lepidoptera species delimitation.

**Keywords:** Insecta, Lepidoptera, Damage, *Quercus brantii*, sequencing, COI barcoding, Phylogenetic tree, Iran.

## Identificación molecular de algunos Lepidoptera inmaduros causantes de la defoliación de *Quercus* L., en la provincia de Lorestán, oeste de Irán (Insecta: Lepidoptera)

## Resumen

*Quercus* L., dominan casi el 40% de la superficie forestal de Irán. El segundo grupo destructivo de insectos son los Lepidoptera defoliables distribuidos en los bosques de Zagros de Irán. Hemos evaluado las comunidades de lepidópteros en los ecosistemas de los bosques de Zagros en la provincia de Lorestan, al oeste de Irán, durante un periodo de unos 2 años que coincidió con los brotes de defoliación experimentados en esta zona. Se recogieron 500 larvas de lepidópteros que se alimentaban de hojas de *Quercus*. La relación filogenética de los Lepidoptera se analizó mediante el programa informático BEAST, basado en el método de la inferencia bayesiana. En total, se identificaron 14 taxones de Lepidoptera a partir de las secuencias de ADN de sus estadios inmaduros. Mediante BLASTn se identificaron seis géneros y ocho especies. Aquí desarrollamos un enfoque basado en el código de barras COI para la delimitación de especies de Lepidoptera.

**Palabras clave:** Insecta, Lepidoptera, Daño, *Quercus brantii*, secuenciación, código de barras COI, árbol filogenético, Irán.

## Introduction

Zagros forests in the Irano-Turanian region with an area of six million hectares occupy almost 40% of the total forests of Iran. These forests in western Iran contain 170 tree and shrub species (Sagheb-Talebi et al. 2014). The genus *Quercus* L. comprises more than 400 species found throughout

the Northern hemisphere (Gil-Pelegrín et al. 2017). *Quercus* forests dominate almost 40% of Iran's forest area between 1,000 and 2,000 m elevation. These forests belong to two phytogeographical regions including Caspian-Arasbaran and Irano-Turanian (Sagheb-Talebi et al., 2014). *Quercus brantii* Lindl., occurs on south-western slopes of central Zagros between altitude 1,800 and 2,000 m.a.s.l. (Jazirehi & Ebrahimi Rostaghi, 2003). These dominant trees play a fundamental role in the Zagros ecological nature, principally through serving as food plants for numerous herbivores in the food chain, which are important resources and shelter refuges for thousands of species of animals (Jafarzadeh et al. 2020). The arthropod herbivore fauna of *Quercus* trees is usually richer than other tree species, and hence the insect groups are a crucial element of biodiversity in their ecological communities (Marquis et al. 2019; Tavakoli, Khaghaninia, et al. 2019).

Insect leaf feeders or defoliators are the largest groups of forest pests (Ciesla, 2011). Insect feeding on foliage can take a variety of forms such as; whole external feeding (all leaf tissue fed except the veins and midribs), external partial feeding (only tender tissue of leaves are eaten), and internal feeding with symptoms such as dead spots and discoloration (Ciesla, 2011). An outbreak of phytophagous insects usually causes a temporary shift from wood to leaf production, large changes in the stream-water chemistry (biogeochemical effects), rapid decrease of the growth and the productivity of the forest (short-term), nitrogen leakage from the forest ecosystem and influence ecosystem-level processes such as nutrient cycling, increase in the death or decline of trees, change in the species composition (long-term) (Eshleman et al. 1998; Reynolds et al. 2000; Swank et al. 1981; Webb et al. 1995). The second group of destructive insects is the *Quercus* defoliator Lepidoptera in the Zagros forests (Abai, 2009). One of the most important challenges facing all those involved in plant protection management of the forest is the lack of accurate knowledge of the defoliator insect species occurring in these areas. In the defoliation process of *Quercus* trees, a complex of different species are involved; the identity of many of which is unknown, and there is always a request from us to identify and naming of these pests (commonly named "a leaf / bud eating worm", "a white-spotted leaf-eater", "a biting leaf-eater", "an awkward silk weaver" and etc. (Rahmani et al. 2022). In most cases, these pests are univoltine (in some cases multivoltine), start herbivory simultaneously and their population size decrease in a short period of time, so it returns to their original state before an outbreak. But in general, they have a negative effect on the annual growth of infested trees and seed production. Many of these pests are native to the region and can be found every year according to climatic conditions (Rahmani et al. 2022). Elevation and tree density, soil variables such as pH, and litter properties were responsible for the insect herbivory in forests of northern Iran (Hajizadeh et al. 2016). Various outbreaks of herbivorous insects were recorded in forests of Iran; *Hyphantria cunea* (Drury, 1773), *Altica viridula* Weise, 1889, the geometrid moths such as *Ennomos quercinaria* (Hufnagel, 1767) and *Erannis defoliaria* (Clerck, 1759) in Caspian regions (Barimani Varandi et al. 2006; Kavosi, 2007; Kavosi & Gninenko, 2007; Kiadaliri et al. 2005), *Lymantria dispar* (Linnaeus, 1758) in northern Zagros (Tavakoli et al. 2018), *Leucoma wiltshirei* Collenette, 1938, in southern Zagros (Jalili, 2022).

