Arch. Biol. Sci., Belgrade, 63 (4), 1225-1234, 2011 DOI:10.2298/ABS1104225D

PARASITIC WASPS AS NATURAL ENEMIES OF APHID POPULATIONS IN THE MASHHAD REGION OF IRAN: NEW DATA FROM DNA BARCODES AND SEM

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Abstract- DNA barcoding is a modern method for the identification of different species, including insects. Among animals, the major emphasis of DNA barcoding is on insects. Due to this global trend we addressed this approach for surveying a group of insects. The parasitic wasps (including primary and hyperparasitoids) of pome fruit orchard aphids were collected from Iran-Mashhad during 2009-2010. Preliminary identification of this group was performed by using morphological and morphometric characters and SEM. The COI gene in the specimens was amplified and sequenced. In this survey, *Aphidius matricariae*, *Binodoxys angelicae, Diaeretiella rapae*, *Ephedrus persicae*, *Lysiphlebus fabarum* and *Praon volucre* parasitoids and *Alloxysta* sp., *Asaphes suspensus*, *Dendrocerus carpenteri*, *Pachyneuron aphidis*, *Syrphophagus aphidivorus* hyperparasitoids were studied. Based on intra-interspecies distances and phylogenetic analysis using NJ, all species possess diagnostic barcode sequences. The results of this study show that the COI sequence could be useful in identification study of this group of insects. Here we have provided the first GenBank data for the COI gene of the above-mentioned hyperparasitoids as well as an initial attempt toward preparing DNA barcodes for Iranian parasitoid and hyperparasitoid aphids.

Key words: Parasitic wasps, aphids, Iran

UDC 632.6/7(55):632.9

INTRODUCTION

Aphidiina e wasps (Braconidae: Aphidiinae) are koinobiont endoparasitoids of aphids with approximately 50 genera and 400 species (Mackauer and Starý, 1967; Starý, 1988). They are a group of small parasitic wasps that are identified by morphological characteristics such as wing venation, petiole, propodeum and ovipositor and often have been discussed as a separate family, Aphidiidae, because of their specialization on aphids, reduced wing venation and a flexible structure between the second and third tergites of the gaster. Several species of these wasps have been used for the biological control of aphids (Stary 1970; Carver 1989). Aphidiines are divided into four tribes, Aclitini, Aphidiini, Ephedrini and Praini (Mackauer and Stary 1967; Stary 1988) are y that exhibit differences based on adult and larval morphology (Mackauer, 1968; O'Donnell, 1989; Finlayson, 1990). The Aphidiini is the largest tribe. This tribe is divided into two subtribes, Aphidiini and Trioxini.

There are different hypotheses about the phylogenetic relationship of the members of this subfamily, but relationships within this subfamily are still unresolved. Recently, it was proposed that morphological and behavioral data are not enough for resolving phylogenetic relationships in this group, so the application embryologic and molecular data are recommended (Smith et al., 1999).

Each of the four recognized tribes mentioned above have been suggested as being basal: Ephedrini, based on its adult morphology, especially wing venation, (Mackauer, 1961; Gradenfors, 1986) DNA

sequence of 28SrDNA (Belshaw and Quicke, 1997) and 18S regions (Sanches et al., 2000); Praini, based on pupation habit (internal or external pupa), venum gland apparatus (Edson and Vinson, 1979) and DNA sequence of NADH1 (Smith et al., 1997); Aphidiini based on its final instar larval morphology (Finlayson, 1990), and Aclitini, based on its morphological characters, behavior (Chou, 1984) and DNA sequencing of 16SrDNA (Kambhampaati et al., 2000). Certainly, only one if any of the four proposals is correct. Because of the ambiguity in the phylogenetic relationship of aphidiine, many molecular studies have been undertaken to solve this problem.

Primary parasitoids limit the populations of the aphids that attack them. The action of primary parasitoids is limited by the intervention of hyperparasitoids. Active hyperparasitoids on Aphidiines belong to the Encyrtidae, Pteromalidae, Megaspilidae and Cynipidae families.

