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Molecular detection of common pistachio psylla (*Agonoscena pistaciae* Burckhardt & Lauterer) in the gut contents of *Oenopia conglobata* Beetles

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Abstract
Agonoscena pistaciae is the important pest of pistachio orchard in the world. In order to detect this pest in the gut contents of arthropod predators, three molecular markers were developed from *A. pistaciae* cytochrome oxidase I fragment in this study. Three designed forward primers could amplify three fragments of different lengths (210, 291, 446bp). Primer sensitivity and detection period for pistachio psylla residue in the gut contents of *Oenopia conglobata* were determined in the laboratory conditions. These molecular markers were able to amplify a very small amount of target DNA in the presence of substantially greater amounts of predator's DNA. The time calculated for median detection success for three markers were determined at 25°C: 16.543h, 18.322h, 18.951h and at 30°C: 14.203h, 16.399h, 17.396h, respectively. DNA of *A. pistaciae* was detected in longer time by using these primers that produce the shorter fragment. These primers have this potential to be used for ecological studies of predator-prey interactions in the field.

Keywords: *Agonoscena pistaciae*, *Oenopia conglobata*, Molecular detection, Prey, gut contents of predator

1. Introduction
Pistachio is one of the economically important crops in Iran, with about 250000 ha of plantations that mostly are located in Kerman province [9]. The particular climatic conditions for pistachio cultivation have caused development of a monoculture system where it has been resulted in outbreaks of pests in pistachio orchard [26].
Numerous phytophagous arthropod pests attack the pistachio trees and damage every part of plant throughout its growing period. Among the pests of pistachio, the common pistachio psylla (CPP), *Agonoscena pistaciae* Burckhardt & Lauterer, 1989 (Hem.: Sternorrhyncha: Psylloidea: Psyllidae: Rhinocolinae) is a native serious pest in Iran and has been recognized as the main problem for pistachio growers. The rapid increase of CPP population usually occurs in early spring through to mid-autumn. Outbreaks in the populations of CPP cause problems during kernel development, such as bud drop and defoliation in pistachio trees [27]. Therefore, the severe damage affects the yields of pistachio trees and subsequently heavy economic losses to pistachio growers. For this reason, the control of this pest has become a basic necessity for pistachio-growers, who insist on spraying to reduce the damage [26, 27].
Recently, integrated pest management (IPM) systems and the use of biological control methods have received more attention in other agricultural systems, according to the prevalence of insecticides resistance in CPP and environmental pollutions which caused by using of insecticides, need to be considered IPM as a potential approach for controlling CPP [27].
There are many opportunities for exploiting the biological control potential of long term or permanent populations of naturally occurring predators of pests in farms and gardens [36]. Many of predator species are polyphagous and are more likely to be exploitable as major biocontrol agents against a particular pest species. Symondson *et al*. reviewed the importance of general predators in biological control programs and stated that these predators significantly reduced pest density in annual crops in about 78% of the cases [35].
The presence of several native biological control agents against CPP in pistachio orchards, have increased hopes to produce a healthy product [24, 25]. However, so far no attempt has been done to explore the real potential of these bio-control agents for controlling of CPP in Iran. Predators form the largest and the most diverse natural enemies of the CPP in Iranian pistachio

growing orchards [25]. These predators are ladybirds, bugs, lacewings and spiders, and coccinellids are the main group of predators of CPP. Among coccinellid beetles, *Oenopia conglobata* (Linnaeus) (Col, Coccinellidae) is considered as the most abundant predatory beetles in the pistachio orchards in Kerman province [28].

The detection of predation in natural habitats is difficult because there are many alternative hosts in nature [10, 29]. For the interpretation of predation, especially when generalist predators can feed on a wide range of prey, different methods have been used including direct observation, predator enhancement or exclusion, direct or biochemical estimation of gut contents [22] and using molecular method for detecting prey DNA in gut contents of predators [2, 14, 34, 17].

