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MORPHOLOGY AND MOLECULAR IDENTIFICATION OF THE LARVAL STAGE OF TWO SPECIES FROM THE GENUS *CHRYSOBOTHRIS* ESCHSCHOLTZ, 1829 (COLEOPTERA, BUPRESTIDAE)

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ABSTRACT

The genus of *Chrysobothris* Eschscholtz, 1829 is one of the most diverse and widespread genera of the family Buprestidae of some 700 described species distributed throughout the world. In Iraq, particularly in the Kurdistan region, about 4 species had been recorded so far, many of these species are sympatric, share larval host plants, and are difficult to reliably separate morphologically. The current study investigates species limits and relationships among the recognized species occurring within the Erbil Province; mitochondrial cytochrome C oxidase (COX I) molecular analysis confirmed the monophyly of two *Chrysobothris* species, *Ch. affinis* (Fabricius, 1794) and *Ch. chrysostigma* (Linnaeus, 1758). Implications of the resultant larval morphology and molecular techniques are discussed. Diagnostic characteristics that are depended to identifying the species within *Chrysobothris* in larval stage were illustrated and then compared with the molecular data.

Keywords: Buprestidae, *Chrysobothris*, Coleoptera, COXI, Molecular, Morphology.

INTRODUCTION

Chrysobothris Eschscholtz, 1829 is a common genus widely distributed throughout the world (MacRae and Basham, 2013). The species group within *Chrysobothris* in the larval stage is separated from each other by a pronotal plate, pronotal sculpture and their asperities, and the inner armament of the proventriculus (Bily, 1999). The larvae develop in the sapwood of the stems of the apricot *Prunus armeniaca* L., 1753, peach *Prunus persica* L., 1801, plum *Prunus domestica* L., 1753, pear *Pyrus communis* L., 1753 and pomegranate *Punica granatum* L., 1753. This genus is one of the most diverse and widespread genera of the family Buprestidae of some 700 described species distributed throughout the world (Hawkeswood,

1995; Bellamy, 2002). In Iraq, particularly in the Kurdistan Region, about 4 species had been recorded so far (Cobos, 1970; Knopf, 1971; Ali, 2007). They are *Ch. affinis* (Fabricius, 1794); *Ch. beesoni* Obenburger, 1926; *Ch. beesoni* kherii Cobos,1970; *Ch. solieri* Castelnau and Gory,1837, and *Ch. parvipunctata* Obenburger, 1914. But recently it has been proven that *Ch. beesoni* and *Ch. beesoni* kherii were synonymous for *Ch. parvipunctata* (Löbl and Löbl, 2016, Bílý *et al*., 2011). Although several studies have already dealt with the adult stages of the species belonged to this genus in different areas (Holynski, 1975; Barr and Westcott, 1976; Westcott, 1983), but there was lack of comprehensive larval morphology. Accordingly, it is necessary for proper identification to provide a detailed larval description which is based not only on the optic but also on molecular technique. Furthermore, these species were found to be common and destructive pests in Kurdistan orchards. Molecular sequencing techniques provide a precise other approach to suppose evolutionary relationships among closely related species (Rubinoff *et al*., 2006; Hansen *et al*., 2015).

The main objectives of this study are to identify species levels using inferences of DNA sequences from cytochrome oxidase (COXI) and to try to determine further morphological characteristics of the larva. Eventually, differences between *Ch. affinis* and *Ch. chrysostigma*, if recognized, will not only yield information that may help in identification of larval stages, but also actually facilitate the timing and placement of insecticides to agree with activity of economically important buprestids and reduce dangerous and overpriced control measures.

MATERIALS AND METHODS

Collection and identification of larval specimens

Last instar larvae are collected from the stem of the apricot *Prunus armeniaca*, peach *Prunus persica*, plum *Prunus domestica*, pear *Pyrus communis* and pomegranate *Punica granatum*. The collection of the larvae has been performed in different locations of Erbil Province; the collected larvae are placed in vials containing 70% and 99% alcohol and then transferred to the laboratory for morphological and molecular study. The adult specimens have been reared from the larvae and identified with the help of the available literature (Cobos, 1986). The identification of the larval stage had been confirmed to the genus level by Dr. Mark G. Volkovitsh and Dr. Svatopluk Bílý. The morphological terminology used in the present paper follows the ones in the papers of Volkovitsh (1979), Bíý (1999) and Bíly and Volkovitsh (2003, 2005).

