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Comparison of Molecular and Conventional Methods for Estimating Parasitism Level in the Pomegranate Aphid *Aphis punicae* (Hemiptera: Aphididae)

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Abstract

Aphidiinae (Braconidae: Aphidiinae) is a subfamily of endoparasitic wasps specialized in parasitizing aphids. Although, to date, different methods have been used to measure parasitism level, obtaining an accurate estimate remains challenging due to several limiting factors. This study was set to: 1) Compare efficiency of conventional and molecular-based methods in estimating parasitism level of the pomegranate aphid *Aphis punicae* (Passerini; Hemiptera: Aphididae), and 2) Estimate seasonal activity of the Aphidiinae parasitoids of the pomegranate aphid. The molecular approach (polymerase chain reaction [PCR]) detected the presence of three main parasitoids *Lysiphlebus fabarum* (Marshal; Hymenoptra: Braconidae), *Binodoxys angelicae* (Haliday; Hymenoptra: Braconidae), and *Ephedrus persicae* (Frogatt; Hymenoptra: Braconidae). The presence of hyperparasitoid and aphid DNAs did not interfere with the outcome, indicating specificity of the selected primers. Minimum concentrations of DNA needed for successful amplifications were 16.33, 28.65, and 22.65 ng μl^{-1}, for *L. fabarum*, *B. angelicae*, and *E. persicae*, respectively. The level of parasitism was significantly higher in spring (28.42%) than both summer and fall; parasitism level during summer (11.89%) and fall (5.86%) formed a homogeneous statistical subset. Although the overall level of parasitism estimated by PCR (22.7%) was more than twofold higher than those estimated by a conventional counting method (10.5%), there was a strong positive correlation between the two approaches. Provided the potential limitations of either method, simultaneous use of both methods was recommended for an objective estimate of the effectiveness of the Aphidiinae parasitoids as biological control agents of *A. punicae*.

Key words: Braconidae, Aphidiinae, parasitoid, parasitic wasps, seasonal parasitism

Aphids are phloem-sap feeders that utilize a wide range of host plants, including those of economic importance. Under most circumstances, aphid populations are expected to remain in check by a wide range of natural enemies, primarily parasitoid wasps (Moawad and Al-Barty 2011). As such, application of broad-spectrum insecticides, which indiscriminately target both pest and beneficial populations, may consequently result in an increase in aphid numbers, by eliminating the natural enemies (Brunner et al. 2001, Thomson and Hoffmann 2007, Rouhani et al. 2013). Therefore, to employ the most effective and relatively more sustainable pest management practices, it is essential to acquire clear understandings of the natural enemy presence, and their efficiency in targeting pest populations, in any given agroecosystem.

Pomegranate, *Punica granatum* (Linnaeus; Myrtales: Punicaceae), is a suitable host for a number of aphid species, which can negatively impact both its yield and production quality. The pomegranate aphid *Aphis punicae* (Passerini; Hemiptera: Aphididae) is a

prominent example, which affects pomegranate trees throughout the Mediterranean Basin (Bayhan et al. 2005, Alford 2007) and further to the east, throughout northeastern Iran (Moawad and Al-Barty 2011, Rouhani et al. 2013). *A. punicae* infests pomegranate leaves, inflorescences and fruits, resulting in reduced plant vigor. Heavy infestations by the aphid may also provide a suitable environment for mold growth, due to excessive secretion of honeydew. However, the use of natural enemies is still a highly recommended approach to manage aphid populations in pomegranate orchards due to environmental concerns regarding pesticide use (Kambrekar and Biradar 2015).

Parasitoid wasps, from the subfamily Aphidiinae, constitute the most important natural enemies of aphids, and are used as biocontrol agents in various agro-ecosystems (Stary 1970, 1979; Sanchis et al. 2000). Predicting the likelihood of success in biological control programs, however, relies on precise identification of parasitoids and estimation of their location-specific level of parasitism (Hajek 2004).

