

## Molecular Identification and Detection of *Lysiphlebus fabarum* (Hym.: Braconidae): A Key Parasitoid of Aphids, by Using Polymerase Chain Reaction

S. Rahimi Kaldeh<sup>1\*</sup>, R. Hosseini<sup>1</sup>, J. Hajizadeh<sup>1</sup>, and M. M. Sohani<sup>2</sup>

### ABSTRACT

All species in the genus *Lysiphlebus* Förster (Hymenoptera: Braconidae, Aphidiinae) are solitary endoparasitoids of aphids, which are one of the most difficult and unknown taxonomic groups to identify. A pair of species-specific primers as a molecular marker was designed to identify the most abundant and important aphid parasitoid wasp, *L. fabarum* Marshall, collected from Guilan province (Iran). This primer pair, which produces a 148 bp fragment length, was developed for *L. fabarum* by using the ATPase 6 gene region of mitochondrial DNA. The lower detection limit to amplify DNA of *L. fabarum* in a singleplex PCR was determined to be 72 pg of parasitoid DNA/μl PCR which was enough to detect this parasitoid in early life stages within its host. The *L. fabarum* eggs cannot be detected within *Aphis fabae* Scopoli (Hemiptera: Aphididae) earlier than 12 hours after oviposition. A pair of species specific primers designed for *L. fabarum* can be used to monitor the wasp populations under field conditions.

**Keywords:** Aphid parasitoid wasp, ATPase 6, Molecular marker, PCR.

### INTRODUCTION

Aphids may transmit viruses from plant to plant. The viruses cause mottling, yellowing, or curling of leaves and stunting of plant growth. They can also produce large quantities of sticky exudates known as honeydew, which often turns black with the growth of a sooty mold fungus as an indirect damage (Hurej and Werf, 1993). In broad bean fields, black bean Aphid, *Aphis fabae* Scopoli (Hemiptera: Aphididae) is a destructive pest which need to be controlled by conventional chemical methods when damage becomes noticeable (Basedow *et al.*, 2006).

The boost of environmental concerns which is associated with the growing

prevalence of insecticide resistance and outbreaks of secondary pests, has led to a high interest in biological control (Ruberson and Williams, 2000). Natural enemies can be very effective in the control of aphids. Aphid parasitoids have an important role in the control of aphid populations and therefore have been used in several biological control programs (Stary, 1976). The most important and economic parasitoid wasps of *A. fabae* are Aphidiinae wasps. *Lysiphlebus* Förster is a relatively complex genus within Aphidiinae, with about 30 described species (Kambhampathi *et al.*, 2000). Although *Lysiphlebus* species are efficient parasitoids for most aphid pests, many closely related species in this genus are difficult to distinguish morphologically.

<sup>1</sup> Department of Plant Protection, College of Agricultural Sciences, University of Guilan, Rasht, Islamic Republic of Iran.

\* Corresponding author; e-mail: S.rahimik@yahoo.com

<sup>2</sup> Department of Biotechnology, College of Agricultural Sciences, University of Guilan, Rasht, Islamic Republic of Iran.



*Lysiphlebus fabarum* is the most abundant and dominant parasitoid of *Aphis fabae*, *Aphis craccivora* Koch and *Brachycaudus cardui* L. in Guilan province. *Lysiphlebus confusus* is a sympatric and abundant parasitoid wasp species in the same area and often parasitizes *Aphis farinosa* Gmel and *B. cardui*. *Lysiphlebus fabarum* and *L. confusus* are polyphagous aphid parasitoids. They are two closely related species which can easily be confused. The most important morphological character for separating *L. fabarum* from *L. confusus* is the lower marginal setae of forewings in females where in *L. confusus*, the lower marginal setae of forewing is longer than those on the surface whereas in *L. fabarum* it is as short as those on the surface (Rakhshani *et al.*, 2005). *L. fabarum* and *L. confusus* are considered to be endoparasitoid wasps and detecting and differentiating their immature stages within their hosts is particularly impossible.

Traditional methods to detect and discriminate parasitoids within their hosts include rearing hosts in the laboratory for adult emergence or dissecting hosts (Traugott *et al.*, 2006). These methods have some disadvantages, e.g. rearing of parasitoids needs space and facilities to maintain living parasitoids on their hosts because sometimes parasitized hosts do not have abilities to produce an adult parasitoid (Persad and Hoy, 2003). Also, there is a time lag between host collection and parasitoid emergence in many cases (Agusti *et al.*, 2005). Host dissection, may produce an accurate estimate but it requires taxonomic expertise. In addition, there are some closely related parasitoid species in a given host that make it very difficult to detect and determine in their immature stages by host dissection (Persad *et al.*, 2004).

In some cases, morphological identification of arthropod species is difficult because of their small size, lack of efficient morphological characteristics and lack of a specialist (Hosseini *et al.*, 2007). Taxonomic identification keys are useful to identify adult aphid endoparasitoids

(Atanassova *et al.*, 1998) but as a limitation, they are not applied to identify immature stages of endoparasitoids and need the knowledge of experts. Therefore, it has been suggested that an efficient method should be developed to make handling of aphid samples easier, so that it would not need host plant production and daily observations that require rearing procedures and taxonomic knowledge.

