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Morphological and Molecular Identification of *Adoretus hirsutus* (Ohaus, 1914) (Coleoptera:Scarabaeidae:Rutelinae) from Erbil Governorate Kurdistan Region- Iraq

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ABSTRACT

This study provides a detailed description of *Adoretus hirsutus* (Ohaus, 1914) (Coleoptera:Scarabaeidae: Rutelinae), as a first record in Iraq. Between March and July 2022, we collected specimens from various weed flowers in different locations within the Erbil Governorate, Kurdistan Region, Iraq. According to molecular analysis, *Adoretus hirsutus* was used as a source of samples for PCR amplification of the fragments (710 bp) of the mtCOI gene for phylogenetic analysis. To compare the nucleotide sequence with those of other insect species, a section of the mtCOI gene from the collected insect was aligned with the NCBI GenBank database using the BLAST tool. The BLAST results showed that the second record in the NCBI GenBank identity of insects. The COI sequence of *Adoretus hirsutus* was submitted to GenBank with accession numbers OQ4288117, OQ428818, and OQ428819. The morphological diagnostic characteristics of the species are; Labrum nearly cup shaped, lateral margins moderately concave with a row of small spines, median apical projection overhanging the mentum. Mandibles irregular shaped, apically with single oval shaped tooth. Terminal maxillary palpomere 1.5 times as long as the 2nd. Antenna brown, consisting of 10 antennomeres ending in a unilateral three lamellated club, equal in length. Outer margin of fore tibia with three acute teeth. Aedagaeus moderately curved, parameres elongated oval, apical part is very acute. Some important parts such as labrum, mandible, antenna, fore legs, elytra, pygidium and male genitalia have been photographed.

Key words: Molecular identification; morphological study; mt COI gene; *Adoretus hirsutus*; sequencing.
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INTRODUCTION

The Scarabaeidae family, in its present definition, encompasses more than 30,000 species of beetles found across the globe. These beetles are commonly referred to as scarabs or scarab beetles. They are characterized by their stout bodies, with many species displaying vibrant metallic colors. One distinguishing feature of these beetles is their clubbed antennae, which are composed of lamellae, specialized plates that can be compressed into a ball or spread out like leaves, enabling them to detect odors. [1]; [2]. Within the family, there are highly destructive beetles that primarily consume live plant matter during adulthood, while they typically feed on plant roots or decaying wood during their larval stage. Another group of scarabs primarily consists of dung-feeders or scavengers. These beetles predominantly feed on dung or decomposing plant and animal matter as adults. In many cases, the larvae also consume the same materials, which are provided to them by the adult beetles. [1]; [3]. The Rutelinae subfamily, commonly known as shining leaf chafers, is a significant group comprising approximately 200 genera and 4,100 species. They have a cosmopolitan distribution, meaning they are found worldwide. [4]. Adults of the subfamily are phytophagous and feed on leaves, flowers, grass pollen and maturing grass seeds, some adults aid in pollination of plants, and the larvae typically feed on roots (berries, corn and grass), compost, decaying vegetation, decaying wood [5]. The adults of this subfamily can be identified by several distinguishing features. Firstly, they have a metallic and robust body structure. Secondly, their tarsal claws are unequal in size, large, and capable of movement. Additionally, their elytra do not fully cover the pygidium (the posterior part of the abdomen) and often have a membranous margin underneath. [6]. *Adoretus* Laborte is a significant genus within the subfamily, known for its elongated and oval shape. These beetles measure approximately 10-12 mm in length and are characterized by a brown coloration with a covering of white cream setae. The labrum displays a central apical projection that extends beyond the mentum. The elytra are irregularly coarsely punctate, and the disc usually has 2-3 raised longitudinal lines [7];[8]Within the genus, there are multiple species that exhibit striking similarities, making it challenging to differentiate them without examining the males' genitalia or utilizing molecular identification tools. Adult *Adoretus* beetles are considered generalist herbivores, as they feed on the leaves of various plant species across a wide range. Several other species are also pests and invasive [9]. Many species of the genus can cause heavy damage in nurseries and young plantations [10]. Species identification of Coleoptera, however, requires a sophisticated technique [11]. Given these facts, DNA testing looks to hold promise for resolving the identification of species conundrum because DNA is durable and stable [12]; [13]. For this purpose, partially genomic conservative areas, such as the mitochondrial COI gene, can really be sequenced [14]; [15]. Various creatures, along with some species of insects, can be accurately identified via DNA sequencing, particularly those who have identical morphologies. Because of its exceptionally high-resolution phylogenetic signal, the mitochondrial cytochrome-c oxidase subunit 1 gene (CO1) is frequently utilized for species separation and investigating the hidden variation in similar species[[16].

