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*Orijinal araştırma (Original article)*

# Molecular and morphological identification of hymenoptran parasitoids from the pomegranate aphid, *Aphis punicae* in Razavi Khorasan province, Iran

Razavi Horasan (İran) bölgesi Nar bitinde (*Aphis punicae*) hymenoptera parazitoitlerinin moleküler ve morfolojik tanılanması

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## Abstract

Parasitoids play the positive role in aphids control management by keeping the population of aphids below the economic threshold. The pomegranate aphid, *Aphis punicae* is attacked by several parasitoids which mostly belong to Aphidiinae subfamily. The identification of parasitoids and hyperparasitoids is problematic due to their small size and ambiguous morphological characteristics. The analysis of molecular data of DNA sequences has been suggested as a complementary approach to the classical methods to identify biocontrol agents. In the present study, active parasitoids and hyperparasitoids of the pomegranate aphid collected from Razavi Khorasan province were identified using morphological characters and analysis of two gene sequences including COI and 28S. Phylogenetic analysis was performed using Maximum parsimony and Bayesian methods. The identified parasitoids were *Binodoxys angelicae*, *Lysiphlebus fabarum*, *Ephedrus persicae*, and the hyperparasitoids were *Syrphophagus aphidivorus*, *Alloxysta* sp. and *Pachyneuron* sp. We demonstrated that the mitochondrial COI gene was superior for the identification of these insects compared to the nuclear 28S (D2- region) and that it was compatible with morphological studies. This is the first study on molecular identification of the parasitoids and hyperparasitoids on pomegranate aphids in Iran.

**Keywords:** Pomegranate, parasitoids, COI, 28S, aphids, identification

## Özet

Parazitoidler yaprak bitleri populasyonunu ekonomik eşiğin altında tutarak kontrol yönetiminde olumlu bir rol oynar. Nar yaprakbiti, (*Aphis punicae)* çoğunlukla Aphidiinae alt familyasına ait çeşitli parazitoitler tarafından saldırıya uğramaktadır. Parazitoitleri ve hiperparazitoidlerin tanımlanması, küçük boyutları ve belirsiz morfolojik özellikleri nedeniyle sorunludur. DNA dizilerinin moleküler veri analizi, biyokontrol ajanlarını tanımlamak için klasik yöntemlere tamamlayıcı bir yaklaşım olarak önerilmiştir. Bu çalışmada, Razavi Horasan ilinden toplanan nar yaprak bitinin aktif parazitoitleri ve hiperparazitoidleri morfolojik karakterler kullanılarak ve iki gen dizisinin COI ve 28S'in analizi de dahil olmak üzere tanımlanmıştır. Filogenetik analiz maksimum parsimoni Bayesian yöntemleri kullanılarak gerçekleştirilmiştir. Tanımlanan parazitoidler *Binodoxys angelicae*, *Lysiphlebus fabarum*, *Ephedrus persicae*, hiperparazitoidler *Syrphophagus aphidivorus*, *Alloxysta* sp. ve *Pachyneuron* sp.'dir. Mitokondriyal COI geninin bu böceklerin tanımlanması için Nükleer 28S'e göre (D2 bölge) üstün olduğu ve morfolojik çalışmalar ile uyumlu olduğu tespit edilmiştir. Bu çalışma, İran'da nar yaprakbiti parazitoit ve hiperparazitoidlerinin moleküler tanımlaması açısından ilk çalışmadır.

**Anahtar sözcükler:** Nar, parazitoitler, COI, 28S, afitler, tanılama

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## Introduction

The pomegranate tree, *Punica granatum* is attacked by a number of aphids influencing quality and quantity of its product. *Aphis punicae* (Passerini) (Hemiptera: Aphididae) is a sap-sucking insect of pomegranate presenting through the Meditranean basin and also further to the east (Bayhan et al. 2005; Alford 2007). The aphid is serious and widespread insect in most pomegranate cultivated area of Iran. This species is well known for its ability to reduce plant vigor, facilitate the growth of the mould on leaves, consequently reduce crop quality and yield. Both adults and nymphs feed on leaves, inflorescences and fruits (Moawad & Al-Barty 2011; Rouhani et al. 2013).

The pomegranate aphid, *A. punicae* is attacked by several parasitoids mostly belonging to the subfamily Aphidiinae (Hym. Braconidae). They are regarded as entirely solitary koinobiont endoparasitoids of aphids comprising of about 50 genera and 400 species (Mackauer & Starý 1967; Starý 1988). They are divided into four tribes, Aphidini, Trioxini, Praini and Ephedrini. Several species of Aphidiinae wasps have been successfully used in biological control programs (Carver 1989) and many aspects of their biological cycle have been studied throughout the world (Starý 1970; Sanchis et al. 2000).

Reliable identification of Aphidiinae is one of the main constraints faced by ecologists when studying insect communities specially for assessing their potential in biological control programs (Gotelli 2004; Bigler et al. 2005). The parasitic Hymenoptra are hyperdiverse, taxonomically understudied and contain numerous cryptic species (Sha et al. 2007). The diagnostic characters separating Aphidiinae species are subtle and difficult to appreciate. Even when keys are available, they are difficult to use and morphological identification of species remains problematic if not impossible for non-specialists (Tomanovic et al. 2003). Consequently, misidentification of parasitoids used in biocontrol may result in serious economic losses (Compere 1961; Rosen & Debach 1973; Zhou et al. 2014).

The use of a standardized DNA region for fast and accurate species identification such as COI has proved to be efficient in hyperdiverse groups for which morphological identification is difficult or impossible (Valentini et al. 2009). Although many examples have shown that the COI is reliable for accurate species identification, some studies revealed that a unique region of mitochondrial DNA does not supply ample resolution and could be misleading (Linares et al. 2009; Derocles et al. 2011). For this reason, a nuclear gene is sometimes required to improve species identification and may also serve as a validating tool during the implementation of the database (Fre´zal & Leblois 2008).

The present study aimed to determine the species of Aphidiinae parasitoids and hyperparasitoid species which emerged from *A. punicae* in Razavi Khorasan province using morphological characters,mitochondrial COI and nuclear 28S genes analysis.