In order to solve the above-mentioned difficulties a new and accurate approach is needed to identify, detect and discriminate parasitoids in their host and estimate the actual parasitism rates in the field. DNA-based techniques are relatively quick and cost-effective tools to detect and discriminate parasitoids within their hosts (Greenstone, 2006). Polymerase chain reaction (PCR) based species-specific primers has proved to be a powerful tool to solve the problems. In each conventional PCR, species-specific primers can detect and amplify a small amount of target DNA. In addition, species-specific primers can be used to check corpse of aphids and primitive specimens if they were parasitized and to determine responsible parasitoid (Jones *et al.*, 2005).

In order to design the species-specific primers, several gene regions have been targeted (Garipey *et al.*, 2007). Among different gene regions, mtDNA is the most widely used. Using mtDNA has two major advantages. First, it can be easily amplified in a variety of species. Second, the mitochondrion has a high evolutionary rate (Hurst and Jiggins, 2005).

Persad *et al.* (2004) successfully developed species-specific primers to identify and distinguish two parasitoids of the brown citrus aphid, *Toxoptera citricida* Kirkaldy (Hemiptera: Aphididae), in Florida. Species-specific primers have been used for the identification of many parasitoid species such as *Trichogramma australicum* Girault (Hymenoptera: Trichogrammatidae) (Amornsak *et al.*, 1998), *Anaphes iole* Girault (Hymenoptera: Mymaridae) (Zhu and Williams, 2002),

*Trichogramma dendrolimi* Matsumura (Zheng and Zue, 2002), *Peristenus stygicus* Loan (Hymenoptera: Braconidae) (Zhu *et al.*, 2004).

In this paper, we describe and evaluate the ability and efficiency of a pair of specific primers to detect *L. fabarum* within its aphid hosts. Firstly, we designed a pair of specific primers for the identification of *L. fabarum* and then it was used to detect and analyze the parasitism levels within field collected aphids.

## MATERIALS AND METHODS

### Insects

Samples of *L. fabarum* and *L. confusus*, two the most abundant aphidiin wasps were collected in spring 2008 from different parts of Guilan province, mostly Rasht<sup>1</sup>, Foman<sup>1</sup> and Shaft (Rasht, Guilan university: 37°11' 38.32' N, 49°38' 18.82' E), Foman (Ghalerodkhan: 37°05' 43.71' N, 49°15' 36.98' E) and Shaft (Emamzade Ebrahim: 37°00' 10.94' N, 49°14' 22.60' E). on different host plants. Mummified aphids on foliage were collected, transferred to laboratory and held in 14×12×5 cm plastic cages at 22±1°C, 70±5% RH and 16:8 (L: D) hours until adult parasitoids emergence. Emerged adult wasps were kept in absolute ethanol. Identification was done based on morphological characteristics by relevant taxonomic keys (Rakhshani *et al.*, 2005) and then compared with the identified and confirmed *L. fabarum* deposited in the Natural History Museum of Guilan University (Rasht, University of Guilan). All identified specimens were stored at -20°C for subsequent molecular assays.

### DNA Extraction

DNA of specimens was extracted from individual *L. fabarum* using the CTAB method of Juen and Traugott (2005) with the

adaptation that a 600 µl extraction buffer (12.5mg CTAB, 360µl ddH<sub>2</sub>O, 62.5µl 1M Tris, 175µl 5M NaCl, 25µl 0.5M EDTA and 1.25µl β-mercaptoethanol.) was used. The DNA pellet was suspended in 50 µl of TE (10mM Tris-Hcl pH 8.0 and 1Mm EDTA) and stored at -20°C.

To evaluate the quality and quantity of extracted DNA, the DNA content of 20 extracts, was measured by ND-1000 NanoDrop spectrophotometer (NanoDrop technologies Inc., Wilmington, DE, USA) according to manufacturer instructions.

### Primer Design

Four species belonging to the genus *Lysiphlebus* including *L. fabarum*, *L. confusus* Tremblay and Eady, *L. cardui* (Marshall) and *L. testaceipes* (Cresson) and two outgroups (*Trioxys angelicae* (Haliday) and *Ephedrus niger* Gautier, Bonnamour and Gaumont) were chosen for this study. For these species, ATPase 6 gene region sequence from mitochondrial DNA was obtained from the GenBank database with accession numbers listed in Table 1. Sequences were aligned by using ClustalW Ver. 1.82 (<http://www.ebi.ac.uk/clustalw/>). Then, a pair of primers was designed for *L. fabarum* based on differences among sequences and evaluated using PrimerQuest (<http://www.idtdna.com/biotools/primer-quest/primer-quest.asp>). This primer pair was designated LFF and LFR. A 148-bp PCR product was produced by using the LFF and LFR primer pair (Table 2).

**Table 1.** Names of species and their accession numbers found in Gene Bank databases used to design the species specific primer.

Species	Accession number
<i>Lysiphlebus fabarum</i>	AJ400594
<i>Lysiphlebus confusus</i>	AJ400598
<i>Lysiphlebus cardui</i>	AJ400597
<i>Lysiphlebus testaceipes</i>	AJ400595
<i>Trioxys angelicae</i>	AJ400612
<i>Ephedrus niger</i>	AJ400617

**Table 2.** Species-specific primer sequences designed from the ATPase 6 mtDNA of *Lysiphlebus fabarum*, optimal PCR annealing temperature, amplification size and %GC.