The main aim of the present work was to identify the caterpillars (immature / larval stages) causing losses due to defoliation in forest-steppe *Quercus* stands for two years period (2018-2020) using COI barcoding and to reconstruct phylogenetic relationships of lepidopterans. In this paper, we showed that *Quercus* leaf defoliating is a phenomenon that does not belong only to one species and several species of lepidopteran larvae are responsible for this event.

## Methods

### STUDY AREA AND SAMPLE COLLECTION

This investigation was conducted from March 2018 to June 2020 in the Zagros forests of Lorestan province, western Iran. The forests of Lorestan province cover one million two hundred thousand hectares and about 44% of the total area of the province in the west, south, southwest of the centre and east of the province. There are about 90 species of trees and shrubs (woody plants) in this province. In

Lorestan, there are two species of *Quercus* including *Quercus brantii* and *Q. infectoria* Oliv. with several growing hybrids to be recognized. A number of 500 lepidopteran larva feeding *Quercus* leaves handy were collected. Caterpillars were caged using a white canvas curtain fabric measuring 2 x 2 meters cover the ground under the tree. Sixteen to 51 trees were analyzed in each station. The specimens were photographed under a stereomicroscope (Wild-Heerbrugg M8Model) equipped with a digital camera (Nikon® Coolpix S7000) (Figure 1).

## DNA extraction and PCR

The genomic DNA of a representative specimen was extracted using CTAB according to Doyle & Doyle (1987). A fragment of the cytochrome oxidase subunit I (*COI*) was amplified by polymerase chain reaction (PCR) using the primers designed by various authors (Table 1). PCR reactions were carried out in a thermocycler (Corbett®, Australia) based on a touchdown temperature profile: 3 minutes at 94°C, 11x [45 s at 94°C, 50 s at 60-50°C, 60 s at 72°C], followed by 24x [45 s at 94°C, 50 s at 50°C, 60 s at 72°C], 3 minutes at 72°C). PCR for each 25 l final volume reaction was performed using 12.5 l RedMaster PCR 2X (Sinaclon®, Iran), 1 l of each primer (10 pM), 4 l gDNA template (100 ng/l), and 6.5 l ddH<sub>2</sub>O. The PCR products were visualized with 1% agarose gel electrophoresis and finally submitted to a third-party service provider (Codon Genetic Group®, Iran) for sequencing using Applied bioSystems-ABI, 3130XL.

**Table 1.** PCR primers (matching result according to primer BLAST) used in molecular analysis and the size of expected *COI* gene fragment targeting different butterflies

Primer name (direction)	Sequence (5'→3')	Taxa (GB acc. no.) / Gene target size (bp)		
		Amphipyra effuse (MW332787) Catocala fraxini (OV884055) Eriogaster sp. (Lasiocampidae) (NC_062175) Pammene albiginana (GQ149501) Apocheima cinerarius (KR478686) Quercusia quercus (KM592971) Erannis defoliaria (FR990095) Euproctis crypsosticta (KY996558) Saxyrum behrii (OP431069) Dicycla oo (Noctuidae) (MZ297463) Tortrix viridana (AY883092) Bena Bicolorana (Nolidae) (NC_062184) Aporophylla lueneburgensis (OW028764) Orthosia cruda (MZ297463)		
C1-J-1718*	GGA GGA TTT GGA AAT TGA TTA G			
C1-N-2191*	CCC GGT AAA ATT AAA ATA TAA ACT TC	524		
LCO1490**	GGT CAA CAA ATC ATA AAG ATA TTG G		701	
HC02198***	CAG GGT GMC CAA AAA ATC		702	

\*From Simon et al. (1994) with minor modification in C1-J-1718 as follows: deletion of –TTCC... from the 5' end; \*\*From Folmer et al. (1994); \*\*\*Modified after

Folmer et al. (1994) with major modification in HC02198 as follows: deletion of –TAAACTT... from the 5' end along with some minor changes.