## Materials and methods

The parasitoids and hyperparasitoids were sampled once a week from infected *A.punicae* on pomegranate trees *P.granatum* during spring, summer and autumn in 2012-2013. The sampling was carried out in the orchards of Mashhad, Kashmar and Neyshabur located in Khorasan Razavi province, Iran. The leaves infested by live or mummified aphid colonies were cut and transferred to the lab and reared in mesh-covered transparent glass vessels. The vessels were maintained in growing chamber for 3-4 weeks at 25 °C, 65% RH (Relative Humidity) and 16:8 h (L:D) photoperiod conditions. The emerged wasps were collected and maintained in 96% ethanol for further examination.

Microscopic slides were prepared using Hoyers medium from different parts of the specimen including mouthpart, antennae, forewings, petiole, propodeum and ovipositor. Morphological characters of all species were drawn under a microscope (Olympus$^{TM}$ $BH_2$ Phase-contrast). Measurements were taken with an ocular micrometer based on slide-mounted specimen.

The genera of specimens were identified using the available keys (Starý 1973; Kavallieratos et al. 2001; Tomanovic & Kavallieratos 2002; Starý et al. 2010; Rakhshani et al. 2011; Rakhshani et al. 2012) and confirmed by recognized specialists. Voucher specimens of parasitoids were deposited at the Department of Plant protection, Ferdowsi University of Mashhad, Iran. Furthermore, identification of the species belonging to Braconidae were confirmed by Petr Starý, Laboratory of Aphidology, Institute of Entomology, Cezech Republic and those belonging to Pteromalidae, Figitidae and Encyrtidae were confirmed by Juli Pujade-Villar, Faculty of Biology, University of Barcelona, Spain. The taxonomic status of the aphid *A. punicae* was kindly confirmed by Olivera Petrovic-Obradovic, Faculty of Agriculture, University of Belgrade, Serbia.

## DNA extraction and sequencing

Genomic DNA was extracted from the verified sample of each specimen. For this purpose, parts of the body which were not used in morphological study including legs and abdomen were used for extraction by Chelex® Ultra 100 molecular biology grade resin. Nitrogen-frozen sample was crushed using a micro pestle in 50 µl buffer and 2 µl proteinas K. The homogenate was incubated at 60°C for 4 hours. The supernatant was extracted and stored at - 20°C for PCR (Sayed et al. 2013).

The primers which used in PCR were presented in (Table 1). PCR was carried out in an Eppendorf Mastercycler gradient (Eppendorf, Hamburg, Germany) in standard 25 µl reaction containing 3 µl DNA template, 3 µl (10X) buffer, 1 µl $MgCl_2$, 0.5 µl dNTPs,1 µl of each primers (10 Pmol/ µl), 0.3 µl *Taq* polymeras (5U) and 15.2µl distil water.

Table 1. List of primers used for PCR in this study

| Target region | Primer and sequence | Reference |
|---|---|---|
| COI | LCO1490: 5´-GGTCAACAAATCATAAAGATATTGG-3´<br>HCO2198: 5´-TAAACTTCAGGGTGACCAAAAAATCA-3´ | (Folmer et al. 1994) |
| 28S-D2 | 28S F: 5´-AGAGAGAGTTCAAGAGTACGTG-3´<br>28S R: 5´-TTGGTCCGTGTTTCAAGACGGG-3´ | (Hancock et al. 1988; Linares et al. 1991; Campbell et al. 1993) |

Temperature conditions of the COI amplification were denaturant at 94°C for 60S, annealing at 55°C for 90S and extension at 72°C for 90S (30 cycles, plus an initial denaturation at 94 °C for 1 minute and a final extension at 72 °C for 8 minute). Temperature conditions of the 28S amplification were similar to COI with the exception of annealing in which was set at 60 °C.

All products were loaded in a 1% agarose gel. Sequencing reactions were performed using a 3730XL DNA analyzer by Macrogen Co. (Seoul, South Korea).

## Sequence analysis and specimen delineation

Sequences were checked using the BioEdit (Hall 1999). Sequences evaluation was performed by inputting the sequence in the nBlast software which was based on a species identification system. The basic statistical analysis was performed using MEGA 5 software (Tamura et al. 2011). Barcoding gap was examined between interspecific and intraspecific distance in 167 taxons of COI and 153 taxons of 28S genes for subfamily Aphidiinae. The histograms were constructed using calculation of pairwise sequence divergence on the basis of kimura-2-parameter distance model (Kimura 1980; Felsenstein 1985) by TAXONDNA (Meier et al. 2006).

For each gene, two different methods including maximum parsimony and Bayesian analyses were used to reconstruction of Aphidiinae phylograms. Maximum parsimony analysis was performed using PAUP* 4.0b10 (Swofford 2002) under equal weighting of all characters. To find the most parsimonious tree, heuristic search (Hillis et al. 1996) was performed with 1000 random addition replicates with TBR option for branch swapping and gaps coded as the fifth character. Statistical support for each node was evaluated by bootstrap analysis (Felsenstein 1985) with 1000 replications. Bayesian analyses were conducted by Mr Bayes 3.04 (Huelsenbeck & Ronquist 2001) using default parameter Marcov chain for 1000000 generations, with sample frequency 10 until average standard deviation of split frequencies getting under 0.01. The frequencies of nodes in these trees were taken as a posteriori probabilities (Huelsenbeck & Ronquist 2001). The COI and 28S sequences of *Xorides* sp. (Ichneumonids) were used as outgroup for the analysis.

## Result

1440 specimens of aphid parasitoids were collected from pomegranate orchards on *Aphis punicae*. The primary parasitoids belonging to Aphidiinae were represented by three species: *Binodoxys angelicae* Haliday, *Ephedrus persicae* Frogatt and *Lysiphlebus fabarum* Marshal. 1010 specimens were collected from hyperparasitoids belonging to 3 families of hymenopterans including *Alloxysta* sp. (Figitidae), *Pachyneuron* sp. (Pteromalidae) and *Syrphophagus aphidivorus* Mayr (Encyrtidae).

## Morphological study

*Lysiphlebus fabarum* (Hym., Braconidae, Aphidiinae)

Female: Body length 1.4-1.6 mm, black, Head dark brown, maxillary palps with 3 palpomers and labial palps with 1 palpomer (Fig. 1 A), Antennae filiform with 13 segments (Fig. 1 B), Petiol widely triangular (Fig. 1 E), Propodeum smooth with two divergent carinae (Fig. 1 D), Forewing venation incomplete, R1 distinctly longer than stigma, M&m-cu vein incomplete, setae on fringe of forewing similar to those on surface (Fig. 1 C), Ovipositor sheath weakly convex dorsally (Fig. 1 F).

