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Using *COI* gene for identifying *Aenasius bambawalei* Hayat (Hymenoptera: Encyrtidae), a parasitoid of *Phenacoccus solenopsis* Tinsley in Iran

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Abstract

Aenasius bambawalei Hayat (Hymenoptera: Chalcidoidea: Encyrtidae) is a solitary endoparasitoid of *Phenacoccus solenopsis* Tinsley. It was first found in Iran in 2011 following the introduction of its host, *P. solenopsis*, in the country in 2009. In order to confirm the identification of *A. bambawalei* which was based on classic taxonomy, the mitochondrial cytochrome oxidase I (*COI*) gene was sequenced in *A. bambawalei* samples collected from southern Iran. The parasitoids were retrieved from the specimens collected from four provinces of Iran, namely Khuzestan, Bushehr, Hormozgan and Fars. The polymerase chain reaction was performed according to standard protocols on the extracted DNA to amplify a fragment of the *COI* gene. Obtained 633 bp *COI* sequences were aligned with five *A. bambawalei COI* sequences available in GenBank from China and Pakistan and two other sequences as outgroups. In addition, a dendrogram was prepared and graphically depicted in a neighbor-joining tree. Our identifications were confirmed by the matches between our sequences and those of the GenBank database. Sequences displayed a BLAST hit and had a sequence similarity percentage of 99%-100% with the GenBank sequence assigned to the same species. The results of *COI* sequence analysis were in agreement with the morphological identifications. The findings of this study pointed out that *COI* gene sequence data is a precise and accurate tool for the rapid identification of *A. bambawalei* species.

Keywords: DNA markers, Mealybug, Molecular identification, Parasitism, PCR

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Introduction

Aenasius bambawalei Hayat (Hymenoptera: Chalcidoidea: Encyrtidae) is a solitary endoparasitoid of *Phenacoccus solenopsis* Tinsley and has been reported in the areas of Pakistan and India (Poorani et al., 2009), in which cotton grows. The species *A. bambawalei* was found as the only dominant and aggressive parasitoid in charge of reducing *P. solenopsis* in the mentioned countries after the initial establishment of this mealybug (Rishi Kumar et al., 2010). Adult female parasites infect the third instar nymphs of *P. solenopsis* (Ashfaq et al., 2010), leading to the death of the host prior to maturity. The first record of the presence of hymenopteran parasitoid *A. bambawalei* on *P. solenopsis* in Iran was from Khuzestan province, southwest Iran. The parasitoid appeared first at low density on *Althaea officinalis* and *Hibiscus rosa-sinensis* in Dezful in June 2011 (Mossadegh et al., 2013). Later it was found on different host plants in Mahshahr and eastern Ahvaz according to Mossadegh et al. (2013). In the absence of the insecticidal control of *P. solenopsis*, a rapid rise in the population density of *A. bambawale* was observed at eastern Ahvaz, leading to 95% parasitism in *P. solenopsis* in 2012 (Mossadegh et al., 2015). Afterwards, *A. bambawalei* was reported by Mossadegh et al. (2015) in provinces Hormozgan, Fars, Bushehr, Kerman, and Kohgiluyeh and Boyer-Ahmad, as well as Gheshm Island. Recently, research has focused on the growth, development, and biological parameters of *A. bambawalei* in Iran (Joodaki et al., 2018; Tamoli Torfi et al., 2019).

Molecular diagnostic techniques provide the required means for rapid, accurate, and inexpensive identification when morphological characters are not sufficient (Armstrong & Ball, 2005). DNA barcoding is a taxonomic method capable of timely identifying the species utilizing a short genetic marker in the mitochondrial DNA of an organism (Ladoukakis & Zouros, 2001; Mehravar et al., 2017). The mitochondrial cytochrome oxidase I (*COI*) gene has received more attention during recent years and leads the use of 500-650 bp from the first half of the *COI* gene in DNA barcoding. In order to confirm the morphological identification of *A. bambawalei*, a fragment of the *COI* gene was sequenced and evaluated in *A. bambawalei* samples collected from southern Iran.

Materials and Methods

We isolated parasitoid specimens out of the materials collected from four provinces of Khuzestan, Bushehr, Hormozgan, and Fars (Table 1). These parasitoids were identified by classic taxonomy as *A. bambawalei* and were preserved in 95% ethanol. The DNA was extracted from the samples using DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the protocols of the manufacturer. The DNA extracts were amplified by PCR for a fragment of the *COI* gene according to standard protocols. A pair of parasitoid-specific primers, AenF1 (5'-GTTTCTCACATAATTTGTAG-3') and AenR1 (5'CCTCGGAGGATAAAAAGAC-3') was applied to amplify a 744-bp fragment of *COI*, as stated by Ashfaq et al. (2010). The *COI* sequences of *A. bambawalei* obtained in FASTA format were imported into the MEGA 11.0 software package for sequence alignment and were compared to some other *A. bambawalei* published sequences. *Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae) (accession number MG850873.1; Iran) and *Aulacus sinensis* He & Chen (Hymenoptera, Evanioidea) (accession number MG923485.1; China) *COI* sequences were downloaded from GenBank and used as outgroups. Multiple sequence alignments were carried out by the ClustalW algorithm using default parameters. Furthermore, genetic distances were calculated by the pairwise Kimura 2-parameter and 1000 bootstrap replicates to calculate standard error utilizing the MEGA 11.0. The dendrogram was derived and graphically displayed in a neighbor-joining (NJ) tree (Saitou & Nei, 1987).

