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Taxonomic Position of Iranian Isolates of *Eretmocerus mundus* (Merect), a Parasitoid of *Bemisia tabaci* (Gennadius)

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ABSTRACT- *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), is one of the most important pest of vegetable and fruit crops. This polyphagous pest has a range of natural enemies including the parasitoid *Eretmocerus mundus* (Merect) (Hymenoptera: Aphelinidae). To determine the molecular profile and taxonomic status of Iranian isolates of *E. mundus*, parasitized *B. tabaci* samples were collected from cotton crops in Maharlu region (Shiraz, Fars province). DNA was extracted from samples and subjected to polymerase chain reaction using a pair of mitochondrial cytochrome oxidase subunit I (*mtCOI*) gene specific primers. A DNA fragment of approximately 850 bp in size was amplified, cloned and sequenced. Comparison of the DNA nucleotide (nt) sequence of the *mtCOI* gene of three Iranian isolates with the same region of Chalcidoid species (Hymenoptera) available in the GenBank indicated that the *mtCOI* gene of Iranian isolates of *E. mundus* shares 98.8-99.5 % sequence homology. Among the Chalcidoid species compared, the Iranian isolates of *E. mundus* were more closely related to two Moroccan isolates of *E. mundus*; their *mtCOI* gene shared 96.7-97.7 % homology, being more distantly related to a *Heterandrium* species (family Agaonidae), and their *mtCOI* gene nt sequence sharing 81.2-83.8 % homology.

Keywords: ***Bemisia tabaci*, *Eretmocerus mundus*, Parasitoid, Mitochondrial Cytochrome Oxidase Subunit I (*Mtcoi*)**

INTRODUCTION

Bemisia tabaci (Gennadius) (Hemiptera: Aleyrodidae) biotype complex is a key pest of many horticultural crops worldwide. This phloem-feeding insect, especially the B biotype which is the predominant biotype in most areas of Iran (16), causes damages to

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crops through direct feeding, contaminations with honeydew and sooty mould and the transmission of plant viruses, primarily begomoviruses (4). Many biological characteristics, including broad host-range, multivoltinism, high reproductive rate, high population size, the ability to vector plant viruses and a propensity to develop resistance to most insecticides have all contributed to the difficulty of controlling this whitefly (15). One of the key challenges posed by *B. tabaci* is its ability to develop resistance against insecticides (4). To decrease the need for applications of insecticides, the use of natural enemies, in particular the parasitic wasp, *Eretmocerus mundus* (Mercet) (Hymenoptera: Aphelinidae) is one of the most important ways to reduce pest population (8).

The interactions between whiteflies and their parasitoids are complex and can be quite diverse in various host parasitoid systems (7). Arthropod parasitoids and predators are present everywhere and operate continuously on all life stages of the whitefly *B. tabaci* (2). *E. mundus* lays eggs on the second-third instar nymph of *B. tabaci* underneath the leaves; after which its first instars hatch and penetrate the host (9). *E. mundus* was identified as the dominant parasitoid species against all whitefly-infected plants in Fars province, southern Iran; its efficiency increasing against parasite *B. tabici* through the end of the season as the population of whiteflies reaches to its highest rate (1).

During the past decade, techniques of molecular and cellular biology have been accepted in molecular entomology by many researchers. The application of genetic diagnostics under the umbrella of classical taxonomy is imperative for the successful development of biological control against *B. tabaci* (19). Morphological and molecular taxonomic criteria that were sufficiently discriminatory to enable the separation of different species of *Eretmocerus* attacking *B. tabaci* and *Trialeurodes vaporariorum* (Westwood) in Australia have been previously described (4). Although *B. tabaci* is a well known pest in cotton fields of Iran and *E. mundus* is identified as the dominant parasitoid species of this important pest (1), most studies have been restricted to characterizing the morphological and biological aspects of these insects (1 and 20). In this paper, the molecular characterization and taxonomic position of *E. mundus* isolates in Iran are evaluated. Nucleotide sequences from the cytochrome oxidase I (*COI*) gene were used to determine its phylogenetic relationships.

MATERIALS AND METHODS

Sample preparation

E. mundus samples (from parasitized nymphs of *B. tabaci*) were collected from a cotton (*Gossypium hirsutium* L.) field in Maharlu region of Shiraz, Fars province during 2008 and stored in vials in 95% ethanol at -20˚C.

Morphological identification

The taxonomy of Aleyrodidae is based almost entirely on the final (fourth) larval instar or puparial stage (the late fourth nymphal stage having red eyes) (1). Therefore, in the

present study *B. tabaci* was identified morphologically by the microscopic examination of slide-mounted puparium or pupal cases of collected samples (17). *E. mundus* was identified using *Ertmocerus* identification key (11).