Detection of aphid parasitism is somewhat challenging because of the small size of both host and parasitoid (Greenstone, 2003). The identification of hyperparasitoids is even more difficult because of their minute size.

Traditional methods of insect identification which are based on morphological characters have some difficulties which encourage researchers to use molecular methods for resolving this problem. Several molecular methods, including allozyme electrophoresis (Walton et al., 1990), RAPD (Kazmer et al., 1995) and specific PCR (Heraty et al., 2007), have been used to differentiate parasitoid wasps. Among these methods, DNA sequencing is the most frequently applied. For this purpose, different gene regions have been used, among which COI has a high application because of its high success rate in distinguishing between species (Hebert et al., 2004).

In recent years, DNA barcoding has become important in studying different groups of insects. DNA barcoding makes it possible to identify samples at all stages of life by using short DNA sequences (e.g. eggs, larvae, nymphs or pupae). It is an approach for identifying many invertebrate taxa for which morphological identification is problematic due to lack of taxonomic keys for immature life stages. The standard sequence used for this purpose is a fragment of the 5′ end of the mitochondrial COI gene that is amplified by "universal primers" (Folmer et al., 1994). Several studies have shown that this is a reliable tool for the molecular identification of Lepidoptera, Hymenoptera, Coleoptera and Diptera species (Hajibabaei et al., 2006; Fisher et al., 2008; Greenstone, 2005; Smith et al., 2007).

Aphidiinae studies performed in Iran are limited to the identification and introduction of species. A total of 59 species of Aphidiinae have been reported from Iran (Rakhshani et al., 2008.; Kazemzadeh et al., 2009).

In this study, the identification of parasitoid and hyperparasitoid wasps was done on pome fruit orchard aphids in northeastern Iran (Mashhad). Specific confirmation was based on the sequences of COI genes, detailed morphological characteristics and SEM. Here, the DNA barcodes for all the studied species of parasitoids and hyperparasitoids are presented. The phylogenetic relationship between these species and other species of Aphidiinae were investigated. This is the first data about DNA barcodes of parasitoids and hyperparasitoids in Iran.

MATERIALS AND METHODS

Collection and preparation of the specimens

During 2009-2010, a survey was carried out to determine the parasitoids of aphids in pome fruit orchards in the Mashhad region located in the northeastern part of Iran. In order to collect the parasitoid wasps, samples were taken from apple, pear and quince tree leaves bearing aphid colonies and transferred to the lab for rearing in transparent glass vessels covered by mesh. The rearing vessels were held at room temperature for 2-3 weeks until the adult parasitoids emerged. The emerged wasps were collected daily with an aspirator and dropped into 96% ethanol for later examinations.

External morphology was illustrated using an Olympus™ BH2 Phase-contrast microscope. Microscopic slides were prepared using Hoyer's medium (Rosen and DeBach, 1979). Measurements were taken with an ocular micrometer. The ratio measurements were based on slide-mounted specimens. Collected specimens were identified to the level of genus using the identification key of "Revision of Far East Asian Aphidiidae" (Stary, 1967) and "Annotated Keys to the Genera of Nearctic Chalcidoidea" (Gibson et al., 1997).

Collected samples were identified as farmuch as possible by classical methods and confirmed by a specialist; the specimens of parasitoids, Pteromalidae, Encyrtidae and Megaspilidae were sent to Peter Stary, Laboratory of Aphidology, Institute of Entomology, Czech Republic; Laszlo Zoltan, Department Taxonomy and Ecology, Babes-Bolyai University Romania; George Japoshvili, Director of Entomology and Biocontrol Research, Centre Ilia State University, Turkey and Hajimu Takada, Laboratory of Entomology, Faculty of Agriculture, Kyoto Prefectural University, Kyoto, Japan, respectively.

The SEM images of the species were obtained with a LEO 1450VP scanning electron microscope (LEO Co. LTD, Germany) after gold coating by mini sputter coater SC7620 (Quorum Technologies).