## BLASTn AND PHYLOGENETIC ANALYSIS

The DNA sequence was manually checked using FinchTV® software to correct any sources of error or ambiguities if present. Homologies with the available sequence data in GenBank were checked using BLASTn analysis and the phylogenetic closeness. The criteria for the identification via BLASTn analysis were 99% - 100% sequence identity with comparable COI sequences of each taxa deposited in

GenBank and an acceptable query cover (more than 90%). In many cases, we used from Bold Systems website (<https://boldsystems.org/index.php/>) as a complementary data bank in order to improve the identification of Lepidoptera taxa. Finally, the sequence was submitted to GenBank, and the accession numbers were achieved. The nucleotide sequence was then used to build a phylogenetic tree supporting the identification. To construct the COI phylogenetic tree, various alignment datasets (according to different families or superfamily) were analysed using BEAST (Ver. 2.6.0) (Bouckaert et al. 2014) based on the Bayesian Inference (BI) method. The phylogenetic trees were summarized and visualized using Tree Annotator and then FigTree (Ver. 1.4.4.), respectively. For this purpose, COI sequences including sequences of the present study, as well as the comparable data sequences of GenBank submitted from various parts of the world including the species of a genus were used. Sequences were selected according to the identity revealed by the BLAST algorithm. Then, the sequences were aligned using SeaView4 software (Gouy et al. 2010). The genetic distances among and between sequences were calculated using Maximum Composite Likelihood (MCL) modelled in MEGA7 (Kumar et al. 2016). The out-groups were chosen from the sister groups as well as successively more distant lineages including some taxa of this study and other taxa. Thus, the genera *Euproctis*, *Erannis*, *Lasiocampa*, *Satyrrium* and *Tortrix* were examined as out-groups.

## Results

### CATERPILLAR IDENTIFICATION VIA BLASTN ANALYSIS

In total, 14 Lepidoptera taxa were identified based on DNA sequences of their immature stages. Six genera and eight species were identified using BLASTn. The genera including *Apocheima* Hübner, [1825], *Catocala* Schrank, 1802, *Eriogaster* Germar, 1810, *Euproctis* Hübner, [1819], *Pammene* Hübner, [1825], *Satyrrium* Scudder, 1876 and species *Amphipyra pyramidea* (Linnaeus, 1758), *Aporophyla nigra* (Haworth 1809), *Bena bicolorana* (Fuessly 1775), *Dicycla oo* (Linnaeus 1758), *Erannis ankeraria* (Staudinger 1861), *Orthosia cruda* ([Denis & Schiffermüller], 1775), *Quercusia quercus* (Linnaeus 1758), *Tortrix viridana* Linnaeus 1758 were found (Table 2). The most identified taxa (5 taxa) were related to the noctuid family and the least taxa were related to Erebidae, Lasiocampidae, Nolidae, each one species (Figure 2). COI sequences were registered in GenBank under the accession numbers MT832310, MT832359, MT833841-2, MT835252-4, MT844020, MT844050-5, MT881717, MT947393, MT948195. After immature rearing and adult emergence of some Lepidoptera such as *Catocala* and *Satyrrium* in laboratory conditions (data not shown), the specimens need to be morphologically identified at the level of species for further investigations.

### PHYLOGENETIC ANALYSIS

Phylogenetic trees were constructed based on partial COI sequence data and several clades were formed according to genetic distances and out-group relationships (Figure 3). The genetic distances between clades are as follows: 9% to 21% (Noctuoidea, seven clades), 14% (Geometridae, two clades), 11% (Lasiocampidae, two clades), 10% (Lycaenidae, two clades), 17% (Tortricidae, two clades). The COI phylogeny of the various lepidopteran genera is rooted in clades of selected out-group genera. The COI phylogenetic tree shows in Noctuoidea (Figure 3A), the clade *Euproctis* is a more distinct evolutionary lineage as ancestral, basal, or a sister group with 19% to 21%, genetic distance compared with other clades.