DNA extraction, amplification and sequencing

DNA was extracted from single, whole specimens using the method described by De Barro and Driver (3) with minor modifications. Specimens were washed briefly in sterile distilled water to remove alcohol prior to homogenisation. Each sample was homogenised in 20 µl lysis buffer (50 mM KCl, 100 mM Tris-HCl, pH 8.0, 45% Tween 20, 45% Triton X 100, 0.2% gelatin, 120 µg/mL proteinase K). Homogenization was carried out using a pipette tip for each sample that had been melted and moulded to fit in a 1.5 µl microcentrifuge tube. After homogenization, a further 20 µl of lysis buffer was added. The homogenate was incubated at 65˚C for 30 min. To inactivate proteinase K, samples were boiled for 10 min. Sterile distilled water was added to yield a final homogenate volume of 40 µl. Samples were then stored at -20˚C for further experiments. To detect *E. mundus*, the *mtCOI* gene was selected as a marker gene. The primers used to amplify the *mtCOI* gene fragment were: C1-J-2195 (5′-TTGATTTTTTGGTCATCCAGAAAGT-3′) and L2-N-3014 (5′-TCCAATGCACTAATCTGCCATATTA-3′) (6). PCR was carried out in a total reaction volume of 25 µl containing 1.0 µl of each DNA template, oligonucleotide primers (each 1 µM), 200 µM each of dCTP, dGTP, dTTP and dATP. 1.5 mM $MgCl_2$ and 0.5 or 0.75 units of *Taq* DNA polymerase (Cinagen, Iran) in the reaction buffer were provided by the same source. The mixture was subjected to a PCR program optimized based on Frohlich *et al.* (6) including a cycle of 5 min at 94˚C and 35 cycles of 30 s at 94˚C, 45 s at 50˚C and 1 min at 72˚C. The final cycle was followed by a 10-min incubation period at 72˚C.

PCR products were subjected to electrophoresis on 1% agarose gel and the amplified DNA fragments were stained with ethidium bromide and visualized by a UV light source. The PCR product containing the single amplified DNA fragment with the expected size (~850 bp) was purified using a QIAquick PCR Purification kit (QIAGEN, Germany) and cloned into a pTZ57R/T plasmid using an InsT/Aclone PCR Product Cloning Kit (Fermentas) according to the manufacturers' instructions and sequenced using M13 universal primers and an ABI PRISM system at Macrogen (South Korea). The nucleotide sequence data obtained from three individual parasitized nymphs of *B. tabaci* (parasitoids) from Maharlu region were submitted to the GenBank Nucleotide Sequence Database (http://www.ncbi.nlm.nih.gov/genbank).

Sequence Analysis

Sequence data were analyzed and compared with related sequences available in GenBank (Table 1) using standard nucleotide-nucleotide BLAST program from the National Center for Biotechnology Information (NCBI). Genetic distances were calculated using the Vector NTI 11 software package (InforMax, Bethesda, MD). Phylogenetic analysis was carried out using MEGA5 (10 and 18) and Megalign softwares. A phylogenetic tree was constructed by Maximum Likelihood Method (MEGA5 software), using the GTR + G model based on 100 replicates.

Table 1. Characteristics of Chalcidoidea super family members used in sequence comparisons with Iranian *E. mundus* isolates. *Eurytoma caninae* was used as an outgroup species.

Species (isolate)	Country of origin	Genomic sequence accession number	Reference
***Eretmocerus mundus* (isolate E1)**	**Iran**	**JN627214**	**present study**
***Eretmocerus mundus* (isolate E2)**	**Iran**	**JN627215**	**present study**
***Eretmocerus mundus* (isolate E3)**	**Iran**	**JN627216**	**present study**
***Eretmocerus mundus* (isolate GDEL_14)**	**Morocco**	**EU017330**	**(5)**
***Eretmocerus mundus* (isolate GDEL_22)**	**Morocco**	**EU017331**	**(5)**
***Eretmocerus cocois* (isolate 44P)**	**France**	**EU017334**	**(5)**
***Eretmocerus desantisi* (isolate GP2411_3)**	**France**	**EU017332**	**(5)**
***Heterandrium* sp. (strain A12.7.w)**	**Panama**	**EF158865**	**(14)**
***Philocaenus warei* (strain PHIL.WAR)**	**Panama**	**AY014967**	**(13)**
***Eurytoma caninae* (voucher H169)**	**France**	**EF525181**	**(12)**

