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Morphological and Molecular Methods in Identification of *Aphidius transcaspicus* Telenga (Hym: Braconidae: Aphidiinae) Parasitoid of *Hyalopterus* spp. (Hom: Aphididae) with Additional Data on Aphidiinae Phylogeny

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ABSTRACT

During 2009-2010, aphid parasitoid wasps (Hymenoptera: Braconidae: Aphidiinae) on stone fruit trees were investigated in Khorasan Razavi province, North East of Iran. In this survey, eight species from six genera, were reared from 26 associations (Aphid-host plant-parasitoid) in different localities in Khorasan Razavi province. *Aphidius transcaspicus* (62%) was the most abundant species of aphid parasitoids, in strict association with Hyalopterus spp. ITS2 rDNA sequence analyses confirmed classical studies. In phylogenetic analyses, our specimen, *A. transcaspicus* Mashhad1, made a single clade with other populations of this species. *A. transcaspicus* showed a good control potential on *Hyalopterus pruni* Geoffroy (Hom: Aphididae) on plum and apricot. So it can be used as a biocontrol agent against the mealy plum aphid in Khorasan Razavi geographical climates.

Key words: Aphidius transcaspicus, *Hyalopterus pruni*, ITS2, Iran.

INTRODUCTION

Aphidiinae are solitary obligatory endoparasitoids of aphids and many species of them have been considered as potential biocontrol agents (Stary 1970). The genera *Aphidius* Nees is one of the most diverse taxa within the subfamily Aphidiinae (Tomanovic *et al*., 2007). In terms of systematics, *Aphidius* is a difficult genus (Mescheloff and Rosen, 1990).

In some species, aphid host knowledge is helpful in identification, some of the morphological characters used for identification may also vary (environmentally-induced variations) (Pungerl, 1986). In *Aphidius transcaspicus*, geographical variability between populations of this species was indicated (Lozier *et al*., 2009). There has been some uncertainty surrounding the taxonomic status of *Aphidius transcaspicus*. It was first described by Telenga (1958) from Uzbekistan. Stary (1975) considered *Aphidius transcaspicus* as synonymous with *Aphidius colemani* and again separated

from *Aphidius colemani* (Kavallieratos and Lykouressis, 1999). Despite the intense effort to separate species from each other, Uncertainties remain, because some of the morphological characteristics (maxillary and labial palps, body color, notaulices and wing venation), are very variable and overlap (Garantonakis *et al.,* 2009). This Mediterranean parasitoid, treated in general papers, is specialized on *Hyalopterus* spp. (Takada, 1998; Kavallieratos *et al*., 2001; Rakhshani *et al*., 2008; Lozier *et al*., 2009**)** and *Melanaphis donacis* Passerini (Waterhouse, 1998; Kavallieratos *et al*., 2006). According to Stary (1965) and Lozier *et al.,* (2008), it is an effective natural enemy of *Hyalopterus pruni* on peach. Erkin (1983), in studying natural enemies of aphids on stone and pome fruit trees in Turkey, found this species only on *H. pruni.* This species currently is under investigation as a potential biological control agent for the mealy plum aphid, *Hyalopterus pruni*, in prune trees in California (Lozier *et al*., 2008; Lathman and Mills, 2010). *A. transcaspicus* is a very common and economically important species in Iraq, which is climatically similar to Iran. It parasitizes *H. pruni* and *Melanaphis donacis* in mass number and was treated in relation to its biology and IPM proposals in Iraq (Al-Rawy *et al.,* 1969). Use of parasitoid wasps as a biocontrol agents at first step requires correct identification (Lozier *et al*., 2006, 2008). Recently, DNA studies, using PCR techniques, have been investigated as a tool for identification and phylogeny (Landry *et al,* 1993). Ribosomal DNA has been an important target for these studies (Orrego and Agudelo Silva, 1993; Pinto *et al.,* 1997). At the species and intraspecific levels, the internal transcribed spacer regions (ITS-1 and ITS-2) are often used as taxonomic tools in many groups of insects (Campbell *et al.,* 1993). A region of these repeats more suitable for genus and species comparisons is the ITS2 region that site between the 5.8S and 28S RNA genes. The ITS2, a non-coding rapidly evolving region, has highly repetitive sequences that can differ among closely related populations, which have been proven useful for comparing closely related insect species, subspecies, or populations (Silva *et al*., 1999). In this survey, identification of *A. transcaspicus* as the most abundant aphid parasitoid that found especially on *Hyalopterus pruni* in apricot and plum trees in Khorasan Razavi province in North East of Iran was performed and phylogenetic analyses with other populations were studied.