## Discussion

### THIS STUDY AS A FAUNAL WORK

In this survey, out of 14 collected taxa (eight species and six genera with unidentified species),

seven taxa including four species and three genera (unknown species) are reported for the first time in Lorestan province. Two species are identified as *Quercus* tree defoliator for the first time from Iran. According to the literatures review, the taxa *Apocheima*, *Aporophyla nigra*, *Bena bicolorana*, *Orthosia cruda*, *Pammene*, *Quercusia quercus* and *Satyrium* have not been reported from Lorestan province, and two species *Aporophyla nigra* and *Orthosia cruda* have not been mentioned as *Quercus* tree defoliator in Iran (Abai, 2009; Mirzayans & Abai, 1974; Mohammadian, 2005; Shahreyari-Nejad et al. 2018). Other taxa which are previously reported in Iran including *Amphipyra pyramidea*, *Orthosia cruda*, *Ap. nigra*, *Dicycla oo*, *Euproctis*, *Catocala*, *Tortrix viridana*, *Erannis*, *Eriogaster*, *Quercusia quercus* and *B. bicolorana*. *Am. pyramidea* distributed in Palearctic from Southern Europe to Turkey, Levant, Iran, and temperate regions of Asia collected on *Quercus* species, polyphagous on various deciduous trees and bushes (Kravchenko et al. 2007). *O. cruda* distributed in West-Palearctic including Morocco, Algeria, Tunisia, Europe, Turkey, Levant, Caucasus, Transcaucasia and Kazakhstan collected on deciduous trees, mainly on *Quercus*, *Salix* and *Populus* L. (Kravchenko et al. 2007). *Aporophyla nigra* distributed in West-Palearctic. Morocco, Tunisia, Libya, Southern and Central Europe, Turkey, Levant, Iran, Iraq and Caucasian region collected on *Astragalus* L., *Crataegus monogyna* Jacq., *Rosa canina* L., *Calluna Salisb.*, *Rumex* L., *Plantago* L., *Stellaria* L., *Deschampsia* P. Beauv., *Epilobium* L., *Genista* L. and *Oxalis* L. (Kravchenko et al. 2007). *D. oo* distributed in European-West Asiatic, Southern, Central and Eastern Europe, Turkey, Levant, Iran, Iraq, and Transcaucasia. Over the past few decades extinct from many parts of Europe, drastically decreased in most of the other regions. This species invades *Q. calliprinos* Webb and *Q. ithaburensis* Decne also other *Quercus* spp. (Kravchenko et al. 2007). The new data of the present study indicate the need for further faunistic studies in different regions of Zagros provinces. Another aspect of the importance of this type of studies is that during the last two decades, due to climate changes, we have witnessed several outbreaks of *Quercus* leaf-eating pests in the forest areas of Iran, and therefore, identifying them seems necessary to control them in the future. The following taxa reported by Mirzayans and Abai (1974) and Abai (2009) on *Quercus* tree in Iran; various *Euproctis* and *Catocala* from Zagros mountains and northern provinces, *D. oo* from Zagros mountains, *Am. pyramidea* from northern and south western provinces, *Tortrix viridana* from different parts, and also, two species of the genus *Erannis* and *Eriogaster*. The following lepidopterans taxa were reported by Mohammadian (2005) in various parts of Iran; thirteen *Catocala*, five *Eriogaster*, five *Euproctis*, seven *Satyrium*, four *Amphipyra* (such as *Am. pyramidea* from Zagros & Elburz Mountains), three *Erannis* (such as *Er. ankeraria* from Northern half of Iran Plateau), *Aporophyla australis* (Boisduval 1829), *Q. quercus* and *T. viridana* from Zagros Mountains, *B. bicolorana* from Caspian Eastern shore, *D. oo* from West & North of Iran, *O. incerta* (Hufnagel, 1766) from Northern half of Iran. Ravan et al. (2016) reported some Noctuidae species on the *Quercus* tree bionomically and morphologically; Some Ravan's reported species such as *Ap. nigra* and *D. oo* are distributed in Khuzestan and Fars provinces located in southwest Iran. Due to the importance of the *Quercus* tree in forest ecology, many studies have been conducted on its *Quercus* defoliator pests in the surrounding countries; the Geometridae family was indicated as the most representative Lepidoptera family feeding on *Quercus* trees in Turkey (Torun & Çalişkan, 2016). Larvae of 54 Lepidoptera species were collected from eight species of *Quercus* in Greece (Kalapanida & Petrakis, 2012). In another study, in addition to the Geometridae family, the highest number of *Quercus* foliage defoliators was reported among Noctuidae (Kalapanida & Petrakis, 2012). Torun and Çalişkan (2016) reported Lepidoptera larval communities feeding on *Q. pubescens* in Turkey including seven Geometridae, four Noctuidae, two Tortricidae, two Thyatiridae and one species for each of the families Gelechiidae, Arctiidae, Gracillariidae, Lasiocampidae, Lymantriidae, Oecophoridae, Pyralidae, Yponomeutidae, Pterophoridae and Lycaenidae. In the present study, the greatest richness of *Quercus* defoliating caterpillars belonged to the Noctuidae family (five taxa) and Noctuoidea superfamily (seven taxa) followed by Geometridae, Lycaenidae, Tortricidae (each family with two taxa) and finally Lasiocampidae (one taxon).