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To date, rearing parasitized hosts (and counting the number of emerged parasitoids), host dissection, and molecular detection of the parasitoid(s) within the parasitized aphids have been utilized as common approaches to detect and identify parasitoids, and to assess the level of parasitism in various systems (Mathé-Hubert et al. 2013).

Although microscopic examination of dissected hosts is helpful in determining parasitoid presence (Jones et al. 2003), it involves a tedious process (Greenstone 2006), that may sometimes fail to provide an accurate estimate of the level of parasitism by a given parasitoid. This is because hyperparasitoids are also commonly present in parasitized populations of aphids, including *A. punicae* (Farrokhzadeh et al. 2014), and dissection methods could potentially lead to an overestimation of parasitism level of the pest population, as a result of misidentifications (Agusti et al. 2005). In addition, taxonomic keys could lead to misidentifications, due to the small size of the parasitoids, as well as ambiguities in some of the key morphological traits (Atanassova et al. 1998).

Rearing parasitized hosts requires ample space, preparation of artificial diet, and is also subject to the possibility of inaccurate estimation of parasitism due to potential pre-emergence mortality resulting from various biotic and abiotic factors (Stuart and Greenstone 1996). Some studies, however, disregarded this pre-emergence mortality (Sheppard and Kissam 1981), providing only an approximation of the level of parasitism (Mathé-Hubert et al. 2013). In an effort to reduce the negative impact of pre-emergence mortality on estimations of parasitism level, a combination of adult emergence and dissection methods has been employed. This approach provided a more accurate assessment, when parasitoid development reached a level identifiable by dissection and when the insect was not decayed beyond recognition. However, these methods often failed to detect parasitism that may have occurred in the field or the laboratory (Ratcliffe et al. 2002).

For the past few decades, molecular techniques have been used to assess parasitism levels by detecting proteins and the DNA associated with parasitoid wasps. Electrophoresis enzyme methods to detect proteins require considerable skill levels and are not sensitive enough to separate closely related species (Castañera et al. 1985, Traugott et al. 2006). Other molecular methods used to assess parasitism levels include microsatellite markers (Masutti and Chavigny 1997) and randomly amplified polymorphic DNA (RAPD) (Landry et al. 1993).

The polymerase chain reaction (PCR), with rapid production of multiple copies of nucleotide sequences (Mullis 1990), can detect immature stages of parasitoids more accurately by detecting small quantities of DNA (Glick and Pasternak 1998). This is particularly advantageous when parasitoids are difficult to be distinguished based on morphological traits during immature stages of development (Greenstone et al. 2005, Traugott et al. 2006). Previous studies showed that the mitochondrial gene 16S rDNA can be used successfully in phylogenetic studies of Aphidiinae (Jones et al. 2005, Derocles et al. 2012), due to its sufficient diversity across closely related species (Kambhampati et al. 2000, Zhu and Williams 2002). Furthermore, the copy numbers of the mitochondrial DNA are relatively higher in early developmental stages and anneal the 16S primer relatively more easily to parasitoids DNA (Hoy 1994, King et al. 2008, Derocles et al. 2012). The objective of the present study was to compare the efficiency of the conventional method of counting adult parasitoids following rearing of the parasitized aphids with a PCR-based approach for estimating seasonal parasitism level by Aphidiinae parasitoids, for the first time in a pomegranate production system.

Materials and Methods

Sampling Procedure

A. punicae were collected from a pomegranate orchard in Kashmar, Razavi Khorasan province of Iran, at longitude 58° 26′ 7.88″ E and latitude 35° 12′ 54.15″ N. The sampling was carried out twice a week, between April and October 2013. To assess the level of parasitism by counting the emerged wasps, one aphid-infested branch (25 cm length) was removed from each of 20 randomly selected trees within an orchard (about 1950 m^2) containing 1,100 trees. The cut branches were immersed in water bottles at the basal ends and caged individually, using the mesh-covered cylindrical cages (described below), before being transported to the laboratory.