MATERIAL AND METHODS

Collection and Deposition of Specimens

Sampling

Sampling on stone fruit trees was carried out during 2009 - 2010 in five different localities of Khorasan Razavi, Iran (Kardeh, Mashhad, Faragard, Golmakan, Torghabe). Samples from the host trees (almond, apricot, peach, plum) bearing aphid colonies consisting of live and mummified aphids and host plants were collected. Branches with leaves from each tree were carefully cut off and 15 branches with 8-10 cm were transported to plastic boxes which were labeled with date of sampling, host plants and location and covered with mesh for ventilation. Boxes were held at room temperature for 2-3 weeks till the adult parasitoids emerged. The emerged wasps were clipped daily using

an aspirator and dropped into 96% ethanol. Selected fresh specimens of aphids were immersed in 75% ethanol (Rezwani, 2001) and preserved there for later identification.

Specimen preparation

Phase contrast microscope

Emerged parasitoid preserved in 10% KOH along 48 hours for cleaning and dissected parasitoid specimens were mounted on slides in Hoyer for later identification. The external structures of emerged parasitoid (antennae, maxillary palps, labial palps, wings and female genitalia) were studied using an Olympus BH2 phase-contrast microscope and illustrated with a drawing tube. The ratio measurements were based on slide-mounted specimens. Terminology follows Kavallieratos *et al*. (2001) and Rakhshani et al. (2008).

Scanning electron microscope (SEM)

Emerged parasitoid left to soak overnight in 100% ethanol to remove any debris. The external structures (antennae, head, maxillary and labial palps, petiole and propodeum) were gold coated with a sputter coater and examined using a scanning electron microscope.

Molecular methods

DNA extraction and amplification

Genomic DNA was isolated from individual wasp by Qiagene kit (www. Qiagen. com). PCR amplification were performed by Primus thermocycler (MWGBiotech) in 25µl volumes including, 1µl DNA, 0.5µl dNTPs (CinaGen), 1µl Mgcl2 (CinaGen), 0.3µl *Taq* polymerase enzyme (100 units/µl) (CinaGen), 2.5µl PCR buffer (CinaGen), 1µl forward and reverse ITS2 primer (10ρmol/µl**)** (Bioneer) and 16.5µl DWH20. The primers that used to amplify the ITS2 region were: 5́-TGTGAACTGCAGGA-CACATG-3́ (forward) and 5́-GTCTTGCCTGCTCTGAG-3́ (reverse) (Stouthamer *et al*., 1999). The PCR cycling program was including 60s at 94°C followed by 30 cycles of 60s at 94°C, 90s at 50°C and 90s at 72°C with 8 min after the last cycle. 4µl PCR products and 1µl DNA Ladder (100bp) (Frementas) were electrophoresed on a 1% agarose gel. Finally gel was stained using ethidium bromide.

Molecular analyses

PCR product was sent to Milligene France Company for sequencing. Resulted sequence was edited and was confirmed as a ribosomal ITS2 by searching the GenBank database of the NCBI using blast protocol. Phylogenetic analyses were performed with similar sequences from GenBank (table1). Alignment of the sequences of the 5.8S, ITS2 and 28S regions was performed by ClustalW Program (Thompson *et al*., 1994)**.** MEGA Program version 4.0 (Tamura *et al*., 2007) were used to estimate evolutionary distances and compute basic statistical quantities. Also maximum likelihood, maximum parsimony and neighbour joining trees from Aphidiinae and using *Alloxysta leunisii* (Hym: Figitidae: Charipinae) as the outgroup were constructed by MEGA 4.0 Program. To assess the support of clades in the phylogenic trees, the

bootstrap test was performed with 1000 replications (Felsenstein, 1985).

Table 1. Similar ITS2 sequences used for phylogenic studies.

