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First record of *Aphelinus paramali* Zehavi and Rosen 1989 (Hymenoptera, Aphelinidae), parasitoid of *Aphis pomi* de Geer (Hemiptera, Aphididae) in Iran, and its phylogenetic position based on sequence data of ITS2 and COI genes

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Abstract

The occurrence of *Aphelinus paramali* (Zehavi & Rosen) (Hym., Aphelinidae) was evidenced from North east Iran, in association with *Aphis pomi* (de Geer). This species is reported from Iran for the first time, and *A. pomi* is introduced as a new host for this parasitoid. Detailed morphological characters were studied with scanning electron microscopy (SEM) photographs. Also, sequences of ribosomal internal transcribed spacer 2 (ITS2) and cytochrome oxidase subunit I (COI) genes were used for determining species boundaries and comparing with other *Aphelinus* species. Different results were obtained in phylogenetic analysis of these two regions. Analysis of COI gene supported the closer relationship of this species with *Aphelinus abdominalis*. This is the first data about comprehensive characterization of a parasitic wasp using morphological characters, SEM and two-locus information from Iran.

Key words: *Aphelinus paramali*, COI, Iran, ITS2, Mashhad, new record.

Introduction

Aphids (Hemi., Aphididae) are among the most important pests (Iversen & Harding 2007), attacked by diverse groups of natural enemies, of which the aphid parasitoids are one of the most important biocontrol agents (Graham 1976). Few species of the aphelinids (Hym., Chalcidoidea, Aphelinidae) are in association with the aphids (Zuparko 1997). The family Aphelinidae include 33 genera and 1168 species placed in seven subfamilies, among them the aphid parasitoids restricted to subfamily Aphelininae (Yasnosh 1976). Species identification of Aphelinidae is rather difficult because of their small size and the paucity of clear morphological differences (Prokrym *et al*. 1998). Studies on Aphelinid species of Iran include a list of 43 species reported by Modarres Awal (1997) and the reports of Fallahzadeh *et al*. (2002), Davoodi *et al*. (2004), Abd-Rabou and Ghahari (2005) and Ghahari *et al*. (2010). Several molecular methods, including allozyme electrophoresis (Walton *et al*. 1990), random amplified molymorphic DNA (RAPD) (Kazmer *et al*. 1995), microsatellite DNA variability (Vanlerberhe-Masutti & Chavigny 1997) and sequences of internal transcribed spacer 2 (ITS2) (Zhu & Greenstone 1999; Zhu *et al*. 2000; Heraty *et al*. 2007), have been used to differentiate *Aphelinus* species. ITS2 was the first molecular marker used to distinguish strains identified as *Aphelinus varipes* (Foerster), *Aphelinus albipodus* (Hayat and Fatima) and *Aphelinus hordei* (Kurdjumov) (Zhu *et al*. 2000). This region is a non-coding and rapidly evolving region which has been commonly used successfully as a molecular marker to discriminate among closely related insect species, where morphological characters bear no significant insights (Campbell *et al*. 1993; Collins & Paskewits 1996; Stouthamer *et al*. 1999; Zhu & Greenstone 1999; van Veen *et al*. 2003), as well as phylogenetic studies and inter-intraspecific relations (Hillis & Dixon 1991).

As a result, in this study we report *Aphelinus paramali* as an aphelinid parasitoid of *Aphis pomi* from Iran (Mashhad) for the first time, of which specific confirmation is presented based on the sequences of ITS2 and cytochrome oxidase subunit I (COI) genes, and detailed morphological characters using scanning electron microscopy (SEM).

