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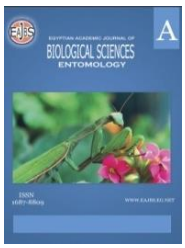

Morphological Variation and DNA Barcoding Identification of *Acanthoscelides obtectus* (Coleoptera: Chrysomelidae: Bruchinae)

Enas H. S. Ghallab, Gawhara M. M. Abu El-Hassan, Radwa M. Azmy, Sohair Gadallah, and Shams Fawki*
Department of Entomology, Faculty of Science, Ain Shams University, Abbassia 11566, Cairo, Egypt
E-mail: shfawki@sci.asu.edu.eg

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ABSTRACT

Acanthoscelides obtectus (Say) is a serious invasive pest that attacks grains and legumes, especially *Phaseolus vulgaris* Linnaeus. It is widely distributed as a pest of *P. vulgaris* in many countries and in Egypt as well. This study applied an integrated approach that combined morphological and DNA barcoding identification of the species. The results yielded direct sequencing of the mitochondrial cytochrome oxidase subunit 1 (COI) gene and defined the morphological variation among individuals of *A. obtectus*. The resulting sequence of the COI gene was compared with the *A. obtectus* gene that was previously documented in GenBank.

INTRODUCTION

Grain legumes are an essential source of nutrition for humans and animals and play a major role in sustainable food production strategy (Calles *et al.* 2019, Vasconcelos *et al.* 2020). Bruchids (pulse beetles) are serious pests that threaten legumes worldwide. Several bruchid species are invasive, having crossed geographical barriers to become widely distributed via human-mediated migrations and the importation or exportation of food grains (Ahmed *et al.* 2019).

Among bruchid genera, *Acanthoscelides* Schilsky is the largest and the most diverse genus of the New World Bruchidae and includes more than 300 species of legume pests (Johnson 1990). The genus was originally divided by Johnson (1983, 1990) and Kingsolver (1968, cited after Johnson 1990) into 14 species groups. The *obtectus* species group was comprised of five species, *A. amplilobus* Johnson, *A. argillaceus* (Sharp), *A. eriosemicola* Johnson, *A. obtectus* (Say), and *A. obvelatus* Bridwell, and is characterized by the following features: a mucro on the hind femur 0.16–0.20 times the length of the first tarsomere, elytra vaguely variegated, similar male genitalia, and pest status on legume plants belonging to subfamily Papilionideae (Romero-Nápoles 2010).

The bean weevil *A. obtectus* is a cosmopolitan beetle that originated in the Neotropics. It is a serious pest that specializes primarily on kidney beans and *Phaseolus vulgaris* L. and also can attack other fabaceous seeds (Thakur 2014, Allotey *et al.* 2016, Gad and Abied 2019). *Acanthoscelides obtectus* exhibits sexual dimorphism represented by variations in the color of antennae and size (Gad and Abied 2019, Budeˇcevi´c *et al.*

2021). The distribution of the species in Egypt was first reported in 2002 in the CABI database (CABI, EPPO 2002). The official record of the species in Egypt was reported by Naroz *et al.* (2019). Gad and Abied (2019) and Ahmed *et al.* (2019) studied morphology, biology, and molecular identification of the species. However, these studies did not include variation among individuals of the species or phylogenetic relationships with closely related species, and the molecular study included only partial sequencing of the COI gene. Accurate identification of invasive pest species is the cornerstone of biomonitoring strategy; hence the need for taxonomic knowledge (Armstrong 2005). Due to the lack of this expertise, new molecular methods have been developed for species identification. Molecular identification depends on molecular markers such as mitochondrial DNA genes (relatively conserved in all taxa/species), which are widely used in molecular investigations, and phylogenetic analyses (Boore 1999, Dowton *et al.* 2002, Zhang *et al.* 2015). DNA barcodes are molecular markers based on the conserved gene sequences of an organism's genetic material (Kuppu *et al.* 2017). DNA barcoding has been successfully integrated with morphological taxonomy for insect species identification in several studies, including Gill *et al.* (2014), Oba *et al*. (2015), Syromyatnikov *et al.* (2017), Yao *et al.* (2017), and Lin *et al.* (2018).

Accordingly, this study aimed to complete the molecular identification of *A. obtectus* using COI gene sequencing and to define the morphological variation among the individuals of the species.