Species	Colone	Abbreviation	Location	Accession Number
Aphidius transcaspicus Telenga	At1	At1G	Greece	DQ504299
Aphidius transcaspicus Telenga	At2	At2G	Greece	FJ495551
Aphidius transcaspicus Telenga	At3	At3G	Greece	FJ495552
Aphidius transcaspicus Telenga	At4	At4G	Greece	FJ495553
Aphidius transcaspicus Telenga	At5	At5G	Greece	FJ49SSS4
Aphidius transcaspicus Telenga	Mashhad1	AtM	Iran	HMS36194
Aphidius colemani Viereck	Aco	AcoU	USA	EUS61659
Aphidius colemani Viereck	Acl	AclG	Greece	DQ504298
Aphidius colemani Viereck	Ac2	Ac2G	Greece	FJ495547
Aphidius colemani Viereck	Ac3	Ac3G	Greece	FJ495548
Aphidius colemani Viereck	Ac4	Ac4G	Greece	FJ495549
Aphidius colemani Viereck	Ac5	Ac5G	Greece	FJ495550
Lysiphlebus testaceipes Cresson	pAJ214	LtpAJ214U	USA	AY498555
Lysiphlebus confuses Tremblay	Strain 1	Lcl 51	Iran	GQ359414
Lysiphlebus fabarum Marshall		LfPsl	Iran	FJ870105
Lysiphlebus fabarum Marshall		Lfcsl	Iran	FJ870106
Alloxysta leunisii Hartig		*Alloxysta leunisii* UK	United Kingdom	AJ309963

RESULTS

Morphologic Study and Distribution

Morphology of *Aphidius transcaspicus.*

Female: Body length 1.7- 2.3 mm, light brown to yellowish., Head dark brown, the tentorial index is about 0.42, face and clypeus with sparse setae, maxillary palps with 4 palpomeres and labial palps with 3 palpomeres (Fig1A, B), Antennae filiform with 16-17 segments (Fig.1C), scape, pedicel and first flagellomere (F1) light brown, rest of flagellum dark brown, the length ratio of F1/F2 is 1.22 (Fig.1D), Metasoma brown, Petiole light yellowish brown with sparse setae in lower level (Fig.1E), Anterolateral area of petiole costate (Fig.1F), Propodeum carinated with 5 areolae, that central areola is narrow and pentagonal (Fig.1H), Forewing venation (incomplete): R1 vein not reaching forewing margin, stigma widely triangular, metacarpus about half of pterostigma length, Rs+M and 2/Rs veins absent, M and m-cu veins united forming M+m-cu vein (Fig.1I), Last terga light brown and ovipositor sheath dark brown (Fig.1J).

Male: Male is similar to the female except that its color is dark brown with black sparse spots and its antennae has 18-19 segments (Fig.1K).