The main aims of this study include, collecting the samples from a wide range of locations in the Erbil Governorate, Kurdistan Region of Iraq; molecularly identifying the species and a detail description of the species with photographing the important parts.

MATERIALS AND METHOD

First: Molecular study

The study utilized three adult dried beetle samples.

1. DNA Extraction

In the study, mtDNA extraction was performed on adult specimens. The DNA extraction was performed using the ZYMO Quick-DNA Tissue/Insects Microprep Kit

(USA-D6015) according to the instructions provided by the manufacturer. Subsequently, the DNA was stored at -20℃ for future use. The Nanodrop (Thermo scientific-UK) was used to quantify and assess the quality of the DNA.

2. Polymerase Chain Reaction (PCR) Amplification

Mitochondrial DNA with primers of a specific gene were manufactured for using the sequences of cytochrome c oxidase subunit I (Table 1), produced by Company of Micro-gene in South Korea, and then the primers were amplified using normal PCR for every species. The band size of amplicons was 710bps. The amplification was prepared in 50µl of ultimate mixtures consist of 2X Master Mix (AMPLIQON A/S Stenhuggervej, 22), 10 Pico-mol of primer pairs, DNase free water and DNA (Table 2), using Bioresearch PTC-200 Gradient thermocycler. The PCR program consisted of the following steps: initial denaturation at 95℃ for 5 minutes, followed by 35 cycles of denaturation at 95℃ for 40 seconds, annealing at 60℃ for 40 seconds, and extension at 72℃ for 1 minute. After completing the cycles, a final extension step was performed at 72℃ for 10 minutes. The PCR products were then stored at -20°C for future use [17]. To analyze the amplicons, a 1.5% agarose gel was prepared and run in 1X TAE buffer for 30 minutes at 70 volts. The gel was stained with ethidium bromide and visualized using a UV trans-illuminator.

Table (1): Mitochondrial COI primers sequences.

Gene name	Primer	Oligonucleotide Sequences	Product size	Reference
Cytochrome Oxidase C- I (COI)	LCO 1490	5’ GCTCAACAAATCATAAAGATATTGG-3’	710bps	(Simon *et al*., 1994)
	HCO 2198	5’ TAAACTTCAGGGTGACCAAAAAATCA-3’		

Table (2): Reaction mixture for the PCR amplification of mtCOI gene.

No.	PCR requirements	Concentration	Volume (µl)
1	Taq DNA, Master-Mix	2x	25
2	Forward Primer	10 Pico-mol	3
3	Reverse Primer	10 Pico-mol	3
4	Dnase free Water	-	15
5	Template DNA	50ng/µl	4
Total			50

3. Mt-COI Sequencing

The Micro-gene Center in Korea sequenced the mtCOI incomplete gene amplicons from the specimens using the Applied Biosystems ABI Prism Terminator Sequencing Kit. The chromatograms of the COI gene were modified and base calls were verified using the Finch TV program.

4. Sequence alignment and submission.

The exploration program utilized partial mt-COI gene sequences for aligning DNA sequences. We utilized the Basic Local Alignment Search Tool (BLAST) accessible on the NCBI (National Center for Biotechnology Information) website.. This tool enabled us to compare and align the query sequences with

previously added biological sequences, thus identifying similarities with other targets.

Second: Morphological study

The present paper is based on 30 specimens which collected during the period March till July 2022 from different localities of Erbil governorate, by using light trap, hand picking and sweeping net. The specimens underwent several preparation steps before examination. Initially, they were immersed in boiling water for 10-15 minutes to soften their parts, followed by the separation of these parts. Mouthparts and male genitalia were then boiled in a 10% KOH solution and placed on a heater source while shaking for approximately 15-20 minutes to dissolve lipids. Subsequently, they were immersed in distilled water for 3-4 minutes to neutralize the alkali.

Next, the parts were immersed in 25% ethyl alcohol and dissected under a dissecting microscope. They were then sequentially transferred to 50%, 75%, and 100% ethyl alcohol for 2 minutes each to remove water content through dehydration. For translucency, the parts were soaked in xylene for two minutes and finally mounted on slides using DPX solution to facilitate subsequent examination under a microscope. Measurements of various body parts were taken using an ocular micrometer [2]; [18]; [19]. Detailed information about the collection localities, host plants, and date was recorded. Identification of the species was accomplished using the taxonomic key of [8] and molecular sequences.