Materials and methods

Collection and preparation of specimens

A survey was carried out during 2009–2010 to determine the parasitoids of *Aphis pomi* in pome fruit orchards in the Mashhad region located in the North eastern part of Iran. In order to collect the parasitioid wasps, we collected apple tree leaves bearing aphid colonies in the field and reared them in transparent glass vessels covered by mesh. The rearing vessels were kept at room temperature for 2–3 weeks until the adult parasitoids emerged. The emerged wasps were clipped daily using an aspirator and dropped into 96% ethanol for further examination. External morphology was illustrated using an Olympus™ BH2 phase-constrast microscope. Microscopic slides were prepared using Hoyer's medium (Rosen & DeBach 1979). Measurements were taken using an occular micrometer. The ratio mesurements were based on slide-mounted specimens. Collected specimens were identified to the level of genus using identification key "Annotated keys to the genera of Nearctic Chalcidoidea (Hymenoptera)" (Gibson *et al*. 1997). The specimens have been sent to James B. Woolley from Department of Entomology, Texas A&M University, College Station, TX, USA, for confirmation of specific identity. The SEM images of the species have been obtained with LEO 1450VP scanning electron microscope (LEO Co. Ltd., Oberkochen, Germany) after gold coating by mini sputter coater SC7620 (Quorum Technologies, East Sussex, England).

Molecular studies

DNA was extracted using AccuPrep Genomic DNA Extraction Kit™ (Bioneer Corporation, Daejeon, South Korea) (http://www.bioneer.com) following the manufacturer's instructions. A single individual wasp, kept at –20°C, was crushed with a micropestle in 200 μL lysis buffer and 20 μL proteinase K. The homogenate was incubated at 60°C for 4 h. The supernatant was extracted and stored at –20°C. Polymerase chain reactions (PCRs) were carried out in an Eppendorf mastercylcer gradient (Eppendorf, Hamburg, Germany) in standard 25 μL reactions containing 1 μL DNA template, 2.5 μL (10X) buffer, 1 μL $MgCl_2$, 0.5 μL deoxynucleotide triphosphate (dNTP), 1 μL forward and reverse primer (10 pmol/μL) and 0.3 μl *Taq* polymerase (5U). The primers used to amplify the ITS2 region were: (forward) 5′-TGTGAACTGCAGGACACATG-3′ and (reverse) 5′-GTCTTGCCTGCTCTGAG3′ (Stouthamer *et al*. 1999). For COI gene amplification, the primer set reported by Folmer *et al*. (1994) including LCO1490: 5′-GGTCAACAAA TCATAAAGATATTGG-3′ (forward) and HCO2198: 5′-TAAAC TTCAGG GTGACCAAAAAATCA-3′(reverse) were used.

Temprature conditions for ITS2 amplification were denaturation at 94°C for 60 sec; annealing at 50°C for 90 sec and extension at 72°C for 90 sec (30 cycles, plus an initial denaturation at 94°C for 1 min and a final extension at 72°C for 8 min). For the COI region these were denaturation at 94°C for 60 sec; annealing at 54°C for 90 sec and extension at 72°C for 90 sec (30 cycles, plus an initial denaturation at 94°C for 1 min and a final extension at 72°C for 8 min).

All products were gel-purified in a 1% agarose gel and then cleaned using a Bioneer gel band purification kit (Bioneer). Sequencing reactions were performed in a 3730XL DNA analyzer in (Macrogen Co., Seoul, South Korea) (http://dna.macrogen.com). Primers for the sequencing reaction were those used in the amplification step. All sequences were confirmed in both directions and repeated.

The sequence chromatograms were checked using Bioedit software (Hall 1999). Sequences for the ingroups and outgroups were provided from EMBL/NCBI GenBanks (Table 1). Sequences were aligned using ClustalX software (Thompson *et al*. 1997) with default settings. MEGA4 (Tamura *et al*. 2007) was used to estimate evolutionary distances based on the Kimura two-parameter (K2P) model and moreover to compute the basic statistical analysis.

Phylogenetic analysis was done using maximum likelihood method with 1000 bootstrap replications (Felsenstein 1985) for COI with the software package PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/).

For phylogenic relationships analysis of COI, *Trichogramma* was used as an outgroup. This selection was based on previous molecular phylogenetic analyses, which had strongly supported *Trichogramma* genus as a sister group of family Aphelinidae (Heraty *et al*. 2007; P. Rugman Jones, pers. comm.).