Development of molecular markers for amplification of specific prey DNA using the polymerase chain reaction has proven to be more specific and sensitive in detecting prey remains in the gut contents of predators. This method easily can detect small amounts of a variety of prey DNA remains in the gut contents of invertebrate predators [40, 6, 14, 34, 4, 13, 20, 15, 16, 17, 18, 7, 8, 30,11].

In this study, we aimed to develop species-specific primers for CPP, to test their specificity and sensitivity against non-target species and other common predators, to test the potential of multiplexing of two primer pairs to assess the detection of target DNA and to explore the effects of time since feeding and temperature on detection of prey DNA (CPP) in singleplex and multiplex PCR.

2. Materials and methods

2.1 Sample collection

Adults of CPP (*A. pistaciae*) were collected by an aspirator on pistachio leaves from a pistachio orchard near Kerman city (30°24'49"N, 56°55'19"E) in Iran. Nymphs of CPP also collected from pistachio leaves by brush. Collected specimens were maintained on pistachio leaves for two days at the laboratory conditions. A few specimens were selected for identification by examining their morphology and the male genitalia following the current relevant taxonomic key [5].

Adults of *O. conglobata* beetles were collected from pistachio orchards by shaking the branches on a white plate and sucking by aspirator. They were transferred to the laboratory and reared on the black bean aphid, *Aphis fabae* Scopoli (Hemiptera: Aphididae) in laboratory conditions (25°C±1, 16:8 L: D photoperiod, 65±5% of relative humidity). Aphids were reared on bean plants in a greenhouse.

Other psylla species tested in this study were collected from Kerman province included *Cacopsylla pyri* (Förster) (Hemiptera: Psyllidae) collected from pear orchards located in Bardsir area (29°55'16"N and 56°37'52"E), *Euphyllura olivina* Costa (Hemiptera: Psyllidae) collected from olive orchard located in urban area of Kerman city (30°19'3"N, 57°4'5"E) and *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) collected from a citrus orchard in Orzooiyeh area (28°23'18"N, 56°21'1"E) by using an aspirator.

2.2 DNA extraction and sequencing

DNA of specimens was extracted individually by following the protocol for animal tissues of the DNP Kit (CinnaGen, Co. Iran). Total DNA was dissolved in 100 µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and its quality and quantity was determined by a spectophotometer (Eppendorf Bio photometer, Germany) and stored at–20 °C for subsequent molecular assay. Two universal primers C1-J-1718 as forward, 5'-GGAGGATTTGGAAATTGATTAGTTCC-3', and C1-N-2191 as reverse primer, 5'-CCCGGTAAAATTAAAATATAAACTTC-3' [33], were used to partially amplify the mitochondrial COI gene by PCR.

Amplification was performed in 25µl reaction volumes containing: 4µl of DNA, 0.5µl dNTPs (15mM), 1µl MgCl2 (50mM), 1µl of each primer (10µM), 0.2µl of *Taq* DNA polymerase (5U/µl) (CinnaGen.Co.Iran) and 2.5µl of 10X manufacturer's buffer.

The reaction mix was put into a 0.2ml PCR tube and amplification was performed in a MyGenie96 thermal Block PCR (Bioneer. Co. Korea) with the following temperature profile: 35 cycles of 94°c for 60s; 54 °C for 60s and 72 °C for 60s. The first cycle of denaturation was carried out at 95 °C for 5 min, and a last cycle of extension at 72°c for 10 min.

PCR products were separated by electrophoresis on a 1% agarose gel in TBE containing Safesatin® for DNA staining. A portion of COI gene from two individuals of each species was sequenced by Seqtech. Co. (U.S.A) in an ABI 3730XL Genetic Analyzer according to the Sanger method in both forward and reverse directions.

2.3 Design of primers and molecular analysis

Sequencing results were reviewed by the Finch TV program (version 1.4.0) (Informer Technologies, Inc. 2015) and edited manually for each species, separately.

