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Identification of Noctuidae and Nolidae (Lepidoptera) Major Crop Pest Species in Iran: A Combination of Morphological and DNA Barcoding Approaches

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ABSTRACT

Larvae of numerous Noctuidae and Nolidae species have significant annual economic losses in agriculture. DNA-based diagnostics have been proposed as an effective way to accelerate the identification and discovery of new species. This study aimed to determine the utility of up to 642 bp Cytochrome c Oxidase subunit I (COI) barcodes for identifying 12 major Iranian Noctuidae and Nolidae crop pests and confirming morphological identifications based on classical taxonomy. We combined molecular and morphological analysis to identify 53 specimens collected from populations throughout Iran. The results indicated the presence of a distinct barcode gap for different pest species. The mean interspecific sequence divergence (Kimura 2-parameter) was an order of magnitude (10.0%) greater than the mean intraspecific sequence divergence (0.29%). This combination of DNA and morphological analyses identified 13 species, one of which was previously unknown and may represent a new previously overlooked *Earias* species. There were no, or very few, sequences from Iran in international databases for some of the test species. Here, we increase the number of specimens from Iran and aid in taxonomic interpretation. The current study will aid in the identification of the most common Noctuidae and Nolidae major pest species in Iran, regardless of the observer's taxonomic skills, developmental stage of the vouchers, as well as sex, or insect preservation condition. Our data enables researchers and practitioners involved in the bio-surveillance of insect pests to identify taxa based on simple DNA sequence comparisons quickly. DNA barcoding in conjunction with morphological identifications can provide secondary evidence supporting morphological identifications and improve taxonomic resolution.

Keywords: Barcoding gap, Genetic distance, Molecular identification, Nucleotide divergence.

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INTRODUCTION

Around a quarter of the approximately 6,000 economically significant Lepidoptera species are members of the superfamily Noctuoidea, divided into over 500 genera (Zhang, 1994). Nolidae and Noctuidae are the two important families of Noctuoidea and the larvae of numerous species have a significant economic impact on agriculture (Kitching, 1984; Zahiri *et al.*, 2013). In Iran, as in other parts of the world, some of these species are highly polyphagous and cause severe damage to several crucial crops, including rice, maize, cotton, sugar beet, sugarcane, beans, sunflowers, tobacco, and vegetables (Khanjani, 2005; 2009).

Accurate pest species identification is the first and most critical step in developing effective pest management strategies (Szalanski *et al.*, 2003). Historically, species diversity measures have been dependent on

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morphology-based species identification for most metazoan groups, and adult male and female genitalia structures in undamaged specimens are required to accurately identify most noctuoid pests (Holloway, 1998; Blaxter and Floyd, 2003). Adults of genera such as *Mythimna* and *Sesamia* have a similar habitus and could easily be misidentified (Holloway, 1998). Numerous misidentifications of stem borer species have resulted in the publication of misleading data that are often perpetuated for decades (Polaszek, 1992). This issue becomes vital when larval identification is considered. Larval setae, for example, are easily broken and frequently absent from alcohol-preserved and deep-frozen material. This complicates identification and produces unreliable results (Meijerman and Ulenberg, 1996). Unambiguous morphological diagnostic keys in females or immature stages are frequently unavailable, and significant overlap in host range and attraction to pheromone blends limit the use of behavioral criteria (reviewed in Pogue, 2002; Meagher *et al.*, 2008).

Developing a method to supplement morphological characteristics is of practical importance for accurately identifying moth pest species (Hebert *et al.*, 2003a; Hajibabaei *et al.*, 2006; Dumas *et al.*, 2015). The DNA barcoding system using the mitochondrial DNA *Cytochrome c Oxidase subunit I* (COI) gene (658 bp) is particularly effective in expediting species identification, determining native provenance, and enhancing the ability of biosecurity agencies in detecting invasive species in insects, particularly for Lepidoptera species (e.g., Hebert *et al.*, 2003b; Nagoshi *et al.*, 2011; Ashfaq and Hebert 2016; Lee *et al.*, 2019).

Although studies developing DNA sequence data for identifying Noctuoidea (Lepidoptera) pest species have previously been conducted in some local regions of Iran (e.g., Mehravar *et al.*, 2017), no comprehensive study across the country includes the major crop pest species. As the first step toward developing a barcode database for moth pest species in Iran, the purpose of this study was to assess the accuracy of morphological identification of major Iranian Noctuidae and Nolidae crop pests using DNA barcoding.

MATERIALS AND METHODS

Specimens

According to the literature, we chose 12 species of Noctuidae and Nolidae that are the most destructive, polyphagous, and almost ubiquitous crop pests in Iran (e.g., Azmayesh Fard, 2014; Khanjani, 2005, 2009): *Agrotis ipsilon* (Hufnagel, 1766), *A. segetum* (Denis & Schiffermüller, 1775), *Autographa gamma* (Linnaeus, 1758), *Spodoptera exigua* (Hübner, 1808), *S. littoralis* (Boisduval, 1833), *Leucania loreyi* (Duponchel, 1827), *Mythimna unipuncta* (Haworth, 1809), *Helicoverpa armigera* (Hübner, 1808), *Heliothis peltigera* (Denis & Schiffermüller, 1775), *Sesamia cretica* Lederer, 1857, *S. nonagrioides* (Lefèbvre, 1827), *Earias insulana* (Boisduval, 1833). Specimens were collected from eight provinces using a light trap from agricultural fields infested with target pests, mostly between 2015 and 2016 (Table 1). We attempted to collect data from as many different regions of Iran as possible. Typically, specimens were stored at -20°C following collection. At least three specimens from each species (a total of 53 specimens) were chosen for molecular analysis. The Insect and Mite Collection of Ahvaz, Department of Plant Protection, Shahid Chamran University of Ahvaz, Iran, housed the materials.