Male: Male is similar to female with the exception of its antennae having 13-15 segments (Fig. 1 B).

*Binodoxys angelicae* (Hym., Braconidae, Aphidiinae)

Female: Body length 2.2-2.6 mm, light brown to yellowish., Head dark brown, maxillary palps with 4 palpomers and labial palps with 2 palpomers (Fig. 2 A), Antennae filiform with 12 segments (Fig. 2 B), Petiole longed, distance between primary and secondary tubercules longer than width at spiracles (Fig. 2 F), Propodeum distinctly carinated (Fig. 2 E), Forewing pterostigma widely triangular, radial vein distinct, extend just beyond the stigma, other veins reduced (Fig. 2 C), Hypopiygium contains prong originating near apex of last sternite with 3-4 setaes on their dorsal aspect ,ovipositor sheath curved downward (Fig. 2 F).

Male: Male is similar to female with the exception of its antennae having 10-11 segments (Fig. 2 B).

*Ephedrus persicae* (Hym., Braconidae, Aphidiinae)

Female: Body length 2-2.2 mm, black., Head black, maxillary palps with 4 palpomers and labial palps with 2 palpomers (Fig. 3 A)., Antennae filiform with 12 segments (Fig. 3 B), Petiol light yellowish with spare setae in lower level (Fig. 3 F), Propodeum distinctly carinate, with a wide central pentagonal areola (Fig. 3 E), Forewing 3Rs reaching the margin of wing, 2Rs longer than 1.5 times as long as width (Fig. 3 C), Ovipositor sheath oval shape and wide towards base (Fig. 3 F).

Male: Male is similar to female except that its color is dark brown.

*Syrphophagus aphidivorus* (Hym., Encyrtidae)

Female: Body length 1.1-1.7mm, black., Head dark brown, maxillary palps with 4 palpomers and labila plalps with 2 palpomers (Fig. 4 A), Antennae 10 segmented with short hair, flagellum 9 segmented consisting of 6 funicle segments and 3 club segments (Fig. 4 B), Forewing large and longer than the body, distal vein enlarged, marginal vein narrow and very short, stigma and postmarginal veins very short (Fig. 4 C), Foreleg with curved tibia spur (Fig. 4 D).

Male: Male is similar to female with the exception of its antenna having 9 segments and each segment bearing long hairs (Fig. 4 B).

*Pachyneuron* sp. (Hym., Pteromalidae)

Female: Body length 1.9-2.3 mm, black., Head black, maxillary palps with 4 palpomers and labial palps with 2 palpomers (Fig. 5 A), Antennae 10 segmented, ring 3 segmented (Fig. 5 B), Forewing with long submarginal vein, relatively long and thickened marginal vein, Postmarginal vein as long as marginal vein, stigma enlarged (Fig. 5 C), Foreleg with curved tibial spur (Fig. 5 D), Ovipositor sheath short and extended as half long as gaster.

Male: Male differs from the female in darker coloration of the gaster, Body length 1.2-1.9 mm.

*Alloxysta* sp. (Hym., Figitidae)

Female: Body length 0.8-1.3 mm, black., Head dark brown, maxillary palps with 4 palpomers and labial palps with 2 palpomers (Fig. 6 A), Antennae filiform with 13 segments (Fig. 6 B), Mesoscutum smooth, round in dorsal view with scattered setae, Forewing large, longer than the body, covered with dense pubescence; radial cell distinct, marginal setae present (Fig. 6 C).

Male: Male is similar to female. Body length has 0.8-1.5 mm.

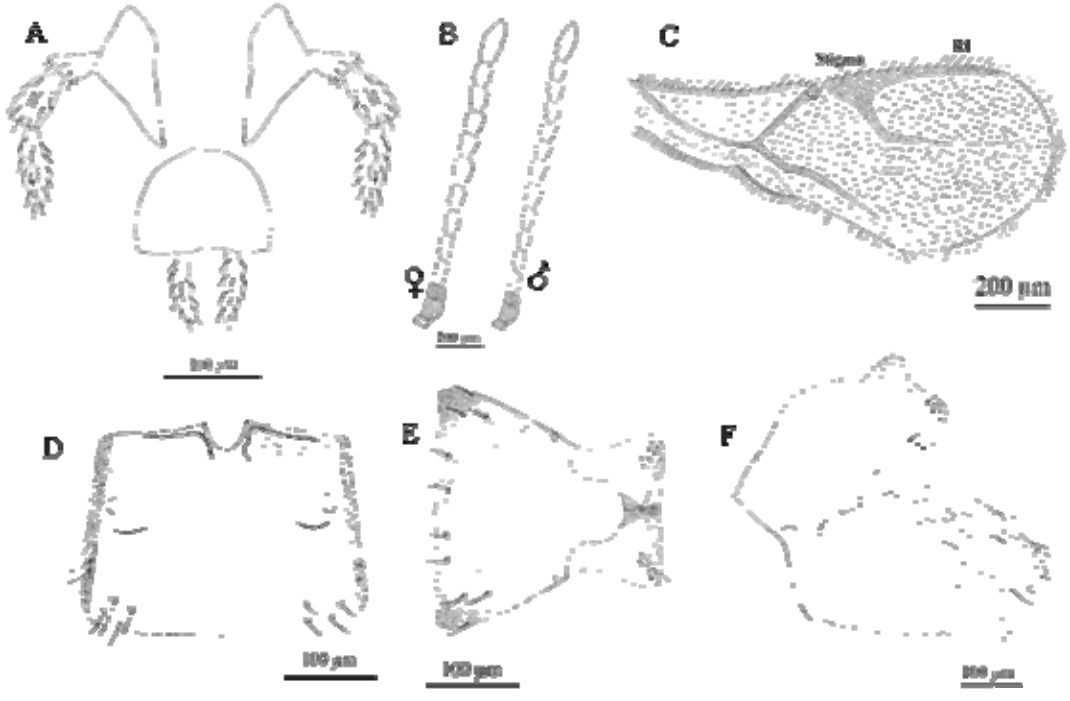



Figure 1. Morphological characters of *Lysiphlebus fabarum*: A) Mouthparts, B) Antennae, C) Forewing, D) Propodeum, E) Petiol, F) Ovipositor sheaths (lateral view).