DNA extraction and sequencing of the COI gene

DNA was extracted with the AccuPrep Genomic DNA Extraction Kit™ (Bioneer Corporation) (http://www.bioneer.com (A single wasp was kept at -20°C, 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.

PCR was carried out in an Eppendorf Mastercylcer gradient (Eppendorf, Hamburg) in standard 25 µl reactions containing 2µl DNA template, 3 µl (10X) buffer, 1µl $MgCl_2$, 0.5 µl dNTPs, 1 µl forward and reverse primer (10 picomoles/µl), 0.3 µl *Taq* polymerase (5U). For COI gene amplification, the primer set reported by Folmer et al., (1994) including LCO1490: 5'-GGTCAACAAATCATAA AGATATTGG-3' (forward) and HCO2198: 5'-TAAAC TTCAGG GTGACCAAAAAATCA-3'(reverse) were used. Temperature conditions for COI amplification were denaturation at 94°C for 60 s, annealing at 56°C for 90 s and extension at 72°C for 90 s (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 Bioneer gel band purification kit (Bioneer co. Korea). Sequencing reactions were performed in a 3730XL DNA analyzer in Macrogen co. (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.

Sequence analysis and specimen delineation

Sequences were aligned using the ClustalX multiple sequence alignment program (Larkin et al., 2007) and subsequently edited manually by eye using BioEdit (Hall, 1999).

Specimen identification was done by inputting the sequence both in the nblast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Barcode of Life Database species identification tool (http://www.barcodinglife.org) which is based on a species identification system. Sequences were compared to identify intra- and interspecific nucleotide differences.

The MEGA 4 (Tamura et al., 2007) was used to estimate evolutionary distances and to compute the basic statistical analysis. Phylogenetic analyses were done using neighbor joining (Saitou and Nei, 1987) for COI with 1000 replications of bootstrap (Felsenstein, 1985). Sequences for the ingroups and outgroups were provided from the EMBL/GenBank with accession number (Table 1). *Bracon* sp. was used as an outgroup in this study.

RESULTS AND DISCUSSION

In this study 2707 specimens of aphid parasitoids

Table 1. List of ingroup and outgroup taxa in phylogenetic analysis and their GenBank accession numbers.

Species	accsion number	Species	accsion number
Aphidius matricariae	GU237130	*Monoctonus* sp	FJ414164
A. matricariae	EF077526	*M. pseudoplatani*	AY935417
A. colemani	FM210127	*P. volucre*	EU819397
A. colemani	FM210126	*P. volucre*	EU819395
A. rhopalosiphi	EU819406	*P. volucre*	EU819394
A. picipes	EU819393	*P. gallicum*	EU819398
A. uzbekistanicus	EU819386	*P. gallicum*	EU819399
A. ervi	EU819385	*P. gallicum*	EU574906
Ephedrus californicus	AY935416	*P. gallicum*	EU819400
E. plagiator	EU819390	*P. unicum*	EU574904
E. incompletus	GU237131	*P. occidentale*	EU574903
Lysiphlebus testaceipe	FM210176	*P. humulaphidis*	EU574905
L. testaceipe	EU294100	*Praon* sp	FJ414906
L. testaceipe	EU294101	*Praon* sp	FJ414907
Monoctonus sp	FJ413773	*Bracon* sp.	FN662420
Monoctonus sp	FJ414765		

and 2313 hyperparasitoids were collected and identified. Theywhich attack five species of aphids of pome fruits (*Aphis pomi*, *Dysaphis affinis*, *D. plantaginea*, *Allocotaphis quaestionis* and *Nearctaphis backeri*). The primary parasitoids belonging to the Aphidiidae family are represented by 6 species: *Aphidius matricariae* Haliday, *Ephedrus persicae* Frogatt, *Praon volucre* Haliday, *Lysiphlebus fabarum* Marshal, *Diaeretiella rapae* McIntosh and *Binodoxys angelicae* Haliday. The hyperparasitoids belonged to 4 families of Hymenopterans: *Alloxysta* sp. (Charipidae), *Pachyneuron aphidis* Bouche and *Asaphes suspensus* Nees (Pteromalidae), *Dendrocerus carpenteri* Curtis (Megaspilidae) and *Syrphophagus aphidivorus* Mayr (Encyrtidae).