RESULTS AND DISCUSSION

First: Molecular study

PCR amplification of partial COI gene

The primers pairs of the specific mitochondrial gene were manufactured for utilizing the COI gene sequences which produced by the company of micro-gene made in South Korea. The primers got a clear band of 710bp. The amplicons were run and pictured via 1.5% Agarose gel Figure 1.

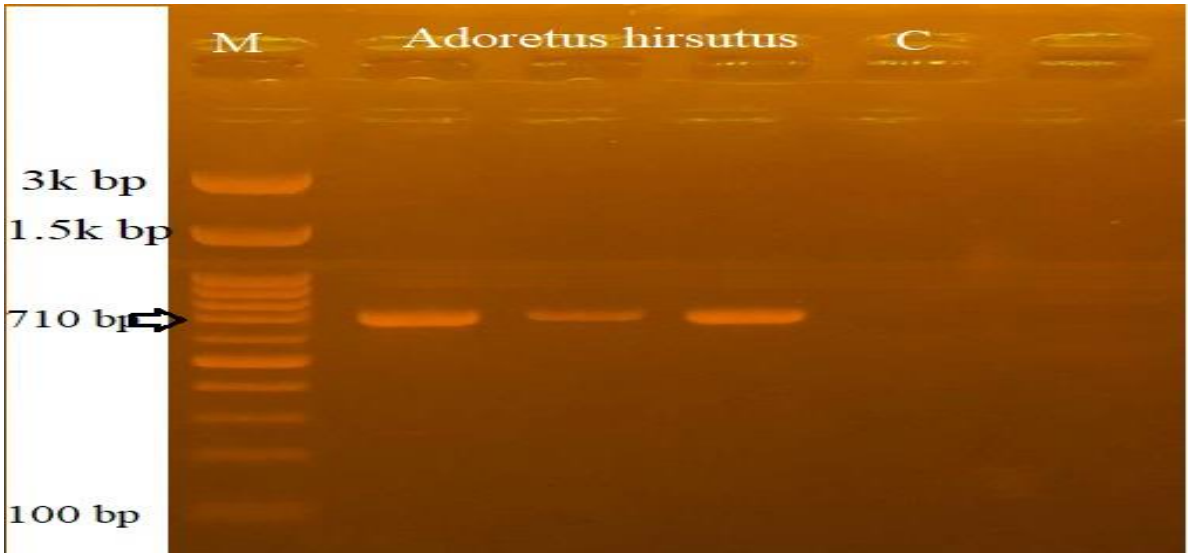

Figure 1: PCR amplification of partial cytochrome C Oxidase I gene from insects *Adoretus hirsutus* . M; indicate: ladder (3K bp-100 bp) and lane 2- 4: 710 bp of PCR products of from insects and C is negative control.

Partial Sequence of COI gene

The sequence of the DNA, a forward primer LCO1940 was achieved in each sample through ABI 3130X genetic analyzer (Applied Biosystem). The DNA template for sequence-specific PCR amplification was derived from the amplicons of the specimen.

Molecular Identification of genus and species

The insect samples' partial COI gene sequences were submitted to the NCBI GenBank through the BLAST program available at (http://blast.ncbi.nlm.nih.gov/). Subsequently, they were compared to other saved insect sequence species. The BLAST

results showed that the second record in the NCBI GenBank identity of insects table 3, had the highest identity to our nucleotide sequences. After obtaining these results, we submitted our query sequences to the NCBI GenBank, where they were assigned accession numbers: OQ428817, OQ428818, and OQ428819.

Table 3 shows the partial COX subunit I gene sequences in NCBI, which were then fed into the same sequences after submission.

Insect identified	Accession Number	Query Cover%	Identic Number%	Accession Number of BLAST Identification	Country
Adoretus hirsutus	OQ428817 OQ428818 OQ428819	100	100	MT129331	Korea

Phylogenetic inferences

Based on the nucleotide sequence of COXI, the phylogenetic analysis showed that the three species of insects investigated were grouped as expected. Based on the sequence divergence similarity data and the constructed phylogeny, it was evident that the species within their respective genera exhibited close relationships with one another

Figure 2: Employing Maximum Likelihood with boost strap of mega 11 program, show phylogenetic positioning of each insect species with similar Gen Bank sequences of COI partial that available in Gen Bank.