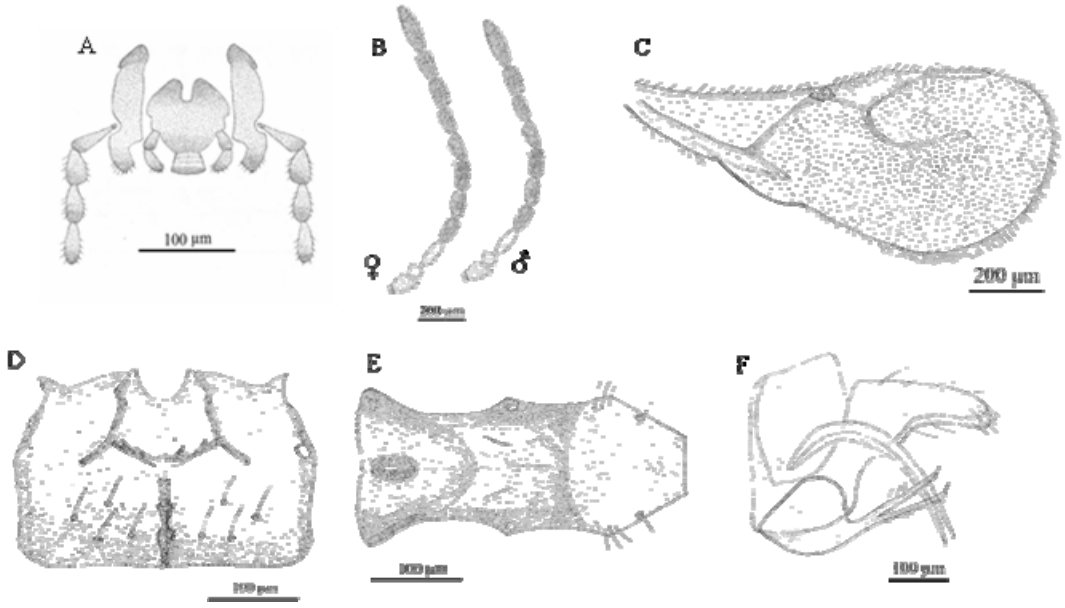



Figure 2. Morphological characters of *Binodoxys angelicae*: A) Mouthparts, B) Antennae, C) Forewing, D) Propodeum, E) Petiol, F) Ovipositor sheaths (lateral view).

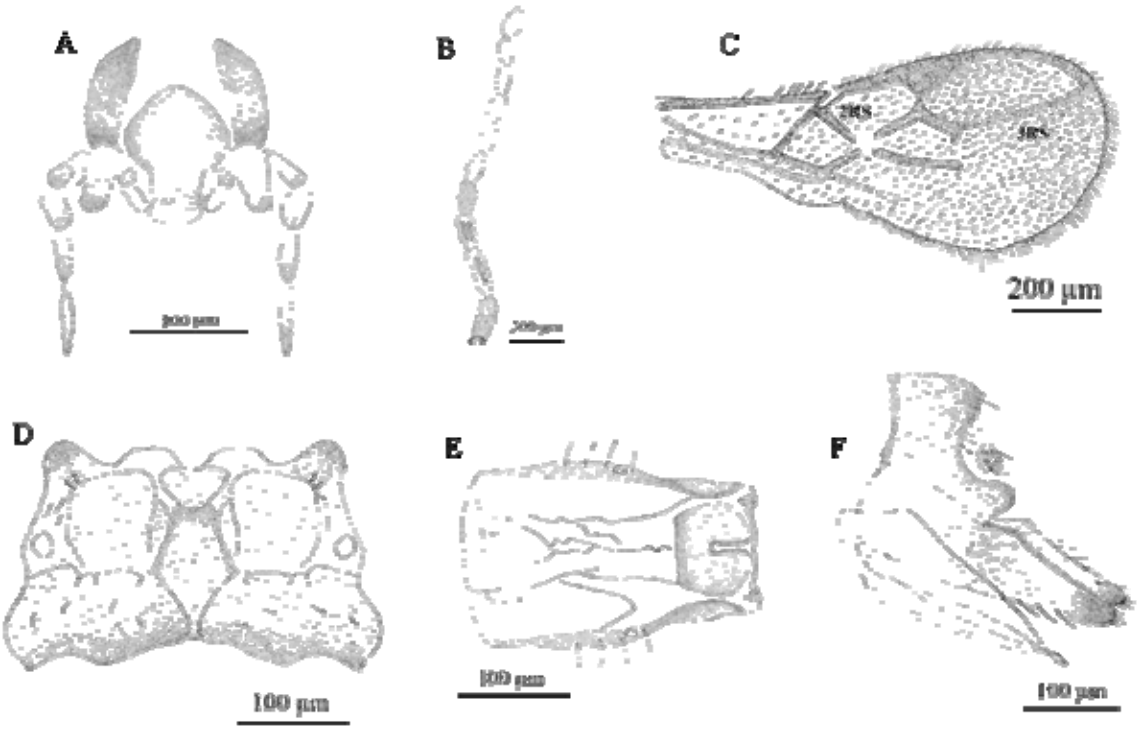



Figure 3. Morphological characters of *Ephedrus persicae*: A) Mouthparts, B) Antennae, C) Forewing, D) Propodeum, E) Petiol, F) Ovipositor sheaths (lateral view).