Species Identification

The abdomen was dissected for genitalia examination to confirm the specimen's identity. Dissections of adult specimens' genitalia were performed and compared to original species descriptions, available taxonomic revisions, and published resources. Apart from *Earias* genus, which is a member of Nolidae, other species belonged to Noctuidae (Table 1). This method combined molecular analysis with an examination of genital morphology.

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Table 1. Collection details of Noctuidae and Nolidae crop pest species in Iran subjected to DNA extraction.

Provisional morphological identification	Specimen code	Province, locality	GPS coordinates	Collection date
Agrotis ipsilon	Ag.i.-IR-Krm	Kerman, Dalfard	28º 56′ N, 57º 39′ E	Summer, 2015
Agrotis ipsilon	Ag.i.-IR-Khu	Khuzestan, Malaqa	31º 35′ N, 50º 00′ E	Spring, 2016
Agrotis ipsilon	Ag.i.-IR-Khr	Khorasan-e Razavi, Mashhad	36º 14′ N, 59º 41′ E	Spring, 2016
Agrotis ipsilon	Ag.i.-IR-Hmd	Hamedan, Hamedan	34º 55′ N, 48º 28′ E	Summer, 2016
Agrotis segetum	Ag.s.-IR-Hmd	Hamedan, Hamedan	34º 55′ N, 48º 28′ E	Summer, 2016
Agrotis segetum	Ag.s.-IR-Khr	Khorasan-e Razavi, Mashhad	36º 14′ N, 59º 41′ E	Spring, 2016
Agrotis segetum	Ag.s.-IR-Krm	Kerman, Omrudoieh	29º 06′ N, 57º 33′ E	Spring, 2015
Agrotis segetum	Ag.s.-IR-Khu	Khuzestan, Malaqa	31º 35′ N, 50º 00′ E	Spring, 2016
Autographa gamma	Au.g.-IR-Krm	Kerman, Hishin	28º 38′ N, 57º 56′ E	Autumn, 2015
Autographa gamma	Au.g.-IR-Khr	Khorasan-e Razavi, Mashhad	36º 10′ N, 59º 43′ E	Spring, 2016
Autographa gamma	Au.g.-IR-Gil	Gilan, Rasht	37º 11′ N, 49º 38′ E	Spring, 2016
Autographa gamma	Au.g.-IR-Khu	Khuzestan, Malaqa	31º 35′ N, 50º 00′ E	Spring, 2016
Autographa gamma	Au.g.-IR-Hmd	Hamedan, Hamedan	34º 53′ N, 48º 29′ E	Summer, 2016
Autographa gamma	Au.g.-IR-Far	Fars, Shiraz	29º 51′ N, 52º 29′ E	Spring, 2015
Spodoptera exigua	Sp.e.-IR-Khr	Khorasan-e Razavi, Mashhad	36º 10′ N, 59º 43′ E	Spring, 2015
Spodoptera exigua	Sp.e.-IR-Krm	Kerman, Dalfard	28º 56′ N, 57º 39′ E	Spring, 2015
Spodoptera exigua	Sp.e.-IR-Hmd	Hamedan, Hamedan	34º 55′ N, 48º 28′ E	Summer, 2016
Spodoptera exigua	Sp.e.-IR-Khu	Khuzestan, Malaqa	31º 35′ N, 50º 00′ E	Spring, 2016
Spodoptera littoralis	Sp.l.-IR-Khu1	Khuzestan, Gotvand	32º 18′ N, 48º 45′ E	Spring, 2016
Spodoptera littoralis	Sp.l.-IR-Krm	Kerman, Dalfard	28º 56′ N, 57º 39′ E	Winter, 2017
Spodoptera littoralis	Sp.l.-IR-Khu2	Khuzestan, Ahvaz	31º 17′ N, 48º 39′ E	Spring, 2015
Spodoptera littoralis	Sp.l.-IR-Far	Fars, Kamfiruz	30º 20′ N, 52º 13′ E	Spring, 2017
Leucania loreyi	L.l.-IR-Khr	Khorasan-e Razavi, Mashhad	36º 10′ N, 59º 43′ E	Spring, 2015
Leucania loreyi	L.l.-IR-Krm	Kerman, Khabr	28º 39′ N, 56º 32′ E	Spring, 2015
Leucania loreyi	L.l.-IR-Khu	Khuzestan, Malaqa	31º 35′ N, 50º 00′ E	Spring, 2016
Leucania loreyi	L.l.-IR-Hmd	Hamedan, Hamedan	34º 53′ N, 48º 29′ E	Summer, 2016
Leucania loreyi	L.l.-IR-Gil	Gilan, Rasht	37º 11′ N, 49º 38′ E	Spring, 2015
Mythimna unipuncta	My.u.-IR-Gls	Golestan, Gorgan	36º 37′ N, 54º 07′ E	Summer, 2013
Mythimna unipuncta	My.u.-IR-Khu	Khuzestan, Malaqa	31º 35′ N, 50º 00′ E	Spring, 2016
Mythimna unipuncta	My.u.-IR-Maz	Mazandaran, Nahar khoran	36º 45′ N, 54º 28′ E	Summer, 2013
Helicoverpa armigera	He.a.-IR-Khu	Khuzestan, Ahvaz	31º 17′ N, 48º 39′ E	Spring, 2016
Helicoverpa armigera	He.a.-IR-Hmd	Hamedan, Hamedan	34º 53′ N, 48º 29′ E	Summer, 2016
Helicoverpa armigera	He.a.-IR-Khr	Khorasan-e Razavi, Mashhad	36º 10′ N, 59º 43′ E	Spring, 2016
Helicoverpa armigera	He.a.-IR-Krm	Kerman, Khabr	28º 39′ N, 56º 32′ E	Spring, 2015
Heliothis peltigera	He.p.-IR-Khu	Khuzestan, Malaqa	31º 35′ N, 50º 00′ E	Spring, 2016
Heliothis peltigera	He.p.-IR-Hmd	Hamedan, Hamedan	34º 53′ N, 48º 29′ E	Summer, 2016
Heliothis peltigera	He.p.-IR-Khr	Khorasan-e Razavi, Mashhad	36º 10′ N, 59º 43′ E	Spring, 2016
Heliothis peltigera	He.p.-IR-Gil	Gilan, Rasht	37º 11′ N, 49º 38′ E	Summer, 2015
Heliothis peltigera	He.p.-IR-Krm	Kerman, Omrudoieh	29º 06′ N, 57º 33′ E	Summer, 2015
Sesamia cretica	Se.c.-IR-Khu	Khuzestan, Ahvaz	31º 04′ N, 48º 20′ E	Autumn, 2015
Sesamia cretica	Se.c.-IR-Far1	Fars, Nurabad	30º 05′ N, 51º 30′ E	Summer, 2011
Sesamia cretica	Se.c.-IR-Far2	Fars, Nurabad	30º 01′ N, 51º 33′ E	Summer, 2011
Sesamia cretica	Se.c.-IR-Far3	Fars, Nurabad	30º 05′ N, 51º 30′ E	Summer, 2011
Sesamia nonagrioides	Se.n.-IR-Khu1	Khuzestan, Ahvaz	31º 06′ N, 48º 37′ E	Autumn, 2015
Sesamia nonagrioides	Se.n.-IR-Khu2	Khuzestan, Ahvaz	31º 04′ N, 48º 20′ E	Autumn, 2015
Sesamia nonagrioides	Se.n.-IR-Far	Fars, Mahkuyeh	29° 00′ N, 52° 33′ E	Summer, 2011
Earias insulana	Ea.sp.-IR-Krm1	Kerman, Jiroft	28° 31′ N, 57° 45′ E	Spring, 2014
Earias insulana	Ea.sp.-IR-Krm2	Kerman, Jiroft	28° 32′ N, 57° 51′ E	Spring, 2017
Earias insulana	Ea.sp.-IR-Krm3	Kerman, Jiroft	28° 32′ N, 57° 51′ E	Spring, 2016
Earias insulana	Ea.sp.-IR-Krm4	Kerman, Jiroft	28° 31′ N, 57° 45′ E	Spring, 2016
Earias insulana	Ea.sp.-IR-Krm5	Kerman, Jiroft	28° 38′ N, 56° 45′ E	Spring, 2016
Earias insulana	Ea.i.-IR-Khu	Khuzestan, Ahvaz	31º 17′ N, 48º 39′ E	Spring, 2016
Earias insulana	Ea.i.-IR-Far	Fars, Kamfiruz	30º 20′ N, 52º 13′ E	Spring, 2016