The Basic Local Alignment Search Tool (BLAST) was used to compare the similarity of nucleotide sequences with sequences present in the Gene Bank database (http://www.ncbi.nlm.nih.gov/blast/). All edited sequences were aligned using Mega 6 [38]. Specific primers were designed for CPP according to different psylla species sequence variations, especially in regions that were unique to this species. Primer design guidelines proposed for the design of efficient and specific primers by Innis and Gelfand [18] and Saiki [31] were followed. The primer-primer interactions were analyzed using the online program "OligoAnalyzer 3.1" [19]. After designing primers, they were ordered to synthesize by Cinnaclon Co. (Iran). For optimization of each primer pair, a gradient PCR program was performed by using gradient thermocycler (Eppendorf-Mastercycler gradient) with the following temperature profile: 35 cycles at 94 ºC for 30s, 50 ºC as the lower temperature and 63 ºC as the higher temperature for 30s, 72 ºC for 60s. A first cycle of denaturation was carried out at 95 ºC for 2 min and a last cycle of extension was performed at 72 ºC for 5 min.

2.4 Primer specificity and sensitivity

The specificity of the primer pairs was separately tested for each of the designed primers by attempting to amplify target DNA (at least 10 individuals) from CPP, four psylla species collected in this study and other insects collected from the Pistachio orchards (Table 1) The sensitivity of each primer was tested by different concentration of CPP DNA. To test if designed primers would amplify specific DNA in the presence of predator DNA even in the higher amounts, extracted DNA of CPP was diluted and mixed with DNA of predator (*O. conglobata*). The concentration of the predator's DNA was 500 ng and constant in all mixtures and different concentration of the prey's DNA added to it. In another test, the total DNA of predators that has fed ten individuals of 5th instar CPP nymphs were tested by each primer.

Table 1: List of insects used to test PCR primers specificity

No.	scientific name	Order: Family	No.	scientific name	Order:Family
1	*Agonoscena pistaciae*	Hem.: Psyllidae	8	*Megacoelum brevirostre*	Hem.: Miridae
2	*Cacopsylla pyri*	Hem.: Psyllidae	9	*Exochomus quadripustulatu*	Col.: Coccinellidae
3	*Diaphorina citri*	Hem.: Psyllidae	10	*Hippodamia undecimnotata*	Col.: Coccinellidae
4	*Euphyllura olivina*	Hem.: Psyllidae	11	*Adalia decempunctata*	Col.: Coccinellidae
5	*Aphis fabae*	Hem.: Aphididae	12	*Coccinella septempunctata*	Col.: Coccinellidae
6	*Aphis gossypii*	Hem.: Aphididae	13	*Oenopia conglobata*	Col.: Coccinellidae
7	*Brevicoryne brassicae*	Hem.: Aphididae	14	*Kermania pistaciella*	Lep.: Tineidae

2.5 Specificity and sensitivity of primers in Multiplex PCR

Beside specificity and sensitivity of primers in the singleplex PCR, a separate assay was performed to test specificity and sensitivity of primers in multiplex PCR. The mixture primer pairs F-2 and F-3 with C1-N-2191 (1:1) were used for multiplexing. Two different DNA templates were tested in multiplex PCR: 1) different concentration of CPP DNA was mixed with DNA of predator, 2) predators that have fed ten individuals of 5th instar CPP nymphs. For multiplexing PCR, annealing temperature used in cycling program was 58 ºC for 30s and the rest of the conditions were the same as singleplex.

2.6 Detection of CPP DNA in the gut contents of *O. conglobata*

O. conglobata that was collected from field were starved at room temperature for at least seven days. After this period, each predator was fed on ten individuals of 5th instar CPP nymphs, afterward they were frozen at–20 ºC two hour since consuming their prey for subsequent molecular assay**.**

2.7 Effect of time and temperature on detection of prey DNA

Five adults of *O. conglobata* were individually confined in 1.5 ml microfuge tubes and starved in two incubators for 24 h at 25 °C and 30 °C prior to testing.