Primers	Sequence	Annealing temp.	Fragment size	% GC <sup>a</sup>
Forward (LFF)	5'-AGGTTTAATAATTTTTGGGTGAG-3'	61	148	30.4
Reverse (LFR)	5'-ACGAATAGATAAAGTAAAAGGACG-3'			33.3

<sup>a</sup> means the percentage of guanine-plus-cytosine

### PCR Amplification

PCR was performed in 20 µl total volume of reaction buffer containing 200 µM dNTPs, 1 µl MgCl<sub>2</sub>, 0.4 µM of each primer, 1 U of Taq DNA polymerase and 2 µl of DNA template (40-80 ng µl<sup>-1</sup>). All PCR reagents were purchased from CinnaGen Co., Iran. The reaction mix was put into a 0.2 ml PCR tube and amplification was performed in a MJ mini<sup>TM</sup> (BIORAD) thermocycler. Cycling conditions were optimized by gradient PCR for a singleplex assay to determine the precise annealing temperature.

The temperature profile was as follows: an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 61°C for 1 min and extension at 72°C for 1 minute, and a final extension at 72°C for 2 minutes.

### Primer Specificity and Sensitivity

The specificity of designed primers was tested with target DNA, DNA of host

species (including *Aphis fabae*, *A. craccivora* Koch, *A. ruborum* Börner), the most important and abundant parasitoid wasps of Aphidiinae in Guilan province and *Coccinella septempunctata* Linnaeus as the predator of *A. fabae* found in the same habitat (Table 3).

A serial dilution of DNA concentrations including 18.4, 9.2, 4.6, 2.3, 1.15, 0.6, 0.3, 0.14, 0.072, 0.036, 0.018, 0.0090 and 0.0045 (ng) was used to determine the sensitivity of *L. fabarum* primer. The PCR conditions were as above.

Detection of *Lysiphlebus fabarum* in Parasitized Nymphs of *Aphis fabae*

In order to determine the ability of the specific primers to detect immature stages of *L. fabarum* inside *A. fabae* nymphs, a separate experiment was performed under laboratory conditions. To obtain mature females, groups of up to 20 newly emerged *L. fabarum* of both sexes were stored in 12×1.5 cm plastic vials for 24 hours before introducing them individually into each of 9 cm diameter Petri dishes each including 5 second to third instars of *A. fabae*. All experiments were visually monitored to check if the parasitoid lays an egg inside the host body. As soon as an

**Table 3.** Parasitoid wasps, their host aphids, host plant and collection area.

Collected species	Host plants	Collection area
<i>Aphis craccivora</i> Koch	<i>Robinia sp.</i>	Rasht
<i>Aphis fabae</i> Scopoli	<i>Faba vulgaris</i>	Pirbazar
<i>Aphis ruborum</i> Börner.	<i>Rubus idaeus</i>	Rasht
<i>Aphidius matricariae</i> Haliday	<i>Calendula sp.</i>	Loshan
<i>Diaeretiella rapae</i> McIntosh	Brassicaceae	Rasht
<i>Ephedrus niger</i> Gautier	<i>Artemisia sp.</i>	Rasht
<i>Lysiphlebus confusus</i>	<i>Salix aegyptiaca</i>	Rasht
<i>Lysiphlebus fabarum</i>	<i>Faba vulgaris</i>	Pirbazar
<i>Trioxys angelicae</i> Haliday	<i>Hibiscus sp.</i>	Rasht
<i>Coccinella septempunctata</i> Linnaeus	<i>Faba vulgaris</i>	Pirbazar

attack occurred, the nymph was removed. In total, 30-40 nymphs attacked by *L. fabarum* were obtained. Parasitized nymphs were either placed immediately in a -20°C freezer or kept for 3, 6, 9, 12, 15, 18, 21, 24, 36, 48 and 72 hours at 22±2°C, 16: 8 hour (Light: Dark) photoperiod, and 70±5% RH in 14×12×5 cm rearing plastic Petri dishes on the leaves of green bean (*Faba vulgaris* L.). For each time interval at least three individuals were used. After each time interval, parasitized nymphs were transferred into a 1.5ml tube and stored at -20°C. DNA was extracted from parasitized nymphs (see DNA extraction) and amplified by using the designed species-specific primer in a singleplex PCR assay.

### Field Sampling

PCR assay was done to detect and analyze parasitism levels within field-collected nymphs of *A. fabae*. The aphids were collected randomly from different parts of plants by hand in a cultivated green bean field at Pirbazar (Rasht) in Guilan province (Iran) on 22 April 2009, 5 May 2009, 9 May 2009 and 23 May 2009, and transferred to the laboratory. Approximately 75 randomly selected aphids were reared on the leaves of green bean in 14×12×5 cm rearing plastic Petri dishes for up to 10 days until mummies developed, then mummified aphids were isolated into a 1.5 ml tube, allowed to emerge, and identified to species by relevant taxonomic keys (Rakhshani *et al.*, 2005). A total of 72 individuals (18 individuals from each time period) of the second and third instar *A. fabae* nymphs were randomly selected from infested plants and stored at -20°C for subsequent molecular assays. Comparisons were performed using T-TEST between the parasitism rate determined by LFF/LFR primers and that determined by key identification of adult parasitoids.