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DNA Extraction and PCR Amplification

DNA was isolated from the legs or abdomen of adults using the Qiagen DNEasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The PCR reaction was carried out according to the protocols and primers described in Hajibabaei *et al.* (2006). Samples were amplified using the primer pair LEP-F1 (5′-ATTCAACCAATCATAAAGATATTGG-3′) and LEP-R1 (5′-TAAACTTCTGGATGTCCAAAAAATCA-3′) for the target 658-bp fragment of COI.

PCRs were performed on a Bio-Rad PCRMJ Mini Gradient Thermal Cycler programmed for an initial denaturation period of 60 seconds at 94°C, six cycles of denaturation for 60 seconds at 94°C, 90 seconds of annealing at 45°C, 75 seconds of elongation at 72°C, 36 cycles of denaturation for 60 seconds at 94°C, 90 seconds of annealing at 51°C, elongation at 72°C for 75 seconds, and a final extension period for 5 minutes at 72°C. Electrophoresis in 2% agarose gel was used to determine the quality of the PCR products. Sequencing was conducted by Macrogen Inc. (Korea). GenBank was used to store all sequences (Accession numbers OM350057-OM350109, see Table 2).

Barcode Analysis and Species Delimitation

We sequenced 53 specimens of target pest taxa for this study. Additionally, we included 57 pre-existed public sequence records from GenBank for the studied species with diverse geographic origins. Multiple Alignment with the Clustal W algorithm was used to align the sequences in the MEGA7 software package (Kumar *et al*., 2016). The alignments that resulted were cropped to a length of up to 642 bp. We used NCBI's BLAST tool to compare our sequence dataset to sequences already published in GenBank. The mean intraspecific and mean interspecific distances were calculated in the same dataset using MEGA7, with Kimura two-Parameter (K2P) distances, uniform rates between sites, and the default 1,000 bootstrap replicates.

A gap between intraspecific and interspecific diversity in the distribution of pairwise differences can be observed in all sequences in the given barcode data set; this gap has been termed the 'barcode gap'. The pairwise distances within and between morphospecies were calculated using a K2P model. The barcode gap was calculated in the manner described by Puillandre *et al.* (2012). The Neighbor-Joining (NJ) tree was constructed using MEGA7 software, and bootstrap analysis was performed on the NJ tree (10,000 replicates) to provide a visual representation of the data, as this tree is not solely utilized to determine phylogenetic information.

RESULTS

Comparison with International Databases

Our identifications were confirmed by comparing our sequences to those in the GenBank database, with the majority of sequences displaying a BLAST hit (and a sequence similarity percentage of between 99 and 100%) with a GenBank sequence assigned to the same species (Table 2). However, five sequences (OM350105-OM350109) morphologically assigned to *E. insulana* had BLAST hits with wildly divergent sequences (~ 6%). They shared only 92–93% similarity with *E. insulana* GenBank sequences, strongly implying that these sequences belong to a different species. As a result, we classified them as *Earias* sp. in the subsequent analysis.