Estimation of Parasitism Level

Counting of Emerging Parasitoids

The aphid-infested branches were individually caged in mesh-covered transparent acrylic cylinders (35 × 20 cm; height × diameter) and maintained in a growth chamber for 3 wk at 25°C, 56% Relative Humidity (RH) and a 16:8 (L:D) h photoperiod (Hill and Hoy 2003). The emerging parasitoids were collected, on a daily basis, and identified according to morphological characteristics of adults using the available taxonomic keys (Stary 1979, Kavallieratos et al. 2002b, Rakhshani et al. 2012). Species identifications of Aphidiinae were verified by Dr. Petr Starý, Laboratory of Aphidology, Institute of Entomology, Czech Republic.

Daily counts were conducted to minimize the risk of repeated parasitism. The percentage of aphids parasitized in each cage was regarded as an experimental unit. The per-cage level of parasitism was calculated by dividing the number of mummified aphids (or the number of emerged parasitoid adults) by the total number of aphids (Tomanovic et al. 1996, Kavallieratos et al. 2004). Aphids were counted under a stereomicroscope (OPTEK MST C-2D), immediately upon arrival to the laboratory.

Molecular Based Assessment

To minimize the risk of failure in detecting parasitized aphids, DNA was extracted 48h after collecting the field samples (Jones et al. 2005). Approximately 100 live aphids were collected off of the fresh leaves and maintained in the laboratory at 25°C, 56% RH and a 16:8 (L:D) h photoperiod, for the 48-h pre-extraction period.

DNA Extraction and Amplification

To estimate the level of parasitism for each sampling date, 14 aphids were removed and extracted individually (total of 770 aphids). DNA extraction was done using Chelex Ultra 100 molecular biology grade resin. Nitrogen-frozen samples were ground using a micro pestle in 25 µl Chelex and 1 µl Proteinase K. The homogenate was incubated at 60°C for 4 h. The supernatant was removed and stored at –20°C for later PCR analysis (Sayed et al. 2013).

Primers from 16S rDNA, specific to Aphidiinae subfamily (Jones et al. 2005), were used to detect parasitoids (Table 1). PCR was carried out in an Eppendorf Mastercycler gradient (Eppendorf, Humburg, Germany). The 25 µl reaction volume contained 3 µl of DNA template, 3 µl (10×) buffer, 1 µl MgCl$_2$, 0.5 µl dNTP$_s$, 1 µl of each of the primers (10 Pmol µl^{-1}), 0.3 µl *Taq* polymeras (5U), and 15.2 µl double distilled water. Adult parasitoid DNA [*Lysiphlebus fabarum* (Marshal; Hymenoptra: Braconidae)] was used as a positive control. Double distilled water was included on plates as a negative control. PCR steps included an initial denaturant at 94°C for 60 s, followed by 30 cycles denaturant at 94°C for 60 s, annealing for 90 s at 52°C for 16S, and 54°C for COI, an extension period at 72°C for 90 s, and a final extension at 72°C for 8 min. PCR products (10 µl) were electrophoresed in a 1% agarose gel and visualized by UV light.

Table 1. The primers used in molecular estimation of parasitism by Aphidiinae

Target region	Primer and sequence	Reference
COI	LCO1490: 5′-GGTCAACAAATCATAAAGATATTGG-3′	(Folmer et al. 1994)
	HCO2198: 5′-TAAACTTCAGGGGTGACCAAAAAATCA-3′	
16S	WaspF1: 5′-ACCTGTTTATCAAAAACATG-3′	(Jones et al. 2005)
	WaspR: 5′-CGAGGTCGCAATCTTTTTTA-3′	

Primer Sensitivity and DNA Dilution Evaluation

Before using molecular procedures to estimate parasitism, primer sensitivity was examined. Total DNA was extracted from adult *A. punicae*, the three parasitoids, *L. fabarum* (Marshal), *Binodoxys angelicae* (Haliday; Hymenoptra: Braconidae), and *Ephedrus persicae* (Forgatt; Hymenoptra: Braconidae), and the three hyperparasitoids, *Syrphophagu aphidivorus* (Mayer), *Alloxysta* sp., and *Pachyneuron* sp. In this assay, five samples were included for each of the tested species.