Results and discussion

Morphological study

In total 27 female and one male specimens of *Aphelinus paramali* (Zehavi and Rosen) were collected in association with *Aphis pomi*.

Table 1 Species used in phylogenetic analysis and their GenBank accession numbers

ITS2		COI	
Spesies	Accession number	Spesies	Accession number
Aphelinus hordei	EU561656	*Eretmocerus eremicus*	FM210161-63
Aphelinus hordei	EU561646-50	*Eretmocerus mundus*	FM210165-69
Aphelinus asychis	DQ350563-66	*Encarsia berlesei*	GQ922199-201
Aphelinus mali	AY941827	*Encarsia diaspidicola*	GQ922196-198
Aphelinus mali	DQ350567	*Encarsia formosa*	FM210158-60
Aphelinus mali	AY941827	*Trichogramma evanescens*	GQ367960
Aphelinus near *mali*	DQ350568	*Trichogramma achaeae*	DQ177918
Aphelinus varipes	DQ350571	*Trichogramma cacoeciae*	DQ177917
Aphelinus varipes	DQ350582-83	*Trichogramma chilonis*	DQ177915
Aphelinus varipes	DQ350573-74	*Trichogramma dendrolimi*	DQ177912
Aphelinus varipes	DQ350576	*Aphelinus abdominalis*	FM210123
Aphelinus varipes	EU561657	*Aphelinus abdominalis*	FM210124
Aphelinus varipes	DQ350578-80		
Aphelinus albipodus	AY603665-66		

Female

Body length about 0.8 mm–1.6 mm. Compound eyes with short inter-ommatidial setae, Fore wing (Fig. 1a) 2.4 times as long as wide; triangle at base separated from linea calva by one complete row of setae, accompanied by 5–10 setae in incomplete rows. Maxillary palps two-segmented (Fig. 1b), labial palps 1-segmented. Antennal scape (Fig. 1c), covered sparsely with semi-erect setae, 4.5 times as long as wide, pedicel twice as long as wide, about half length of scape; first (F1) and second (F2) funicular segments short, subequal, 0.8 times as long as their maximum width, slightly narrower than pedicel; third funicular segment 1.2–1.5 times as long as wide, slightly longer than F1 and F2 together, bearing one elongate sensillium; club 3 times as long as wide, bearing 4–10 elongate sensillae. Thorax (Fig. 1d–e) reticulated dorsally, mesoscutum 1.5 times as long as scutellum, bearing about 40 short setae, and 2 long setae at posterior margin, scutellum with 2 pairs of long setae, each axilla bearing 3–5 short setae; each parapsis bearing one long seta. Mid-tibial spur 0.8 times length of the basitarsus.

Gaster finely reticulate, slightly shorter than head and thorax together. Ovipositor (Fig. 1f–g) complex at rest 3 times as long as wide and as long as middle tibia; sheaths 0.35–0.40 times of ovipositor length (Zehavi & Rosen 1989).

Coloration

Head, thorax and propodeum black; 1–3 abdominal segments yellow, the rest dark brown to black. Wings hyaline. Scape and pedicel dark, other segments yellow. All coxae black. Fore leg femur with varying amounts of dark centrally, pale on apical; middle femur dark (except apical portion), hind femur entirely yellow, fore tibia yellow, middle tibia with varying amounts of dark centrally, hind tibia dark except apices; all tarsi yellow, except dark hind basitarsus (Zehavi & Rosen 1989).

Male (based on one male specimen)

Similar to the female, differing mainly in structure of antennae. Length about 0.9 mm. Scape 3 times as long as wide, bearing 4 tubercles, truncate sensillae on its ventral view; third funicular segment twice as long as wide, club as long as or slightly longer than scape. Mid-tibial spur slightly shorter than basitarsus.

Molecular study

ITS2 gene was amplified with the flanking region of 5.8S and 28S rDNA. The boundaries of the ITS2 were determined using the conserved sequence of the flanking regions by comparison with other sequences extracted from GenBank. The sequence of this gene as well as the COI DNA region were submitted in GenBank (http://www.ncbi.nlm.nih.gov) with accession numbers HQ438286 and JF521493, respectively.