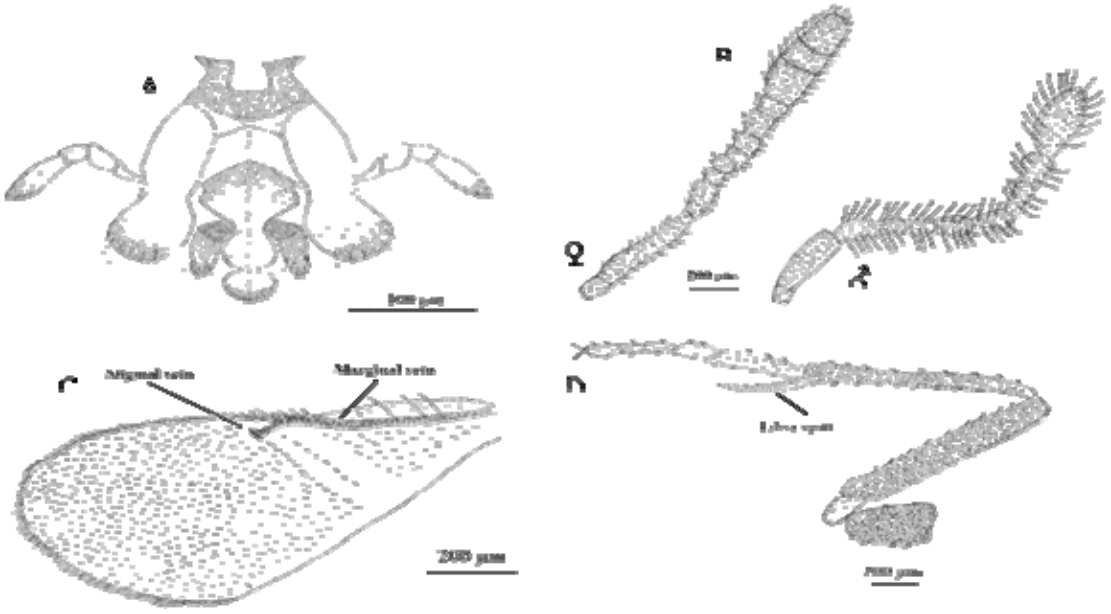
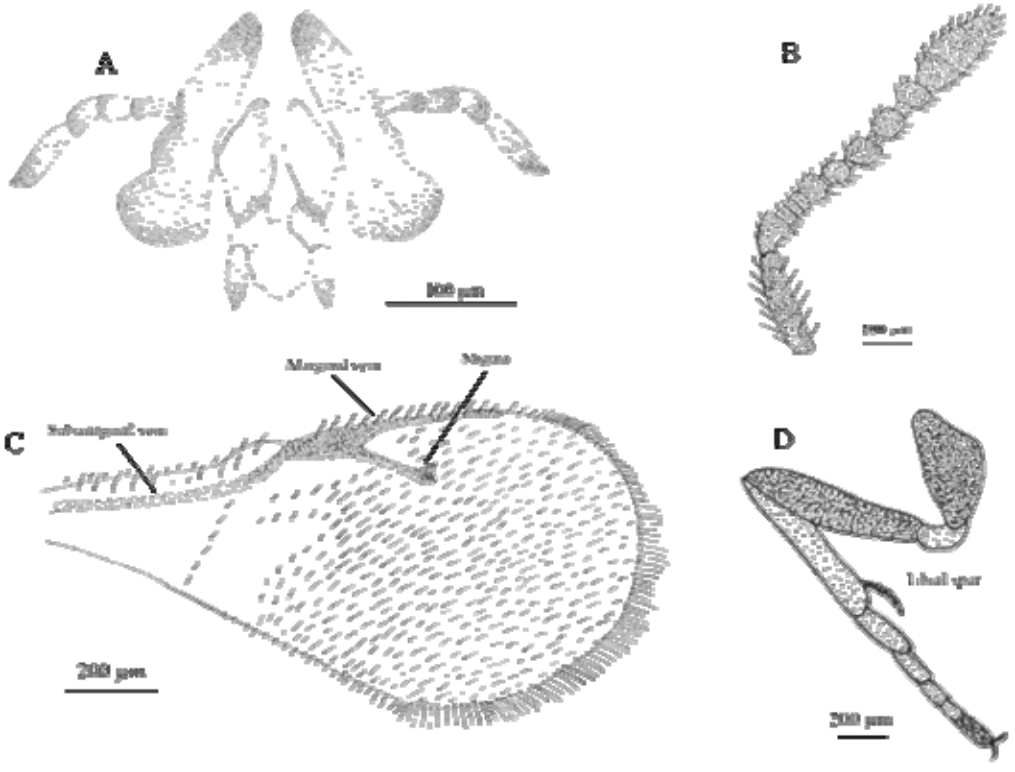


Figure 4. Morphological characters of *Syrphophagus aphidivorus*, A) Mouthparts, B) Antennae, C) Forewing, D) Foreleg.




Figue 5. Morphological characters of *Pachyneuron* sp., A) Mouthparts, B) Antennae, C) Forewing, D) Foreleg.

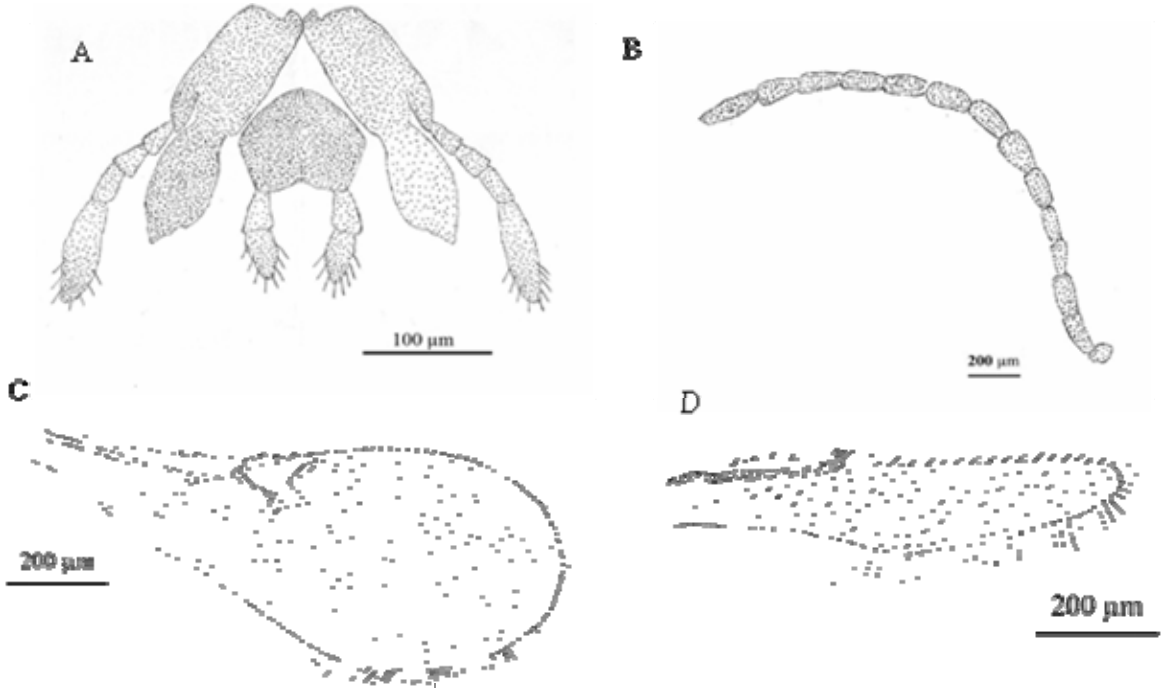



Fig. 6. Morphological characters of *Alloxysta* sp., A) Mouthparts, B) Antennae., C) Forewing., D) Hindwing.