The primer sensitivity test was carried out in three steps. First, DNA was amplified using the universal COI primer (Table 1) to confirm DNA extraction success. Then, the extracted DNA was amplified using 16S rDNA primer to determine its specificity for parasitoids. Finally, the mixed DNA of parasitoids, host and hyperparasitoids were amplified using 16S rDNA primer to simulate a homogenate representing a scenario, which could occur under natural circumstances. The PCR was conducted as previously described, with the exception that the total volume of DNA was 6 µl (i.e., 3 µl of parasitoid and 3 µl of either aphid or hyperparasitoid) with 12.2 µl of double distilled water.

The dilution test of parasitoid DNA was performed to determine the concentration sensitivity of the primer. The final volume of mixture templates consisted of 3 µl, as undiluted DNA, and 2, 1, 0.7, 0.5, 0.3 µl of the parasitoids DNA diluted using 0, 1, 2, 2.3, 2.5, 2.7 µl of double distill water, respectively. Concentration of the DNA template was determined using a NanoDrop 2000c. The sensitivity and dilution tests were repeated three times.

Statistical Analyses

Repeated measures analysis of variance (ANOVA) was used to compare estimated levels of parasitism based on the different methods over time. Estimation method was treated as a fixed factor and sampling time was included as a random factor. Mean comparisons were performed using Least Significant Difference (LSD). The association between parasitism level from the two estimation methods was examined using Pearson correlation. The statistical analyses were carried out using GLM option of SAS software, version 9.1 (SAS 2003).

Results

Parasitoid Identification

There were three species of parasitoid wasps identified throughout our study. The three species were *L. fabarum* (Marshal), *B. angelicae* (Haliday), and *E. persicae* (Forgatt) all of which belong to the subfamily Aphidiinae. Voucher specimens of parasitoids were deposited at the Department of Plant Protection, Ferdowsi University of Mashhad, Iran under the voucher numbers listed in Table 2.

Estimation of Parasitism Level

The highest level of parasitism was detected on 8 June 2013 (Fig. 1). The estimated parasitism levels by the two methods, counting of emerging adults (26.97%) and molecular detection of parasitoids (64.26%), differed significantly ($F_{(1, 104)}$ = 14.67; P = 0.052; Fig. 2) and varied throughout the sampling seasons ($F_{(2, 104)}$ = 19.30; P = 0.049) (Fig. 3).

Table 2. The voucher specimens and voucher numbers

Species	Voucher number
Aphis punicae	(KA. 8941)
Lysiphlebus fabarum	(KA. 8942)
Binodoxys angelicae	(KA. 8943)
Ephedrus persicae	(KA. 8944)
Syrphophagus aphidivorus	(KA. 8945)
Alloxysta sp.	(KA. 8946)
Pachyneuron sp.	(KA. 8947)

The level of parasitism was significantly higher in spring (28.42%) than both summer and fall (LSD, P < 0.05). However, parasitism level during summer (11.89%) and fall (5.86%) formed a homogeneous statistical subset (LSD, P < 0.05). The lowest level of parasitism (0%) was recorded on 3 August 2013. No significant interaction was observed between the estimation method and season ($F_{(2, 104)}$ = 2.33; P = 0.102), indicating that the relative efficiency of the evaluated methods was not impacted by seasonal changes. There was a significantly positive relationship between the two methods as shown by Pearson correlation test (r = 0.905, N = 110, P < 0.001).

During our study, the time of parasitoid activity showed two distinct peaks. The first increase started in April and peaked in the late May and early June, followed by a drop towards late June. The second peak was observed in early September, which was followed by a drop in late September (Fig. 1). These peaks were confirmed by both molecular and conventional estimation methods.

Molecular Approach Sensitivity Evaluations

The results of individual PCR analyses confirmed DNA extraction success and validated the sensitivity of the COI primer used in this study (Fig. 4). Moreover, the 16S primer successfully amplified parasitoid DNA(s), but not those of aphids and hyperparasitoids, confirming its specificity and suitability for effective estimation of parasitism level (Fig. 5). Host/parasitoid and parasitoid/hyperparasitoid mixed DNA had no adverse effect on the accuracy of parasitoid DNA detection with the 16S primer (Fig. 6).