Second: Morphological study

***Adoretus hirsutus* (Ohaus, 1914)**

Description

Body (Fig.3a): Elongate oval, brown covering with covering of white/cream setae. Body length 13.0 – 16.5 mm and the width 6.5- 7.9 mm.

Head: Brown-dark brown, length 2.6-3.1 mm and the width 3.8-4.4 mm, surface covering by densely of short, yellow setae. Frons brown, slightly convex, margin reflexed and round at angles, surface covering by moderate dense of short yellow setae. Clypeus brown, pentagonal with a reflexed margin, surface covered with coarsely punctures and with sparse of short yellow setae. Genae, narrow. Eye black brown, oval, length 1.0 – 1.5 mm, canthus visible, carinate reaching to 1/3 of eye width, with moderate dense of brown setae. Labrum (Fig. 3b) cup shaped with a median apical projection overhanging the mentum, lateral margins with row of short thick robust spines, surface with short dark spines and sparse of short yellow setae. Mandible (Fig.3c) irregular shaped, apically with single oval shaped tooth, dorsal surface with sparse of short yellow setae, molar area with comb of short yellow setae. Maxilla (Fig 3 d) brown - black, stipes nearly cup shaped covered with dense of short-long brown setae; cardo brown, triangular with sparse of brown setae, galea black height sclerotized, distal part with four denticles, the palpomeres are brown, 1st palpomere small, filiform shaped, 2nd–3rd palpemeres cup shaped, 2nd palpomere1.2 times as long as the 3rd, terminal maxillary palpemere elongated oval, 1.4 times as long as the 3rd palpemere. Labium (Fig. 3e) brown – dark brown, mentum trapezoidal, covered with height dense of long pale yellow

setae, labium consist of three labial peplomers, 1st and 2nd palpemeres cup shaped, 2nd 1.3 as long as the 1st , apically with 4-5 long, brown setae, terminal labial palemere cylindrical, 0.9 as long as the 2nd and bare. Antenna (Fig. 3 e) brown - dark brown, consisting of 10 antennomeres ending in a unilateral three lamellated club, equal in length, with sparse of short yellow setae, 1st antennomere long, cup shaped 5 times as long as the 2nd, 3th and 4th antennomeres same length, 5th antennomere 0.8 times as long as 6th ,7th antennomere small and rectangular, 8th -10th antennomeres lamellate shaped.

Thorax: Pronotum brown – dark brown, nearly hexagonal shaped; surface coarsely and densely punctured wit short pale-yellow setae; surface elevated, anterior margin slightly sinuate; sides medially angulate; posterior margin slightly convex; front angles acute and hind angles obtuse. Scutellum brown, obtusely triangular; sides slightly curved; apex blunt; surface coarsely and densely punctured wit short pale-yellow setae. Elytra (Fig.3g) brown with distinct humerus, length 7.4- 9.2 mm, Coarsely and densely punctured. Epipleuron rounded, with moderate dense of brown setae. Hind wing hyaline, yellow length 6.7 – 8.0 mm, veins dark brown, stigma (Radial cell) small, oval, low sclerotized, medial spur and M veins reaching to the posterior margin of the wing. Fore leg (Fig.3h) Brown, Fore coxae tubular shaped, trochanter nearly oval, fore femur cylindrical, inner and outer margins with moderate dense of long yellow setae, fore tibiae nearly tubular, outer margin sharply tridentate, apical dentils slightly longer than the others, subterminal with single short spur, surface covering with moderate dense of short, pale yellow setae, tarsus five segmented, 1st -4th segments cup shaped,1st segment 1.8 times as long as the 2nd , 2nd – 4th segments nearly same length, apically with 3-4 spines, 5th segment tubular shaped, slightly curved, 4 times as long as 4th , inner margin moderately concave with 3 yellow seta protarsal claws unequal, longer one of fore and mid cleft, with tips far away. Middle legs resemble to the fore legs except, mesocoxae conical shaped, mesotibia without denticles, truncate and fringed with short spines at extremity, terminally with two unequal spurs. Hind legs resemble to the fore legs except, metacoxae rectangular shaped, metatibia without denticles, surface carinated with short pale-yellow setae, terminally with row of very short spines and 2 unequal spurs.