Barcode Gap between Intra- and Interspecific Distances

For all 53 Iranian sequences included in the study, the relative frequency distribution

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Table 2. The blast hit comparisons of our sequences to those in the NCBI GenBank database.

Specimen code (GenBank accession number#)	Identification (DNA+Morphology)	Best GenBank Blast hit	Corresponding taxon	Similarity %	Coverage (bp)
Ag.i.-IR-Krm (OM350057)	*Agrotis ipsilon*	MZ959071.1	*Agrotis ipsilon*	99.84	642
Ag.i.-IR-Khu (OM350058)	*Agrotis ipsilon*	MZ959071.1	*Agrotis ipsilon*	99.84	642
Ag.i.-IR-Khr (OM350059)	*Agrotis ipsilon*	MZ959071.1	*Agrotis ipsilon*	99.53	642
Ag.i.-IR-Hmd (OM350060)	*Agrotis ipsilon*	MZ959071.1	*Agrotis ipsilon*	99.84	642
Ag.s.-IR-Hmd (OM350061)	*Agrotis segetum*	MF059277.1	*Agrotis segetum*	100.00	642
Ag.s.-IR-Khr (OM350062)	*Agrotis segetum*	MF059277.1	*Agrotis segetum*	99.84	642
Ag.s.-IR-Krm (OM350063)	*Agrotis segetum*	MF059277.1	*Agrotis segetum*	100.00	642
Ag.s.-IR-Khu (OM350064)	*Agrotis segetum*	MF059277.1	*Agrotis segetum*	100.00	642
Au.g.-IR-Krm (OM350065)	*Autographa gamma*	LR989881.1	*Autographa gamma*	100.00	642
Au.g.-IR-Khr (OM350066)	*Autographa gamma*	LR989881.1	*Autographa gamma*	100.00	642
Au.g.-IR-Gil (OM350067)	*Autographa gamma*	LR989881.1	*Autographa gamma*	100.00	642
Au.g.-IR-Khu (OM350068)	*Autographa gamma*	LR989881.1	*Autographa gamma*	100.00	642
Au.g.-IR-Hmd (OM350069)	*Autographa gamma*	LR989881.1	*Autographa gamma*	100.00	642
Au.g.-IR-Far (OM350070)	*Autographa gamma*	MF679519.1	*Autographa gamma*	99.34	302
Sp.e.-IR-Khr (OM350071)	*Spodoptera exigua*	MT449725.1	*Spodoptera exigua*	100.00	642
Sp.e.-IR-Krm (OM350072)	*Spodoptera exigua*	MT449725.1	*Spodoptera exigua*	100.00	642
Sp.e.-IR-Hmd (OM350073)	*Spodoptera exigua*	MZ297463.1	*Spodoptera exigua*	100.00	642
Sp.e.-IR-Khu (OM350074)	*Spodoptera exigua*	MZ297463.1	*Spodoptera exigua*	100.00	642
Sp.l.-IR-Khu1 (OM350075)	*Spodoptera littoralis*	MN803323.1	*Spodoptera littoralis*	99.69	642
Sp.l.-IR- Khu2 (OM350076)	*Spodoptera littoralis*	MN803323.1	*Spodoptera littoralis*	100.00	642
Sp.l.-IR- Krm (OM350077)	*Spodoptera littoralis*	MN803323.1	*Spodoptera littoralis*	100.00	642
Sp.l.-IR-Far (OM350078)	*Spodoptera littoralis*	MN803323.1	*Spodoptera littoralis*	99.38	321
L.l.-IR-Khr (OM350079)	*Leucania loreyi*	MK860952.1	*Leucania loreyi*	100.00	642
L.l.-IR-Krm (OM350080)	*Leucania loreyi*	MK860952.1	*Leucania loreyi*	99.84	642
L.l.-IR-Khu (OM350081)	*Leucania loreyi*	MK860952.1	*Leucania loreyi*	99.84	642
L.l.-IR-Hmd (OM350082)	*Leucania loreyi*	MK860952.1	*Leucania loreyi*	100.00	642
L.l.-IR-Gil (OM350083)	*Leucania loreyi*	MK860952.1	*Leucania loreyi*	99.84	642
My.u.-IR-Gls (OM350084)	*Mythimna unipuncta*	KX281211.1	*Mythimna unipuncta*	100.00	635
My.u.-IR-Khu (OM350085)	*Mythimna unipuncta*	MG468368.1	*Mythimna unipuncta*	100.00	302
My.u.-IR-Maz (OM350086)	*Mythimna unipuncta*	MG468368.1	*Mythimna unipuncta*	100.00	302
He.a.-IR-Khu (OM350087)	*Helicoverpa armigera*	MG437196.1	*Helicoverpa armigera*	100.00	642
He.a.-IR-Hmd (OM350088)	*Helicoverpa armigera*	MH190451.1	*Helicoverpa armigera*	100.00	642
He.a.-IR-Khr (OM350089)	*Helicoverpa armigera*	MH190451.1	*Helicoverpa armigera*	100.00	642
He.a.-IR-Krm (OM350090)	*Helicoverpa armigera*	MF051184.1	*Helicoverpa armigera*	100.00	642
He.p.-IR-Khu (OM350091)	*Heliothis peltigera*	EU768928.1	*Heliothis peltigera*	100.00	642
He.p.-IR-Hmd (OM350092)	*Heliothis peltigera*	EU768928.1	*Heliothis peltigera*	99.84	642
He.p.-IR-Khr (OM350093)	*Heliothis peltigera*	EU768928.1	*Heliothis peltigera*	100.00	642
He.p.-IR-Gil (OM350094)	*Heliothis peltigera*	EU768928.1	*Heliothis peltigera*	99.84	642
He.p.-IR-Krm (OM350095)	*Heliothis peltigera*	EU768928.1	*Heliothis peltigera*	99.84	642
Se.c.-IR-Khu (OM350096)	*Sesamia cretica*	MH851121.1	*Sesamia cretica*	100.00	632
Se.c.-IR-Far1 (OM350097)	*Sesamia cretica*	MH851121.1	*Sesamia cretica*	99.84	632
Se.c.-IR-Far2 (OM350098)	*Sesamia cretica*	MH851112.1	*Sesamia cretica*	100.00	632
Se.c.-IR-Far3 (OM350099)	*Sesamia cretica*	MH851121.1	*Sesamia cretica*	100.00	632
Se.n.-IR-Khu1 (OM350100)	*Sesamia nonagrioides*	MK566730.1	*Sesamia nonagrioides*	100.00	641
Se.n.-IR-Khu2 (OM350101)	*Sesamia nonagrioides*	MK566730.1	*Sesamia nonagrioides*	100.00	641
Se.n.-IR-Far (OM350102)	*Sesamia nonagrioides*	KU891970.1	*Sesamia nonagrioides*	99.33	300
Ea.i.-IR-Khu (OM350103)	*Earias insulana*	MK636811.1	*Earias insulana*	99.69	642
Ea.i.-IR-Far (OM350104)	*Earias insulana*	MK636811.1	*Earias insulana*	99.27	273
Ea.sp.-IR-Krm1 (OM350105)	*Earias* sp.	MH886650.1	*Earias vittella*	93.84	632
Ea.sp.-IR-Krm2 (OM350106)	*Earias* sp.	MK636803.1	*Earias vittella*	93.86	585
Ea.sp.-IR-Krm3 (OM350107)	*Earias* sp.	MH886650.1	*Earias vittella*	93.68	632
Ea.sp.-IR-Krm4 (OM350108)	*Earias* sp.	MH886650.1	*Earias vittella*	93.92	624
Ea.sp.-IR-Krm5 (OM350109)	*Earias* sp.	MH886650.1	*Earias vittella*	93.95	627