Mean (±SE) stock concentrations of DNA extracted from *L. fabarum*, *B. angelicae*, and *E. persicae* were 68.0 ng µl⁻¹ (6.50), 122.8 ng µl⁻¹ (3.60), and 87.1 ng µl⁻¹ (11.88), respectively. As we increased the dilution rate of the amplified DNA, the intensity of bands formed on the agarose gel decreased (Fig. 7).

The minimum DNA concentration for reliable detection of *L. fabarum* was estimated at 16.33 ng µl⁻¹. The Minimum required DNA concentrations for successful detection of *B. angelicae* and *E. persicae* were 28.65 and 22.65 ng µl⁻¹, respectively.

Discussion

This study was conducted to compare the efficiency of conventional and molecular methods in estimating level of parasitism of the aphid *A. punicae* by the Aphidiinae parasitoids in a pomegranate host throughout the production season in Iran.
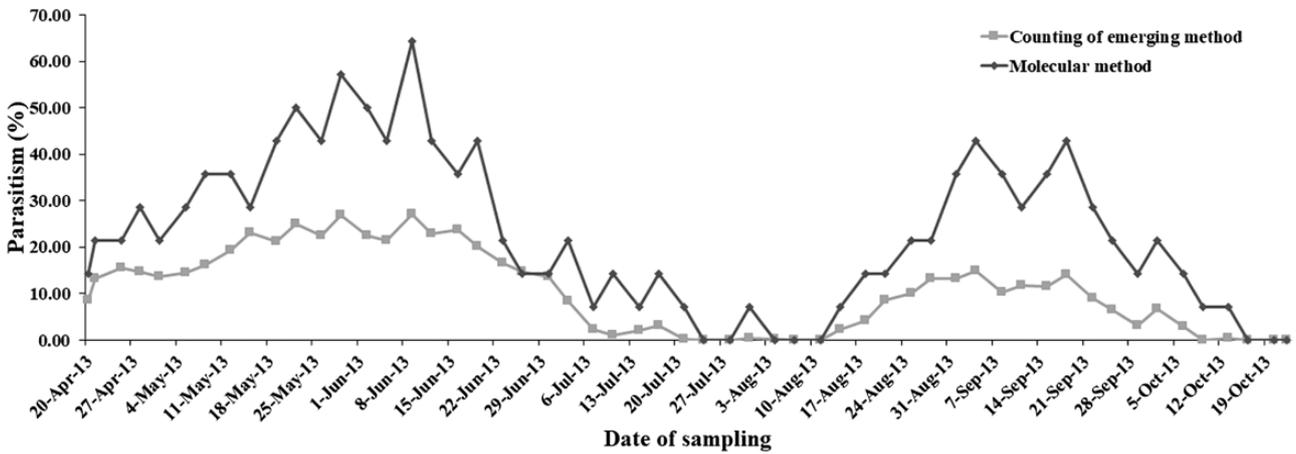
Fig. 1. Mean parasitism level of Aphidinnae on *Aphis punicae* in Kashmar during 2013 estimated by two methods.
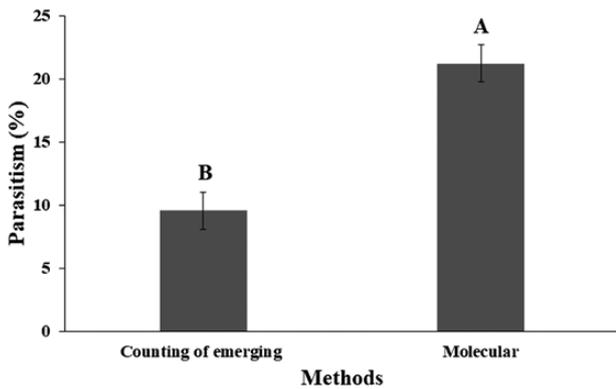