**Table 2.** Caterpillars collected in this study and their GenBank data

No. of station	Collection site (county)	GCS	Altitude m.a.s.l.	Taxa identified (GB acc. no.)						
				Noctuoidea			Geometroidea	Bombycoidea	Papilionoidea	Tortricoidea
				Noctuidae	Erebidae	Nolidae	Geometridae	Lasiocampidae	Lycaenidae	Tortricidae
1	Meleh-ye Shabanan (Khorramabad)	48°09'40"E 33°32'07"N	1250	<i>Am. pyramidea</i> (MT832310) <i>Catocala</i> sp. (MT881717) <i>D. oo</i> (MT835254) <i>O. cruda</i> (MT833841)			<i>Apocheima</i> sp. (MT844054) <i>Er. ankeraria</i> (MT844050)		<i>Qi. quercus</i> (MT844051)	<i>Pammene</i> sp. (MT844055)
2	Eyvandar (Aleshtar)	48°15'54"E 33°40'27"N	1525		<i>Euproctis</i> sp. (MT832359) <i>O. cruda</i> (MT833842)				<i>Satyrium</i> sp. (MT844052)	
3	Nozhian (Khorramabad)	48°34'35"E 33°13'45"N	1345	<i>Catocala</i> sp. (MT835253)	<i>Euproctis</i> sp. (MT844020)			<i>Eriogaster</i> sp. (MT844053)		
4	Sarkanah (Sepiddasht)	48°43'16"E 33°15'45"N	1327			<i>B. bicolorana</i> (MT947393)				<i>T. viridana</i> (MT948195)

GCS: geographical coordinates system, m.a.s.l.: meters above sea level, GB acc. no.: GenBank accession number, Am.: *Amphipyra*, Ap.: *Aporophyla*, B.: *Bena*, D.: *Dicycla*, Er.: *Eranis*, O.: *Orthosia*, Qi.: *Quercusia*, T.: *Tortrix*

THE CONTEXT AND IMPORTANCE OF STUDY

The most important aspect of this study was the attendance of researcher at the time and place of which the defoliation by caterpillars was occurred. As in the authors' previous studies (Tavakoli et al. 2017, 2018; Tavakoli, Hosseini-Chegeni et al. 2019), trees damaged by pest insects were significantly more defoliated and the affected trees exhibited heavily aggregated caterpillars feeding on the foliage. The sampling technique consists of shaking the tree twigs to fall various larvae on a white cloth spread under the tree over the ground and then the direct manual collection of larval samples. Furthermore, the larva that feed on the *Quercus* trees (particularly *Quercus brantii* in this study), showing a richer species diversity, with high level of homochromy and homomorphy among the larva with tree parts (trunk, branches, and foliage) that live on it. They cannot be easily seen and collected by amateurs and even some insect collectors, so this method was used. A constraint of our study is that most of the present defoliator pests are univoltine species, i.e., their damaging and larval stages appear at certain times (Glavendekiæ & Medarevia, 2010; Kalapanida & Petrakis, 2012). Then, researchers can monitor and study them at certain times. Following the defoliator there has been an ecological succession of wood boring beetles and the *Quercus* decline (dieback) occurrence in the areas where we have had the outbreak of insect defoliators (Csóka & Kovács, 1999; Domingue et al. 2011; Netherer & Schopf, 2010; Rouault et al. 2006). Rearing caterpillars to adult stage in several forestry lepidopteran species are more difficult at laboratory conditions, and usually due to the poor data related to behaviour, biology and ecology, the breeding and hatching of larva is very difficult (laboratory observations). Inevitably, we had to use molecular methods for diagnosis. Unlike some polyphagous pests such as *Lymantria dispar* which is previously reported in Zagros forests of Iran (Tavakoli et al. 2018), the larvae of the present study are monophagous and feed exclusively on *Q. brantii*. The authors could not find them on other hosts. Due to the lack of basic knowledge on the emergence time of adult insects and rich species diversity of leaf-feeder Lepidoptera of *Quercus* trees, shrubs, and pastures, it was impossible for us to use light traps and obtain adult Lepidoptera that are feeding on leaves. When using light traps and collecting Lepidoptera, its host is not known. A set of these pests are native to Zagros forests of Iran and so are not invasive, while in the climate change context, the concern is more about invasive than native pests (Simmons et al. 2014). The Zagros forests of Lorestan province are highly damaged i.e., under the severe pressure due to the agricultural and animal husbandry activities, as well as successive droughts, the leaf feeders and wood borings were dominated (Attarod et al. 2016; Beiranvand et al. 2015; Jazirei & Ebrahimi Rostaghi). As a global perspective human and none human factors may be the most destructive agents in the forestry ecosystems (Kulman, 1971; McCullough et al. 1998). Here we developed a COI barcoding-based approach to lepidopteran species delimitation.