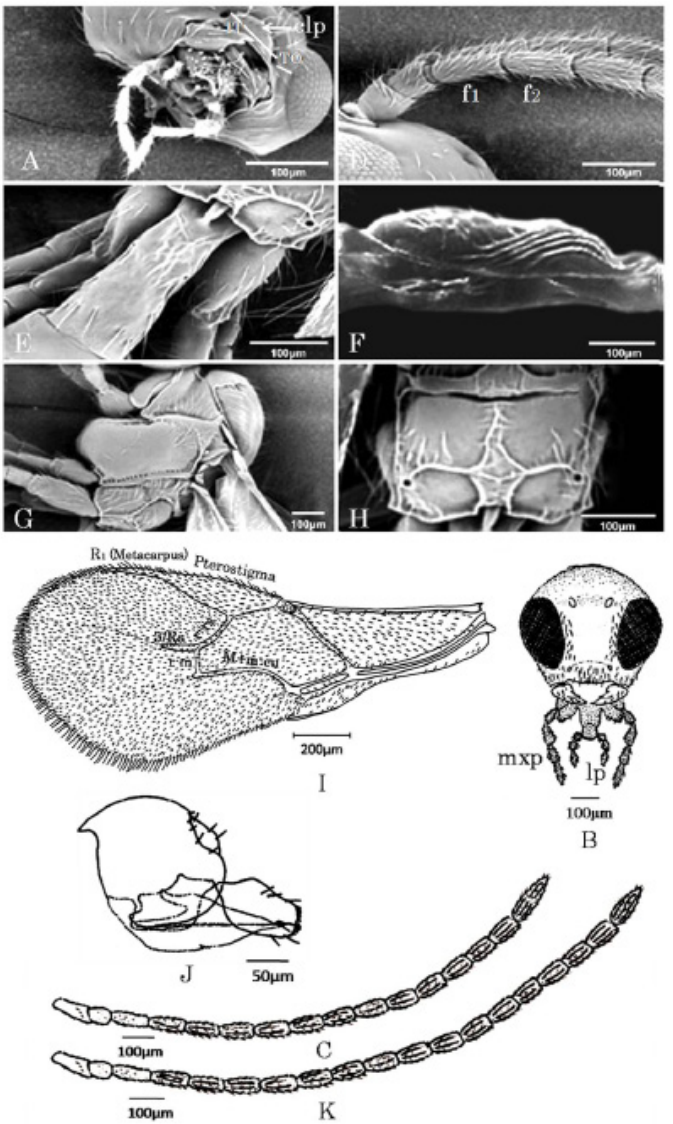

Fig. 1. Morphological characters of *Aphidius transcaspicus* Telenga, A- Head, TO: tentorio-ocular line, IT: intertentorial line, clp: clypeus., B- Head, mxp: maxillary palps, lp: labial palps., C- Female antennae., D- Basal part of antennae, f1: first flagellomere, f2: second flagellomere., E- Petiole., F- lateral aspect of petiole., G- Thorax (lateral aspect)., H- Propodeum., I- Forewing., J- ovipositor sheaths (lateral aspect)., K- Male antennae.

Aphid, parasitoids and plant association

We have determined the presence of eight parasitoid species from six genera

reared in associations of aphids on stone fruit trees at different localities of Khorasan Razavi province, North East of Iran

***Aphidius matricariae* Haliday, 1834**

Brachycaudus divericata (Shaposhnikov) on *Prunus domestica*: Khorasan Razavi-Kardeh, 3 May 2010, (2♀♀).

Brachycaudus helichrysi (Kaltenbach) on *Prunus armeniaca*: Khorasan Razavi-Mashhad, 5 May 2010, (2♀♀); Khorasan Razavi-Torghabe, 12 May 2010, (32♀♀, 11♂♂); on *Prunus domestica*: Khorasan Razavi-Torghabe, 5 May 2010, (2♀♀);

Hyalopterus amygdali (Blanchard) *Prunus amgygdalinus*: Khorasan Razavi-Torghabe, 12 May 2010, (5♀♀, 2♂♂).

Myzus persicae (Sulzer) on *Prunus armeniaca*: Khorasan Razavi-Mashhad, 27 April 2010, (2♀♀, 1♂♂); on *Prunus persica*: Khorasan Razavi-Torghabe, 20 April 2010, (4♀♀, 2♂♂); 12 May 2010, (8♀♀, 3♂♂).

***Aphidius transcaspicus* Telenga, 1958**

Hyalopterus amygdali (Blanchard) on *Prunus amgygdalinus*: Khorasan Razavi-Golmakan, 29 May 2010, (2♀♀,1♂); Khorasan Razavi-Torghabe, 20 April 2010, (11♀, 5♂♂); 9 May 2010, (12♀♀, 6♂♂); 12 May 2010, (9♀♀, 5♂♂); 19 May 2010, (17♀♀, 8♂♂); on *Prunus armeniaca*: Khorasan Razavi-Mashhad, 5 May 2010, (8♀♀, 6♂♂); on *Prunus persica*: Khorasan Razavi-Faragard, 30 April 2010, (5♀♀, 3♂♂).