Dissecting, slides preparation and imaging

First, the larvae were dissected and boiled in 10% KOH aqueous solution until the soft tissues are dissolved. After that the samples were rinsed in distilled water (Alexeev, 1960; Chamorro *et al*., 2012; Volkovitsh and Bílý, 2015). Second, the specimens were mounted on slides, the DPX media (a mixture of polystyrene, tricresyl phosphate and xylene) that acts as a clearing agent to decompose soft tissue was used. The dissected parts were dehydrated by rinsing in serial concentrations of alcohol (30%, 50%, 70%, 90%, and 100 %) and then they were rinsed in xylole. Then the prepared structures were placed in DPX drops using 0.5 mm micro needle and covered with coverslips. The slides were placed horizontally on a hot plate at 36˚C for 24–48 hours until they got dried for the avoidance of bubble formation. Third,

images were taken by a compound microscope (Huma scope premium) with mounted LCD camera, and scaling of the displayed structures were measured by a stage micrometre.

Molecular Study

For the molecular study, we followed the procedure of Asghar *et al*. (2015), in which the larval specimens that had been collected and preserved in absolute ethanol and stored in refrigerator (-20) $\overset{\circ}{C}$ until DNA extraction could be performed. A total sample size of (12) larvae were used for this experiment.

DNA Extraction

The genomic DNA of a specific tissue (i. e. thorax and abdominal segments) was extracted from a pooled sample consist of three larvae of each species. Animal and fungi DNA preparation kit (Jena Bioscience GmbH .07749 - Germany), was used.

Quantification of extracted DNA

The spectrophotometer model NanoDrop 1000 manufactured by Thermo Scientifics designed for measuring nucleic acid concentrations in sample volumes of one microliter was used. The instrument is driven by a PC, which allows 280 nm. This ratio is used to assess the purity of DNA and RNA (Kumar *et al*., 2007).

Agarose gel electrophoresis

Agarose gel electrophoresis was the procedure used to separate DNA fragments based on their molecular weight and intrinsic. The technique consists of three basic steps:

1. Preparation of agarose gel.
2. Electrophoresis of DNA fragments.
3. Visualization of DNA fragments (Lee *et al*., 2012).

Preparation to the amplification of partial Mt- COXI gene

The PCR amplification for COXI partial gene was prepared in 25 µl of the reaction mixture for 50 samples containing genomic DNA and 2x Taq polymerase master mix (AMPLIQON), 20 Picomol of CJ-C1-J-1718 forward primer: (5'- GGA GGA TTT GGA AAT TGA TTA GTT CC -3'), with 20 pmol of reverse primer (5'- ACT GTA AAT ATA TGA TGA GCT CA -3'), DNase free water and template DNA by Bioresearch PTC-200 Gradient thermocycler. Temperature profile included step one is an initial denaturation at 95 Cº for 5 min, step two followed by 35 cycles of denaturation at 95Cº for 35 seconds, a primer annealing at 50Cº for 45 seconds, an extension at 72 Cº for 1 min and final step is an extra extension at 72 Cº for 5 min (Tabs 1, 2) (Hansen *et al*., 2015).

Table (1): Primer COXI used for identifying the species of the genus *Chrysobothris***.**

Primer	Direction	Target	Tm C	Primer sequences	Reference
C1-J-1718	Forward	Mt-COXI	52	5´GGAGGATTTGGAAATTGATTAGTTCC3´	Hansen *et al*. (2010)
C1-J-1718	Reverse			5´ACTGTAAATATATGATGAGCTCA 3´	

Table (2): PCR agents with their concentration and volumes.

No.	PCR components	Concentration	Volume (µl)
1	Master Mix	2x	12.5
2	Forward Primer	20 Pmol	2
3	Reverse Primer	20 Pmol	2
4	DNase free Water	-	6.5
5	Template DNA	50ng/µl	2
Total			25

DNA Sequencing

The samples of the PCR product of the COXI mt-DNA partial gene had been sequenced by ABI Prism Terminator Sequencing Kit (Applied Biosystem) at the Immuno gene Center, Physician's Street in Erbil. Chromatograms of primers gene were edited and also checked using Finch TV and chromas program software. Some of the products were prepared and sent to the Republic of Korea (South), and sequenced by the molecular and genetic company; Macro Gene Company.