Abdomen: Expanded oval, consist of seven brown visible segments, surface with moderately dense of short yellow setae.1st - 6th abdominal sternites transverse, 1st abdominal sternite membranous, 0.9 times as long as 2nd, 3 sternite 1.1 times as long as 4th, 5th sternite 0.9 times long as 4th, 6th sternite transverse oval,1.1 times as long as 6th, 7th sternites small and cup shaped. 1st -5th abdominal tergites membranous and transverse; 6th and 7th tergites height sclerotized;4th tergite 1.2 times as long as the 5th; 5th and 6th tergites same length; pygidium (Fig.3i) cup shaped, rugosely punctate, covering with height dense of short yellow setae. Spiculum gastrale (Fig. 3j), low sclerotized. Invert Y- shaped, apical arm 1.3 times as long as the laterals.

Male genitalia: Aedeagus brown – dark brown, moderately sclerotized. Length 3.7-4.5 mm. Phallobase invert cup shaped; in dorsal view (Fig. 3k), parameres short and broad, slightly convex at the base; sides slightly curved and sharply angulated near the base; inner margin curved; apex medially notched and bilobed; in (Lateral view) (Fig. 3l) Phallobase nearly rectangular, apex of parameres needle like. peins tubular, slightly shorter than parameres.

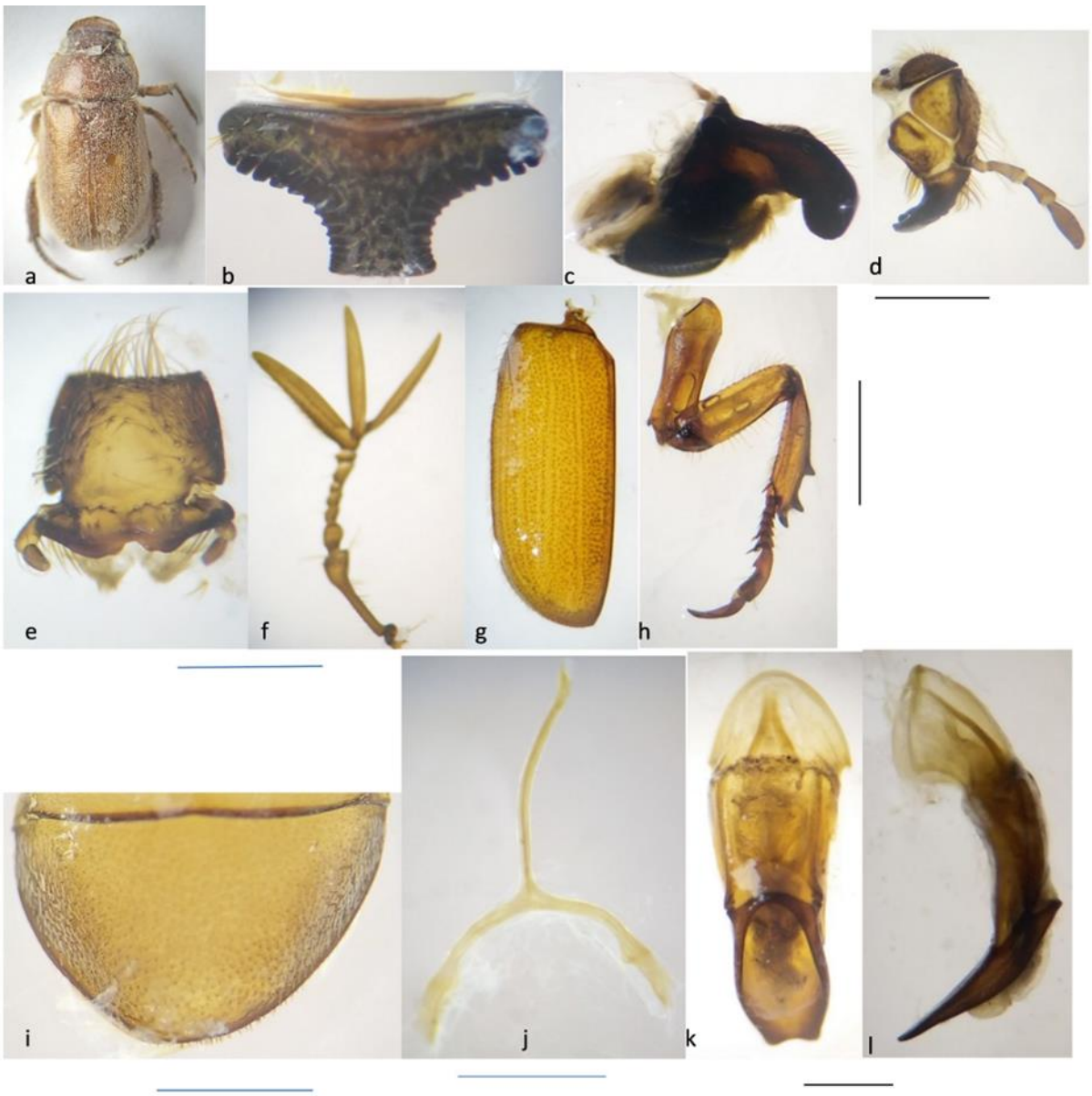

Fig. 3 Adoretus hirsutus a. Habitus (3X) b. Labrum c. Mandible d. Maxilla e. Labial palp f. Antenna g. Fore leg h. Elytra i. Pygidium j. Spiculum gastrale k. Aedeagus (Dorsal view) l. Aedeagus (Lateral view). Scale bar: 1mm

CONCLUSIONS

This study provides a comprehensive description of *Adoretus hirsutus*(Ohaus, 1914), which was recorded for the first time in Iraq. Molecular analysis using PCR amplification of a mtCOI gene fragment was conducted on the collected specimens, and comparison with sequences in the NCBI GenBank database identified a new species based on the mitochondrial COI gene, which represents the second record of insects in the database. The obtained COI sequence of *Adoretus hirsutus* has been deposited in the GenBank database under accession numbers OQ4288117, OQ428818, and OQ428819, providing a valuable resource for future research and biodiversity studies. The morphological study includes, a detailed description of the species