### RESULTS

Analysis of ATPase 6 gene region of mitochondrial gene for chosen species following alignment of sequences showed variations among species sequences which allowed designing a pair of diagnostic primers for *L. fabarum* (Figure 1). Annealing temperature was determined to be 61°C by using gradient PCR.

Species specificity test for LFF/LFR primer pairs indicated that amplification of the expected size was possible only with the DNA of target species while no cross reaction was observed with other tested DNA samples (Figure 2). Sensitivity test of LFF/LFR primer pairs to amplify DNA of *L. fabarum* in singleplex PCR showed a lower detection limit of 72 pg (Figure 3).

Results showed that the DNA of *L. fabarum* could be detected by singleplex PCR in the parasitized *A. fabae* nymphs tested under laboratory conditions. Detection time threshold for LFF/LFR primer pairs were determined to be 12 hours while no detection was observed shorter than this period. All time intervals beyond the 12 hours (including 15, 18, 21, 24, 36, 48 and 72 hours) were also able to detect target DNA (Figure 4).

Field-collected aphids (n= 72) were screened to estimate the levels of parasitism by *L. fabarum*. PCR analysis of *L. fabarum* revealed parasitism rates of 33.33, 66.66 and 72.22% on the second, third and fourth sampling dates (see above), respectively while no detection of parasitoid was made for the first sampling date. No mummified aphids were observed at the first sampling date while the levels of parasitism based on the number of identified adult parasitoids were estimated to be 22.66, 58.66 and 81.33% on the second, third and fourth sampling dates, respectively. Samples reared to parasitoid emergence were only parasitized by *L. fabarum*. Statistical analysis revealed that the percentage of hosts parasitized by *L. fabarum* as detected by PCR was not statistically different from



```

*           20           *           40           *           60
AJ400594 : .....T.....TT.A..... : 60
AJ400598 : -----G.....G..... : 31
AJ400597 : ....A...G.....G.TA...A.....AN..A : 60
AJ400595 : .....G.....G.....G.....G.....C : 60
AJ400612 : .....G.....G.....G.....C : 60
AJ400617 : .....G..T...T...G.TAA..T...A.....G..T.T.AT... : 60
          TGAATTTCAACATTAATTGGTTATTTAATTATTCCCTCAATTATATTGATTAATAATAATCT

*           80           *           100          *           120
AJ400594 : .....T.....G..... : 120
AJ400598 : ..T..A.T.A.....T..C.....AT.....G..... : 91
AJ400597 : .....A.T...T.A.....G..T.....G..... : 120
AJ400595 : ..T.....G.....G.....G..... : 120
AJ400612 : ..T.....G.....G.....G..... : 120
AJ400617 : .....A.T.A...G.....C.....TAT...C...GGGG.....G..... : 120
          CGAATTTTATTTTATAATTAATTTTATAATAATTTTATGAATAGAAATTTAAAATTTAT

*           140          *           160          *           180
AJ400594 : .....G.....C..... : 180
AJ400598 : .....C..A.....T.....A.....AGGCA.....T..... : 151
AJ400597 : .....G.....T.....A.A.....A..... : 180
AJ400595 : .....G.....G.....G..... : 180
AJ400612 : .....G.....G.....G..... : 180
AJ400617 : ..G...C.....T.....A.....AGG.A.....T..... : 180
          TTAAAAATAAATTTTATAATAAATAATTTTATTTTATATTTCTTTATTTATTTATTTAT

*           200          *           220          *           240
AJ400594 : T.....T...A.....T..... : 240
AJ400598 : .....G..G.....T..A...T..... : 211
AJ400597 : .....T.....G..G.....T..... : 240
AJ400595 : .....T.....G.....G..... : 240
AJ400612 : .....T.....G.....G..... : 240
AJ400617 : .....T.....T.....T..A..... : 240
          ATAATAAATAAATTTTATAGGCTTATTTCCCTTATATTTTACAAGGTCAAGACATTTAATT

*           260          *           280          *           300
AJ400594 : .....A.....G..... : 300
AJ400598 : ....TC.....G..G..A...T.....T..... : 271
AJ400597 : ....T...C.....T...A...A...T...A...T... : 300
AJ400595 : .....GG.....T.....G.....G..... : 300
AJ400612 : .....GG.....T.....G.....G..... : 300
AJ400617 : ....T.....T.....T.....A.T... : 300
          TATTCATTAAGTTTATCATTACCTATATGATTAGGTTAATAATTTTGGTTGAGTAAAA

*           320          *           340          *           360
AJ400594 : .....T.....T.....T..... : 360
AJ400598 : .....T.....N.....N..N..... : 331
AJ400597 : ....T.....T.....C..... : 360
AJ400595 : .....G.....T.....G..... : 360
AJ400612 : .....G.....T.....G..... : 360
AJ400617 : .....A.....ATG.....C.....T.....T.....C...T... : 360
          AATACAAACTTTTATATTTGCTCATTAGTTCCCTCAAGGAACACCATTGTTTTAATATTT

*           380          *           400          *           420
AJ400594 : ..C...A.....T..... : 420
AJ400598 : ..N...N..N.....TT.G.....A : 391
AJ400597 : ..A...A.....T...T.....C..... : 420
AJ400595 : .....G.....G.....G..A..... : 420
AJ400612 : .....G.....G.....G..A..... : 420
AJ400617 : .....A..G..G..N...G..T.....A.....C...G..... : 420
          TTTATAGTTTTAATTGAATCATTAGAAATATTATTCGTCCTTTTACTTTATCTATTTCGT

*           440          *           460          *           480
AJ400594 : .....T..A..... : 480
AJ400598 : ....T.....G...G.....G..G...G..G..T..T...C...A : 451
AJ400597 : .....T.....T..A..... : 480
AJ400595 : ....T..T.....C... : 480
AJ400612 : ....T..T.....C... : 480