Results and Discussion

In the current study, four morphologically identified *A. bambawalei* specimens belonging to four southern provinces of Iran were successfully sequenced for the mitochondrial *COI* gene. A 633-bp *COI* sequence fragment was used after trimming for analysis. Our identifications were confirmed by matching our sequences with those in the GenBank database (Table 2). Sequences were shown to have a BLAST hit and a sequence similarity percentage of 99%-100% with a GenBank sequence assigned to the same species (Table 2). The results of *COI* sequence analysis coincided with the morphological features.

Pairwise genetic distance values ranged from 0 (between Ab_B and Ab_H) to 0.014 (between Ab_F and Ab_H) (Table 3).

Our obtained sequence data were analyzed and compared with five *A. bambawalei COI* sequences available in GenBank from China and Pakistan along with two outgroups. In order to provide a visual representation of the data, we generated an NJ tree based on the number of nucleotide differences between *COI* sequences (Figure 1). However, this tree does not provide phylogenetic information.

As pointed out in the introduction section, the mealybug pest was observed in Pakistan in 2005 and *A. bambawalei* was described in 2009 as an

Table 1. Details of studied materials: Specimen codes, collected locality, identification, parasitized host, and collection date of the samples used for DNA extraction

Specimen code	Locality	Identification (morphology)	Parasitized host	Collection date
Ab_H	Hormozgan prov. (Bandar_Abbas)	*Aenasius bambawalei*	*Phenacoccus solenopsis*	Nov 2013
Ab_F	Fars prov. (Lar)	*Aenasius bambawalei*	*Phenacoccus solenopsis*	Nov 2013
Ab_B	Bushehr prov. (Bushehr)	*Aenasius bambawalei*	*Phenacoccus solenopsis*	Aug 2014
Ab_A	Khuzestan prov. (Ahvaz)	*Aenasius bambawalei*	*Phenacoccus solenopsis*	Oct 2011

Table 2. Blast hits between our sequences and those from the NCBI GenBank database: specimen codes (GenBank accession number), blast hits, corresponding taxon, similarity percentage, and coverage

Specimen code (GenBank accession #)	Identification (Morphology)	Best GenBank BLAST hit	Corresponding taxon	Similarity %	Coverage (bp)
Ab_H (OL716106)	*Aenasius bambawalei*	KP686434.1	*Aenasius bambawalei*	99.05%	630
Ab_F (OL716107)	*Aenasius bambawalei*	KP686434.1	*Aenasius bambawalei*	99.53%	633
Ab_B (OL716108)	*Aenasius bambawalei*	KP686434.1	*Aenasius bambawalei*	99.00%	603
Ab_A (OL716109)	*Aenasius bambawalei*	KP686434.1	*Aenasius bambawalei*	100%	633

Table 3. Pairwise genetic distances (lower matrix) and standard error estimates (upper matrix) observed between *Aenasius bambawalei* samples

	Ab_H (OL716106)	Ab_F (OL716107)	Ab_B (OL716108)	Ab_A (OL716109)
Ab_H (OL716106)		0.005	0	0.004
Ab_F (OL716107)	0.014		0.004	0.003
Ab_B (OL716108)	0	0.012		0.004
Ab_A (OL716109)	0.010	0.005	0.010	