Hyalopterus pruni (Geoffroy) on *Prunus armeniaca*: Khorasan Razavi-Faragard, 20 September 2009, (12♀♀, 5♂♂); Khorasan Razavi-Mashhad, 25 April 2010, (6♀♀, 2♂♂); 27 April 2010, (6♀♀, 11♂♂); 3 May 2010, (4♀♀, 3♂♂); 5 May 2010, (3♀♀, 2♂♂); 11 May 2010, (27♀♀, 22♂♂); 18 May 2010, (126♀♀, 79♂♂); 20 May 2010, (17♀♀, 9♂♂); 23 May 2010, (15♀♀, 12♂♂); Khorasan Razavi-Torghabe, 5 May 2010, (9♀♀, 7♂♂); 11May 2010, (4♀♀, 4♂♂); 18 May 2010, (3♀♀, 1♂); on *Prunus persica*: Khorasan Razavi-Faragard, 23 October 2009, (6♀♀); 30 April 2010, (6♀♀, 2♂♂); Khorasan Razavi-Golmakan, 29 May 2010, (2♀♀, 1♂); on *Prunus domestica*: Khorasan Razavi-Faragard, 10 September 2009, (10♀♀, 6♂♂); 25 October 2009, (8♀♀, 3♂♂); 9 May 2010, (6♀♀, 4♂♂); Khorasan Razavi-Kardeh, 29 October 2009, (6♀♀, 5♂♂); Khorasan Razavi-Mashhad, 5 May 2010, (13♀♀, 7♂♂); 18 May 2010, (4♀♀, 1♂).

***Binodoxys acalephae* Marshall, 1896**

Brachycaudus helichrysi (Kaltenbach) on *Prunus domestica*: Khorasan Razavi-Mashhad, 3 May 2010, (7♀♀, 6♂♂); 5 May 2010, (9♀♀, 5♂♂).

***Binodoxys angelicae* Haliday, 1833**

Brachycaudus helichrysi (Kaltenbach) on *Prunus domestica*: Khorasan Razavi-Mashhad, 3 May 2010, (3♀♀, 2♂♂); 5 May 2010, (5♀♀, 4♂♂).

***Diaeratiella rapae* M'Intosh, 1855**

Hyalopterus pruni (Geoffroy) on *Prunus armeniaca*: Khorasan Razavi-Mashhad,

27 April 2010, (1♀♀, 1♂); on *Prunus domestica*: 5 May 2009, (2♀♀, 1♂).

Myzus persicae (Sulzer) on *Prunus persica*: Khorasan Razavi-Faragard, 30 April 2010, (2♀♀).

***Ephedrus persicae* Froggatt, 1904**

Hyalopterus amygdali (Blanchard) on *Prunus Prunus amgygdalinus*: Khorasan Razavi-Torghabe, 3 May 2010, (9♀♀, 4♂♂); 5 May 2010, (18♀♀, 9♂♂); 9 May 2010, (13♀♀, 6♂♂); 11 May 2010, (20♀♀, 11♂♂); 12 May 2010, (7♀♀, 1♂♂); 17 May 2010, (6♀♀, 3♂♂); 18 May 2010, (10♀♀, 8♂♂); 19 May 2010, (10♀♀, 6♂♂).

Brachycaudus amygdalinus (Schout) on *Prunus Prunus amgygdalinus*: Khorasan Razavi-Torghabe, 9 May 2010, (5♀♀, 3♂♂); 11 May 2010, (5♀♀, 4♂♂); 12 May 2010, (19♀♀, 11♂♂); 17 May 2010, (6♀♀, 3♂♂); 18 May 2010, (3♀♀, 1♂); 19 May 2010, (4♀♀, 2♂♂).

***Lysiphlebus fabarum* Marshall, 1896**

Hyalopterus pruni (Geoffroy) on *Prunus armeniaca*: Khorasan Razavi-Mashhad, 5 May 2010, (1♀, 1♂); on *Prunus domestica*: Khorasan Razavi-Mashhad, 27 April 2010, (3♀♀, 1♂); 5 May 2010, (4♀♀, 2♂♂).

Brachycaudus helichrysi (Kaltenbach) on *Prunus domestica*: Khorasan Razavi-Mashhad, 10 May 2009, (2♀♀, 1 ♂).

***Praon volucre* Haliday, 1833**

Myzus persicae (Sulzer) on *Prunus persica*: Khorasan Razavi-Faragard, 30 April 2010, (2♀♀, 1♂).

Hyalopterus pruni (Geoffroy) on *Prunus armeniaca*: Khorasan Razavi-Mashhad, 25 April 2010, (3♀♀, 2 ♂♂); 27 April 2010, (2♀♀, 1♂).

Brachycaudus helichrysi (Kaltenbach) on *Prunus armeniaca*: Khorasan Razavi-Mashhad, 5 May 2010, (5♀♀, 6♂♂); on *Prunus domestica*: Khorasan Razavi-Mashhad, 5 May 2010, (1♀♀, 1♂); on *Prunus persica*: Khorasan Razavi-Kardeh, 30 April 2010, (2♀♀, 1♂).