```

**Figure 1.** Clustal W alignment of partial sequences from *L. fabarum*, *L. confusus*, *L. cardui*, *L. testaceipes*, *T. angelicae*, and *E. niger* ATPase 6 gene, obtained from NCBI. The locations of the priming sites for the conserved forward primer LFF and the conserved reverse primer LFR are in highlighted color. A= Adenine; C= Cytosine; G= Guanine, T= Thymine.

```

AJ400617 : .....T....G....G..N.....G.....T..... : 480
          TTAACAGCAAATATAATTGCAGGTCATTTATTATTAACCTTATTAAGAAGATTATTCCCT

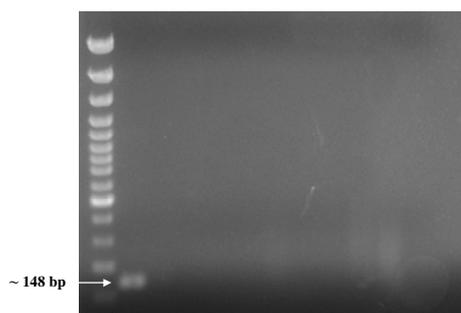
          *           500           *           520           *           540
AJ400594 : .....A.....G..G..... : 540
AJ400598 : G...A..TA.T.....N...G...G..... : 511
AJ400597 : .....A.....G...G..... : 540
AJ400595 : .....C.....G..... : 540
AJ400612 : .....C.....G..... : 540
AJ400617 : G..A.A..TG...A.....G.....AG.....A..G..... : 540
          AATTTTTATTATTTTATTTAATTGTTTTATTTTACAATTAATTTATTAATTTTAGAA

          *           560           *           580           *           600
AJ400594 : .....T..... : 600
AJ400598 : .....T..... : 571
AJ400597 : .....T..... : 600
AJ400595 : .....A..TT..... : 600
AJ400612 : .....A..TT..... : 600
AJ400617 : A..TC.....TT.....A..... : 600
          GTTAGTGTTCATAAATTCATATGTTTTTGTAATTTTAATAATTTTATATTTAAAA

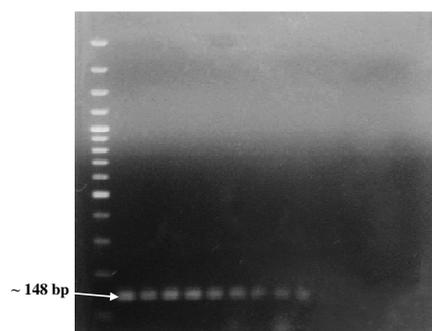
          *           620
AJ400594 : ..... : 624
AJ400598 : ..... : 595
AJ400597 : ..... : 624
AJ400595 : ..... : 624
AJ400612 : ..... : 624
AJ400617 : ..... : 624
          GAAACAAATTATGATAAAATTTAA

```

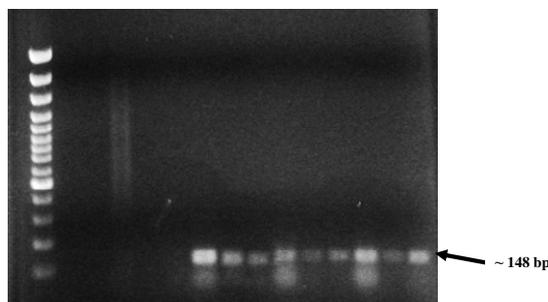
Figure 1. Continued.



**Figure 2.** Diagnostic PCR using the species specific primer for *Lysiphlebus fabarum*, *Lysiphlebus confusus*, *Aphidius matricariae*, *Diaeretiella rapae*, *Ephedrus niger*, *Trioxys angelicae*, *Aphis craccivora*, *Aphis fabae*, *Aphis ruborum*, and *Coccinella septempunctata*, respectively. Lane 1= 100 bp DNA marker, Lane 12= Negative control (no DNA).



**Figure 3.** Diagnostic singleplex PCR using 18.4, 9.2, 4.6, 2.3, 1.15, 0.6, 0.3, 0.14, 0.072, 0.036, 0.018, 0.0090 and 0.0045 (ng/μl) of DNA template from *Lysiphlebus fabarum* adults (serial dilutions). Lane 1= 100 bp marker, Lane 15= Negative control (no DNA).