MATERIALS AND METHODS

Insect Culture:

Adult *A. obtectus* were initially obtained from an infestation of kidney beans obtained from a local store in Cairo, Egypt (2018). The colony was maintained on kidney bean seeds under 27°C ± 2°C, 35% ± 5% relative humidity, and a L16: D8 photoperiod in a laboratory located in the Entomology Department, Faculty of Science, Ain Shams University.

Identification of the Species:

The species was identified based on studies by Johnson (1990), Kingsolver (2004), Alvarez *et al.* (2005), Romero-Nápoles (2010), and Naroz *et al.* (2019). Individuals were examined using a stereomicroscope at 100–400x magnification to determine the variation in morphological characteristics among individuals of the species. Body parts of all sampled individuals were examined to determine morphological variation. Representative live variants were identified and used for molecular study.

DNA Extraction:

Genomic DNA was extracted from individuals freshly anesthetized *A. obtectus* in a −20°C freezer using a Qiagen DNeasy Blood & Tissue Extraction Mini Kit (Cat. No. 69504, Germany) according to the manufacturer‘s protocol.

Sequencing and Bioinformatics Analyses of Cytochrome Oxidase C subunit I:

The universal primers used for the amplification of the mitochondrial cytochrome oxidase C subunit I (COI) gene, which was selected according to Folmer et al. (1994) and Simon *et al.* (1994), were LCO1490 (GGTCAACAAATCATAAAGA TATTGG) and HCO2198 (TAAACTTCAGGGTGACCAAAA AATCA). The PCR reaction ingredients for a final volume of 20 µL were: 1× PCR buffer, 3.2 pmol each of the forward and reverse primers, 0.5 unit of Taq polymerase (HotStar Taq DNA Polymerase, Qiagen), and 200 µM deoxynucleotide triphosphate (dNTP).

To optimize the PCR assay, a gradient PCR assay with different annealing temperatures ranging from 55–65°C for 40 cycles was developed. A 1.5% agarose gel

containing ethidium bromide (0.375 µg/ mL) was used for PCR product visualization. Hyperladder II (Bioline) was the DNA marker used.

The optimum thermocycling reaction conditions were as follows: one cycle at 94°C initial denaturation for 30 sec., 35 cycles of denaturation at 94°C for 30 sec., annealing (at 48°C for 30 sec., extension at 72°C for 60 sec.), and a final extension cycle at 72°C for 10 min.

Sequencing and Bioinformatics Analyses of COI:

Direct Sequencing:

The purified PCR products of the target COI gene were direct-sequenced in both directions according to the Sanger dideoxy sequencing method (Sanger *et al.* 1977) under the following conditions: denaturation from 1°C to 96°C, followed by 25 cycles of 30 sec at 96°C, annealing at 50°C for 15 sec, and an extension at 60°C for 5 min. To confer the sequence identity, each single amplified sequence was pairwise aligned against homologous sequences in the GenBank database using NCBI BLASTn (Zhang *et al.* 2000).

RESULTS

Diagnostic Morphological Characteristics of *A. obtectus* (Say 1831):

Body grayish brown; antennae 11-segmented, segment 1 blackish, 2–5 reddish-brown, 6–10 blackish, serrated, segment 11 reddish, knobbed acute apically; eyes rounded, black with a median fringe of golden setae, post ocular setae orange; frons narrower than setae; pronotum rectangular, notched at posterior, separated along the separation line, covered with brownish pubescents, elytra length nearly two times the width, with whitish and brownish setae on anterior margin and golden brown fringe posteriorly; legs reddish-brown, covered with whitish hairs, mid and hind femora black ventrally, femur emarginated ventrally without a carina, hind femur with one mucro, tibia with dorsal, lateral, ventral, and ventrolateral carina; pygidium broad anteriorly, narrow posteriorly.

Sexual Dimorphism:

Male smaller than female, with a vertical pygidium; female bigger with sub-vertical pygidium.

Morphological Variations:

The bean weevil *A. obtectus* displays extensive variation in the number of denticles on the ventral side of the metafemur. The number of these spines can vary greatly among individuals within a population: there may be one, two, or three. Denticles and their variation in number are prominent features.