To determine the effect of temperature on detectability of prey DNA, two temperatures were selected, 25 and 30 °C. Individual starved predators were placed in a small Petri dish with wet filter paper and allowed to consume ten individuals of 5th instar CPP nymphs. Afterward, the predators that had consumed their meals were either immediately frozen (t=0) or maintained for 2, 4, 6, 8, 10, 12, 14, 15, 16, 17, 18,19, 20, 22, 24, 36 and 48h in a constant temperature incubator at 25 °C or 30 °C, separately. After these times, these predators were frozen at–20 °C for subsequent molecular assay. Additional predators were starved for 24 h and then frozen for use as negative controls in the PCR. For each time and temperature interval, at least 20 individuals were used. For detection of CPP DNA in the gut contents of predators the primer pairs F-1/C1-N-2191, F-2/ C1-N-2191, F-3/ C1-N-2191 and mixture of F-2 and F-3 with C1-N-2191 were used.

2.8 Statistical analysis

Effects of time since feeding and temperature on the frequency of detection of prey DNA were analyzed using logistic regression. Time for median detection success for testing predator was estimated from the final fitted curve. This value characterizes the detection period for prey DNA. Data were analyzed using STATGRAPHICS Centurion XVI version 16.1.11 (StatPoint, Inc., Herndon, VA, USA) and Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA, USA). The ability of primers for detection of DNA of CPP was statistically compared by SPSS V.20 (IBM Co**.,** USA).

This research was done for one year (2014) in the laboratories of Agricultural Research Center of Kerman and University of Guilan, Iran.

3. Results

3.1 Primer design and specificity and sensitivity

Four species of psylla including *A. pistaciae*, *Cacopsylla pyri*, *Euphyllura olivina* and *Diaphorina citri* that commonly occur in Kerman Province were identified morphologically. The middle section of the cytochrome oxidase subunit I coding region was successfully amplified for these identified species of psylla. Amplified fragments varied in length from 513 to 540 bp. Edited sequences were submitted to the National Centre for Biotechnology Information GenBank (National Center for Biotechnology Information, U.S. National Library of Medicine (Accession numbers shown in Table 2).

Table 2: Accession numbers submitted in NCBI

No.	The Scientific Name of Psylla	Accession No. In Ncbi
1	*Cacopsylla Pyri*	KP192848
2	*Euphyllura Olivina*	KR052011
3	*Diaphorina Citri*	KR063658
4	*Agonoscena Pistaciae*	KP192847

The COI sequences of psylla species were aligned and compared, so on the basis of diagnostic differences among sequences, three different species-specific primers were designed for *A. pistaciae* (Table 3).

Table 3: Species-specific primers designed from the COI sequence of Agonoscena pistaciae*

Primer name	Sequence (5'–3')	Annealing temperature (Ta) °C	Fragment size (bp)
F-1	ACAACCTAAGATTCTGACTG	60	446
F-2	CTCCACTTAGCAGGTATC	60	291
F-3	CCTATAGAAACACTTCCTCTG	58	210

* Designed forward primers were used in combination with C1-N-2191 (Simon *et al*. 1994) as reverse primer

Optimized annealing temperatures ranged from 58ºc to 60ºc for each primer pairs (Table 3). Three designed forward primers in combination with a universal reverse primer could successfully amplify three different fragment lengths (210, 291, 446bp) (Table 3, Fig. 1). Each primer pairs proved to be highly specific against non-target DNA and could amplify the expected fragment size only in the presence of the target species DNA (Fig. 1).