**Figure 4.** Diagnostic singleplex PCR using the eleven different time spans after oviposition by *Lysiphlebus fabarum* in *Aphis fabae*, Lane 1= 100 bp marker; Lane 2= negative control (no DNA); Lane 3-14= 0, 3, 6, 9, 12, 15, 18, 21, 24, 36, 48 and 72 hours, respectively, and Lane 15= as positive control (*Lysiphlebus fabarum* adult).



parasitism percentage as determined by rearing aphids ( $F= 1.17$ ,  $df= 3$ ,  $P= 0.90$ , SAS PROC T-TEST).

## DISCUSSION

At first a part of COI and ITS2 were used to identify two closely related species *L. fabarum* and *L. confusus* collected from Guilan province, Iran. The sequencing with a similarity about %99.5 showed that COI is not able to separate *L. fabarum* from *L. confusus*. Compared to COI, the ITS2 region of the rDNA was able to partly separate the two closely related species with the similarity of about (%96.5-98.5). A search among other gene regions present in GeneBank database (Encoding elongation factor 1 alpha and 18S rDNA) to separate *L. fabarum* and *L. confusus* showed, none of abovementioned genes were able to distinguish these two species except ATPase subunit 6 of mtDNA (Unpublished data). Therefore, ATPase subunit 6 was selected to design a pair of species-specific primers for *L. fabarum*.

Many studies demonstrated that molecular identification methods such as PCR-based identification are not affected by life stage, size and sex of the samples (Hinomoto *et al.*, 2004; Hosseini *et al.*, 2007; Saccaggi *et al.*, 2008; Traugott *et al.*, 2008). Our results also showed the possibility of detection and identification of *L. fabarum* eggs within aphid body without the consideration of target species gender or life stage.

In many studies utilizing the polymerase chain reaction (PCR) has proved that the detection and identification of parasitoids are possible at high specificity and sensitivity (Greenstone, 2006). Species-specific primers were designed to detect and identify *Lysiphlebus testaceipes*, *Lipolexis scutellaris* Mackauer (both Hymenoptera: Braconidae) and *Aphelinus gossypii* Timberlake (Hymenoptera: Aphelinidae) as the parasitoids of *T. citricida* (Weathersbee *et al.*, 2004). Persad *et al.* (2004) also developed a species-specific primer to

separate two parasitoid species that attack the brown citrus aphid, *L. testaceipes* and *Lipolexis oregmae* Gahan (*L. scutellaris*). In our results, a species-specific primer was successfully developed for the identification of *L. fabarum* as the most important parasitoid species of Aphidiinae in Guilan province. The designed primer produced discernible DNA bands of expected size. Detection threshold of the designed primer was determined to be  $72 \text{ pg } \mu\text{l}^{-1}$  of target DNA which is enough to detect an individual wasp egg among a great amount of non-target host DNA. Traugott *et al.* (2006) designed species-specific PCR primers, for two braconids, *Cotesia glomerata* L. and *Cotesia rubecula* (Marshall), and one ichneumonid, *Diadegma semiclausum* Hellén. The sensitivity of species-specific primers was determined as little as 1 and 11.8 pg of parasitoid DNA which was comparable with our result.

Results indicated that the designed primer can distinguish parasitized aphid nymphs within 12 hours since oviposition by *L. fabarum*. Persad *et al.* (2004) were able to detect *L. testaceipes* DNA in %34 of brown citrus aphids within 6 hours after oviposition and in %100 of samples after 24 hours, while Jones *et al.* (2005) using *L. testaceipes*-specific primer managed to detect parasitoid DNA as early as 48 hours post-parasitism. In a detection time threshold test, species-specific PCR primers for *L. testaceipes* were capable to detect parasitoid DNA in %8 of *Toxoptera citricida* at least 2 hours post-parasitism and in %100 of samples after 72 hours (Weathersbee *et al.*, 2004). Detection of target DNA by species-specific primers from different parasitoids and hosts may be affected by several factors. The percentage of parasitoid to aphid DNA would vary among samples because of differences in their physiological stages. In addition, differences in detection results by species specific primers may be affected by different primers that amplify different genomic regions with different fragment sizes. Jones *et al.* (2005) suggested that a reason for the

lag time necessary for PCR to detect parasitoid DNA such as *L. testaceipes* might be that DNA of parasitoid eggs is surrounded by a tough and flexible egg chorion which prevents release of DNA from its cell. In addition, it has been proved that the presence of PCR inhibitors with very low levels of parasitoid DNA reduces detection sensitivity (Traugott *et al.*, 2006).

Our designed primer was capable to detect the presence of *L. fabarum* DNA within its hosts as early as 12 hours after the parasitoid laid an egg, as a consequence other developmental stages of parasitoid would also be distinguishable. Results showed that all laboratory parasitized *A. fabae* tested by the species-specific primer after 12 hours produced discernible DNA bands of the expected size; which shows the presence of enough target DNA and high sensitivity of primer pairs despite the presence of large amounts of non targeted host DNA.

The designed primer can be used to monitor natural population of *L. fabarum* and also to determine parasitism rate by *L. fabarum* in the field without delay. Jones *et al.* (2005) were able to estimate levels of parasitism of *L. testaceipes* in winter wheat fields using species specific primers and observed that the estimated parasitism rate by species specific primers was not statistically different from estimated parasitism rate by reared aphids.