One long mucro is always present on the ventrolateral margin of the metafemur in addition to 1–3 denticles. The three detected phenotypes were:

1. In phenotypes with only one denticle, the mucro may be attached to the denticle (Fig. 1) or separated (Fig. 2).
2. In the phenotype having two denticles, the denticles may be separated or attached, and the size of the first inner denticle maybe 1/4 or 1/2 the length of the mucro. The last two denticles may be of the same length, or the exterior one may be smaller. The ventrolateral margin of the metafemur may be smooth or serrated (Figs. 3–6).
3. In phenotypes with three denticles, the denticles may be attached (Fig. 7) or separated (Fig. 8).

There is one more characteristic that appears to vary in the detected phenotypes. The color of the ventral side of the mouthpart is black in insects possessing three denticles with the first denticle equal to half the length of the mucro. In the other phenotypes, the mouthparts are reddish-brown ventrally. None of the detected phenotypes are sex-related.

The mitochondrial COI region of all collected samples was successfully amplified using

PCR. The length of the expected PCR product was 710 bp, however, due to technical issues, we had successfully amplified ± 660 bp from *A. obtectus*.

The resultant sequences were searched against the BLAST database (Zhang *et al.*, 2000), as well as against the BOLD system "Barcode of Life Data System V4" (Ratnasingham and Hebert 2007). The highest similarity scores for our *A. obtectus* sequenced the gene in the BLASTn search were 98.7% with the *A. obtectus* complete mitochondrion genome (KX825864.1). The sequences acquired in this study were deposited in the GenBank database under accession numbers OM057841–OM057843.