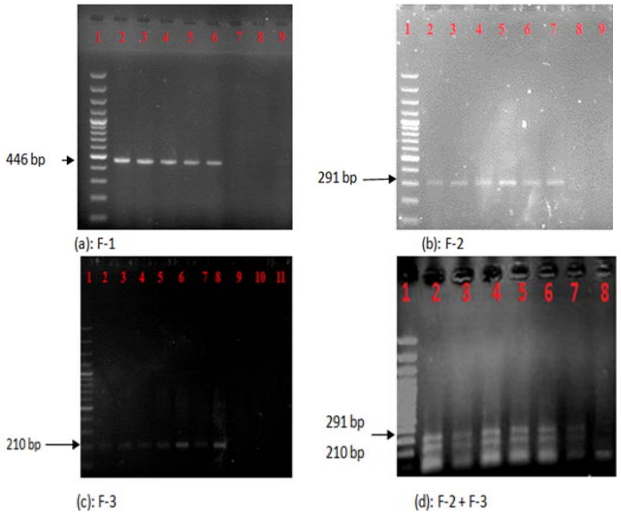

Fig 1: Agarose-gel eletrophoresis (1%) of PCR amplified DNA using the COI specific primers: (a) F-1/C1-N-2191 (446 bp); (b) F-2/C1-N-2191 (291 bp); (c) F-3/C1-N-2191 (210 bp); (d) F-2 +F-3/C1-N-2191 (291 and 210 bp). Lane 1, molecular-size marker 100 bp and other lane different DNA concentration of CPP.2) 320 ng 3)160 ng 4)80 ng 5) 40 ng 6)8 ng 7)4 ng 8) 3.2 ng 9)2.4 ng 10) 1ng 11)0 ng

The sensitivity threshold for each primer pairs in singleplex PCR are showed in Table 4. (These primers can provide specific product in the mixture of DNA of CPP and *O. conglobata*. The results also indicated that these primers could detect DNA of CPP in crude extracted DNA of predators which has fed on 5th instar CPP nymphs (table 4).

Table 4: Sensitivity of CPP primer pairs in singleplex and multiplex PCR*

Primer name	Sensitivity of CPP DNA(ng)		Sensitivity of CPP DNA+ Predator DNA (500ng)	
	Singleplex PCR	Multiplex PCR	Singleplex PCR	Multiplex PCR
F-1	8	-	8	-
F-2	4	4	4	8
F-3	2.40	4	4	8

* Designed forward primers were used in combination with C1-N-2191 (Simon *et al*. 1994) as reverse primer

3.2 Specificity and sensitivity of Multiplex PCR

Due to different length of the fragments made by primer pairs, those primers where could amplify smaller fragments were selected to be used in multiplex PCR [12]. Multiplex PCR with F-2 + F-3/ C1-N-2191 was proved to be specific against non-target species and demonstrated the possibility of detection of CPP DNA in the predator gut contents (Fig. 1).

Detection sensitivity of multiplexing of F- +F-3/ C1-N-2191 primers are shown in Table 4.

3.3 Effect of time and temperature on detection of prey DNA

The results showed that time and temperature can affect the detection of CPP DNA in the predator gut contents (Table 5).

Table 5: The percentage (± SE) of detectability of CPP DNA at different time intervals and temperatures by each primer pair.

Time	F-1		F-2		F-3		F-2 & F-3	
	25 ˚C	30 ˚C	25 ˚C	30 ˚C	25 ˚C	30 ˚C	25 ˚C	30 ˚C
0	100	100	100	100	100	100	100	100
2	100	100	100	100	100	100	100	100
4	100	100	100	100	100	100	100	100
6	100	100	100	100	100	100	100	100
8	100	90±10	100	90±10	100	100	100	100
10	100	80±13.33	100	90±10	93.33±6.66	90±10	93.33±6.66	90±10
12	89.66±9.08	70±15.27	100	80±13.33	86.66±9.08	80±13.33	93.33±6.66	80±13.33
14	77.77±10.08	60±16.32	88.88±7.62	80±13.33	88.88±7.62	80±13.33	88.88±7.62	80±13.33
15	61.11±11.12	50±16.66	77.77±10.08	60±16.32	83.33±9.03	70±15.27	88.88±7.62	70±15.27
16	50±12.12	40±16.32	66.66±11.43	60±16.32	72.22±10.86	60±16.32	77.77±10.08	60±16.32
17	38.88±11.82	20±13.33	52.63±11.76	50±16.66	61.11±11.82	50±16.66	66.66±11.43	60±16.32
18	33.33±11.43	10±6.88	42.10±11.63	33.33±14.21	50±12.12	50±16.66	50±12.12	50±16.66
19	26.66±11.81	10±6.88	42.66±13.33	40±10	46.66±13.33	40±16.32	53.33±13.33	40±16.32
20	20±10.69	0	33.33±12.59	24±8.71	40±13.09	30±15.27	40±13.09	40±16.32
22	13.33±9.08	0	26.66±11.81	20±8.16	26.66±11.81	10±6.88	33.33±12.59	30±15.27
24	10±6.88	0	20±13.33	10±6.88	30±15.27	10±6.88	20±13.33	10±6.88
32	0	0	0	0	0	0	10±6.88	0
48	-	-	-	-	-	-	0	-