PCR-based approaches may overestimate the influence of parasitoids on their hosts. This is because molecular detection of immature parasitoids DNA in a host does not necessarily indicate parasitoid survival, as host immune response may neutralize immature stages of the natural enemy (Traugott *et al.*, 2006). In contrast, the parasitism rate might have been underestimated by using species-specific primers because we were unable to detect parasitoid DNA within 0 to 12 hours since oviposition of wasps into the aphid body.

In ecological study of parasitoids, all conventional methods including rearing and dissection of parasitized hosts have their own advantages and limitations. In this

study, it was proved that the species-specific primer could be used as a powerful tool to detect *L. fabarum* as an endoparasitoid wasp within its hosts. Although PCR-based identification method is faster and more accurate than previously available methods such as rearing to monitor parasitism within aphid populations, it has some limitations such as problems with over or underestimation rate of parasitism. As a consequence, it is suggested that a combination of molecular and conventional methods be considered to have a better understanding of parasitoid and host interactions.

#### ACKNOWLEDGEMENTS

We are deeply grateful to Pter Stary (Biology Centre, AS CR, and Institute of Entomology), Jakob Damgaard (Zoological Museum, Copenhagen) and Ahad Sahragard (Department of Plant Protection, University of Guilan) for their helpful comments.

#### REFERENCES

1. Agusti, N., Bourguet, D., Spataro, T., Delos, M., Eychenne, N., Folchers, L. and Arditi, R. 2005. Detection, Identification and Geographical Distribution of European Corn Borer Larval Parasitoids Using Molecular Markers. *Mol. Ecol.*, **14**: 3267-3274.
2. Amornsak, W., Gordh, G. and Graham, G. 1998. Detecting Parasitized Eggs with Polymerase Chain Reaction and DNA Sequence of *Trichogramma australicum* Girault (Hymenoptera: Trichogrammatidae). *Aust. J. Entomol.*, **37**: 174-179.
3. Atanassova, P., Brookes, C. P., Loxdale, H. D. and Powell, W. 1998. Electrophoretic Study of Five Aphid Parasitoid Species of the Genus *Aphidius* (Hymenoptera: Braconidae), Including Evidence for Reproductively Isolated Sympatric Population and a Cryptic Species. *Bull. Entomol. Res.*, **88**: 3-13.
4. Basedow, T., Hua, L. and Aggarwal, N. 2006. The Influence of *Vicia faba* L. (Fabaceae) by *Aphis fabae* (Scop.)



- (Hymenoptera: Aphidiidae) under the Influence of Lamiaceae (*Ocimum Basilicum* L. and *Satureja hortensis* L.). *J. Pest. Sci.*, **79**: 149-154.
5. Garipey, T. D., Kuhlmann, U., Gillot, C. and Erlandson, M. 2007. Parasitoids, Predators and PCR: The Use of Diagnostic Molecular Markers in Biological Control of Arthropods. *J. Appl. Entomol.*, **131**: 225-240.
  6. Greenstone, M. H. 2006. Molecular Methods for Assessing Insect Parasitism. *Bull. Entomol. Res.*, **96**: 1-13.
  7. Hinomoto, N., Muraji, M., Noda, T., Shimizua, T. and Kawasaki, K. 2004. Identification of Five *Orius* Species in Japan by Multiplex Polymerase Chain Reaction. *Biol. Control.*, **31**: 276-279.
  8. Hosseini, R., Keller, M. A., Schmidt, O. and Framenau, V. W. 2007. Molecular Identification of Wolf Spider (Araneae: Lycosidae) by Multiplex Polymerase Chain Reaction. *Biol. Control.*, **40**: 128-135.
  9. Hurej, M. and Werf, W. 1993. The Influence of Black Bean Aphid, *Aphis fabae* Scop and Its Honeydew on the Photosynthesis of Sugar Beet. *Ann. Appl. Biol.*, **122**: 189-220.
  10. Hurst, G. D. and Jiggins, F. M. 2005. Problems with Mitochondrial DNA as a Marker in Population, Phylogeographic and Phylogenetic Studies: The Effects of Inherited Symbionts. *Proc. Royal Soc. B.*, **272**: 1525-1534.
  11. Jones, D. B., Giles, K. L., Chen, Y. and Shufran, K. A. 2005. Estimation of Hymenopteran Parasitism in Cereal Aphids by Using Molecular Markers. *J. Econ. Entomol.*, **98**: 217-221.
  12. Juen, A. and Traugott, M. 2005. Detecting Predation and Scavenging by DNA Gut-content Analysis: A Case Study Using a Soil Insect Predator-prey System. *Oecologia*, **142**: 344 -352.
  13. Kambhampati, S., Volkl, W. and Mackauer, M. 2000. Phylogenetic Relationships among Genera of Aphidiinae (Hymenoptera: Braconidae) Based on DNA Sequence of the Mitochondrial 16S rRNA Gene. *Syst. Entomol.*, **25**: 437- 445.
  14. Persad, A. B. and Hoy, M. A. 2003. Manipulation of Female Parasitoid Age Enhances Laboratory Cultures of *Lysiphlebus testaceipes* reared on *Toxoptera citricida*. *Fla. Entomol.*, **86**: 429-436.
  15. Persad, A. B., Jeyaprakash, A. and Hoy, M. 2004. High-fidelity PCR Assay Discriminates between Immature *Lipolexis oregmae* and *Lysiphlebus testaceipes* (Hymenoptera: Aphidiidae) within Their Aphid Hosts. *Fla. Entomol.*, **87**: 18-27.
  16. Rakhshani, E., Talebi, A. A., Kavallieratos, N. G., Rezwani, A., Manzari, S. and Tomanovic, Z. 2005. Parasitoid Complex (Hymenoptera, Braconidae, Aphidiinae) of *Aphis craccivora* Koch (Hemiptera: Aphidoidea) in Iran. *J. Pest. Sci.*, **78**: 193-198.
  17. Ruberson, J. R. and Williams, L. H. 2000. Biological Control of *Lygus* sp.: A Component of Area Wide Management. *South. Entomol. Supp.*, **23**: 96-110.
  18. Saccaggi, D. L., Kruger, k. and Pietersen, G. 2008. A Multiplex PCR Assay for the Simultaneous Identification of Three Mealybug Species (Hemiptera: Pseudococcidae). *Bull. Entomol. Res.*, **98**: 27-33.
  19. Stary, P. 1976. Aphid Parasites (Hymenoptera: Aphidiidae) of the Mediterranean Area. W. Junk Publishers, The Hague, **95** PP.
  20. Traugott, M., Zangerl, P., Juen, A., Schallhart, N. and Pfiffner, L. 2006. Detecting Key Parasitoids of Lepidopteran Pests by Multiplex PCR. *Biol. Control.*, **39**: 39-46.
  21. Traugott, M., Bell, J. R., Broad, G. R., Powell, W., Van Veen, F. J. F., Vollhard, I. M. G. and Symondson, W. O. C. 2008. Endoparasitism in Cereal Aphids: Molecular Analysis of a Whole Parasitoid Community. *Mol. Ecol.*, **17**: 3928-3938.
  22. Weathersbee, A. A. I., Shufran, K. A., Panchal, T. D., Dang, P. M. and Evans, G. A. 2004. Detection and Differentiation of Parasitoids (Hymenoptera: Aphidiidae and Aphelinidae) of the Brown Citrus Aphid (Homoptera: Aphididae): Species-specific Polymerase Chain Reaction Amplification of 18S rDNA. *Ann. Entomol. Soc. Am.*, **97**: 286-292.
  23. Zheng, X. L. and Zuo, R. S. H. 2002. PCR-based Technique for Identification and Detection of *Trichogramma* spp. (Hymenoptera: Trichogrammatidae) with Specific Primers. *Acta. Entomol. Sinica.*, **9**: 9-16.
  24. Zhu, Y. C. and Williams, L. 2002. Detecting the Egg Parasitoid *Anaphes iole*