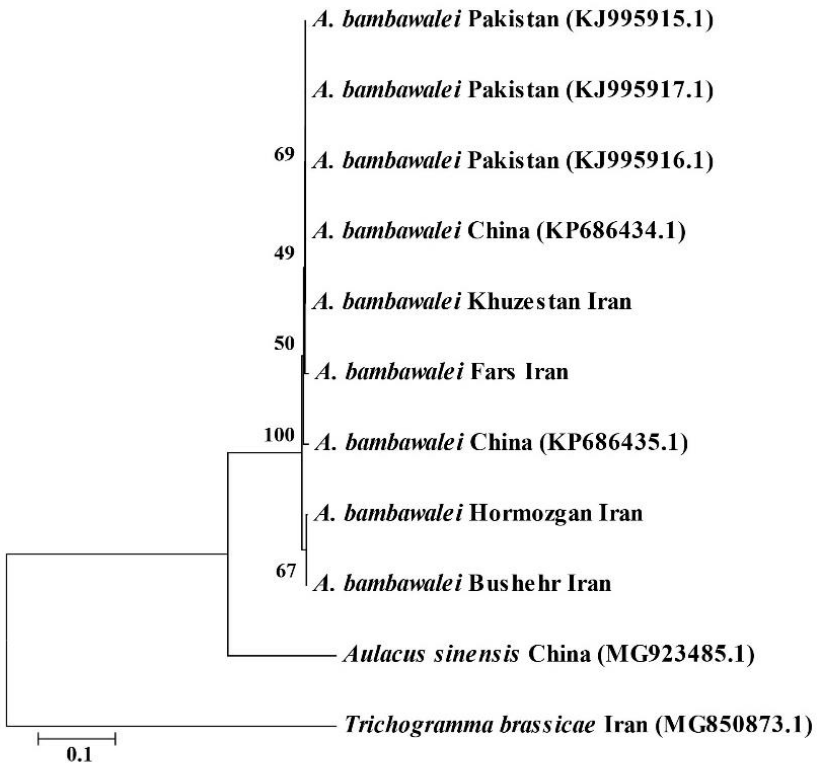

Figure 1. Neighbor-joining tree constructed using distances for the *COI* DNA sequences (K2P model, bootstrap=1000) of different *A. bambawalei* isolates. *Trichogramma brassicae* and *Aulacus sinensis* were used as outgroups

effective endo-parasitoid of this pest (Ashfaq et al., 2010). In Iran, *P. solenopsis* and *A. bambawalei* were recorded for the first time in 2009 and 2011, respectively. It could be suggested that the mealybug pest was imported from Pakistan to Iran by agriculture products in 2009 (Moghadam & Bagheri, 2010). In terms of genetic distances, Iranian sequences were placed close to *A. bambawalei* sequences published in GenBank from Pakistan and China. Therefore, the morphological identifications were confirmed. *A. bambawalei* might have entered Iran together with *P. solenopsis* from Pakistan and spread throughout the country. However, investigations on larger samples from distinct parts of Iran and neighboring countries are recommended to further assess this hypothesis. The findings of this study revealed that the *COI* gene sequence data is a precise and accurate tool allowing rapid identification of *A. bambawalei*.

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کاربرد ژن *COI* در تشخیص زنبور *Aenasius bambawalei* Hayat (Hymenoptera: Encyrtidae)، پارازیتوئید *Phenacoccus solenopsis* Tinsley در ایران

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چکیده

زنبور *Aenasius bambawalei* Hayat (Hymenoptera: Chalcidoidea: Encyrtidae) یک پارازیتوئید داخلی انفرادی روی شپشک *Phenacoccus solenopsis* Tinsley است که در سال ٢٠١١ و پس از ورود شپشک در ٢٠٠٩ به ایران، در استان خوزستان ثبت وگزارش شد. به منظور تأیید شناسایی انجام شده در گزارش زنبور که بر اساس تاکسونومی سنتی بود، اقدام به توالی یابی ژن میتوکندریایی سیتوکروم اکسیداز یک (*COI*) در نمونه‌هایی از زنبور *A. bambawalei* در نواحی جنوبی ایران گردید. برای این منظور نمونه‌هایی از زنبور پارازیتوئید از استان‌های خوزستان، بوشهر، هرمزگان و فارس برای مطالعه انتخاب شد. دی ان ای استخراج شده از این نمونه‌ها در معرض واکنش PCR قرار گرفت و قطعه‌ای از ژن *COI* بر اساس پروتکل‌های استاندارد رایج، تکثیر و توالی یابی شد. داده‌های یک توالی ٦٣٣ جفت بازی از ژن *COI* از نمونه‌های مذکور به همراه پنج توالی از کشورهای چین و پاکستان که از بانک ژن استخراج گردید و دو اوت گروپ مقایسه و تحلیل شدند. بر این اساس یک دندروگرام استخراج و به صورت گرافیکی توسط درخت neighbor-joining نمایش داده شد. توالی‌های ما با توالی‌های موجود در بانک ژن از گونه *A. bambawalei* بر اساس جستجوی Blast تطابق داشت و شباهتی بین ٩٩/٠٠٪ تا ١٠٠٪ نشان دادند. در نهایت نتایج توالی یابی ژن *COI* شناسایی‌های مورفولوژیک زنبور که بر اساس تاکسونومی سنتی بود را تأیید کرد. نتایج این مطالعه خاطر نشان می‌سازد که توالی ژن *COI* می‌تواند یک ابزار دقیق و صحیح برای شناسایی سریع این زنبور فراهم سازد.

کلیدواژه‌ها: PCR، پارازیتیسم، شپشک، نشانگرهای DNA، شناسایی مولکولی

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