RESULTS

Chrysobothris affinis (Fabricius, 1794)

Larval morphology

Body shape and length: Larval body (Pls. 1 a, b) relatively large, long and slender, and belongs to the usual Buprestoid larval type (morpho-ecological group 2) (Volkovitsh, 1979). Length of mature larva 25-27 mm; prothorax strongly enlarged, its width 5.5-6 mm.

Head: Conical-shaped, nearly rounded anterior margin. Epistome (Pl. 1 c), heavily sclerotized, and about five times as wide as long; anterior margin slightly emarginated between globular condyles, posterior margin bisinus; lateroposterior corners sharp, epistomal sensilla centrally located, and each group of epistomal sensilla, consisting of long trichosensilla and one basiconic sensillum. Antennae (Pl. 1 d), basal antennomere nearly round, campaniform sensillum located on dorsal side; apical crown of microspinulae weakly developed with sparse and short microspinulae. Apical antennomere shorter and smaller, two times as wide as long; apical crown of microspinulae with sparse and short microspinulae; apical trichosensilla long and sharp; apical cavity shallow, and provided with sharp sensory appendage, two palmate and one tiny basiconic sensillum that very close bases.

Mandibles (Pl. 1 e): Triangular, dark brown; strongly sclerotized, posterior part have two very small pores and short, thick seta. Apical part with three sharp teeth; cutting edge with two obtuse denticles. Ante-clypeus and labrum (Pl. 1 f), membranous, smooth, and about thrice as wide as long. Labrum trapezoid, longer than wide, anterior margin noticeably rounded and covered by a narrow field of microsetae; palatine sclerite well developed, lateral branches carried long trichosensilla; medial branches with a pair of long apical trichosensilla that prolonged to anterior margin, two campaniform sensilla located between lateral and medial branches. Labio-maxillary complex, prementum, expanded and detached with basal sclerite of

cardo. Labium (Pl. 1 g), bottle-shaped; anterior margin widely rounded, corner sclerite of prementum well sclerotized and carried long sharp seta, with five campaniform sensilla; medial part with two zones of dense microspinulae. Maxillae (Pl. 1 h), cardo slightly rounded and glabrous; stipes bowed inward with densely microspinulae. Basal palpomere of maxillary palpus, sub-cylindrical, wider than long; anterior margin with few short microspinulae and long lateral seta. Apical palpomere, conical, with acutely curved sensillum, and one campaniform sensillum; upper part provided with five tiny sensory cones. Mala, thick and slightly cylindrical; well sclerotized, with six external long sensilla, and one campaniform sensillum.

Thorax, prothorax wide and flattened, pronotal plate well- developed, and sculptured with dark, dense, asperities and sparse micro teeth (Pl. 1 i); prosternal plate with same features but differ from by narrowing of border and forming concavity. Pronotal groove (Pl. 1 j) inverted letter V; prosternal groove, straight and narrow, not extended to the outer border (Pl. 1 k); mesothorax, transverse and four times as wide as long with medial narrowing; metathorax, transverse and slightly narrower and longer than mesothorax.

Spiracles: Mesothoracic spiracles (Pl. 1 L), reniform, and about 3.5 times as wide as long with dense trabeculae. Abdominal spiracles (Pl. 1 m) smaller and spherical shaped with widely reniform.

Proventriculus: Inner armaments of proventriculus developed as a single long protrusion with long and sharp spines (Pl. 1 n).

Abdomen (Pl. 1 a): Relatively long and extremely flattened, first abdominal segment nearly ovoid without lateral folds; segments 2-8 slightly equal in width and length, dorsolateral folds well developed; anal segment sub conical with vertical anal rim; outer surface of the body, covered with dense, short microspinulae and long setae (Pl. 1 o).