RESULTS AND DISCUSSIONS

Detection of *Eretmocerus Mundus* in Parasitized Nymphs of *B. Tabaci*

DNA extracted from parasitized nymphs of *B. tabaci* collected from cotton crops in Maharlu region in Shiraz, Fars province, Iran, was analyzed by PCR for the presence of parasitoids using one specific primer pair designed to amplify the *mtCOI* gene. A PCR product with an expected size (~850 bp) was amplified from parasitized nymphs of *B. tabaci* (Fig. 1, lanes 2 and 3). No DNA fragment was obtained with water, used as negative control in PCR reaction (Fig. 1, lane 1). The amplified fragments were cloned into plasmid vector pTZ57R/T. The sequence of three independent clones was determined. The size of the amplified fragments, excluding the primers, was found to be exactly 822 bp. Comparison of the resulting nucleotide sequences with those available in GenBank using BLAST program revealed that these sequences were related to the *mtCOI* gene sequences of members of *Eretmocerus* genus and had the highest level (96.7-97.7 %) of identityto *mtCOI* gene sequences of *E. mundus* species (Fig. 2) indicating that the collected *B. tabaci* nymphs were parasitized by *E. mundus*. The nt sequences of *COI* gene of the three independent Iranian *E. mundus* isolates have been deposited in GenBank under the accession numbers JN627214, JN627215 and JN627216, respectively, sharing 98.8-99.5% nucleotide sequence homology (Fig. 2).

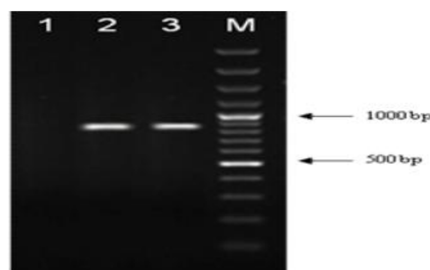

Fig. 1. Electrophoresis pattern of PCR product amplified from DNA extract of parasitized nymphs of *B. tabaci* (lanes 2 and 3) using one specific primer pair designed to amplify the *mtCOI* gene. No DNA fragment (lane 1) was amplified from a PCR reaction when water was used as a template (negative control). M= 100 bp molecular weight marker (Fermentas).

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10		
1		22.0	22.3	22.5	8.0	19.6	23.9	10.0	8.5	8.3	**1**	EF525181\France
2	109.0		98.8	99.3	85.5	83.3	84.4	87.9	97.2	96.7	**2**	JN627216\Iran
3	109.7	0.5		99.5	85.9	81.2	84.8	89.6	97.7	97.5	**3**	JN627215\Iran
4	109.7	0.0	0.5		86.1	83.8	85.0	89.6	97.7	97.5	**4**	JN627214\Iran
5	107.4	17.1	16.6	16.9		83.2	84.2	94.2	89.8	89.5	**5**	EU017334\France
6	112.4	19.1	18.9	19.3	18.9		87.3	85.6	87.7	87.8	**6**	EF158865\Panama
7	104.6	18.1	17.4	17.9	18.8	13.9		86.9	88.2	88.0	**7**	AY014967\Panama
8	108.3	11.0	11.2	11.2	6.1	15.7	15.2		89.2	89.0	**8**	EU017332\France
9	111.8	2.4	2.4	2.4	10.9	13.3	12.9	11.7		99.3	**9**	EU017330\Morocco
10	111.0	2.4	2.4	2.4	11.1	12.9	12.9	11.7	0.5		**10**	EU017331\Morocco
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**		

Fig. 2. Nucleotide percent identity and divergence between selected Chalcidoid species isolates used in present study

Relationship of Iranian isolates of *E. mundus* with other Chalcidoid species and its taxonomic position

Comparison of the nt sequence of *COI* gene of Iranian isolates of *E. mundus* with those of other Chalcidoid species for which nt sequence was available (Table 1), indicated that Iranian isolates of *E. mundus* are most similar to two Moroccan isolates of *E. mundus*, their *COI* gene nt sequence sharing 96.7-97.7 % homology (Fig 2). However, the similarity of these sequences with the same region of two other species of the same genus [i.e. *E. cocois* and *E. desantisi* (5)] was less than 90 percent (Fig. 2). Among the species of the Chalcidoidea super family used in this analysis, *E. mundus* was more distantly related to a *Heterandrium* species (family Agaonidae), their *COI* gene nt sequence sharing 81.2-83.8 % homology (Fig. 2).

