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Research Article
Morphological, Molecular and Biological Studies on Common Bean Weevil *Acanthoscelides obtectus* (Say) in Egypt

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Abstract

Background and Objective: The bean weevil, *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae) is a serious Neotropical origin insect pest on kidney beans, *Phaseolus vulgaris* L. and other legume seeds. The objective of this study was to discriminate *A. obtectus* through morphological, molecular and biological studies. **Materials and Methods:** All stages of *A. obtectus* were morphologically described and their biology was performed. The adults of both *A. obtectus* and *Callosobruchus chinensis* were characterized by molecular analysis. **Results:** All the developmental stages were morphologically described. The morphological description of antennae, legs and wings of the adults indicated that the insect is *A. obtectus*. The partial sequence of COI gene of *A. obtectus* revealed 99% similarity with *A. obtectus* that previously recorded in GenBank and located in a separate glade in the phylogenetic tree. The biological parameters such as eggs per female, hatchability and durations of the developmental stages were also estimated. **Conclusion:** The morphological description, the partial sequence of COI gene and the biological parameters discriminated *A. obtectus* obviously from *C. chinensis*.

Key words: Beetles, bruchidae, bean weevil, *Acanthoscelides obtectus*, *Phaseolus vulgaris* L., legume seeds, kidney beans, *Callosobruchus chinensis*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Actual human population growth and global climate change may affect the food reserves and the availability of seeds for growing plants in the near future. Post-harvest loss caused by pests may exceed 20% in poorly developed and tropical countries due to inadequate management practices and environmental conditions that allow rapid reproduction of pests, especially in developing countries[1]. Bruchids, commonly known as pulse beetles are a serious threat to legumes worldwide. Many of the bruchid species have crossed the geographical boundaries and have become cosmopolitan in distribution through human-mediated migrations and import/export of food grain. This made these pest species highly adaptive and hence is distributed from temperate to tropical climates. They infest seeds of many grain legumes, both in the field and in storage. The Neotropical origin bean weevil, *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchinae) is a serious pest of kidney beans, *Phaseolus vulgaris* L., *P. lunatus* L. and other legume seeds in Africa[2,3], Australia[4], Europe[5,6], America[7,8], the Mediterranean area[9,10] and various other parts of the world[11]. *A. obtectus* infests the different hosts in the fields and stores and rendered unfit for consumption[12-16]. Populations of *A. obtectus* are most commonly detected in stores of dried legumes and their life cycle appears well adapted for reproduction in a storage environment. The females lay eggs in clusters under or nearby a single seed. The first instar larvae burrow into a seed where the beetles spend their larval and pupal stages. The final instar larvae excavate chambers just below the seed and presence of a larva may be detected by a small "window"[17-19]. This insect causes damage by reducing the mass and/or volume, reducing the physiological quality and germination capacity, increasing the temperature and water content of the seeds[20]. Unlike most of the other bruchids, *A. obtectus* Say's reproductive cycle is continuous, without imaginal diapause for temperatures between 14-35°C and it attacks the beans in fields as well as stored seeds[21]. Larvae feed on beans and cause losses more than 30% of stored products[22]. This insect completes its entire life cycle within stored dry beans without returning to the field[21]. *A. obtectus* may adapt to several leguminosae: its host-plant, *P. vulgaris* L. and some non-host plants such as *Vigna unguiculata* (Walp.), *Cicer arietinum* L. and *Vicia faba* L.[23].

During investigation heavy infested *P. vulgaris* seeds by weevils, this study expected to find *C. chinensis* but some morphological features were different. Therefore, the objectives of this study were: (1) Discriminating of *A. obtectus* morphologically, (2) Characterizing of *A. obtectus* molecularly by the Polymerase Chain Reaction (PCR), sequence analyses and phylogenetic tree targeting the gene cytochrome c oxidase subunit I (COI) and 3) Studying the biology of the insect reared on phaseolus seeds grown in Egypt under laboratory conditions.

MATERIALS AND METHODS

Insect culture: The *Acanthoscelides obtectus* strain was collected from infested kidney beans obtained from field that belongs to Giza Governorate, Egypt during the summer season 2016. The insect was maintained on kidney bean seeds *Pharseolus vulgaris* under 27±1°C, 65±5% relative humidity and L16: D8 photoperiod.