Molecular Study

Sequence data and divergence

The size of PCR product of *Aphidius transcaspicus* strain Mashhad1 on agarose gel closely was 700bp. Aligned sequence of 5.8S-ITS2-28S region is 553bp and ITS2 region is 384bp in length. Sequence is available from Gen Bank with HM536194 accession number. In distance matrix, *A. transcaspicus* AtM strain with 0.012 % divergence is mostly similar to *A. transcaspicus* At2G strain (Table 2).

Phylogenetic relationship

Trees obtained from neighbour joining and maximum likelihood analyses have similar topology to maximum parsimony analysis (neighbour joining data not shown)

(Fig. 2, 3). This indicates strong support for the clades. In description of maximum parsimony phylogram, all species and strains of *Aphidius* and *Lysiphlebus* formed a monophyletic group. Strains of *Aphidius colemani* and *Aphidius transcaspicus* were more closely related to each other than *Lysiphlebus species*. Within *Aphidius*, two clades were obvious. One with *A. transcaspicus* strains and the other containing *A. colemani* Viereck strains with 99% bootstrap value support (Fig. 2). *A. transcaspicus* Mashhad1 sited with other population of this species in a single clade. In alignment of the sequences data, insertion and deletion events as by turn transition and transversion happened which high similarity characters among *A. transcaspicus* strains were shown (Fig.4).

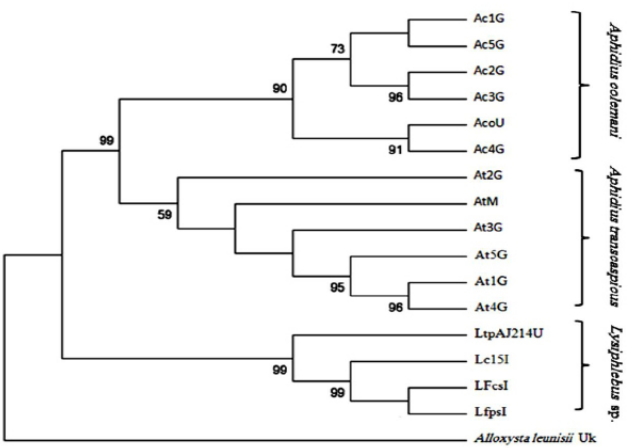

Fig. 2. Phylogenetic analysis of *Aphidius transcaspicus* and related species of this genus based on ITS2 sequence and maximum parsimony analysis, *Alloxysta leunisii* is as outgroup.

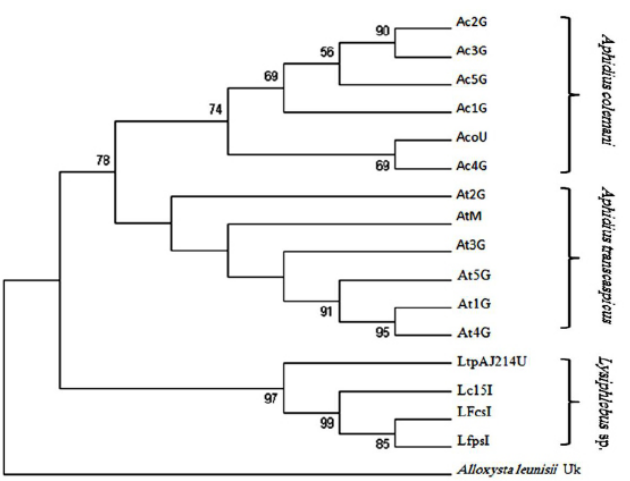

Fig. 3. Phylogenetic analysis of *Aphidius transcaspicus* and related species of this genus based on ITS2 sequence and maximum likelihood analysis, *Alloxysta leunisii* is as outgroup.