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of K2P pairwise distances was calculated, and the automatic barcode gap discovery revealed the presence of a barcode gap at 1.4-4.3% (Figure 1). Distance values indicated a gap of approximately 4% between intraspecific and interspecific distances.

Intra- and Interspecific Nucleotide Divergence

On average, interspecific divergences between target species pairs exceeded intraspecific divergences by order of magnitude. Interspecific nucleotide divergence ranged from 4.8% to 17.3% between the 13 species, with an average of 10.0% (Table 3). Between *S. exigua* and *E. insulana*, the longest distance of 17.3%, and between *A. ipsilon* and *A. segetum*, the shortest distance of 4.8% was observed. Divergences within species ranged from 0.0 to 0.9%, with a mean of 0.29%. With *S. nonagrioides*, the maximum intraspecific nucleotide divergence was found to be 0.9% (Table 3). The distance between *E. insulana* and *Earias* sp. was 8.1%, corroborating the results of the blast hits.

Neighbor-Joining Tree

We used the NJ method to analyze the clustering pattern of 53 sequenced specimens of target pest species and 57 sequence records of studied species from GenBank. The K2P/NJ tree in Figure 2 illustrates the patterns of sequence divergence among the 110 barcode records.

The species analyzed were classified into distinct single-species clades. However, specimens provisionally identified based on morphology as *E. insulana* recovered two distinct species clusters and provided compelling evidence for their separation based on blast hits (Table 2) and nucleotide divergence data (Table 3). Our two *E. insulana* individuals and four from GenBank formed the first species cluster, a sister clade to another *Earias* species. The second *Earias* cluster consisted of five individuals of an unknown species of *Earias*.

BOLD returns a species identification if the query sequence differs by less than 3% species gap from the reference sequence (Ratnasingham and Hebert, 2007). When the BOLD identification engine was used in the Barcoding of Life Database (available at http://www.boldsystems.org/), the five *Earias* sequences were clustered with unidentified private *Earias* sequences from the United Arab Emirates. Morphological re-examination of the genitalia of five unidentified *Earias* species collected in Jiroft, Kerman Province, revealed subtle morphological differences with *E. insulana*. The primary distinction may be that in unknown *Earias*, the posterior edge of the cucullus is broader than in *E. insulana* (Figure 3).

DISCUSSION

Overall, we propose the retention of 13 species based on molecular and morphological evidence, as examining specimens' genitalia corroborated the molecular results.

Our findings indicated that a COI-based identification system holds great promise for identifying Noctuidae and Nolidae pest species in Iran. Moreover, it could unveil a previously unknown species in the genus *Earias* in Iran. When we included 57 sequences from GenBank, test sequences consistently clustered more closely with their conspecific than with any other species. COI sequences have previously been shown to be capable of identifying Lepidoptera (Hebert *et al.*, 2003a) and other animal taxa (Hebert *et al.*, 2003b; Ratnasingham and Hebert, 2013) through DNA barcoding.

The gap between the maximum intraspecific and the minimum interspecific distances has been used to delimit species in various animal groups (Hebert *et al.*, 2004; Meier *et al.*, 2006; Puillandre *et al.*, 2012; Tahir *et al.*, 2018). Numerous studies have

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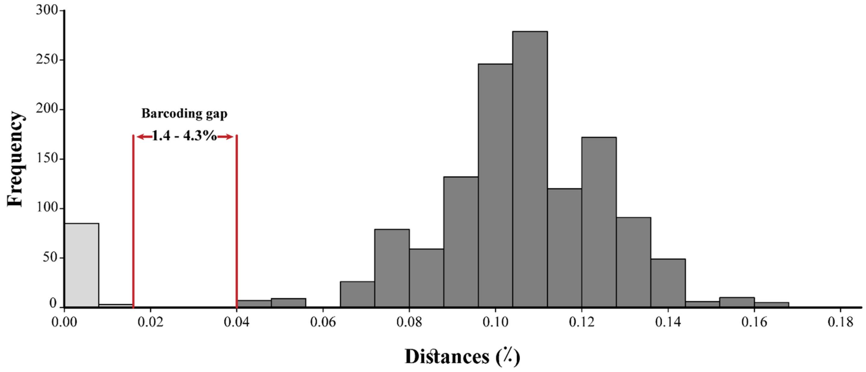

Figure 1. The histogram depicts the barcode gap between the frequency distributions of intraspecific (gray) and interspecific (black) pairwise distances calculated using Kimura two-parameter pairwise distance analysis.