## Molecular study of parasitoids

The COI and 28S genes of three specimens of parasitoids and three hyperparasitoid specimens were amplified by PCR. The fragments of COI gene with 543 bp and 28S gene with 411 bp were submitted to the Genbank (Table 2).

Table 2. The accession number of submitted sequences into the GenBank

| Species | Sampling location | Accession number | |
|---|---|---|---|
| Parasitoids | | COI | 28S |
| *Binodoxys angelicae* | Mashhad, Iran | KF894407.1 | KF894415.1 |
| *Lysiphlebus fabarum* | Mashhad, Iran | KF894405.1 | KF894413.1 |
| *Ephedrus persicae* | Kashmar, Iran | KF894408.1 | KF894416.1 |
| Hyperparasitoids | | | |
| *Syrphophagus aphidivorus* | Mashhad, Iran | KF894410.1 | KF894418.1 |
| *Pachyneuron* sp. | Mashhad, Iran | KF894412.1 | KF894420.1 |
| *Alloxysta* sp. | Mashhad, Iran | KF894411.1 | KF894419.1 |

The base composition of the COI gene in parasitoids and hyperparasitoids had a strong bias toward adenine and thymine, constituting averagely 72.9%. These bases comprised averagely 54.23% of the 28S gene composition (Table 3).

Table 3. Nucleotide frequencies of COI and 28S genes composition of parasitoides and hyperparasitoids

| Species | COI (%) | | | | | | 28S (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | C | G | T | AT | GC | A | C | G | T | AT | GC |
| *Binodoxys angelicae* | 29.3 | 10.6 | 16.0 | 44.1 | 73.4 | 26.6 | 31.2 | 13.1 | 15.5 | 40.2 | 71.4 | 28.6 |
| *Lysiphlebus fabarum* | 31.0 | 10.2 | 15.1 | 43.7 | 74.7 | 25.3 | 28.3 | 16.0 | 18.1 | 37.6 | 65.9 | 34.1 |
| *Ephedrus persicae* | 26.5 | 17.7 | 16.5 | 39.4 | 65.8 | 34.2 | 24.9 | 20.6 | 22.0 | 31.5 | 56.5 | 42.6 |
| *Syrphophagus aphidivoraus* | 33.1 | 10.8 | 12.8 | 43.3 | 76.4 | 23.6 | 18.5 | 26.3 | 30.9 | 24.3 | 42.9 | 57.1 |
| *Pachyneuron* sp. | 30.5 | 14.2 | 12.7 | 42.6 | 73.1 | 26.9 | 21.6 | 25.0 | 31.6 | 21.9 | 43.4 | 56.6 |
| *Alloxysta* sp. | 32.0 | 13.0 | 13.0 | 41.9 | 74.0 | 26.0 | 17.9 | 24.8 | 29.5 | 27.7 | 45.6 | 54.4 |

## The results of nBlast analysis

Sequence evaluation against nBlast software confirmed the classical identification of the parasitoids and hyperparasitoids (Table 4).

Table 4. Results of molecular analysis using nBlast via NCBI database

| Morpho-species | NCBI | |
|---|---|---|
| | COI | 28S |
| *Binodoxys angelicae* | *B. angelicae* {JF730315}† (99) ¥ | *T. pallidus* {Z83588} (99)<br>*B. angelicae* {JQ248908} (94) |
| *Lysiphlebus fabarum* | *L. fabarum* {JX507442} (98) | *L.confiusus* {Z83586} (99)<br>*L. fabarum* {JQ248911} (98) |
| *Ephedrus persicae* | *E. persicae* {JF30312} (92) | *E. persicae* {Z83598} (99) |
| *Syrphophagus aphidivorus* | *S.aphidivorus* {JX507455} (99) | *S. aphidivorus* {HQ599566} (98) |
| *Alloxysta* sp. | *Alloxysta* sp. {DQ012618} (97) | *Alloxysta* sp. {DQ012577} (96) |
| *Pachyneuron* sp. | *Pachyneuron* sp. {HG599572} (99) | *Pachyneuron* sp. {AY876060} (90) |

†The accession number is shown in brackets
¥ The similarity value in percent with target species is shown in parentheses.

## Phylogenetic relationships

Phylogenetic analysis and reconstructed trees resulted from maximum parsimony analyses showed similar topology to Bayesian analyses (Bayesian data not shown) (Figures 7 and 8). In description of maximum parsimony phylograms, all species of Aphidiinae subfamily formed a monophyletic group. The resulted Phylogram based on COI gene showed that *Lysiphlebus fabarum*, *Binodoxys angelicae* and *Ephedrus persicae* sited in single clade with *L. fabarum* (JX507442), *B. angelicae* (JF730315) and *E. persicae* (JF730312), respectively. Whilst, the Phylogram obtained based on 28S gene sequences showed that they sited in a single clade with *L. confuses* (Z83586.1), *T. pallidus* (Z83588.1) and *E. persicae* (Z83598.1), respectively.