In the current work, ITS2 sequence analyses showed high similarity in some of *A. varipes*, *A. hordei* and *A. albipodus* strains, which is supported by 100% bootstrap values. Analysis results based on ITS2 gene did not provide enough information about monophyly of different taxons.

Such is the case for the results obtained by Zhu and Fang (2009). Similar results were gathered when another gene, 16S was used. The 16S rDNA sequences showed sufficient similarity for *A. varipes*, *A. albipodus* and *A. hordei* with

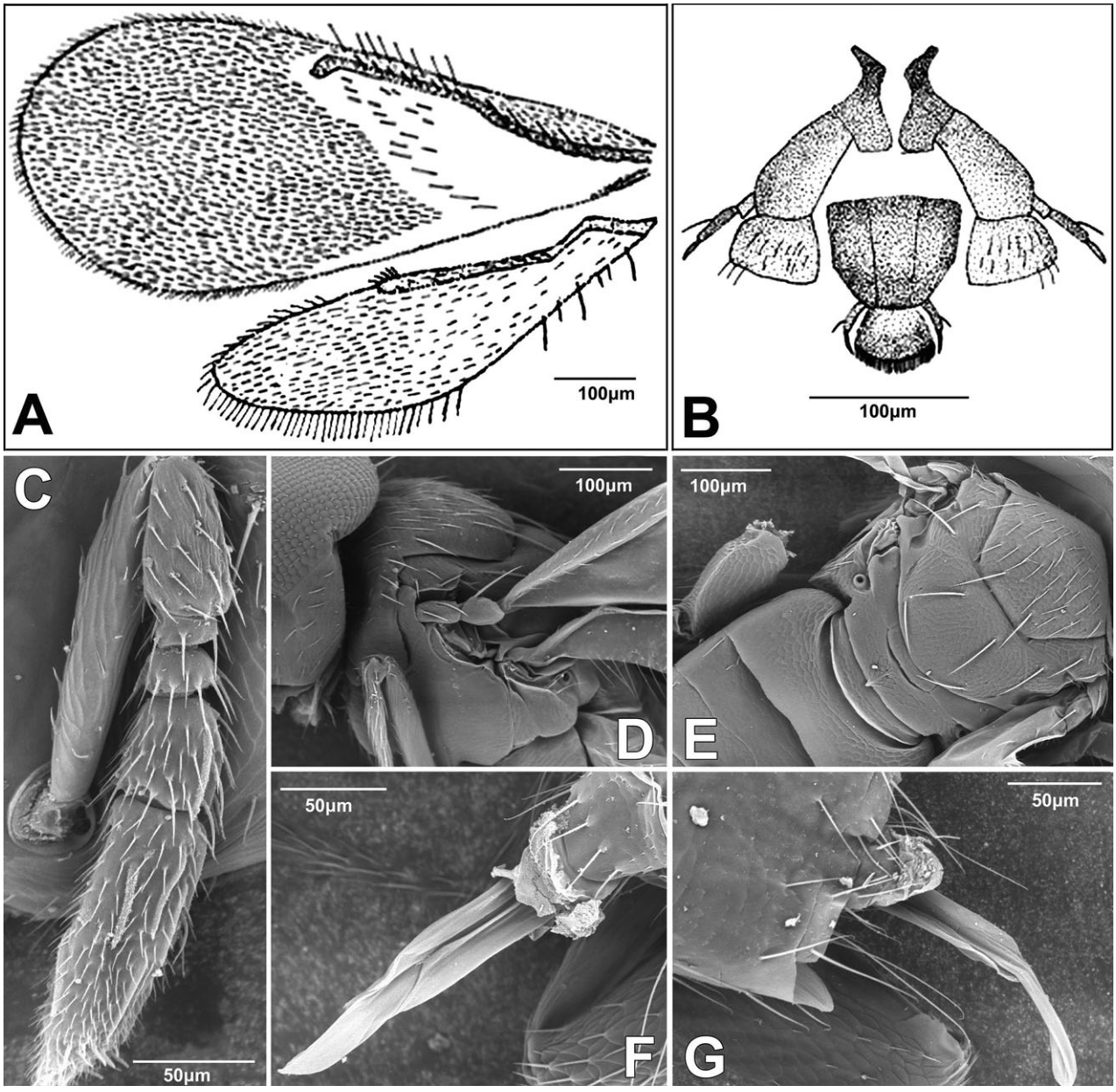

Figure 1 Morphological characters of *Aphelinus paramali* (Zehavi and Rosen) female. (a) Fore- and hind wings; (b) mouth parts; (c) antenna; (d) lateral view of thorax; (e) dorsal view of thorax; (f) dorsal view of ovipositor; (g) lateral view of ovipositor.

100% bootstrap values and these species could be the same species or complex species, while they were distinguishable from *A. asychis* (Chen *et al*. 2002).

Pairwise distance of ITS2 sequences calculated by the Kimura 2-parameter (K2P) model showed about 0.009% nucleotide difference for *A. paramali* and *A. varipes.* As regard to this, there was no record for the 5′ end of the COI gene sequence in GenBank from other species of *Aphelinus*; phylogenic analysis of this gene was performed at the family level. A 402 bp fragment was aligned among them; 60% of sites were conserved and 40% and 33% were variable and parsimony-informative sites, respectively. Phylogenetic relationships analysis based on the COI sequence, using the maximum likelihood method, showed five clades: the first one contained these species of Trichogrammatids; the second contained *Encarsia* sp.; the third included two specimens of *Aphelinus abdominalis* and *A. paramali*; the fourth contained *Eretmocerus eremicus*; and the fifth contained *E. eremicus*. COI sequence analysis showed similarity of *A. paramali* with *A. abdominalis* by 75% bootstrap support (Fig. 2).