Table 2. ITS2 distance matrix (%) (above) and number of differences (below) on pairwise comparison among Aphidiinae population and *Alloxysta leunisii* as outgroup.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1.AtM		0,014	0,012	0,013	0,015	0,014	0,013	0,013	0,014	0,013	0,014	0,013	0,024	0,024	0,024	0,024	0,114
2.At1G	0,060		0,013	0,012	0,005	0,009	0,013	0,014	0,015	0,015	0,014	0,014	0,026	0,026	0,026	0,026	0,112
3.At2G	0,042	0,046		0,008	0,014	0,012	0,008	0,009	0,011	0,010	0,010	0,009	0,022	0,022	0,022	0,022	0,112
4.At3G	0,049	0,039	0,021		0,011	0,012	0,009	0,009	0,011	0,011	0,011	0,009	0,022	0,022	0,022	0,023	0,117
5.At4G	0,068	0,007	0,053	0,039		0,010	0,014	0,014	0,016	0,016	0,015	0,014	0,026	0,026	0,026	0,026	0,112
6.At5G	0,060	0,024	0,042	0,042	0,031		0,013	0,013	0,014	0,014	0,014	0,013	0,024	0,024	0,024	0,024	0,124
7.AcoU	0,049	0,053	0,021	0,028	0,060	0,049		0,005	0,008	0,007	0,005	0,005	0,022	0,022	0,022	0,023	0,120
8.Ac1G	0,049	0,053	0,021	0,028	0,060	0,049	0,007		0,006	0,005	0,006	0,000	0,023	0,023	0,023	0,023	0,122
9.Ac2G	0,060	0,064	0,031	0,039	0,072	0,060	0,017	0,010		0,003	0,009	0,006	0,024	0,024	0,024	0,024	0,128
10.AC3G	0,057	0,061	0,028	0,035	0,068	0,057	0,014	0,007	0,003		0,008	0,005	0,024	0,024	0,024	0,024	0,124
11.Ac4G	0,057	0,060	0,028	0,035	0,068	0,057	0,007	0,014	0,024	0,021		0,006	0,023	0,023	0,023	0,023	0,121
12.Ac5G	0,049	0,053	0,021	0,028	0,060	0,049	0,007	0,000	0,010	0,007	0,014		0,023	0,023	0,023	0,023	0,122
13.Ls15	0,155	0,168	0,126	0,139	0,168	0,151	0,135	0,139	0,151	0,147	0,143	0,139		0,000	0,000	0,013	0,125
14.LfCs	0,155	0,168	0,126	0,139	0,168	0,151	0,135	0,139	0,151	0,147	0,143	0,139	0,000		0,000	0,013	0,125
15.LfPs	0,155	0,168	0,126	0,139	0,168	0,151	0,135	0,139	0,151	0,147	0,143	0,139	0,000	0,000		0,013	0,125
16.LfPa	0,159	0,172	0,130	0,142	0,172	0,155	0,138	0,142	0,155	0,151	0,147	0,142	0,053	0,053	0,053		0,127
17.Alloxysta leunisii	0,988	0,976	0,964	1,000	0,985	1,019	1,016	1,019	1,049	1,032	1,025	1,019	1,069	1,069	1,069	1,072	