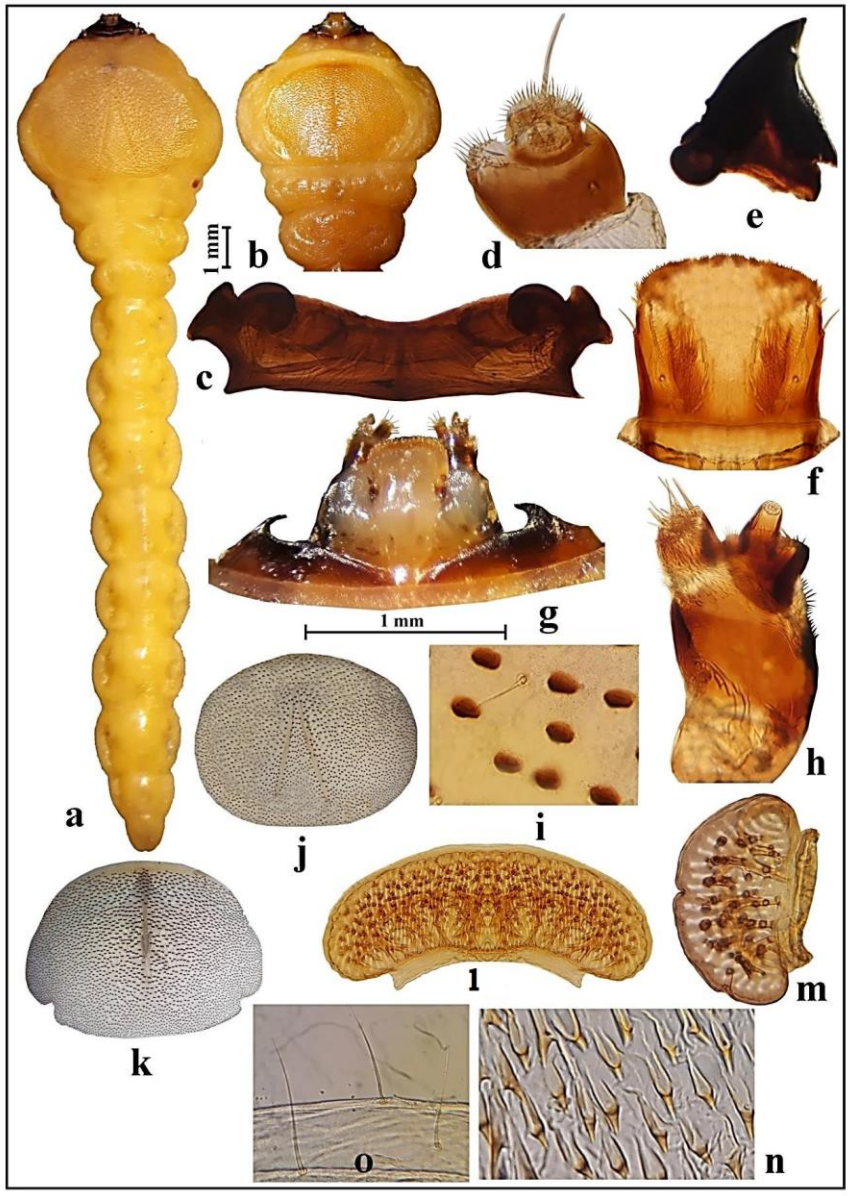

Plate (1): Habits and morphological characteristics of the larva of *Ch. affinis*; (a) Dorsal view of larval body, (b) Ventral view of thorax, (c) Epistome, (d) Antenna, (e) Mandible, (f) Labrum, (g) Labiomaxillary complex, (h) Maxilla, (i) Pronotal asperities, (j) Pronotal plate with groove, (k) Prosternal plate with groove, (l) Mesothoracic spiracle, (m) abdominal spiracle, (n) Inner armament of proventriculus, (o) Body surface.

Chrysobothris chrysostigma (Linnaeus, 1758)

Larval morphology

Body length and shape (Pls. 2 a, b) larva, relatively large, and flattened with pale yellow color; pronotal, and prosternal plate well developed, with nearly circular border; length of mature larva about 25-28 mm; width of prothorax about 5-5.5 mm.