Fig. 2. Mean (±SE) parasitism level of Aphidinnae on *Aphis punicae* estimated by two methods in Kashmar. Means with dissimilar letter are significantly different (LSD; $P < 0.001$).
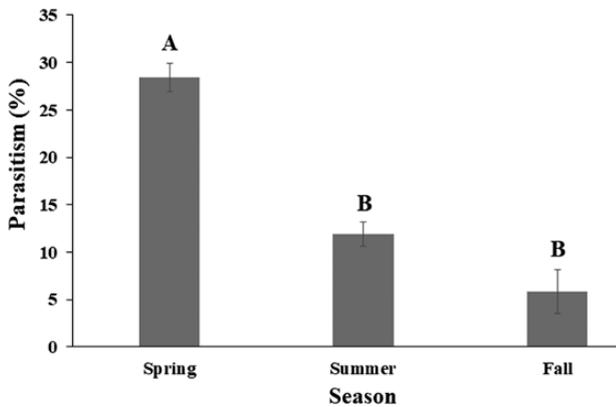

Fig. 4. The PCR products of COI primer: *Aphis punicae* (Lane 1), *Lysiphlebus fabarum* (Lane 2), *Ephedrus persicae* (Lane 3), *Binodoxys angelicae* (Lane 4), *Alloxysta* sp. (Lane 5), *Syrphophagus aphidivorus* (Lane 6), *Pachyneuron* sp. (Lane 7), and negative control (Lane 8).

Fig. 3. Mean (±SE) parasitism level of Aphidinnae on *Aphis punicae* in different seasons in Kashmar. Means with similar letter are not significantly different (LSD, $P < 0.001$).

Fig. 5. The PCR products of 16S primer: *Lysiphlebus fabarum* (Lane 1), *Ephedrus persicae* (Lane 2), *Binodoxys angelicae* (Lane 3), *Aphis punicae* (Lane 4), *Alloxysta* sp. (Lane 5), *Syrphophagus aphidivorus* (Lane 6), *Pachyneuron* sp. (Lane 7), and negative control (Lane 8).

A single primer pair from a nucleotide gene, known to be specific to Aphidiinae subfamily, successfully amplified the 16S rDNA of parasitoid species, providing an accurate, and relatively rapid, estimation of parasitism level. Although, Mathé-Hubert et al. (2013) showed that comparing levels of parasitism by different parasito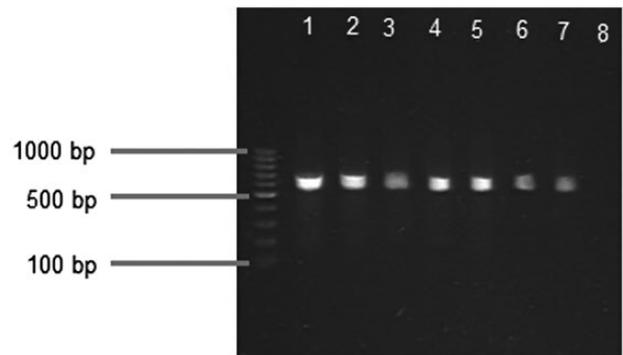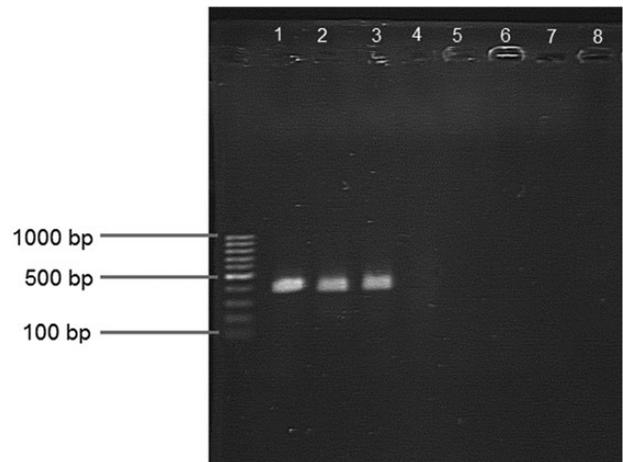id wasp species, using PCR with a single pair of primers, is expected to make comparisons more reliable, the lack of primers with adequate specificity may still lead to misinterpretation of results, if not accompanied with accurate taxonomic studies.