Morphological description: Eggs were examined to identify the shape and size with the aid of a stereo microscope. The eggs were mounted in Hoyer's solution as described by Sharkawi[24], precise description of the egg stage was given. Continuous daily dissections of infested seeds collected after harvesting carried out to study the larval morphological characteristics. The criteria used to determine the larval instars were the head capsule measurements of each instar (10 larvae/instar) by using temporary mounting in Hoyer's solution. A number of infested kidney beans were examined to follow up the development of pre-pupae and pupae. The criteria used to determine the adult measurements were the body measurements of 15 adults (males and females). A stereo microscope was used to examine adults of *A. obtectus* which were selected randomly.

Molecular characterization: The DNA was isolated using ZR Genomic DNATM-Tissue MiniPrep (Zymo Research, USA). According to the manufacturer's manual, cytochrome oxidase subunit I (COI) gene[25]. Folmer region was amplified using My *Taq*TM Red DNA Polymerase (BioLine, UK). The primers used for the amplification of the COI gene were 5'-TCA ACC AAC CAC AAA GAC ATT-3' and FishR1 5'-TAG ACT TCT GGG TGG CCA AAG AAT CA-3'. The thermal program consisted of initial step of 2 min at 54°C followed by 35 cycles of 40 sec at 94°C, 40 sec at 54°C and 1 min at 72°C followed in turn by final extension of 10 min at 72°C. The PCR products were visualized on 1.2% agarose gels, purified and sequencing. Amplicons were purified using DNA clean and concentrator TM-25. Products were labelled using the BigDye Terminator V.3.1 Cycle sequencing kit (Applied Biosystems, Inc.) and sequenced bidirectionally using ABI 3730 Automated Sanger sequencer (Macrogen, Inc.). Sequence was evaluated,

assembled and aligned using Geneious V8.1 software. Refined sequence was used to identify the species using DNA related database (BOLD and BLASTn). Phylogenetic analysis was performed using Mega[26] version 5.1.

Biological determination: The biology of *A. obtectus* was conducted in an incubator under the optimum constant laboratory conditions of 27±1°C and 65±5% R.H. Twenty newly laid eggs were carefully transferred to glass vials (4×10 cm). Ten vials (replicates) were used and checked daily until egg hatching. The incubation period and hatchability percentages were calculated. Furthermore, three glass jars of one pound capacity each were provided with sterilized kidney beans at a rate of ½ kg/jar. Every jar was infested by newly laid eggs obtained from the stock culture at a rate of 4000 eggs. After 3 days, random sample of 10 seeds was taken daily from every jar and immersed in water about 20 min and then carefully examined under a stereo microscope then carefully dissected to collect larvae, pre-pupae and/or pupae. Seeds daily dissection continued until all larvae transformed into pupae. Collected larvae and pre-pupae were preserved in 70% ethyl alcohol and glycerol (1:1 by volume). Preserved larvae and pre-pupae were separately mounted on slides using Hoyer's media and examined under a stereo microscope. Measurements of body length, length and width of the head capsules were taken. Moreover, 20 fresh pupae (<24 h old) were removed carefully from the dissected seeds and transferred into glass vials (4×10 cm). Ten vials (replicates) were, incubated under the previously mentioned optimum conditions and checked daily until adult emergence to record the pupal duration. Additionally, 10 pairs (one female and one male each) of the newly-emerged adults were individually transferred into glass vials each measuring 4×10 cm. Vials were examined daily to remove the eggs using sieving until the females died. Eggs were kept into Petri dishes and pre-oviposition, oviposition and post- oviposition periods, adult longevity and the number of eggs laid/ female were recorded.

Statistical analysis: The F-value (one-way ANOVA at $p<0.05$) and Duncan tests using SPSS computing program version 20 were adopted for calculating biological aspects of *A. obtectus* using ANOVA as described by Snedecor and Cochran[27].

RESULTS

Morphological description: All the eggs were loosely onto the seeds. Freshly laid eggs were milky white color and elongate in shape (Fig. 1). Figure 1a shows the group of *A. obtectus* eggs and Fig. 1b shows the high magnification of a single egg. The larva has six instars (Fig. 2). Figure 2(a-f) show head capsules of the 1st, 2nd, 3rd, 4th, 5th and 6th instar larva of *A. obtectus,* respectively. On the other hand, the corresponding whole body were in Fig. 2(g-l). The head capsule and body measurements of the larval instars were provided in Table 1. There are significant differences between all larvae instars in the length of head capsule and body. However, the width of head capsule and body may record insignificant differences between two or more successive larval instars. The pre-pupa is white in color, while, the pupa is exerts type with white yellowish in color (Fig. 3). Figure 3a and b show the lateral and dorsal view of pre-pupae, respectively. Whereas, Fig. 3c and d show the ventral and lateral view of the pupa, respectively. The body