The values of the detection half-life were calculated through the best fitted logistic equation obtained in 25 or 30 °C for each primer pairs (Table 6).

Table 6: The time for median detection success

Primer	The time for median detection success (T50)	
	25 °C	30 °C
F-1	16.5432 a*	14.2036 b
F-2	18.3229 c	16.3990 bc
F-3	18.9513 c	17.3693 bc
F-2&F-3	19.6642 c	18.0400 bc

* Dunkan ranking, Alpha=0.05

There are significant difference among three tested primers for detection of CPP DNA in time since feeding test in 25 and 30 °C (df= 2, Ms= 16525.846, sig.=0.027; df= 2, Ms= 8960.784, sig.=0.001) but no significant difference was observed between F-2 and F-3 primers. The mean (%) of detection of primers in two temperatures is shown in Table 7. No significant difference was observed between interaction of primers and times (df= 32, Ms=42394.608, sig= 0.997; df= 32, Ms=456.687, sig= 1). The detection CPP DNA in the predator gut contents at 25 °C was more than 30 °C (df= 1, Ms= 1601.455, sig= 0.009) but comparison of detection DNA CPP by one primer at 25 °C and 30 °C showed no significant effect between treatments (Table 7).

Table 7: Comparison mean% detection CPP DNA by primers

primers	Total comparison mean(±SE) %detection CPP DNA		Comparison mean between two temperature (T-test)
	25 °C	30 °C	
F-1	57.446± 3.232 a*	48.823±3.845 a	F= 3.58, t= 1.71, Sig= 0.086 ns
F-2	66.667±3.068 bc	58.823± 3.785 bc	F= 8.98, t= 1.62, Sig= 0.106 ns
F-3	68.511± 3.036 c	62.941± 3.715 bc	F= 5.08, t= 1.16, Sig= 0.243 ns
F-2 & F-3	68.163± 2.982 bc	65.294± 3.661 bc	F= 1.43, t= 0.610, Sig= 0.542 ns

*Dunkan ranking, Alpha=0.05

4. Discussion

Results showed that the developed primers were specific to the target DNA (*A. pistaciae*). These designed molecular markers, could exclusively detect *A. pistaciae in* the gut contents of *O. conglobata*.

The F-2/C1-N-2191 and F-3/ C1-N-2191 primers could detect CPP DNA for a longer time from F-1/ C1-N-2191 primer in the gut contents of *O. conglubata*. However, no significant difference was observed between F-2 and F-3 primers in detection of target DNA.

While the prey DNA in predators' gut is fragmented by digestion enzymes, the detection time of prey DNA depends on the length of the amplification product [14, 2]. The larger fragments become undetectable in the gut more rapidly than the smaller ones [40, 3, 15]. Chen *et al.* [6] found no difference in the detection rates of *S. avenae* fragments shorter than 246 bp and also Juen and Traugott [20] showed no difference in detection rates of DNA fragments between 175 and 387 bp in feeding experiments with cockchafer prey. The results of this study are confirmed by other researchers. The F-2 and F-3 primers which produce fragments smaller than 291 bp could detect CPP DNA for a longer time in the gut contents of predators compared to F-1 that amplifies a 471 bp fragment size.