The observed estimated level of parasitism by molecular approaches might be biased due to various factors. The minimum time required after oviposition, until DNA extraction, is important for accurate detection of eggs, larvae and pupae inside the host. This has been attributed to the early mechanical resistance by the flexible egg chorion against the release of DNA (Jones et al. 2005). To overcome this potentially limiting factor, in this study, DNA was extracted 48 hrs after sampling; it has been shown previously that the minimum time for successful detection of the parasitoid DNA in eggs is 48 hrs after oviposition (Jones et al. 2005). The efficiency of DNA detection in eggs increases with time and is estimated to be more than 98% within 24 h of oviposition (Derocles et al. 2012). Our results demonstrated that the estimated level of parasitism by PCR was more than twofold higher than the conventional method. This finding was consistent with those of Jones et al. (2005) and Agusti et al. (2005). Studies in other systems, such as parasitism of *Lygus* spp. populations by *Peristenus* spp., also revealed a similar pattern of higher efficiency for the PCR approach than conventional methods (Tilmon et al. 2000, Ashfaq et al. 2004). Since the extraction method used in our study was non-specific, it is possible that the extracted DNA might have also included that of the host or other parasitoids. Alternatively or in addition, the primary parasitoids could have been parasitized by hyperparasitoids, which may lay more than one egg in a single host (Fisher 1961). While these factors could potentially lead to over-estimation of parasitism

level, our results from the sensitivity experiment demonstrated that our primers were specific to the Aphidiinae subfamily, only amplifying those of the parasitoids. Stuart and Greenstone (1996) pointed out that *Microplitis croceipes* (Cresson; Hymenoptera: Braconidae) maintained under laboratory conditions are prone to pathogenic infections, and may also be impacted by host immune response or other unknown causes of mortality. Regardless of the underlying cause, pre-emergence mortality may negatively impact emergence success of the adult parasitoids. A molecular approach is expected to detect some of the parasitoids prior to degradation, again providing a relatively more reliable estimate than the conventional approaches, as PCR techniques capable of amplifying DNA fragments from very minute sample quantities (Zhu and Greenstone 1999).

The formation of a visible band on agarose gel requires a minimum DNA quantity, which may vary among parasitoid species. The knowledge of minimum required DNA concentration for a reliable detection would also help to evaluate accuracy of the estimated parasitism level within a pest population. In this study, the concentration of parasitoid DNA required for generating a visible band on agarose gels was determined by examining a series of prepared extraction (DNA) dilutions. The minimum DNA quantities required for the detection of *L. fabarum*, *B. angelicae*, and *E. persicae* were substantially greater than the 0.275 ng/µl concentration reported for the parasitoid species *Anaphes iole* (Girault; Hymenoptera: Mymaridae) (Zhu and Williams 2002). The difference in minimum detectable DNA concentrations between the two studies can be explained by the specificity of the primers. While primers in Zhu and Williams (2002) were designed to specifically target *A. iole*, in our study primers were specific to the subfamily level. It is also important to note, although our findings presented the minimum DNA detection limits for the selected primer pairs, they may not reflect the minimum DNA quantities detectable in field samples containing both the parasitoid and host DNA. This is because our laboratory evaluations were based on pure parasitoid DNA, and not those mixed with host and other parasitoids and hyperparasitoids (Gariepy et al. 2005).