Table 3. Pairwise distances (K2P) between the Noctuidae and Nolidae species studied.[a]

Species	1	2	3	4	5	6	7	8	9	10	11	12	13
Heliothis peltigera (0.002±0.001)		0.026	0.031	0.019	0.028	0.037	0.026	0.030	0.024	0.025	0.037	0.029	0.029
Spodoptera littoralis (0.005±0.003)	0.110		0.026	0.025	0.018	0.033	0.024	0.025	0.024	0.027	0.039	0.029	0.026
Leucania loreyi (0.002±0.001)	0.131	0.108		0.028	0.028	0.028	0.026	0.028	0.024	0.022	0.032	0.030	0.020
Helicoverpa armigera (0.002±0.001)	0.077	0.105	0.117		0.028	0.033	0.027	0.030	0.024	0.025	0.037	0.036	0.023
Spodoptera exigua (0.002±0.001)	0.118	0.074	0.118	0.115		0.033	0.024	0.023	0.026	0.031	0.043	0.032	0.026
Autographa gamma (0.002±0.001)	0.155	0.137	0.118	0.138	0.141		0.031	0.031	0.033	0.035	0.035	0.033	0.028
Sesamia cretica (0.002±0.001)	0.108	0.099	0.108	0.113	0.100	0.129		0.020	0.023	0.024	0.038	0.028	0.026
Sesamia nonagrioides (0.009±0.004)	0.124	0.100	0.115	0.124	0.094	0.125	0.083		0.026	0.026	0.043	0.032	0.028
Agrotis ipsilon (0.002±0.001)	0.103	0.103	0.103	0.099	0.110	0.136	0.099	0.111		0.012	0.030	0.029	0.023
Agrotis segetum (0.001±0.001)	0.105	0.114	0.092	0.106	0.132	0.145	0.100	0.109	0.048		0.035	0.029	0.022
Earias insulana (0.007±0.005)	0.153	0.158	0.136	0.153	0.173	0.143	0.153	0.171	0.123	0.143		0.021	0.036
Earias sp. (0.002±0.001)	0.123	0.122	0.128	0.151	0.133	0.138	0.116	0.133	0.120	0.123	0.081		0.024
Mythimna unipunctata (0.000±0.000)	0.117	0.102	0.077	0.088	0.102	0.110	0.101	0.107	0.089	0.086	0.142	0.094	

[a] Intraspecific divergence is indicated by parenthesis (mean±SE).

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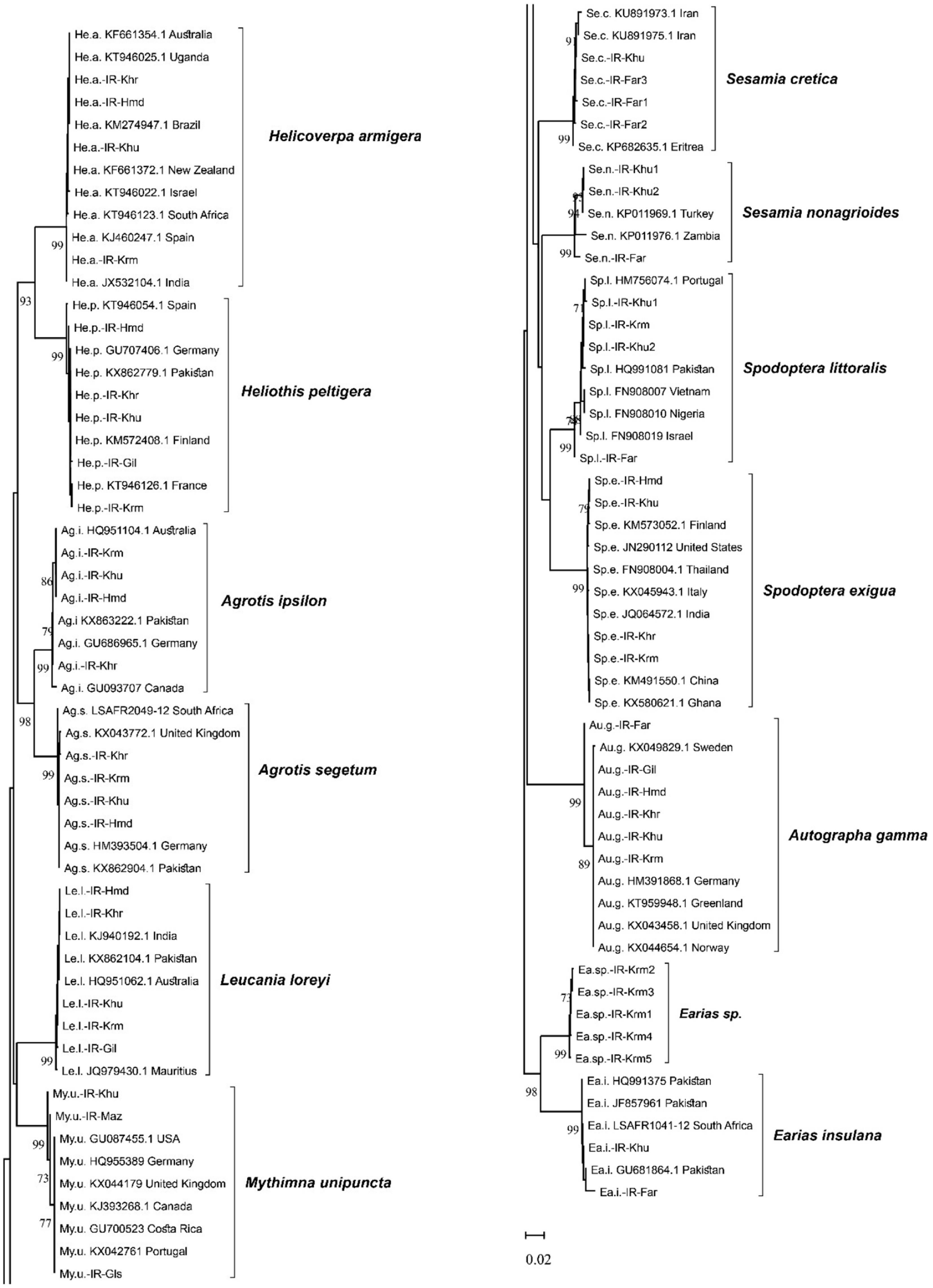