This study built a basic framework to identify and thus the application of suitable control measures for each Lepidoptera species causing *Quercus* defoliation in Lorestan province, western Iran.

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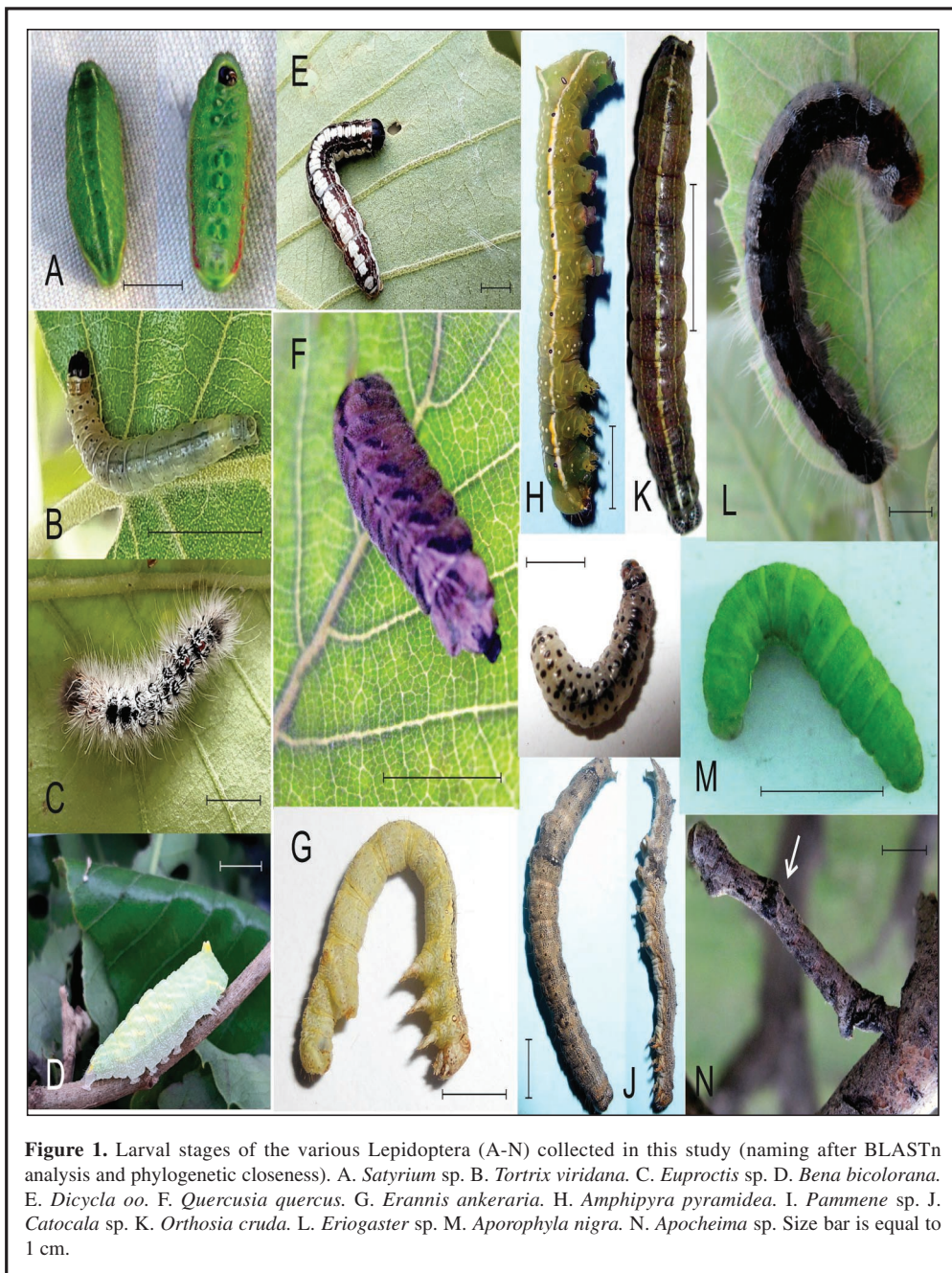
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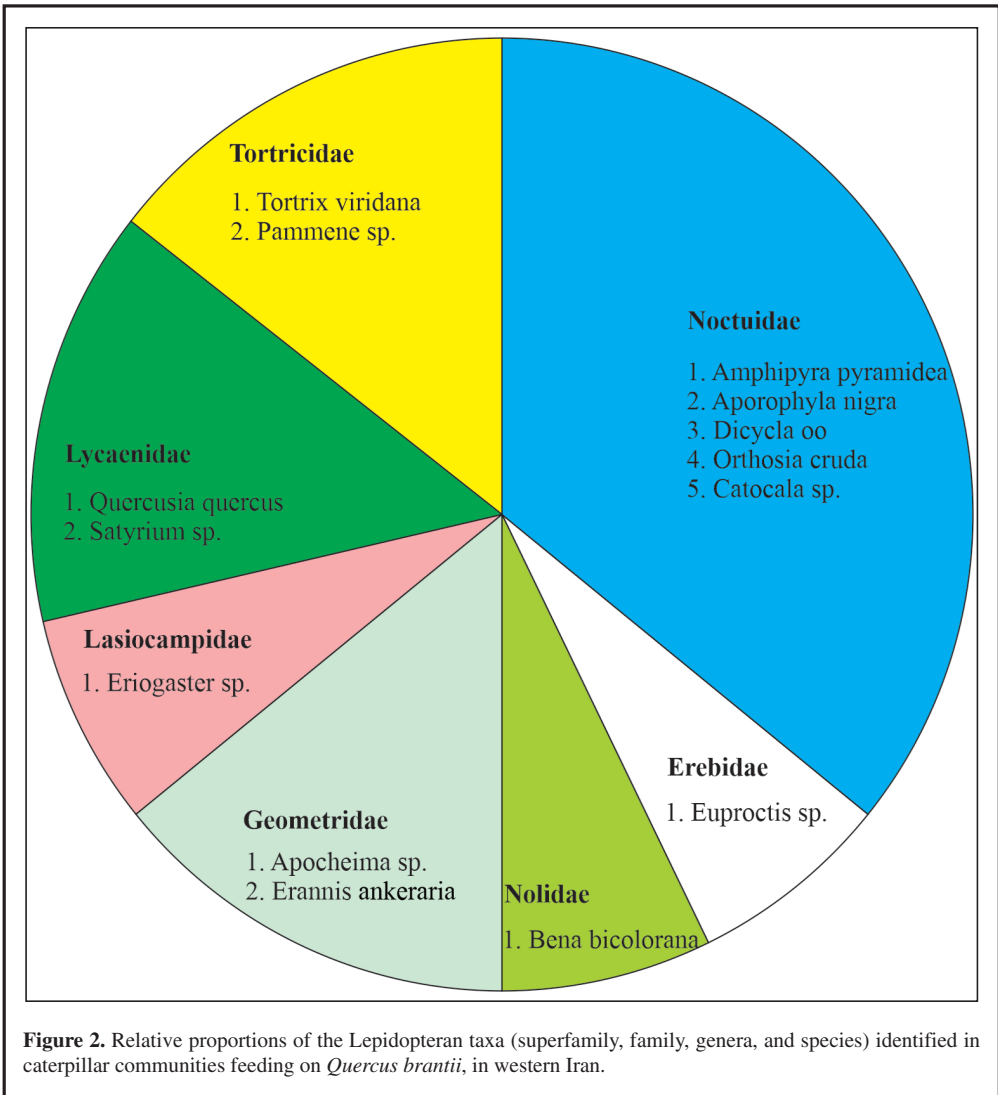