The sensitivity thresholds of species-specific primers are important because it shows that a small amount of prey that consumed by a predator is sufficient for detection [1]. In this study, the sensitivity thresholds of primers were determined. Hosseini *et al.* [16] determinated the sensitivity thresholds of specific primers designed for *Hellula hydralis* (Lep.: Crambidae) and *Plutella xylostella* in range from 0.02 pg to 16.4 pg and Admassu *et al.* [1] that showed the sensitivity of designed primers of earthworm was 0.15 ng/µl.

In the present study, the relative sensitivity of F-2 and mixture of F-2 and F-3 primers sets (291 and 210 bp in size) has been tested and no difference was found in their sensitivity. Thus, the sensitivity of the PCR seems to be the same, at least for this range of fragment sizes, which are useful for other studies. Some workers used another approach to determine the sensitivity threshold of primers. For example, Agusti *et al.* [4] in their study defined the sensitivity of designed primers at 10^{-5} dilution of a target sample and Chen *et al.* [6] detected 10^{-7} aphid equivalents of DNA. However, concentration of the extracted DNA depends on the size of sample and extraction method used. Therefore, results will vary with smaller or larger specimens. Multiplex PCR showed the same sensitivity compared to singleplex PCR. Meanwhile detection sensitivity of F-2 +F-3/ C1-N-2191 primers in the mixture of CPP and *O. conglobata* DNA showed a lower sensitivity compared to singleplex PCR. Results showed that the presence of non-target DNA in PCR does not influence the detectability of target DNA in all primers pair's experiments. This enables us to use the whole body of a predator for DNA extraction. Likewise, Sheppard *et al.* [32] showed that, despite the presence of predator tissue, there was no evidence that non-target DNA could mask the detection of small amounts of prey DNA. Eitziger *et al.* [8] also showed that predators' body size does not affect prey DNA detection by primers that make fragment under 300bp.

Logistic regression equations were fitted to describe the decay in the percentage of positive detection as a function of time. Hosseini *et al.* [16] and Waldner *et al.* [39] also have considered logistic regression equations in their studies.

This research showed that F-2, F-3 primers and multiplexing of these two primers could detect CPP DNA in the gut contents of predator for a longer time, but significant difference in retention half-lives was not observed between 25 and 30°c (Table 6). The previous PCR- based analyses of predator gut contents have found retention half-lives much less than 24 h [6]and Hosseini *et al.* [15] showed that this parameter in *Hippodamia variegata* that feed on *Plutella xylostella* was 17.1h, which is comparable with this result. Results also indicated that the temperature could be effect on detection half-life. Hoogendoorn and Heimpel [14] used four pairs of primers for *Ostrinia nubilalis* to study predation in the ladybird, *Coleomegilla maculata*. They showed that temperature has a negative effect on detectability of prey in predator gut contents. Sopp and Sunderland [37] reported that the detection period generally declines with increasing temperature. In an experiment performed at five different temperatures, the proportion of positive responses to prey residues and duration of median detection intervals of prey (pink bollworm egg) decreased as temperature increased in a ladybird [12]. Hosseini *et al.* [15] and Logan *et al.* [21] demonstrated that temperature is an important factor and should be considered in the evaluation of predation data obtained from field samples. McMillan *et al.* [23] in an experiment showed that decreasing temperature from 21 °C to 14 °C was caused prolongation of digestion time in larvae of the two-spotted ladybeetle (*Adalia bipunctata* L.) which were fed with the bird cherry-oat aphid (*Rhopalosiphum padi* L.) but their results indicated that no significant difference could be found on molecular detection prey in the gut contents of *A. bipunctata*. In the current study, we showed that a 5 °C difference in detection experiments has no significant effect on molecular detection CPP DNA in the gut contents of *O. conglubata* by using each primer pairs.

The primer sets designed and developed in this study could potentially provide a very useful tool for ecological studies of the pistachio psylla in the field. Until now studies on predators of pistachio psylla have been done by the direct observation method in Iran and result of this research could be helpful to determine the key predators of CPP in natural habitats. Determination of the proportion of field-collected predators with detectable DNA from a specific prey, e.g. Pistachio psylla, will be the first step in identifying potential key predators or trophic linkages.

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