Table 1: Length and width measurements of head capsule and body of different larval instars of *A. obtectus*

	Range (Mean±SE)			
	Head capsule		Body	
Larval instar	Length(mm)	Width (mm)	Length (mm)	Width (mm)
1st	0.317±0.023[a]	0.309±0.023[a]	0.406±0.041[a]	0.146±0.016[a]
	(0.28-0.45)	(0.28-0.40)	(0.325-0.52)	(0.130-0.195)
2nd	0.458±0.005[b]	0.367±0.019[ab]	0.659±0.026[b]	0.509±0.017[b]
	0.47-0.63	0.29-0.43	0.585-0.78	0.45-0.58
3rd	0.500±0.001[c]	0.367±0.019[ab]	0.864±0.031[c]	0.650±0.00[b]
	0.50-0.90	0.33-0.73	0.78-0.975	0.65-0.65
4th	0.609±0.015[d]	0.434±0.042[b]	1.641±0.05[d]	1.048±0.095[c]
	0.64-0.79	0.56-0.80	1.43-1.82	0.65-1.365
5th	0.725±0.005[e]	0.634±0.015[c]	2.016±0.032[e]	1.430±0.051[d]
	0.74-0.88	0.57-0.77	1.95-2.15	1.30-1.69
6th	0.807±0.015[f]	0.663±0.017[d]	2.23±0.06[f]	1.644±0.044[d]
	0.69-0.87	0.56-0.71	2.08-2.54	1.56-1.88
F-value	201,159	44,776	187.58	55.589
p-value	<0.001	<0.001	<0.001	<0.001

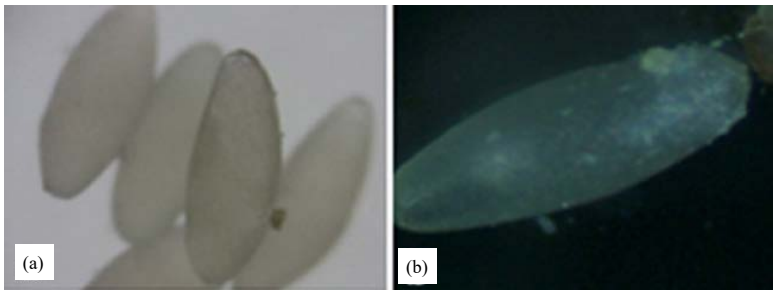

Fig. 1(a-d): Egg of *Acanthoscelides obtectus* (a) Group, (b) High magnification

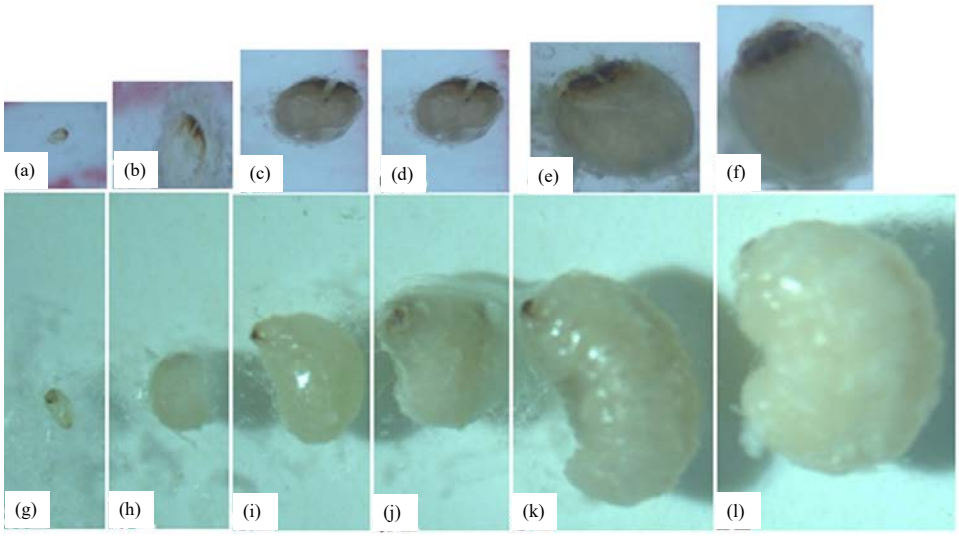

Fig. 2(a-l): *Acanthoscelides obtectus*: head capsules (a) 1st, (b) 2nd, (c) 3rd, (d) 4th, (e) 5th and (f) 6th and their whole body of larval instars, (g) 1st, (h) 2nd, (i) 3rd, (j) 4th, (k) 5th and (l) 6th