The provided SEM images from different parts of parasitoids species are shown in Figs. 1 and 2.

Molecular study of parasitoids

The COI genes from the six parasitoid species and five hyperparasitoids were amplified by PCR. The length of the sequenced COI gene fragment for all species was invariant at 648 bp which were submitted to GenBank (Table 2).

Of the eleven submitted sequences of the COI gene, the only species with this gene sequences already recorded were *A. matricariae* and *P. volucre* from the parasitoids and *D. carpenteri* from the hyperparasitoids, while the other submitted data were new for the GenBank.

The base composition of the COI gene had a strong bias toward adenine and thymine, which constituted approximately 73.7% of the total (table 3).

The multiple alignment of the DNA sequence of COI for 37 taxa showed that 274 sites were conserved, 256 sites were variable and 197 sites were parsimony informative sites, respectively.

Analysis results from the BOLD system

Sequence-comparison against the BOLD system confirmed the morphological identification of two species

– *A. matricariae* and *P. volucre*. In the other cases, due to absence of DNA barcodes, species identification

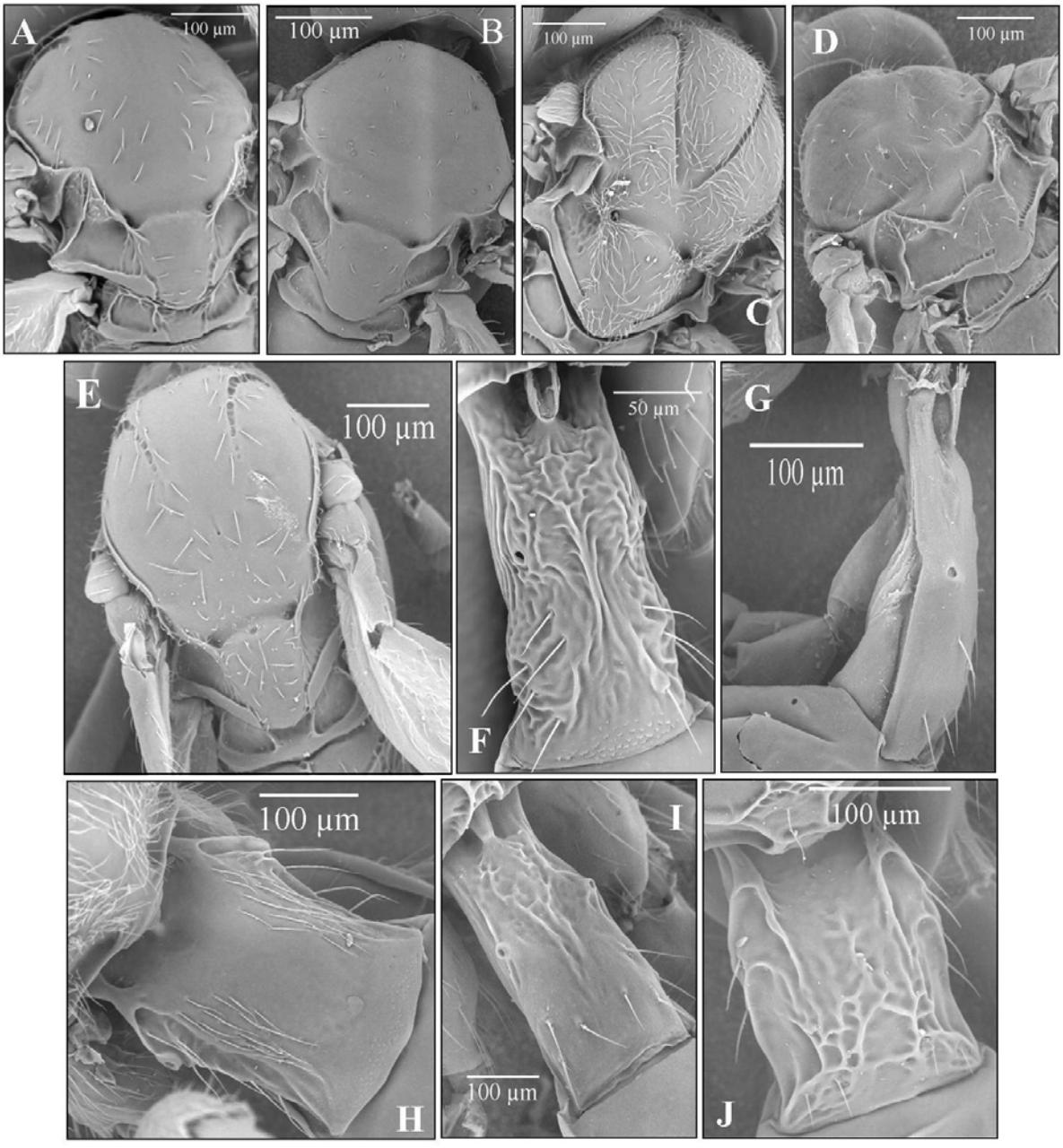

Fig. 1. A, Dorsal aspect of the mesoscutum of *A. matricariae*; B, Dorsal aspect of the mesoscutum of *L. fabarum*; C, Dorsal aspect of the mesoscutum of *P. volucre*; D, Dorsal aspect of the mesoscutum of *D. rapae*; E, Dorsal aspect of the mesoscutum of *E. persicae*; F, Dorsal aspect of the petiole of *A. matricariae*; G, Dorsal lateral aspect of the petiole of *L. fabarum*; H, Dorsal aspect of the petiole of *P. volucre*; I, Dorsal aspect of the petiole of *D. rapae*; J, Dorsal aspect of the petiole of *E. persicae*.

