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

S. Rahimi Kaldeh 1*, R. Hosseini 1, J. Hajizadeh 1, and M. M. Sohani 2

**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.
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., 2006). 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 (Gariepy 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),
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Table 1. Names of species and their accession numbers found in Gene Bank databases used to design the species specific primer.

<table>
<thead>
<tr>
<th>Species</th>
<th>Accession number</th>
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<tbody>
<tr>
<td>Lysiphlebus fabarum</td>
<td>AJ400594</td>
</tr>
<tr>
<td>Lysiphlebus confusus</td>
<td>AJ400598</td>
</tr>
<tr>
<td>Lysiphlebus cardui</td>
<td>AJ400597</td>
</tr>
<tr>
<td>Lysiphlebus testaceipes</td>
<td>AJ400595</td>
</tr>
<tr>
<td>Trioxys angelicae</td>
<td>AJ400612</td>
</tr>
<tr>
<td>Ephedrus niger</td>
<td>AJ400617</td>
</tr>
</tbody>
</table>

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\(^1\), Foman\(^1\) 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\(_2\)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 2. Species-specific primer sequences designed from the ATPase 6 mtDNA of *Lysiphlebus fabarum*, optimal PCR annealing temperature, amplification size and %GC.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Annealing temp.</th>
<th>Fragment size</th>
<th>% GC a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward (LFF)</td>
<td>5´-AGGTTTAATAATTTTTGGGTGAG-3´</td>
<td>61</td>
<td>148</td>
<td>30.4</td>
</tr>
<tr>
<td>Reverse (LFR)</td>
<td>5´-ACGAATAGATAAAGTAAAAGGACG-3´</td>
<td></td>
<td></td>
<td>33.3</td>
</tr>
</tbody>
</table>

a 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 MgCl₂, 0.4 µM of each primer, 1 U of Taq DNA polymerase and 2 µl of DNA template (40-80 ng µl⁻¹). 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™ (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* Borner), 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
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
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.
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Figure 1. Continued.

Figure 2. Diagnostic PCR using the species specific primer for Lysiphlebus fabarum, Lysiphlebus confusus, Aphidius matricariae, Diaretiella 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 pg µl⁻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.

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*Lysiphlebus fabarum* (Hym.: Braconidae)

شناختی و فردای موپولکولی زنبور پاژیبکنندگی به دسته مامیلاریه (Hymenoptera: Mymaridae) در بیگانه‌های میخ‌کنیان شناسایی نشده است. به این دلیل، مطالعه‌ای به شمار می‌آید که جفت آغازیگر اختصاصی می‌دانند. مولکولی زنبور پاژیبکنندگی *L. fabarum* (Marshall) ممتنی در تکثیر نیمه‌نواحی نیمه‌نواحی، فعالیتی که به روش PCR با استفاده از روش DNA استفاده می‌شود.

*L. fabarum* DNA از میکسیدرایی کادر به تکثیر فلزه 1485bp از زنبور پاژیبکنندگی *fabarum* و DNA بود. جفت آغازیگر اختصاصی طراحی شده کادر به دیدایی زنبور پاژیبکنندگی *L. fabarum* در حضور حداقل 77 پیکوگرم می‌بایست و نیز قادر به دیدایی نختم این زنبور در بدن *Aphidocta fabae* (Hemiptera: Aphididae) تخم نمود. طراحی شده و ابزار از نیمه‌نواحی نیمه‌نواحی طراحی شده از *L. fabarum* ابزار ابزار مورد استفاده در تحقیق و درآمد مستمر یافته می‌نماید.