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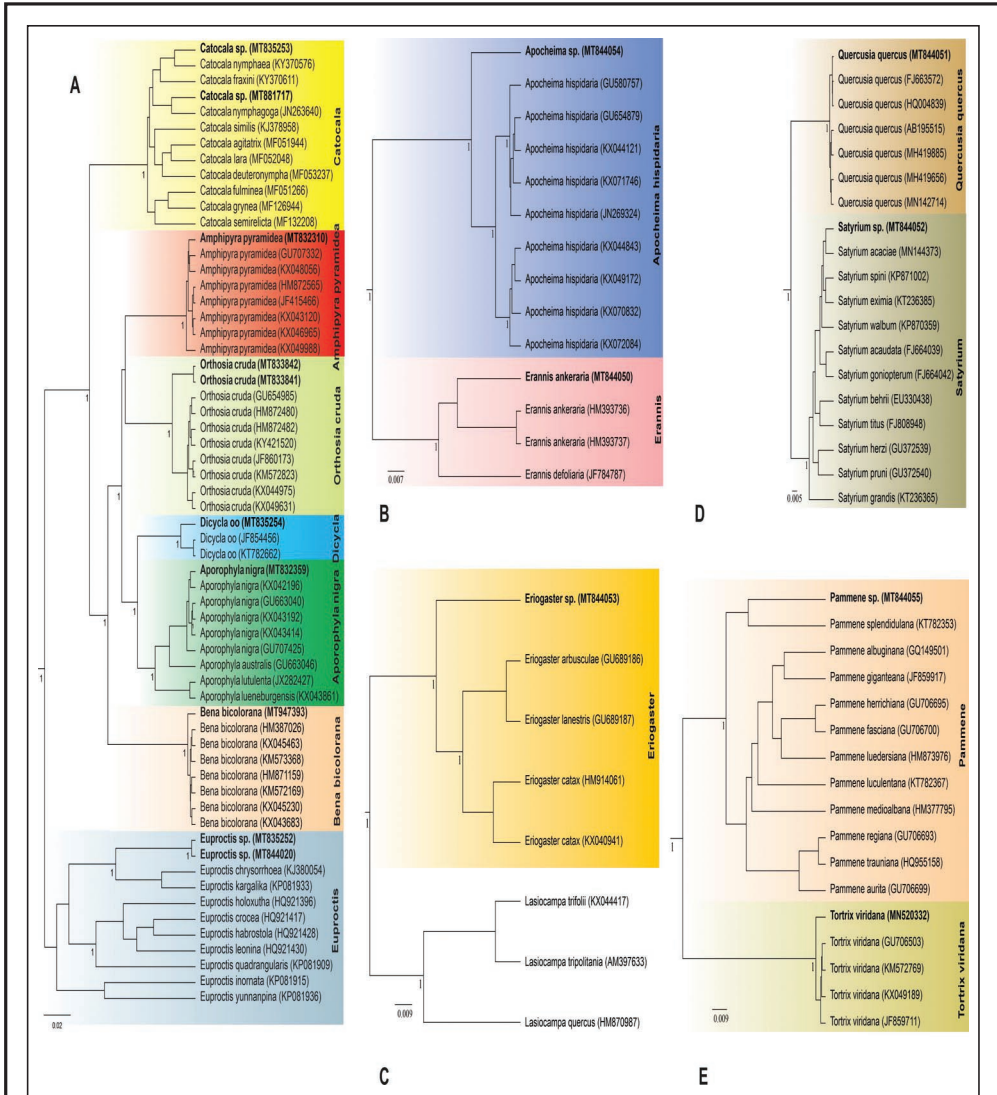
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**Figure 3.** Phylogenetic relationships among Lepidoptera in the Zagros forests of Lorestan province derived from the Bayesian inference (BI) generated based on analysis of partial COI sequences; Numbers below each node show posterior probability value (10 million reiterations). Taxon labels give the species name followed by GenBank accession numbers in parentheses. The taxon sequenced in the present study is highlighted in bold. The clades are well marked with color boxes according to superfamily or families including Noctuoidea containing Erebidae, Noctuidae, Nolidae (A), Geometridae (B), Lasiocampidae (C), Lycaenidae (D), Tortricidae (E).