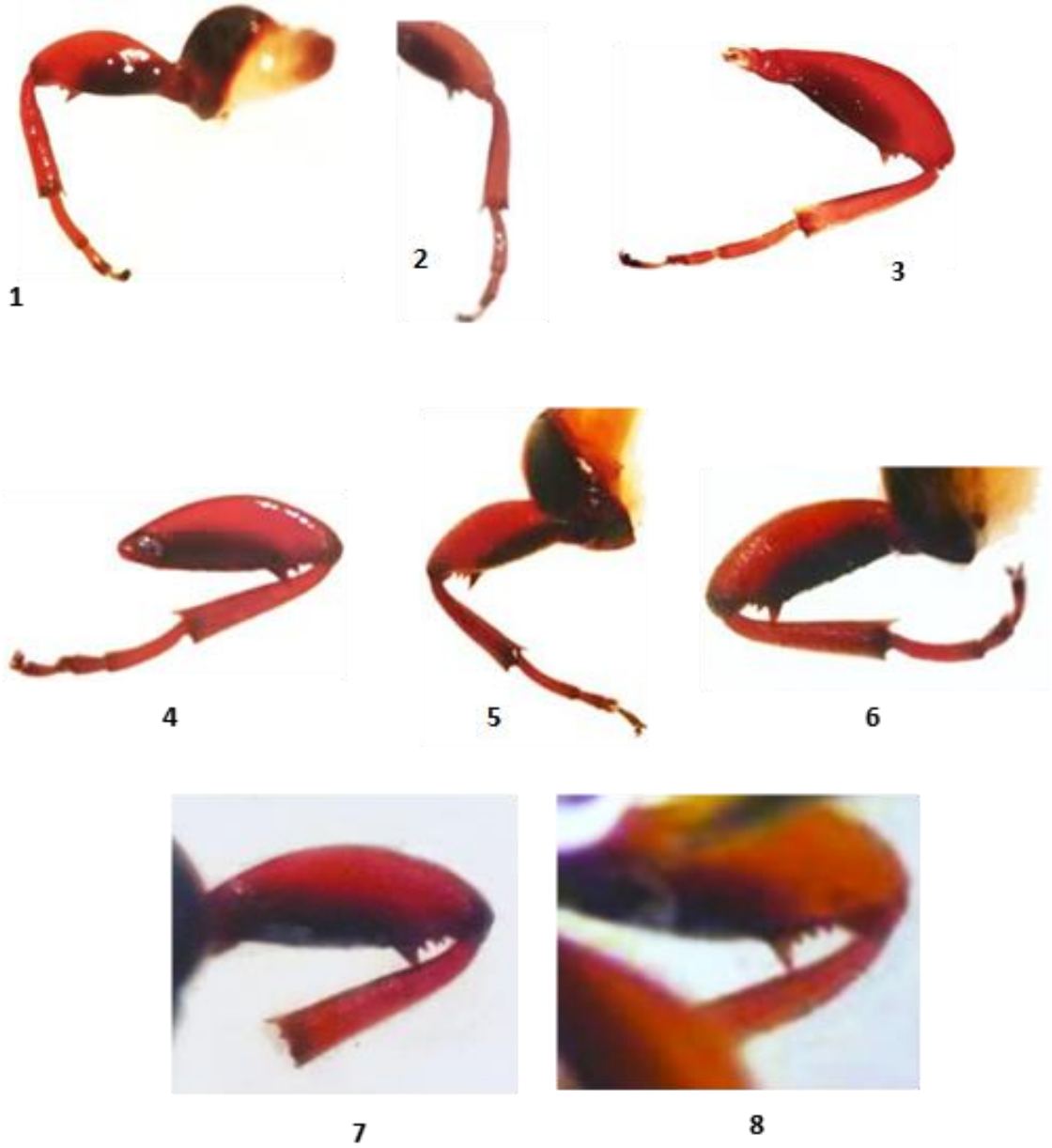

Fig. 1-8. Hind femur. (1) With one denticle attached to mucro; (2) with one denticle separated from mucro; (3) two unequal denticles, the length of the second denticle equal to 1/4 length of mucro; (4) two unequal denticles, the length of second denticle equal to 1/2 length of mucro; (5) two equal denticles as long as 1/4 length of mucro, femur with smooth inner margin; (6) two denticles, femur with serrated inner margin; (7) with three denticles beside the mucro, the last two denticles attached; (8) with three separated denticles beside the mucro.

DISCUSSION

The correct identification of invasive pests is essential in IPM programs (Ahmed *et al.* 2019). Classical taxonomy and DNA barcoding both have specific characteristic advantages and shortcomings. The synergistic use of the two approaches as integrative taxonomy contributes to biodiversity preservation in a rational timeframe and overcomes their individual weaknesses (Sheth and Thaker 2017). Accordingly, the current study combined both morphological and molecular taxonomic approaches to accurately identify the bean weevil *A. obtectus* as an economically important pest.

A morphological examination of the species resulted in the same diagnostic morphological characteristics that were previously mentioned by Johnson (1990), Alvarez *et al.* (2005), Romero-Nápoles (2010), Thakur (2012), Ahmed *et al.* (2019), Naroz *et al.* (2019), and Budeˇcevi´c *et al.* (2021), except for the variation in the numbers of denticles detected in this study. *Acanthoscelides obtectus* exhibits morphological diversification among individuals, with the number of denticles on the ventral side of metafemur varying from 1–3; this is in agreement with Kingsolver (2004), who constructed a key to Hawaiian Bruchidae and characterized the bean weevil as having one long and two or three short denticles on the ventrolateral margin of metafemur. This represented only two forms of the variants that were detected in this study with three or four spines. For every 20 specimens examined, 16 individuals had one mucro and two denticles, and the remaining individuals were divided evenly into the other two forms, having one mucro and one or three denticles. These findings may explain why most of the aforementioned literature defined the species as having only three spines (one mucro + two denticles).

In order to better understand if the COI sequence differs among individuals, it was necessary to amplify it and view its characterization. Our DNA barcoding analyses of the COI gene confirmed the morphological identification. Our results were similar to those obtained by Alvarez *et al.* (2005), Yao *et al.* (2017), and Ahmed *et al.* (2019). The partial sequence of the *A. obtectus* COI gene revealed 98.7% similarity with the reference mitochondrial genome of *A. obtectus* (KX825864.1). The results of this molecular study revealed that there is no difference in the sequencing of COI gene in the aforementioned morphological variants.

One of the possible explanations for this is that even though we amplified a partial region of the gene, it might not have been large enough to reveal the differences between closely related individuals, and a wider range might need to be amplified. This was supported by the results of Dibangou *et al.* (2021), who developed a study that used the 12S rRNA marker as an alternative to COI to identify bruchids for the first time.

In general, more molecular investigations of stored product insects are recommended for accurate rapid identification.

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Ethical Approval: This research paper was approved by the research ethics committee from Faculty of Science, Ain Shams University (ASU-SCI/ENTO/2022/11/2).

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