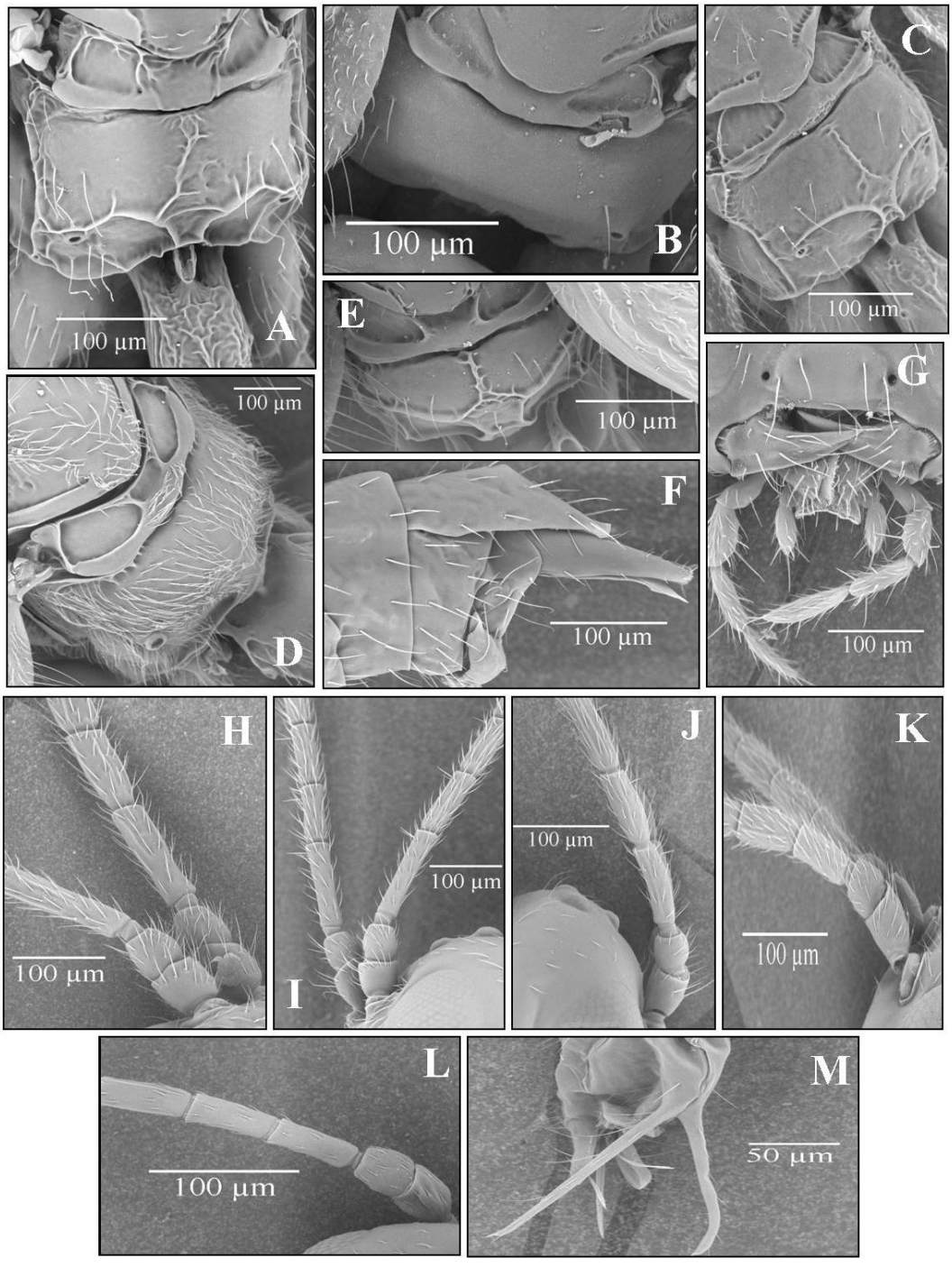

Fig. 2. A, Dorsal aspect of the propodeum of *A. matricariae*; B, Dorsal aspect of the propodeum of *L. fabarum*; C, Dorsal aspect of the propodeum of *D. rapae*; D, Dorsal aspect of the propodeum of *P. volucre*; E, Dorsal aspect of the propodeum of *E. persicae*; F, lateral aspect of the ovipositor of *E. persicae*; G, Mouth parts of *B. angelicae*; H. Antenna of *E. persicae*; I, Antenna of *P. volucre*; J, Antenna of *B. angelicae*; K, Antenna of *A. matricariae*; L, Antenna *of L. fabarum*; M, ventral aspect of ovipositor of *B. angelicae.*

Table 2. Accession numbers of submitted sequences in GenBank.

	Species	Isolate	accession number
Hyperparasitoids	*Pachyneuron aphidis*	FUM11	JF906503
	Asaphes suspensus	FUM12	JF906504
	Syrphophagus aphidivorus	FUM13	JF906507
	Dendrocerus carpenteri	FUM14	JF906505
	Alloxysta sp.	FUM15	JF906506
Parasitoids	*Aphidius matricariae*	FUM17	JF730311
	Praon volucre	FUM18	JF730313
	Ephedrus persicae	FUM19	JF730312
	Lysiphlebus fabarum	FUM20	JF730314
	Diaeretiella rapae	FUM21	JF730316
	Binodoxys angelicae	FUM22	JF730315

Table 3. Nucleotide frequencies of the COI gene in primary and secondary parasitoids.