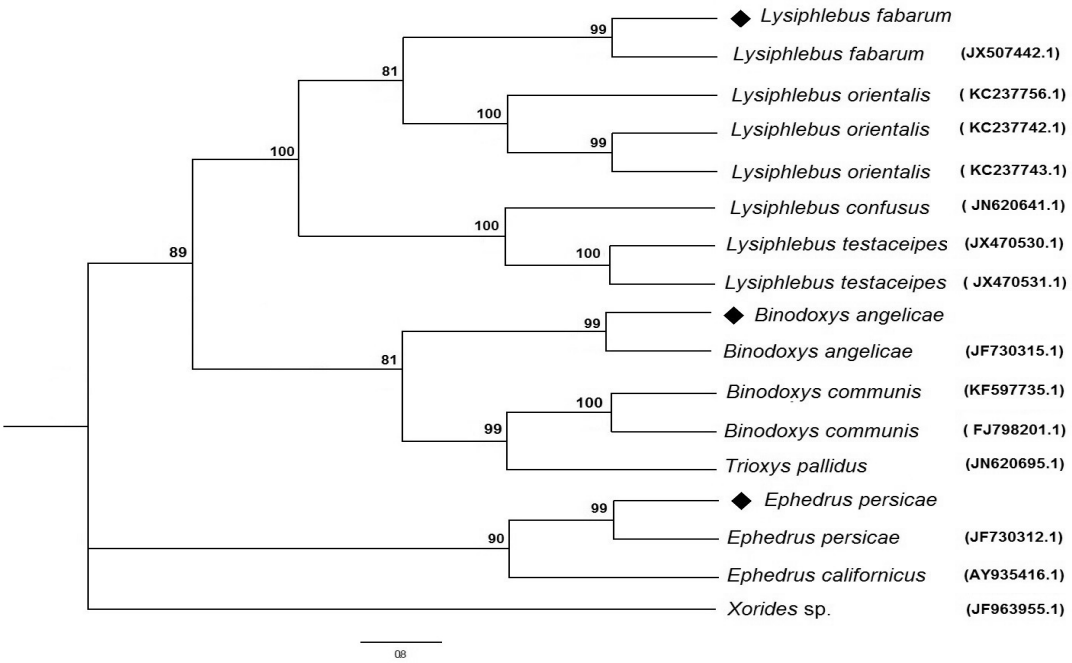



Figure 7. Phylogenetic relationship among Aphidiinae species based on the COI gene using MP method. Numbers at nodes represent the percent bootstrap values. The accession number is shown in parentheses. The samples of this study are shown with square symbols.

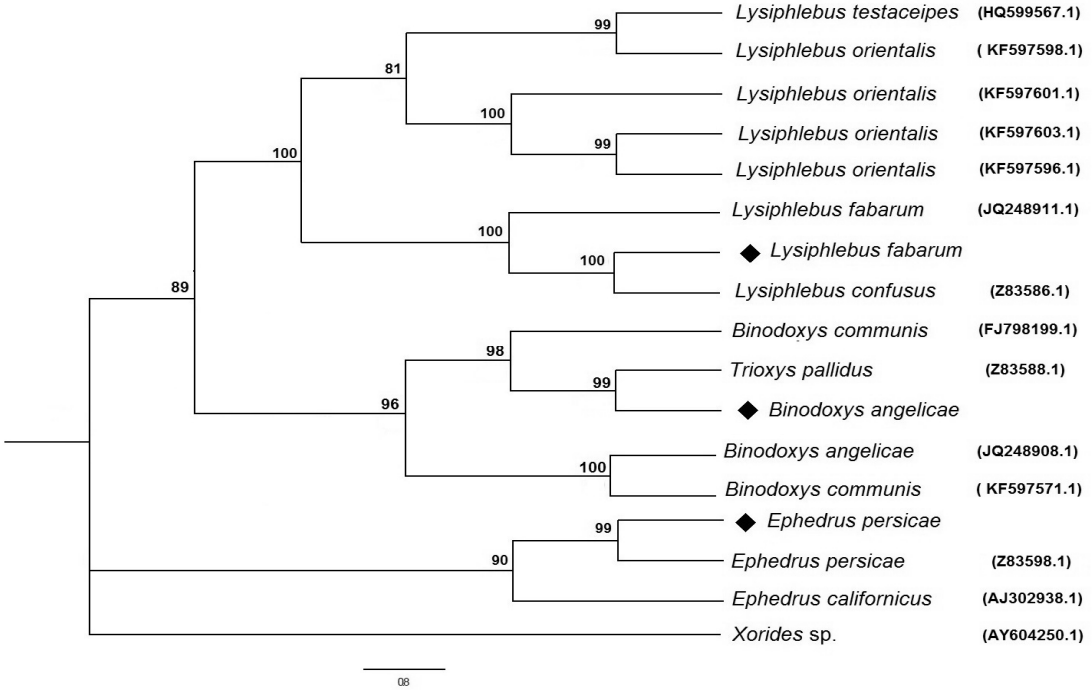



Figure 8. Phylogenetic relationship among Aphidiinae species based on the 28S gene using MP method. Numbers at nodes represent the percent bootstrap values. The accession number is shown in parentheses. The samples of this study are shown with square symbols.

## Gap barcoding in Aphidiinae

In order to assess the effectiveness of barcoding scale in species identification of Aphidiinae, the COI and 28S sequences of other species of this subfamily in Genbank were retrieved and along with the present data were analyzed. The results detected a gap between intra-and-interspecies with COI gene sequences (Figure 9) but not for 28S gene sequences (Figure 10). This indicates that the COI gene can be served as a more reliable marker for gap barcoding than the 28S gene.

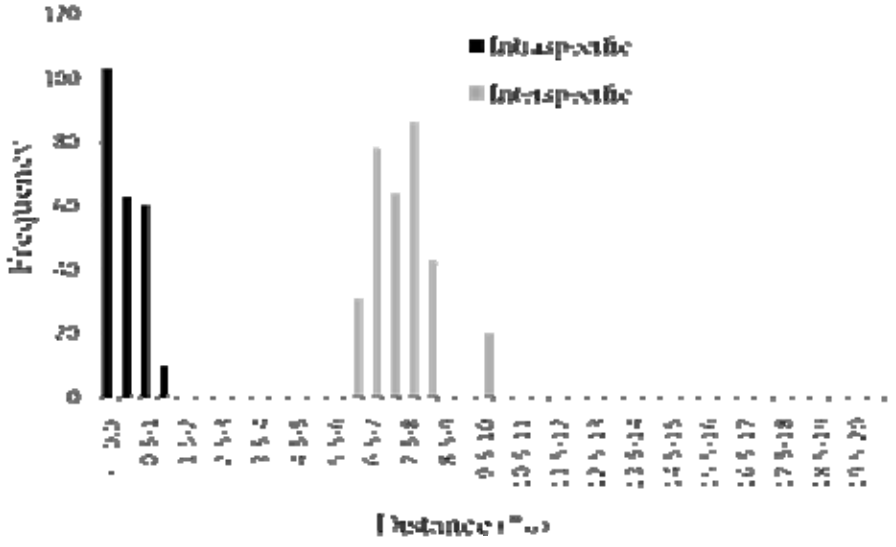



Figure 9. Intraspecific (black) and interspecific (gray) distance between the COI gene sequences of Aphidiinae species. Distance calculation performed using K2P.