Head: Flattened prominent, expanded at base; surface provided with short and dense microspinulae. Epistome (Pl. 2 c), transversely elongated, strongly sclerotized. 4.5 times as wide as long; anterior margin darkened and deeply incurved between semi-rounded condyles, posterior margin slightly bisinus with sharp posteriolateral corners, epistomal sensilla near with each other, and each group with two rather short trichosensilla and one basiconic sensillum. Antennae (Pl. 2 d), basal antennomere transversely, sub-cylindrical, and nearly 1.2 times as long as wide, with campaniform sensillum on the dorsal side; slightly narrowed, basally. Apical antennomere short and wide and about thrice as wide as long, the apical crown of microspinulae well developed, with long and dense microspinulae; apical trichosensilla curved long; apical cavity shallow, well developed and provided with short, sharp sensory appendage, two palmate sensilla, and one tiny basiconic sensillum. Mandibles (Pl. 2 e), triangle-shaped, strongly sclerotized, and expanded basally; apical teeth with three sharp teeth; hind seta short and thick. Ante-clypeus and labrum (Pl. 2 f), membranous, smooth, and about thrice as wide as long. Labrum, trapezoid, longer than wide; anterior margin slightly arched toward mouth cavity, and covered by dense and short microsetae; anterolateral corners round with one long seta and one campaniform sensillum; anterolateral lobes weakly developed; lateral branches strongly developed with long, thick tricosensilla, medial branches with long, sharp apical seta, in which nearly prolonged to anterior microspinuled area. Labio-maxillary complex (Pl. 2 g), prementum, transverse, anterior margin with medial depressing. Labium oblique; anterior margin widely rounded, corner sclerite, tubular, well developed, with long sharp seta and five campaniform sensilla; medial part with two microspinulae zones. Maxillae (Pl. 2 h), cardo round, with few short setae. Stipes strongly sclerotized, anterior margin of stipes with a bundle of long setae, and prolonged to near bases of maxillary palpus. Basal palpomere of maxillary palpus, barrel-shaped, longer than wide, with medial campaniform sensillum; anterior margin with dense and short microspinulae; apical palpomere, cylindrical; two times as long as wide, with four tiny sensory cones. Mala, sub-conical shaped, well sclerotized, longer than wide, with six long and thick tricosensilla, and fine dens microsetae.

Thorax (Pls. 2 a, b): Prothorax ovoid bordered, and well expanded, both pronotal and prosternal plates well-developed and covered by dense, dark brown and, dash shaped asperities with micro sculpture, (Pl. 2 i); pronotal groove inverted “V” letter, and branches connected in apical part, and remarkably diverged posteriorly (Pl. 2 j), prosternal groove straight with tightly tapering ends (Pl. 2 k). Mesothorax, ring-shaped; short, and four times as wide as long; metathorax, slightly transverse, about thrice as wide as long with slightly medial depressions, and basal narrowing; leg rudiments absent.

Spiracles: Mesothoracic spiracles (Pl. 2 l) reniform, three times as wide as long, and arcuated medially; with dense, vertical trabeculae. Abdominal spiracles (Pl. 2 m), slightly spherical

with fewer branches of trabeculae and expanded peretreme. Proventriculus (Pl. 2 n), inner armament of frontal and basal part provided with long and dense microspines; medial part with irregular sculptures in which developed as micro tubercles and carried 2-4 short and sharp micro spines.

Abdomen (Pl. 2 a): Large, and relatively long flattened, first abdominal segment nearly ovoid and distinctly narrower than metathorax and second abdominal segment; segments 2 - 8 slightly wider than long; dorsolateral folds well developed; last segment triangular, and opened with vertical anal rim; whitish dense and short microspinulae covered the whole abdominal surface with presence sparse singular, long bristles (Pl. 2 o).