Estimating parasitism and mortality levels is not only essential in predicting and evaluating success of any biological control program (Mathé-Hubert et al. 2013), but is also regarded as one of the main criteria in deciding whether chemical control is necessary. While counting the emerged adults resulted in underestimating levels of parasitism, the PCR method might be unable to account for the exact mortality due to limiting biotic and abiotic factors. Combining information on parasitism success and parasitoid mortality level will likely provide an accurate measure of the effectiveness of the natural enemy in managing aphid populations. This conclusion supports that
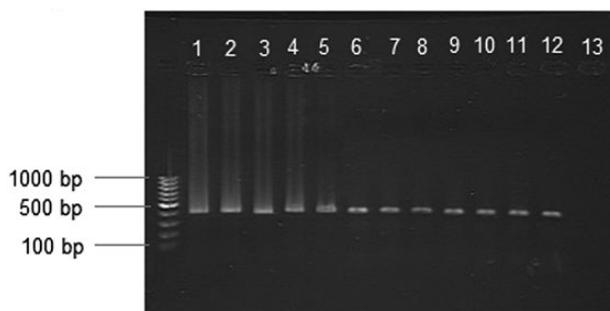

Fig. 6. The PCR products of DNA combinations by 16S primer *Lysiphlebus fabarum+Aphis punicae* (Lane 1), *L. fabarum+Alloxysta* sp. (Lane 2), *L. fabarum+Syrphophagus aphidivorus* (Lane 3), *L. fabarum+Pachyneuron* sp. (Lane 4), *Ephedrus persicae+A. punicae* (Lane 5), *E. persicae+Alloxysta* sp. (Lane 6), *E. persicae+S. aphidivorus* (Lane 7), *E. persicae+Pachyneuron* sp. (Lane 8), *Binodoxys angelicae+A. punicae* (Lane 9), *B. angelicae+Alloxysta* sp. (Lane 10), *B. angelicae+S. aphidivorus* (Lane 11), *B. angelicae+Pachyneuron* sp. (Lane 12), and negative control (Lane 13).

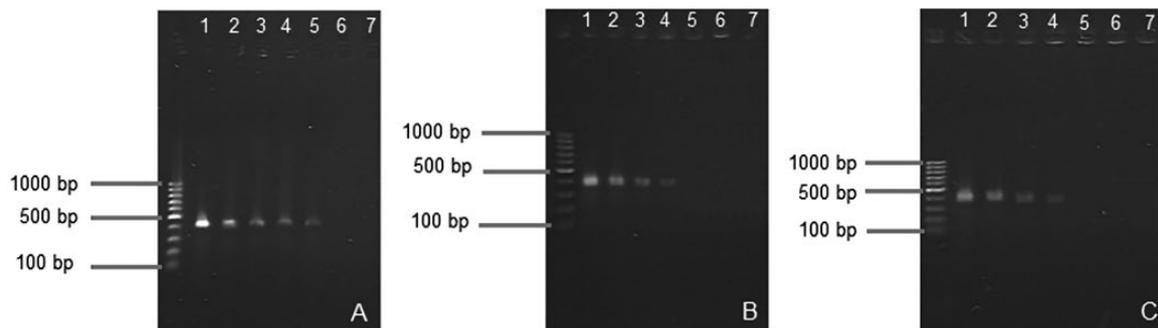**Fig. 7.** The PCR products of different DNA concentrations. **(A)** *L. fabarum*, Lanes 1 to 6 representing 68.0, 65.33, 32.66, 22.86, 16.33, 9.8 ng µl⁻¹. **(B)** *B. angelicae*, Lanes 1 to 6 representing 122.8, 81.86, 40.93, 28.65, 20.46, 12.28 ng µl⁻¹. **(C)** *E. persicae*, Lanes 1 to 6 representing 87.1, 64.73, 32.36, 22.65, 16.18, 9.71 ng µl⁻¹. For all Lane 7 represents negative control.

of (Mathé-Hubert et al. 2013), who also recommended combining the two methods to account for the mortality levels of both host and parasitoids, as well as the potential host recovery when mortality of the parasitoids happens at early stage of development (i.e., egg).

Variations in parasitism level were detected throughout our study. These variations can be attributed to the impact of abiotic environmental variables, such as elevated temperatures and reduced moisture, both of which are known to trigger fluctuations in aphid populations and, consequently, the number of their associated parasitoids (Kavallieratos et al. 2004, Bayhan et al. 2005). Nevertheless, combining conventional methods of counting emerging parasitoids and PCR molecular analysis proved more effective than either method alone in evaluating and predicting the effectiveness of biological control.

Acknowledgments

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