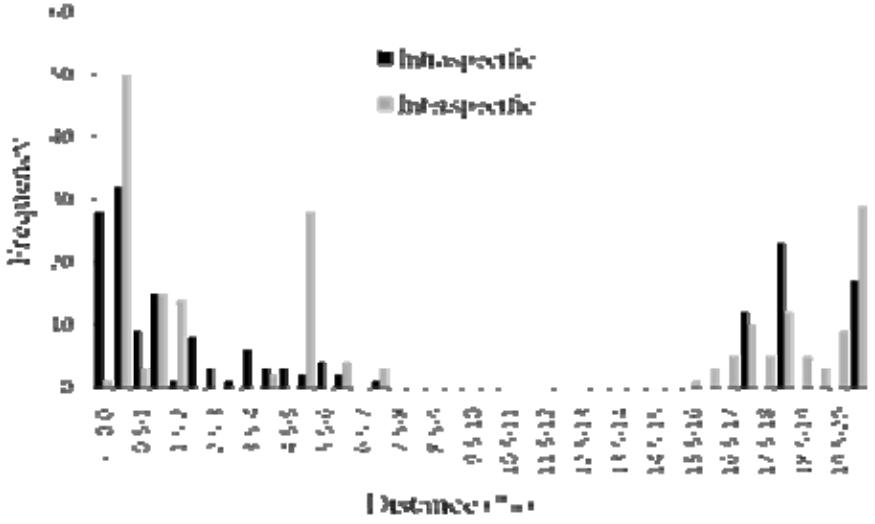



Figure 10. Intraspecific (black) and interspecific (gray) distance between the 28S gene sequences of Aphidiinae species. Distance calculation performed using K2P.

## Discussion

*Aphis punicae* is serious and widespread pest in pomegranate orchards (Alford 2007). Given environmental concerns regarding pesticide use in pomegranate orchards, the use of natural enemies for biological pest control has been regarded as a preferred approach to *A. punicae* management. Nazari et al. (2012) and Rakhshani et al. (2012) have recently reviewed the parasitoid fauna of *A. punicae* in western and northeastern of Iran, respectively and introduced the species *Aphidius matricariae*, *Diartiella rapae*, *Lysiphlebus fabarum* and *Praon necans*. Murat, et al. (2004) and Starý and Havelka (2008) have reviewed the parasitoids of this aphid in other countries and introduced the species *Binodoxys angelicae*, *Ephedrus persicae* and *Aphidius matricariae*.

In morphological studies, ambiguous results might be achieved as it is sometimes difficult to conclude whether lost structure are due to homologous or convergent events (Gibson 1985). In the last decade, the molecular technique has been emerged to facilitate accurate identification of hymenopteran parasitoids (Dowton & Austin 1997; Gimeno et al. 1997). In the present study, the molecular method revealed that the Aphidiinae population in comparison with *Xorides* sp. (outgroup) were monophyletic. Our results were corresponding with the literature (Smith et al. 1999; Sanchis et al. 2000; Shi & Chen 2005).

The previous studies reported *L. fabarum* as a common parasitoid of *A. punicae* in Iran (Talebi et al. 2009) and other countries (Murat et al. 2004; Starý & Havelka 2008). However, its taxonomic character is still considered problematic, due to overlapping with *Lysiphlebus confiusus*. The present study identified *L. fabarum* based on morphological characters and COI gene sequences inferred by nBlast analysis and clade position on phylogram. Based on the results of nBlast analysis and phylogram for 28S gene, the similarity of *L. fabarum* was confirmed with *Lysiphlebus confiusus* (Z83586). This indicated that the 28S is not appropriate marker to separate these two species. Derocles et al. (2011) and Zhou et al. (2014) also demonstrated that these species were very closely related and that the 28S gene was unable to differentiate them.

The results of morphological characters and COI gene sequence analysis for identification of *Binodoxys angelicae* had conflict with result of 28S gene. The morphological characters and COI gene analysis confirmed the identification of *Binodoxys angelicae* but nBlast analysis of 28S gene via NCBI showed it also had similarity with *Trioxys pallidus* (Z83588). The phylogenetic tree resulted from 28S gene sequences showed that *B. angelicae* and *T. pallidus* sited in single clade. The similarity of the genus *Binodoxys* with *Trioxys* has been shown in other studies (Tomanovic & Kavallieratos 2002; Zumoffen et al. 2013). Working on phylogenetic analysis of 28S gene, Belshaw & Quicke (1997) demonstrated that *Binodoxys* and *Trioxys* sited in a single clade.

*Ephedrus persicae* was verified by both COI and 28S genes. This species has been reported as one of *A. punicae* parasitoids complex (Barahoei et al. 2014). The gap barcoding analyses demonstrated that there was gap between intra-species and inter-species in the COI gene of Aphidiinae but not in the 28S gene. This may suggest that COI gene to be served as a more reliable marker than the 28S gene for gap barcoding in Aphidiinae. Similar results were obtained in Melolonthinae subfamily (Karimi et al. 2012) and some taxa of Braconidae family (Smith et al. 2013).

The results indicate that the COI gene is required for reliable identification of Aphidiinae. The lake of enough variable in 28S gene has been shown leading to misidentification of closely related species (Linnen & Farrell 2008). In the present study, these results were corroborated by the absence of a gap barcoding in 28S gene.

Although in most cases transitions are observed more frequently than transversions (Simon et al. 1994), present study findings revealed that AT-transversions occur much more frequently than any other type of changes in Hymenoptran COI gene as shown similarly on some parasitoids (Dowton & Austin 1997). The AT-transversions, however, did not occur in 28S gene (Table 3). This may be due to the higher content of AT bases compared to GC ones. Other authors have reported high occurrence of AT-transversions in the mitochondrial genes (Crozier et al. 1989; Pederson 1996; Darsouei et al. 2011) and this trend seems to be even higher in Hymenoptera (Simon et al. 1994; Dowton & Austin 1997).

## Conclusion

The molecular approach greatly assisted the identification of the parasitoids complex of *A. punicae*. However, the lake of variability in 28S gene suggests that it should be accompanied with other gene markers or morphological traits. Molecular tools based on DNA sequences of certain locus have been successfully used to detect immature stages of parasitoids inside aphids (Traugott et al. 2008). The recommendation for future work on the parasitoids is to employ specific primers for correct detection of Aphidiinae inside *A. punicae*.

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