A dendrogram obtained by phylogenetic analysis with the MEGA5 program indicates three distinct clusters (Fig. 3). The first cluster is divided into two sub-clusters, represented by Iranian and Moroccan isolates of *E. mundus*, respectively. The second cluster (Fig. 3, Cluster II) is represented by two non-*Eretmocerus* species used in this study including *Philocaenus warei* (13) and a species of *Heterandrium* (14). The third cluster (Fig. 3, Cluster III) includes the other two species of *Eretmocerus*, i.e. *E. cocois* and *E. desantisi* (5), and seems to be closer to *E. mundus* isolates (Cluster I) than to species of the second cluster (Fig. 2).

The outcome of this analysis indicated that *COI* gene sequence data can be used to discriminate species of the Chalcidoidea super family and is a precise and accurate tool to provide rapid identification of different taxons.

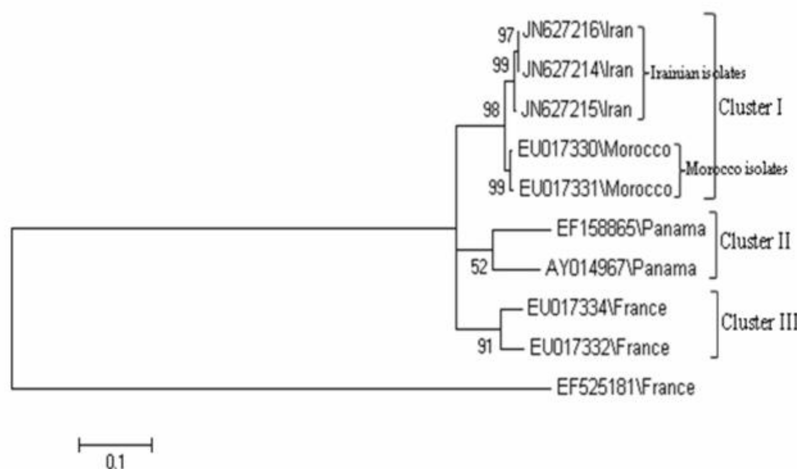

Fig. 3. Phylogenetic tree constructed from the alignment of nucleotide sequences of 10 Chalcidoid species isolates using Maximum Likelihood Method (MEGA5 software) based on 100 replicates. The numbers indicate bootstrap percentages. See Table 1 for genomic sequence accession numbers

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جایگاه تاکسونومی جدایه های ایرانی *Eretmocerus mundus* (Merect)¤ پارازیتوئید سفیدبالک پنبه *Bemisia tabaci* (Gennadius)

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چکیده- سفیدبالک پنبه *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) از مهم‌ترین آفات محصولات زراعی و باغی است. این آفت پلی فاژ دارای مجموعه‌ای از دشمنان طبیعی است که یکی از مهم‌ترین آنها زنبور پارازیتوئید *Eretmocerus mundus* (Merect) (Hymenoptera: Aphelinidae) می‌باشد. در این تحقیق به منظور تعیین مشخصات مولکولی و جایگاه تاکسونومی *E. mundus*، از سفید بالک‌های پارازیت شده یک مزرعه پنبه در منطقه مهارلو در استان فارس جهت استخراج دی ان ای و انجام آزمون واکنش زنجیره‌ای پلیمراز استفاده شد. با استفاده از یک جفت آغازگر اختصاصی ژن زیر واحد شمارهٔ یک سیتوکروم اکسیداز (*COI*) میتوکندریایی، قطعه‌ای در حدود ۸۵۰ جفت باز تکثیر شد. مقایسه همردیف سازی چند گانه ترادف نوکلئوتیدی این ناحیه از ژنوم سه جدایه ایرانی *E. mundus* با ناحیه مشابه از ژنوم زنبورهای بالا خانوادهٔ Chalcidoidea نشان داد که میزان تشابه در ژن *COI* میتوکندریایی جدایه های ایرانی *E. mundus* مورد استفاده در این مطالعه بیش از ۹۹/۵ -۹۸/۸ درصد است. ژن *COI* میتوکندریایی جدایه های ایرانی *E. mundus* بیشترین شباهت (۹۷/۵- ۹۶/۷ درصد) را با جدایه های همین حشره از مراکش نشان داد. در حالی که در میان زنبورهای بالا خانوادهٔ Chalcidoidea مورد استفاده در این مطالعه، جدایه های ایرانی *E. mundus* کمترین میزان تشابه (۸۳/۸-۸۱/۲ درصد) را با یک گونه ی جنس *Heterandrium* (از خانواده Agaonidae) نشان دادند.

واژه‌های کلیدی: ***Bemisia tabaci*؛ *Eretmocerus mundus*؛ پارازیتوئید؛ زیر واحد شمارهٔ یک سیتوکروم اکسیداز میتوکندریایی(*mtCOI*)**

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** مکاتبه کننده