Figure 2. Neighbor-joining tree of COI sequence divergences (K2P) in major Noctuidae and Nolidae species crop pests in Iran (Bootstrap values of < 75% not shown).

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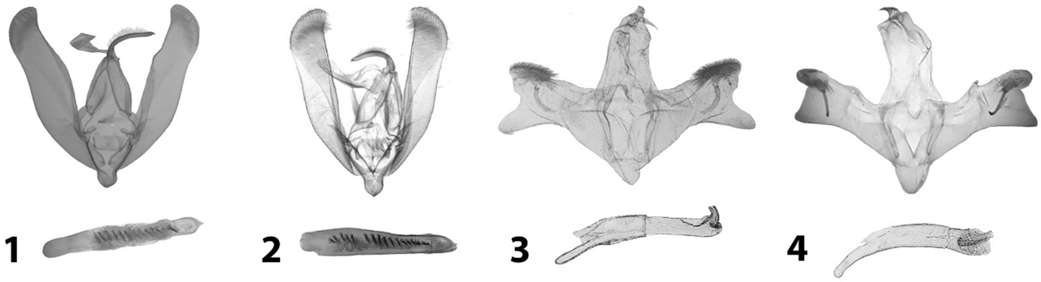

Figure 3. Male genitalia of: 1. *Helicoverpa armigera*, 2. *Helicoverpa zea* (after Pogue, 2004), 3. *Earias insulana*, and 4. *Earias* sp.

used barcode gap analysis in conjunction with other methods of species delimitation to aid in species discrimination (e.g., Ashfaq *et al.*, 2013; Lee *et al.*, 2019). Extensive studies of various groups have established a critical limit of approximately 2-3% interspecific divergence for species identification (Zhu *et al.*, 2017; Huemer *et al.*, 2019). Previous research (e.g., Hebert *et al.*, 2003a; Ball and Armstrong, 2006; Rajaei *et al.*, 2013) demonstrated that mean interspecific divergences between moth species were an order of magnitude greater than mean intraspecific divergences, as measured by K2P distances. Our findings corroborate these results: the mean intraspecific sequence divergence was low (0.29%), whereas the mean interspecific divergence was an order of magnitude greater (10.0%).

The effectiveness of DNA barcoding in revealing previously unknown species of Lepidoptera global pests is well documented (Ball and Armstrong, 2006; Dumas *et al.*, 2015; Zahiri *et al.*, 2017). The divergence of the two *Earias* clusters (8.1%) was large enough to classify them as two distinct species. This level of divergence exceeds the interspecific threshold of 3% proposed by Hebert *et al.* (2003a) for Lepidoptera, implying that specimens from Kerman Province belong to a different species. This remarkable species shares phenotypic characteristics with the widely distributed *E. insulana*, with which it was initially confused. However, the DNA barcodes of five specimens collected in Kerman Province (Jiroft) are distant from those of any *Earias* species recorded in BOLD. This species is currently unidentified at the species level and is likely to be one of the Iranian fauna's additional and previously overlooked species. Based on available barcode data, the original descriptions, and other pertinent literature (e.g. Wiltshire, 1961; Goater, 1994; Fibiger *et al.*, 2009; Alexej Matov, personal comm.), we observed that our unknown *Earias* differs from all *Earias* species in Europe, Western, and Central Asia. It should be noted that the 3% threshold is a guideline and that additional ecological data, such as host plant and other biological characteristics, should be combined with morphological characters and DNA barcodes (including private *Earias* sequences on BOLD) to assign a name to our unknown and possibly new *Earias* species. Thus, additional research into the occurrence of overlooked species of the genus *Earias* in Iran appears necessary.

Once DNA libraries are generated using reference sequences from easily identifiable adults, COI barcodes will cost a few dollars and take a few hours to generate, providing a reliable method of specimen identification for taxa with well-defined species boundaries, such as those considered in this study. Given that many pest species are intercepted as eggs or young larvae that are difficult or impossible to identify, DNA barcoding may be the only method for accurately and rapidly identifying exotic pest species in some circumstances (Armstrong and Ball, 2005). For several of the test species, there were no (e.g., *Earias insulana)* or very few sequences in international databases from Iran. Depositing our barcode records in databases increases the number of specimens from diverse geographical areas and aids in taxonomic classification decision-making.