Species	COI									
	A	C	G	T	A%	C%	G%	T%	AT%	GC%
Aphidius matricariae	182	70	94	268	29.6	11.4	15.3	43.6	73.3	26.7
Praon volucre	196	66	119	289	34.3	11.6	13.5	40.6	74.9	25.1
Ephedrus persicae	193	65	76	228	29.3	9.9	17.8	43.1	72.4	27.6
Lysiphlebus fabarum	189	65	94	268	30.7	10.6	15.3	43.5	74.2	25.8
Diaeretiella rapae	194	65	106	311	28.7	9.6	15.7	46	74.7	25.3
Binodoxys angelicae	305	114	68	193	44.9	16.8	10	28.4	73.2	26.8
Pachyneuron aphidis	196	78	78	276	31.2	12.4	12.4	43.9	75.2	24.8
Asaphes suspensus	190	81	68	266	31.4	13.4	11.2	44	75.4	24.6
Syrphophagus aphidivorus	210	78	98	287	31.2	11.6	14.6	42.6	73.8	26.2
Dendrocerus carpenteri	203	123	88	237	31.2	18.9	13.5	36.4	67.6	32.4
Alloxysta sp.	197	88	91	273	30.4	13.6	14	42.1	72.4	27.6

using this system failed. Searching for the COI sequences of *A. matricariae* and *P. volucre* in the BOLD system revealed a very high similarity between the first species and *A. matricariae* (EF077526) (98.97% similarity) and with *P. volucre* (EU819395) (98.94% similarity). NBLAST analysis for *B. angelicae* and *L. fabarum* attributed 91% and 92% similarities with *Lysiphlebus testaceipes* (FM210176) and *Binodoxys communis* (FJ798201), respectively. In this case, DNA barcoding was reliable at the genus level.

Distance and neighbor joining phylogram

The mean pairwise distance of the COI sequences was 0.161% (range. 0.004 - 0.285%) calculated by the K2P model (not shown).

Intraspecific variations between the *A. matricariae* populations and *P. volucre* were 0.013 (range 0-0.019%) and 0.028 (range. 0.002-0.064%), respectively. There was no DNA barcode for the other species Therefore, the diversity between the populations was not calculated. Nucleotide distances for the different genera were determined separately. The percent nucleotide divergence difference between *L. fabarum* and *L. testaceipes* isolates was 0.038 (range 0-0.077%) while there was no intraspecific difference be-