with many new characteristics previously not mentioned by other researchers; especially in mouthparts and male genitalia. Some important parts of the species which used in identification, particularly, labrum, mandible, antenna, fore legs, Elytra, Pygidium and male genitalia have been photographed. The species is one of the economic importance and mostly widespread in Kurdistan Region- Iraq. Therefore, it is necessary to comprehensive survey of different regions of Iraq, to collect the samples and update the database of the family Scarabaeidae.

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التشخيص الجزيئي والدراسة المظهرية ل *Adoretus hirsutus* (Ohaus, 1914) (رتبة غمدية الأجنحة: عائلة الجعال: تحت عائلة الجعال) في محافظة أربيل أقليم كوردستان العراق

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• تاريخ استلام البحث 2023/08/05 وتاريخ قبوله 2023/09/03

الملخص

تقدم هذه الدراسة وصفا مفصلا ل *Adoretus hirsutus* (Ohaus, 1914) (Coleoptera:Scarabaeidae: Rutelinae) كأول تسجيل في العراق. تم جمع العينات من بعض ازهار الاعشاب للفترة مابين شهر اذار الى تموز 2022 واستخدمت كمصدر لتفاعل البوليمراز المتسلسل PCR لجزء 710bp من جين mt COI لتحليل النشوء والتطور بهدف مقارنة تسلسل النيكليوتيدات فيها مع نظيراتها في الحشرات الاخرى. تمت مقارنة ومحاذاة قسم من جين mt COI من الحشرة المجمعه مع قاعدة بيانات للبنك الوراثي NCB1 باستخدام اداة BLAST، اضهره نتائج BLAST بانها تسجيل الثاني للحشره في البنك الوراثي ل NCBI. تم تقديم تسلسل COI الخاص ب *Adoretus hirsutus* الى البنك الوراثي بارقام انضمام OQ4288117 ، OQ428818 و OQ428819 . ذكر الصفات التشخيصية للنوع وهي :الشفه العليا كأسي الشكل ، حوافها الجانبية مقعرة بدرجة متوسطة مع وجود صف من الأشواك القصيرة وأمتداه من الوسط ليغطي الذقن . الفكوك العليا غير منتظمة الشكل، في قمتها سن بيضوي الشكل . عقلة الملمس الفكي الأخير 1.5 مرة بقدر طول العقلة الثانية . اللآمس بني يتكون من 10 عقل تنتهي بثلاث عقل جانبية ورقية الشكل متساوية في الطول. يحتوي الحافة الخارجية لساق الرجل الأمامي على ثلاث اسنان حادة. السوءة الذكرية متوسطة الأنحناء ، المقابض الجانبية بيضوية متطاولة، جزءهما القمي حاد جدا. تم تصوير بعض الأجزاء المهمة مثل الشفه العليا. الفكوك العليا. الملمس الفكي . اللآمس .ساق الرجل الأمامي . الغمد. ظهر الحلقة البطنية السابعة والسوءة الذكرية.

الكلمات المفتاحية: التشخيص الجزيئي، الدراسة المظهرية، الجين mtCOI *Adoretus hirsutus* التسلسل.