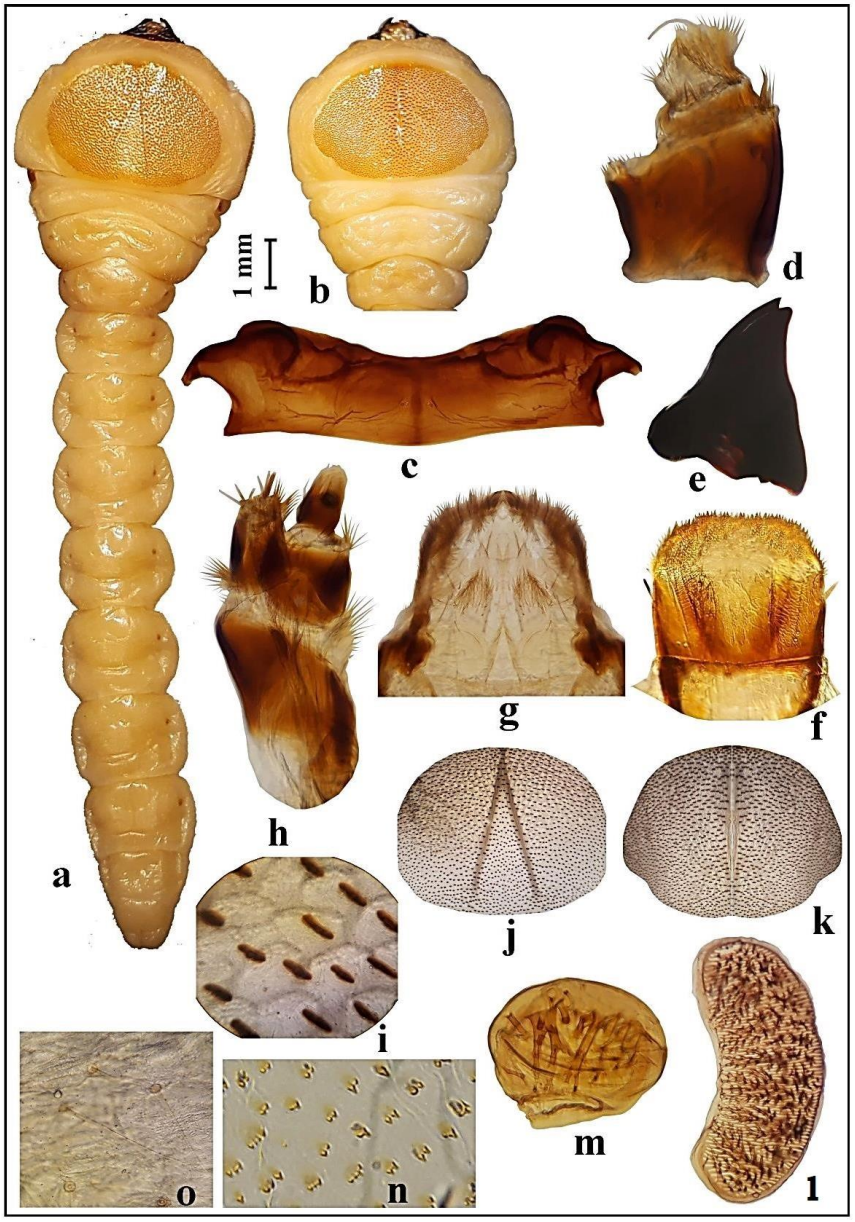

Plate (2): Habits and morphological characteristics of the larva of *Ch. Chrysostigma*; (a) Dorsal view of larval body, (b) Ventral view of thorax, (c) Epistome, (d) Antenna, (e) Mandible, (f) Labrum, (g) Labium, (h) Maxilla, (i) Pronotal asperities, (j) Pronotal plate with groove, (k) Prosternal plate with groove, (l) Mesothoracic spiracle, (m) abdominal spiracle, (n) Inner armament of proventriculus, (o) Body surface.

Molecular study

Genomic DNA for the two specimens belonging to the genus *Chrysobothris* includes *Ch. affinis* (Fabricius, 1794) and *Ch. chrysostigma* were isolated. The purity was also found acceptable ranging between (1.7–1.8) determined by spectrophotometer ratio A_{260}/A_{280}. The molecular weight of the genomic DNA samples was estimated using 1% agarose gel electrophoresis containing λ DNA samples as control and it was found to be the range 50 kb (Pl. 3). The lanes from 1-2b represent the two species respectively; lane M represents unrestricted λ DNA as a standard molecular weight marker ~50 kb. Partial of the COXI gene segments for all the collected specimens was amplified by a universal primer (Wild and Maddison, 2008). The molecular size of DNA amplicons was estimated using 1.5% agarose gel electrophoresis. By using this primer, one single and strong intense band of amplified product of the mt-DNA gene had been produced across the studied species, with molecular weight approximately 650 bp, and clearly could be noted as shown in (Pl. 3).