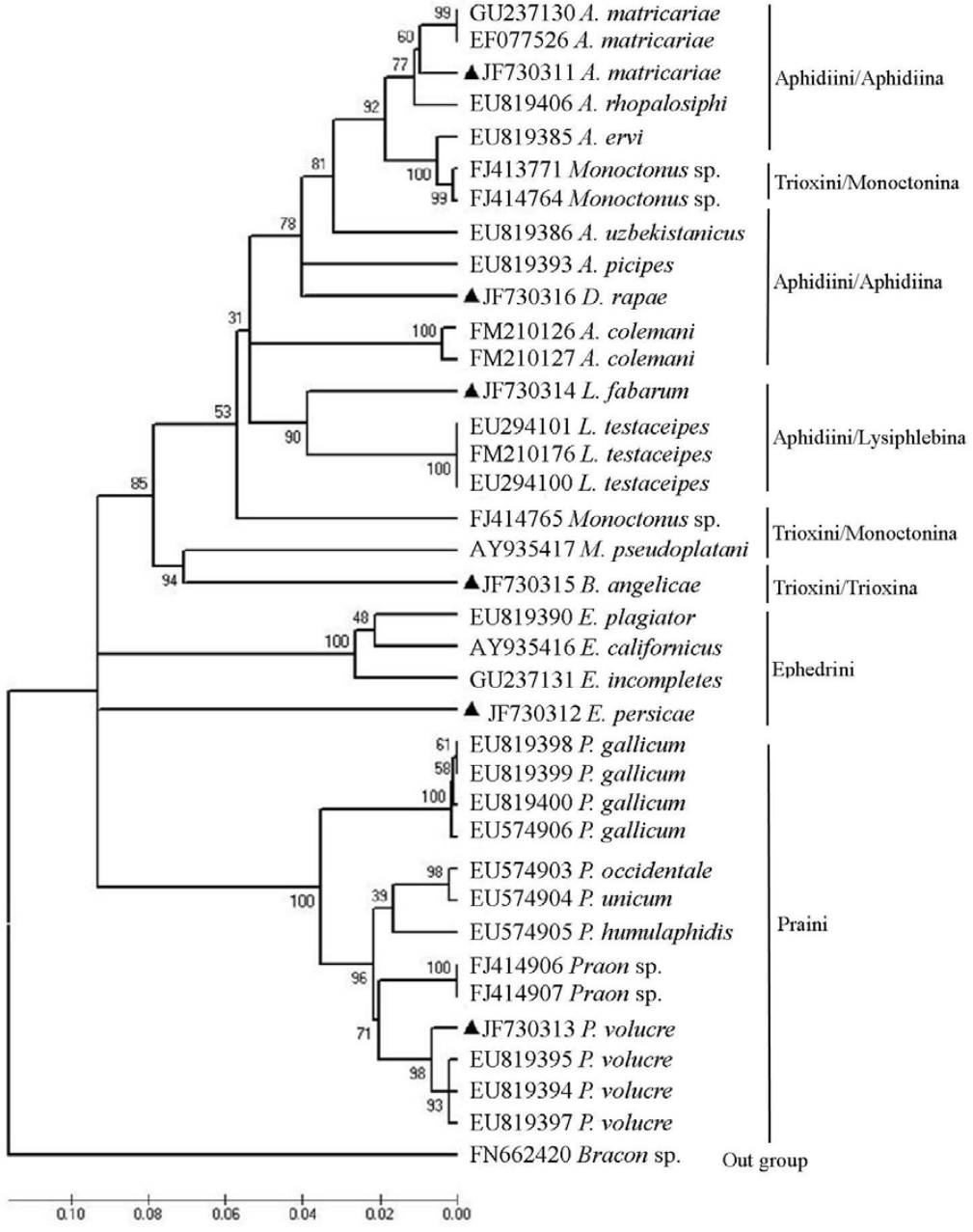

Fig. 3. Neighbor joining tree calculated by MEGA 4.0. The distance measure was set to Kimura 2-parameter of CO1 sequences.

tween the *L. testaceipes* populations. Shufran et al., 2004 calculated nucleotides divergence between *L. fabarum* and *L. testaceipes* species to be 3.45 (range. 0-8.4%).

The mean interspecific nucleotides divergence of *Aphidius*, *Ephedrus*, *Praon* were calculated as 0.073 (range 0-0.118), 0.073 (range 0.041-0.103) and 0.052 (range 0-0.081) respectively.

The neighbor joining tree for parasitoids species is given in Fig. 3.

Molecular study of hyperparasitoids

Comparison of the *D. carpenteri* sequence of Iran with *D. carpenteri* (EU819389) indicated that these two populations differed from each other in the transition of three nucleotides. Nblast results attributed an unknown sequence to the *D. carpenteri* by 99% similarity. This difference was intraspecific in 16S gene and there was 90.5% similarity between individuals (Chen et al., 2006)

The effective application of COI sequence data to molecular diagnostics depends on the patterns of nucleotide substitution and the rate of variation among sites (Blouin et al., 1998). In summary, this study has provided the first COI barcodes for Iranian parasitoid and hyperparasitoid aphids. COI sequence differences between the species were almost 3.72 times higher than the average differences within species,. This might be because of the lack of barcoding of this group of insects. It is hoped that by increasing the numbers of samples and sampling area, more accurate studyies will be performed on this group of parasitoids.

Acknowledgments - We thank Dr Stary, Dr Zoltan, Dr Takada and Dr Japoshvili for confirming the identification of species. We thank P. Stary and Paul Rugmen Jones for their constructive help. We also thank the staff of the Institute of Plant Science, Ferdowsi University of Mashhad, and especially Dr. Mohammad Farsi and Mrs. Mirshahi.

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