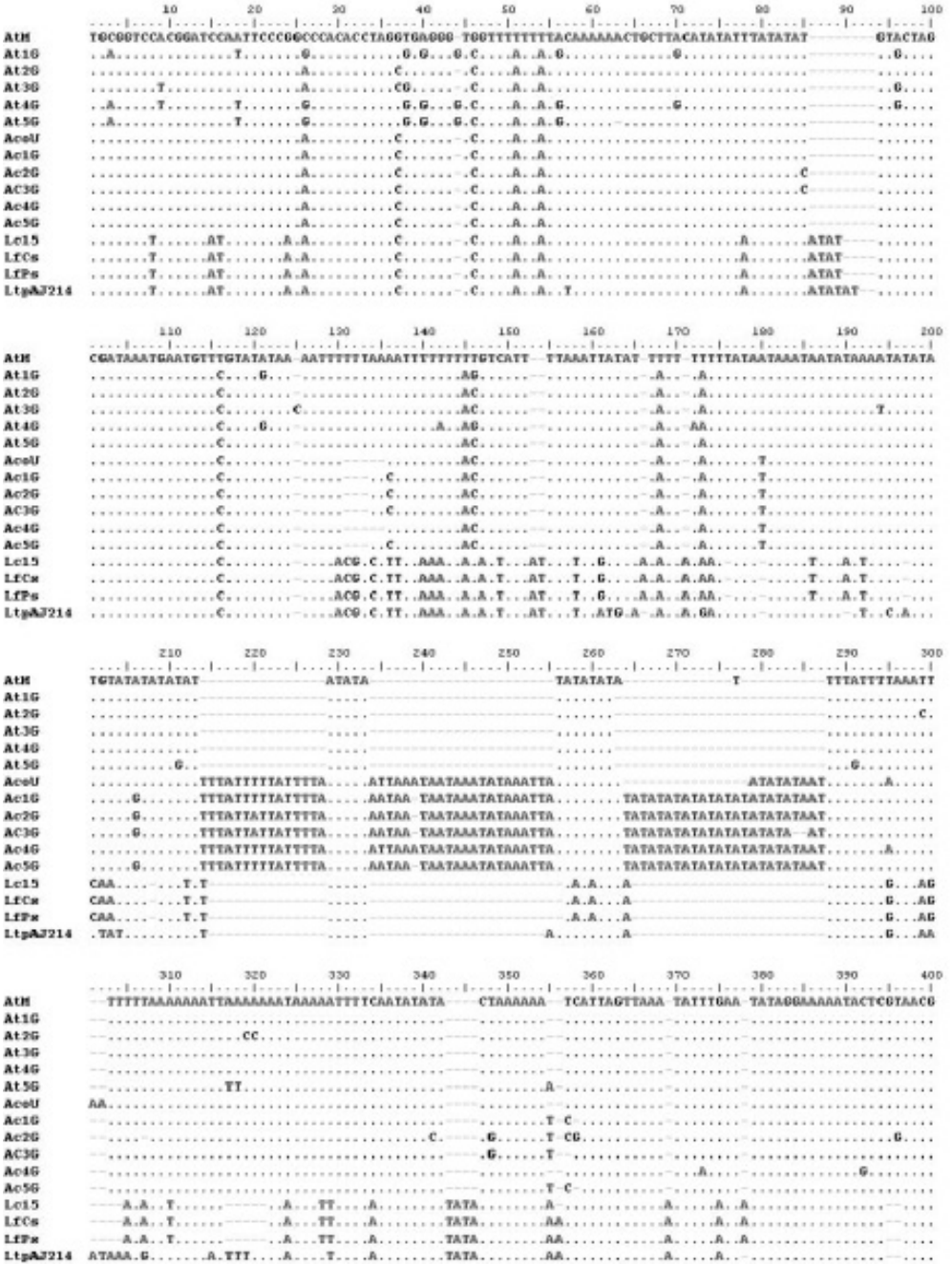

Fig. 4. Alignment of the sequences of the 5.8S, ITS2, and 28S regions of the Subfamily Aphidiinae.

DISCUSSION

In this survey the most abundant aphid was *Hyalopterus pruni* (Hom: Aphididae) and this species was also the most frequently parasitized. In the parasitoids, *Aphidius transcaspicus*, with 62%, was the most abundant parasitoid. This species showed a high biological potential to parasitize *Hyalopterus pruni* in Mashhad geographical climates. So it can be used as a biocontrol agent against the mealy plum aphid.

In molecular studies, The Aphidiinae population in comparison with *Alloxysta leunisii* (outgroup) are monophyletic. Phylogenetic studies on Aphidiinae, based on DNA sequence, show that this subfamily is monophyletic (Smith *et al*., 1999; Sanchis *et al*., 2000; Shi and Chen, 2005). *Lysiphlebus* species are separated from other Aphidiinae and located in a separated clade. Mackaur (1961) based on the morphological studies, considered *Lysiphlebus* as a member of subtribe Lysiphlebina within the tribe Aphidiini and *Aphidius* as a member of subtribe Aphidiina within the same tribe. Sanchis *et al*., (2000), by studding 18S rDNA phylogeny, showed that Lysiphlebina is as a sister clade to Aphidiina. 16s rDNA Phylogeny (Chen *et al*. 2002) confirmed our result. Separation of *A. transcaspicus* and *A. colemani* from each other confirmed capacity of ITS2 region to separation species that morphologically are very similar. Analysis of the 5.8S, ITS2, and 28S regions show that *A. colemani* and *A. transcaspicus* populations are compatible and genetically very similar (Garantonakis *et al.* 2009).

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