Table 2: Body length and width of pre-pupa, pupa and adult of *A. obtectus*

	Length (mm)		Width (mm)	
Stages	Range	Mean±SE	Range	Mean±SE
Pre-pupa	3.8-3.9	3.87±0.03	1.54 -1.75	1.62±0.07
Pupa	3.5-3.8	3.66±0.05	1.65-1.95	1.80±0.06
Adult	2.9-3.2	3.00±0.05	1.45-1.65	1.50±0.05

length and width of pre-pupa, pupa and adult were provided in Table 2. The adult head is grayish brown and its mouth parts are blackish in color (Fig. 4a-c). Antennal segments 1-4 filiform and segments 5-10 broadened and more serrated and the segment 11 non-serrated and acute apically. The color of antennal segments 1-4 is grey, 5-10 is dark blackish, while the color of the segment 11 is yellowish brown (Fig. 4c). Elytra about twice as long as broad and covered with patterned brown and gray (Fig. 4d). Hind wings were hepatitis with spot black (Fig. 4e). Legs reddish brown, except mid venter femur of meso and meta legs, which are yellow in color. Inner ridge of ventral margin of hind femur legs has three teeth-like and spine at the posterior end (Fig. 4f-h). Anterior tooth twice as long as the posterior teeth, with a slight posterior inclination. Posterior teeth equal in size and have similar posterior inclination (Fig. 4h). Ovipositor elongated and supported by 2 pairs of dorsoventrally sclerotized rods, which unite sub-terminally. The ovipositor orifice is supported by a single pair, along with numerous setae of variable size (Fig. 4i-j). Figure 5 shows the heavy infested kidney beans by *A. obtectus* which had one or more holes (Fig. 5a) and newly emerged adult (Fig. 5b).

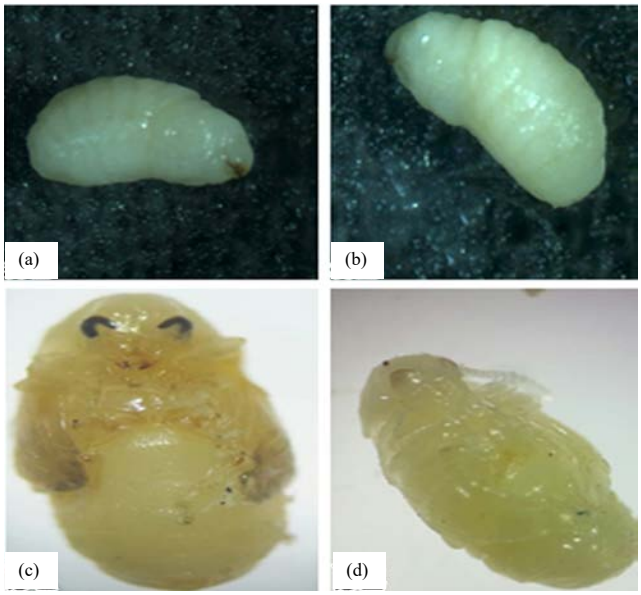

Fig. 3(a-d): (a-b) Pre-pupa and (c-d) Pupa of *Acanthoscelides obtectus*

Fig. 4(a-j): Adult of *Acanthoscelides obtectus*, (a) Females, (b) Male, (c) Mouth part and its antennae, (d) Fore wings, (e) Hind wings, (f) Fore leg, (g) Middle leg, (h) Hind leg, (i) Genitalia in a female and (j) Genitalia in a male

Fig. 5(a-b): (a) Heavy infested kidney beans by *Acanthoscelides obtectus* and (b) Newly emerged adult of *A. obtectus*