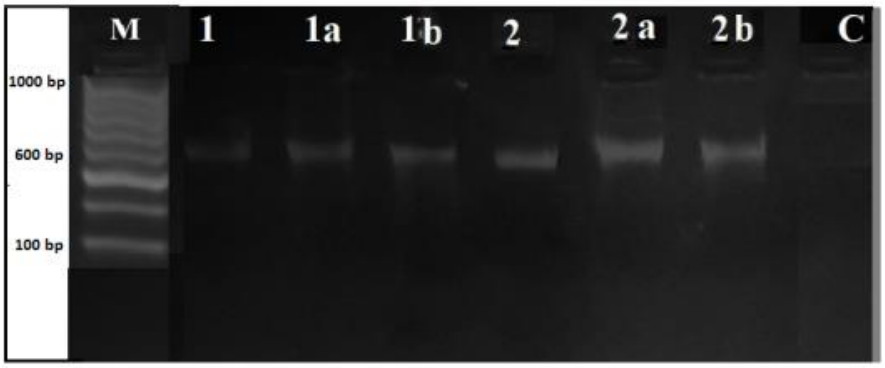

Plate (3): Amplification results obtained by using universal primer complementary to regions encoding mt COXI gene. Electrophoresis was performed on (1.5 %) agarose gel for min. Lane 1 indicates DNA molecular weight 100 bp size ladder. The lanes from 1-2b represent the studied species.

Sequence Alignment

The gene sequences were applied to basic local alignment search tool (BLAST) to blast the sequences and alignment which is available at the national center for biotechnology information (NCBI) website to comparing and alignment query sequence with other biological sequences to find out the similarity with the matched species (Ellis *et al*., 2009).

DISCUSSION

Being compatible with the characteristics used in the keys of the Buprestidae larvae published by Cobos (1986) and Bilý (1999), several morphological characters were selected to describe and identify the larvae of *Chrysobothris* species. This study is based on more morphological details of the larval stage of *Ch. affinis* and *Ch. chrysostigma* which contribute significantly to the accuracy of larval description and identification. Molecular study has rarely used for the confirmation of the identification of buprestid larvae. Earlier studies indicate that the molecular technique is reliable and important for the verification of

morphological description of larval stage and its correct differentiation from the other species of the same genus (Hansen *et al*., 2015). Usually, the immature stages of the insect species are very difficult to distinguish and correctly identify based on external characters. The results of our study provide additional characteristics to the morphology and diagnosis of two different species in the larval stage. We recommend, however, caution that absent the presence of more informative nuclear sequence data, discrimination of these species group members using the repertoire of anatomical structures in current use is insufficient to reliably place specimens into taxonomically defined entities. We suggest that future molecular investigations of species limitations of *Chrysobothris* group members should implement a population genetics approach using several speedily evolving nuclear genes, which would be most definitely acquired successive to a NextGen sequencing campaign.

CONCLUSIONS

In this study the larval stage of two different species of the genus *Chrysobothris* were described and illustrated morphologically. The larval specimens were collected in Erbil Province in the Iraqi Kurdistan Region. Two complementary methods of identification (morphological description and molecular technique) were utilized for analyzing the species identification in larval stage and recognizing as two dangerous horticultural pests in the future. Two species *Ch. affinis* and *Ch. chrysostigma* have been recorded for the first time in GeneBank with their accession numbers (MN066129 and MN066128) respectively that confirmed the identification of these species precisely.

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CONFLICT OF INTEREST STATMENT

The authors have no conflicts of interest to declare.

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المظهر الخارجي والتشخيص الجزيئي لنوعين من يرقات الجنس *Chrysobothris* Eschscholtz, 1829 (Coleoptera, Buprestidae)

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الخلاصة

الجنس *Chrysobothris* Eschscholtz, 1829 هو احدى أكثر الأجناس تنوعًا وانتشارًا في عائلة Buprestidae حيث سجل لها حوالي 700 أنواع موصوف منتشرة في جميع أنحاء العالم. اما في العراق، سجل 4 أنواع ضمن إقليم كردستان؛ وهذه الأنواع مستوطنة وعوائلها النباتية مشتركة ويصعب تميزهم مظهريًا على نحو موثوق.

بحثت الدراسة الحالية عن مدى الأنواع وكذلك العلاقات بين الأنواع الاخرى المشخصة ضمن محافظة أربيل؛ حيث ان تحليلات COXI أُكِدت الأنعزال الجزيئي بين نوعين من جنس *Chrysobothris* و هما كل من: *Ch. affinis* (Fabricius, 1794) و *Ch. chrysostigma* (Linnaeus, 1758)؛ كما وُضِحَ الصفات المظهرية لتشخيص الأنواع في الطور اليرقي الاخير وتم مقارنتها مع البيانات الجزيئية.