- (Hymenoptera: Mymaridae) in Tarnished Plant Bug (Heteroptera: Miridae) Eggs by Using a Molecular Approach. *Ann. Entomol. Soc. Am.*, **95**: 359-365.
25. Zhu, Y. C., Riddick, E. W., Williams, L., Schotzko, D. J., Logarzo, G. A. and Jackson, C. G. 2004. Potential of Detection and Identification of Nymphal Parasitoids (Hymenoptera: Braconidae) of *Lygus* Bugs (Heteroptera: Miridae) by Using Polymerase Chain Reaction and ITS2 Sequence Analysis Techniques. *Ann. Entomol. Soc. Am.*, **97**: 743-752.

### شناسایی و ردیابی مولکولی زنبور پارازیتوئید کلیدی شته‌ها *Lysiphlebus fabarum* (Hym.: Braconidae) با استفاده از روش PCR

س. رحیمی کلد، ر. حسینی، ج. حاجی زاده و م. م. سوهانی

#### چکیده

زنبورهای پارازیتوئید جنس *Lysiphlebus* Förster (Hymenoptera: Braconidae, Aphidiinae) به عنوان پارازیتوئید داخلی-انفرادی شته‌ها، یکی از گروه‌های بسیار مشکل و ناشناخته از لحاظ تاکسونومیک به شمار می‌آیند. در این مطالعه یک جفت آغازگر اختصاصی به منظور شناسایی مولکولی زنبور پارازیتوئید (*L. fabarum* (Marshall)، مهمترین و فراوان‌ترین گونه پارازیتوئید فعال روی شته‌ها، جمع‌آوری شده از استان گیلان طراحی شد. جفت آغازگر طراحی شده بر اساس ناحیه ژنی ATPase 6 از DNA میتوکندریایی قادر به تکثیر قطعه ۱۴۸bp از DNA زنبور پارازیتوئید *L. fabarum* بود. جفت آغازگر اختصاصی طراحی شده قادر به ردیابی DNA زنبور پارازیتوئید *L. fabarum* در حضور حداقل ۷۲ پیکوگرم بر میکرولیتر بوده و نیز قادر به ردیابی تخم این زنبور در بدن شته سیاه باقلا (*Aphis fabae* (Hemiptera: Aphididae)) پس از گذشت ۱۲ ساعت از قرار دادن تخم در بدن شته میزبان بود. استفاده از جفت آغازگر اختصاصی طراحی شده امکان ارزیابی سطوح پارازیتیسیم ایجاد شده توسط زنبور پارازیتوئید *L. fabarum* را در شرایط مزرعه فراهم می‌نماید.