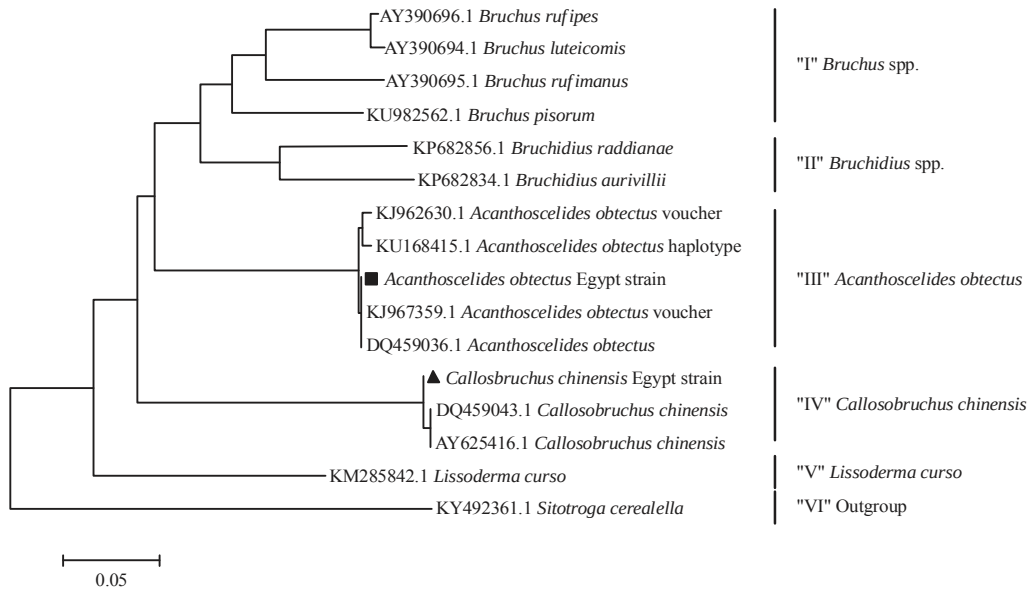

Fig. 6: Phylogenetic tree of *Acanthoscelides obtectus* (■) and *Callosobruchus chinensis* (▲) for the COI gene, reconstructed by Bootstrap test-neighbor joining. The Lepidopteran insect *Sitotroga cerealella* is used as outgroup

Molecular characterization: This study is considered the first attempt to differentiate *A. obtectus* beetle from *C. chinensis* beetle those found in Egypt by sequencing the COI barcode region. The partial sequence of COI gene of *A. obtectus* revealed 99% similarity with *A. obtectus* that previously recorded in GenBank and located in a separate glade "cluster III" in the phylogenetic tree (Fig. 6). The bean weevil *A. obtectus* found in Egypt was closely related to the Serbian and Finland strains. However, *C. chinensis* found in Egypt locate in another clade "cluster IV" far away from *A. obtectus* (Fig. 6).

The biology: The mean number of eggs was 11.9 eggs/female. The mean of incubation period was 6.10±0.28 days. The mean of hatchability was 96.00±2.00%. The duration mean was 7.37±0.82, 5.00±0.00, 3.00±0.37, 3.13±0.44, 3.53±0.40 and 3.33±0.23 days for the 1st, 2nd, 3rd, 4th, 5th and 6th instar larva, respectively. The mean duration of pre-pupa and pupa was 2.81±0.28 and 4.17±0.21 days, respectively. The longevity attained 7.2 and 8.9 days in male and female, respectively. The pre-oviposition, oviposition and post-oviposition periods of matted female attained 1.2, 5.6 and 2.1 days, respectively (Table 3).

Table 3: Some biological parameters of *A. obtectus* reared on kidney beans under laboratory conditions

Biological parameters	Minimum	Maximum	Mean±SE (days)
Incubation period	5	7	6.10±0.28
Hatchability (%)	80	100	96.00±2.0
1st larval instar	3.00	10.00	7.37±0.82
2nd larval instar	5.00	5.00	5.00±0.00
3rd larval instar	2.00	4.00	3.00±0.37
4th larval instar	2.00	5.00	3.13±0.44
5th larval instar	2.00	7.00	3.53±0.40
6th larval instar	2.00	4.00	3.33±0.23
Total larval period	16.00	35.00	25.36±2.26
Per-pupa period	2.00	5.00	2.81±0.28
Pupal stage	3.00	5.00	4.17±0.21
Male longevity	6.00	12.00	7.20±0.69
Female longevity	3.00	14.00	8.90±1.19
Pre-oviposition period	1.00	2.00	1.20±0.13
Oviposition period	2.00	10.00	5.60±0.88
Post-oviposition period	0.00	3.00	2.10±0.35
No. of egg/female	2.00	35.00	11.90±3.27
Total life cycle	24.00	59.00	41.24±3.94