We have previously witnessed the introduction of pest species from neighboring countries into Iran's bordering provinces (e.g., Baniameri and Cheraghian, 2011; Moghaddam *et al.*, 2015). As a result, reference libraries of DNA barcodes are instrumental in identifying pest species in Iran. Increased global trade in plants and plant products has significantly increased the risk of introducing novel pest species, threatening agriculture and forestry with significant economic losses. Due to the scarcity or inadequacy of taxonomists in the majority of insect families in Iran, barcoding identification approaches may be an appropriate tool to assist diverse user groups in identifying species (e.g., inspection at ports of entry for regulatory agencies) (Cook *et al.*, 2010; Madden *et al.*, 2019; Watts *et al.*, 2019). As specimen identification for biosurveillance is commonly time-sensitive and frequently requires a rapid response, DNA barcoding of specimens enables rapid identifications in addition to confirming the accuracy of morphological identifications and revealing source regions (Bellis *et al.*, 2015) and introduction patterns (Blacket *et al.*, 2015; Nagoshi *et al.*, 2011; Mastrangelo *et al.*, 2014).

Integrated Pest Management (IPM) requires accurate identification of target species and frequently involves farmers, crop pest managers, and quarantine agents for monitoring its effectiveness. In Iran, the misidentification of noctuid pest species has resulted in the publication of erroneous data (Esfandiari *et al.*, 2011; Ravan *et al.*, 2015). *Helicoverpa zea* presence, for instance, has been reported from northern and central Iran (Khanjani, 2009), even though it does not occur in Iran (Matov *et al.*, 2008; Ravan *et al.*, 2015) (Figure 3). DNA barcoding is gaining widespread use in IPM and biosurveillance (Armstrong, 2010; Jones *et al.*, 2013; Etzler *et al.*, 2014) because it can distinguish between introduced and native pests (Chown *et al.*, 2008) and aid in species identification in agricultural systems (Frewin *et al.*, 2014; Mehravar *et al.*, 2017).

In lepidopterans, species discrimination is based on sequence divergence at COI values> 2% (Hebert *et al.*, 2003b). However, in some cases of low interspecific divergence, DNA barcoding with COI will fail to discriminate species successfully (Zahiri *et al.*, 2017; Huemer and Mutanen, 2022). COI, for example, cannot be used to detect recent hybridization events because it is a mitochondrial gene that is inherited maternally in the vast majority of animals. When hybridization existence is possible, nuclear genes must be used (Tóth *et al.*, 2017). COI is likely to be effective for identifying most animal species, given the rarity of animal hybrids compared to plant hybrids. Furthermore, a COI-based identification system is unlikely to discriminate between pairs of very young species (Sperling and Hickey, 1994; Sperling *et al.*, 1999). Minor interspecific divergences, on the other hand, are uncommon (Ball and Armstrong, 2006).

In summary, it can be concluded that the current study will aid in the identification of the most prevalent Noctuidae and Nolidae major pest species in Iran, regardless of the observer's taxonomic abilities, developmental stage, sex, or state of preservation of the insect. Our data enables researchers and practitioners involved in the biosurveillance of insect pests who have access to DNA sequencing facilities to identify taxa based on simple DNA sequence comparisons quickly. The use of DNA barcoding in conjunction with morphological identifications can provide secondary evidence supporting morphological identifications and improving taxonomic resolution.

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شناسایی گونه های آفات مهم محصولات زراعی از خانواده های Noctuidae و Nolidae (Lepidoptera) در ایران، ترکیبی از روش های مورفولوژیک و DNA بارکدینگ

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چکیده

لاروهای گونه های متعددی از خانواده های Noctuidae و Nolidae خسارات مهم اقتصادی روی محصولات کشاورزی دارند. شناسایی های مبتنی بر DNA به عنوان روشی موثر برای تسریع شناسایی گونه ها و کشف گونه های جدید پیشنهاد شده است. هدف مطالعه حاضر تعیین کاربرد یک توالی تا ۶۴۲ جفت بازی (بارکد) از ژن cytochrome c oxidase subunit I (COI) برای شناسایی ۱۲ گونه آفت مهم محصولات

زراعی از خانواده های Noctuidae و Nolidae در ایران به منظور تأیید شناسایی های مورفولوژیک صورت گرفته با تاکسونومی کلاسیک بود. ما برای شناسایی ۵۳ نمونه جمع آوری شده از جمعیت های مختلف در سرتاسر ایران از ترکیب مطالعه مورفولوژیک و مولکولی استفاده کردیم. نتایج نشان از وجود یک بارکد گپ مشخص در بین گونه های مختلف داشت. میانگین واگرایی توالی ها بین گونه ها (۱۰%) بر اساس Kimura 2-parameter بزرگتر از میانگین واگرایی توالی ها درون گونه ها (۲۹/۰%) بود. بر اساس ترکیب شناسایی مورفولوژیک و مطالعه DNA تعداد ۱۳ گونه شناسایی شد که یکی از انها تاکنون ناشناخته بوده و ممکن است گونه ای جدید از جنس Earias باشد. برای برخی از گونه های مطالعه شده قبلاً تعداد بسیار کم و یا هیچ گونه توالی در بانک های داده بین المللی ثبت نشده بود. این مطالعه تعداد توالی های مذکور از ایران را در بانک های داده افزایش داد که این امر به تصمیم گیری های تاکسونومیک کمک می کند. مطالعه حاضر در شناسایی مهم ترین گونه های آفات از خانواده های Noctuidae و Nolidae در ایران کمک می کند، فارغ از اینکه توانایی تاکسونومیک کاربر، مرحله رشدی نمونه و نیز جنسیت و نحوه نگهداری نمونه چگونه بوده است. یافته های ما، محققان و متخصصان درگیر در امور نظارت زیستی بر حشرات آفت را قادر می سازد تا گونه ها را بر اساس مقایسه توالی DNA به سرعت و سادگی شناسایی کنند. ترکیب دی ان ای بارکدینگ با شناسایی مورفولوژیک می تواند تأییدی دیگر بر شناسایی های مورفولوژیک بوده و دقت تاکسونومی را بهبود بخشد.

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