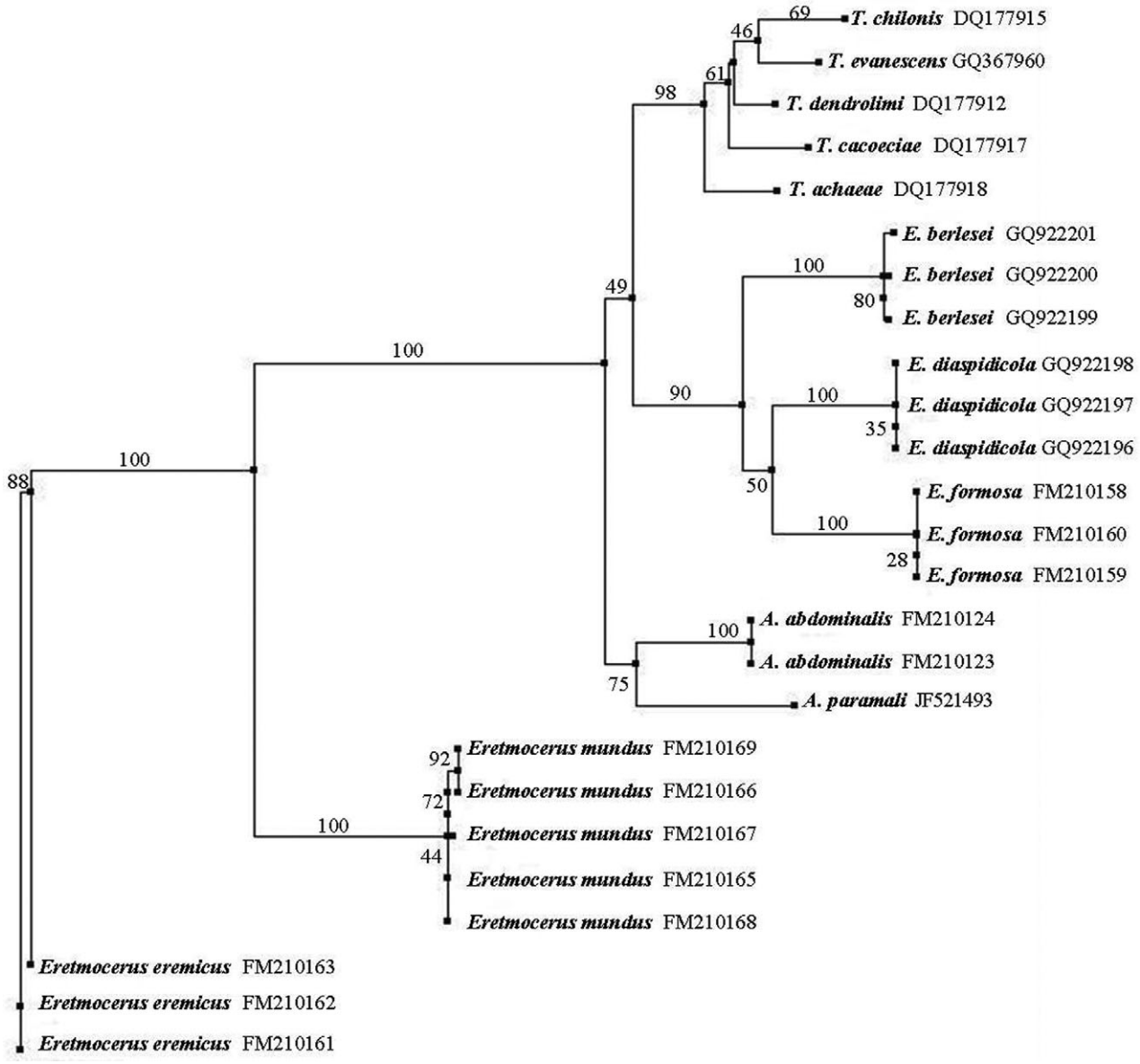

Figure 2 Phylogenetic relationship among *Aphelinus* and related genera from neighbor taxons based on cytochrome oxidase subunit I (COI) sequences using maximum likelihood method.

Mean pairwise distance of COI sequences was 0.155% (range. 0.00–0.262%) which was calculated by the K2P model. This nucleotide difference between *A. paramali* and *A. abdominalis* was 0.101% while there was no intraspecific difference between *A. abdominalis* populations.

Aphelinus paramali was originally reared from *Aphis gossypii* (Glover) and *Aphis punicae* (Passerini) from Israel (Zehavi & Rosen 1989), subsequently recorded from the same aphid in California (Godfrey & McGuire 2004) under the name of *A.* near *paramali*. This form was also recorded from Egypt (Abd-Rabou 2005) and Angola (Prinsloo & Neser 1994). This is a first record of *A. paramali* from Iran together with a new host record. On the other hand, different aphids, including *Myzus persicae* (Sulzer), *Toxoptera aurantii* (Boyer de Fonscolombe) and *Aphis spiraecola* (Patch) have been suggested as tentative hosts for *A.* near *paramali* in California (Godfrey & McGuire 2004). Up to now, some aphid parasiotids including *Aphidius matricariae* (Haliday) (Rakhshani *et al.* 2008) and *Binodoxys angelicae* (Haliday) (Starý *et al.* 2000) have been recorded as major parasitoids of *A. pomi* in Iran.

Many *Aphelinus* species are morphologically similar (DeBach 1969; Darling & Werren 1990; Heraty *et al*. 2007).

Zuparko (1997) suggested that *A. paramali* and *A. mali* have high similarity with each other. Zehavi and Rosen (1989) placed *A. paramali* with *A. prociphili*, *A. campestris*, *A. gossypi* and *A. mali* in the *mali* group.

Generally the genus *Aphelinus* Dalman is a complex taxon bearing many sibling species (Chen *et al*. 2002). Very little is known about taxonomy and host specificity of the species (Zuparko 1997), as well as distributional patterns and economic importance (Michel 1971; Kobs *et al*. 1997). Our results indicated the phylogenetic position of *A. paramali* among other closely related species, in part as above mentioned. Therefore, further investigations using other nuclear and mitochondrial DNA is necessary to confirm the specific identity of *Aphelinus* species, as well as a complete matrix of the allied species.

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