DISCUSSION

Current, it is believed that leguminous crops in Egypt are infested by *Callosobruchus* spp.[28,29] because they seem to very close morphologically with *A. obtectus* which needs more qualified taxonomist to differential it from *Callosobruchus* spp. (e.g., *C. chinensis*). Therefore, the accurate identification of exotic and potentially invasive taxa is very important in IPM programs. Traditionally, identification has been based on morphological diagnoses provided by taxonomic studies. Only experts such as taxonomists and trained technicians can identify taxa accurately because it requires special skills acquired through extensive experience. The DNA barcoding has become increasingly common since it was proposed in 2003, this simple technique has attracted attention from taxonomists, ecologists, conservation biologists, agriculturists, plant quarantine officers and others and the number of studies using the DNA barcode has rapidly increased[30].

This is the first intensive study on the dried bean beetle, *A. obtectus* Say (Coleoptera: Chrysomelidae, Bruchinae) using both morphological description and molecular characterization. Hence, the combination of morphology and molecular results can provide reliable identifications. Bean weevil is a serious pest of kidney beans and food legumes that has strong adapt-ability to the environment. It has multiple hosts in some parts of the Mediterranean area[9,10]. Similar morphological characters of *A. obtectus* have been reported by Say[31], Johnson[32], Kingsolver[7], Alvarez *et al.*[33] and Thakur[34,35]. Morphological characters of *A. obtectus* were resemble structure of genitalia (male and female) of the Indian population of *A. obtectus* are similar to the native population of America described by Johnson[36] and Kingsolver[7]. The results of sequence analyses and phylogenetic tree of the gene COI confirmed the morphological identification. The partial sequence of COI gene of *A. obtectus* revealed 99% similarity with *A. obtectus* that previously recorded in GenBank and located in a separate glade in the phylogenetic tree.

The results of the biology were similar to Thakur and Renuka[12] and Godfrey and Long[37] that freshly laid eggs were milky white and ellipsoidal in shape. Since most of the eggs were not glued onto the seeds it is essential for the freshly hatched first instar larva to find and select the host seeds for the remaining stages of development and food requirements. The data of Parsons and Credland[38], Paul *et al.*[3] and Thakur[34], who studied with the eggs were not glued onto the seeds. The females lay eggs in clusters under or nearby a single seed. Similar observations on biology, ovipositor and larval-pupal development of bruchids have been observed by Southgate[11] and Thakur and Banyal[39]. Oviposition lasted for 7-10 days and the incubation period was 8-10 days and larval development was completed in 14-20 days[12].

It is, however, very likely that in *A. obtcctus* the males pass on nutrients to the males, which would extend female longevity under aphagous conditions. This may plain why mated females without beans lived longer than the virgins; the mated males get extra nutrients but do not spend it on eggs. This hypothesis is supported the report of Fox[40] who found in another aphagous bruchid beetle, *Callosobruchus maculutus*, that ejaculated-derived nutrients contributed to female somatic maintenance[14]. Similar results have already been reported for adults do not feed on the seeds. *A. obtectus*, as other bruchid beetles, is physiologically suited for adult aphagy and females emerge with adequate energy to develop and lay most of their potential eggs. Thus, adults need neither food nor water to reproduce[14]. The optimal generational development of *A. obtectus* occurred at 30°C, although the

insect completed its development within a range of 20-32°C in 34-63 days[41]. In general, more molecular investigations are recommended in stored products insects for accurate identification. This will help better understanding the insect species in their products and environment and benefit for their controlling programs.

CONCLUSION

The insect *A. obtectus* adapted itself on the environmental conditions in Egypt and became a key pest on legume seeds beside other bruchid beetles. This insect could be discriminated from *C. chinensis* by its specific morphological characteristics and partial sequence of COI gene. It is recommended to take account consideration the presence of *A. obtectus* as a key pest in the control program of leguminous insects in Egypt.

SIGNIFICANCE STATEMENT

This study discovered the new key pest *A. obtectus* beside *C. chinensis* on leguminous seeds in Egypt that can be beneficial for integrated pest management programs for insect pests of leguminous seeds. This study will help the researchers to uncover the critical areas of distinguish *A. obtectus* from *C. chinensis* that many researchers were not able to explore. Thus a new theory on insect pests on leguminous in Egypt may be arrived at the consideration of an imported *A. obtectus* as a key insect pest on leguminous seeds in Egypt.

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