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A Simple and Rapid Molecular Method for Simultaneous Identification of Four Economically Important Thrips Species

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ABSTRACT

Quick and authentic identification of exotic and potentially invasive taxa with capability of causing high economic losses or detriments is essential prerequisite for effective plant quarantine and biological control initiatives. The order Thysanoptera includes several agricultural pest species that, not only because of their minute size but also due to their cryptic behavior, incline to undetected transport through international trade of plants. Identification of thrips, particularly at species level, is pretty demanding and requires expertise in knowledge about Thysanoptera. Moreover, in most cases, identification of larval Thysanoptera to species is impossible without presence of adults. Hence, there is a great desire for a facile, accurate, and highly reliable technique for thrips identification. The present study describes species-specific primers for four pest thrips species, and the use of a multiplex PCR assay to detect and to distinguish between the four target species. Five primers were used to simultaneously amplify a specific region of the mitochondrial DNA and produce species-specific fragments. Results indicated that the primers were capable of detecting these four species and amplifying uniquely sized, species-specific PCR products. Furthermore, using a multiplex PCR assay, the primers maintained specificity and sensitivity, and allowed detection of each of the four species in a single reaction. The stringency of the method was tested using specimens of different developmental stages and consistent results were obtained for all of the examined samples. This method is simple enough to be implemented by non-experts and also can be extended to any organism for which quick and reliable identification is needed.

Keywords: Mitochondrial DNA, Multiplex polymerase chain reaction, Species identification, Thripidae, Thysanoptera.

INTRODUCTION

Thrips (Thysanoptera: Thripidae) are tiny insects with worldwide distribution. Of the more than 5,500 thrips species, a few hundred are crop pests causing serious damage to growing crops and harvestable products and less than 0.2% of them are known to be associated with tospoviruses (Mound, 2001). Tospoviruses and their thrips vectors are considered as a main threat to agricultural and ornamental crops that necessitate control measures to avoid disastrous losses. Until now, at least 15 different species of Thysanoptera have been reported as tospoviruses` vectors, among which, *Thrips tabaci* Lindeman and *Frankliniella occidentalis* (Pergande) are reputed to be the most notorious ones. *Thrips palmi* Karny and *Frankliniella intonsa* (Trybom) are also infamous as major virus vectors (Oliver and Whitfield, 2016). Thrips minute size and cryptic behavior make them difficult to detect either in the field or in fresh vegetation transported through international trade of plants or plant materials. In particular, these insects can readily spread around the world in the form of eggs, larvae or adults through

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transport of fresh plant materials (Kirk and Terry, 2003).

Plant quarantine diagnosis and treatments against insect pests require the ability to identify taxa quickly and precisely, as a basic fundamental step. Traditionally, these taxa are identified using morphological characters. Although the generic diagnosis of thrips is fairly straightforward (Mound and Palmer, 1981; Johansen and Mojica-Guzman, 1998; Moritz *et al*. 2004); but identification to species level is much more difficult and requires expert knowledge of the genus (Mound and Palmer, 1981; Mound and zur Strassen, 2001; Hoddle and Mound, 2003). An accurate examination of the minute structural details, which are important in diagnosis of thrips species, is impossible without fully cleared and expertly slide-mounted specimens (Mound and zur Strassen, 2001; Hoddle and Mound, 2003). Moreover, identification of larval Thysanoptera to species is impossible without the presence of adults (Brunner *et al*., 2002). The inability to precisely identify thrips species brings about deficient control and also a substantial decline in exports due to the presence of species of quarantine importance.

The utilization of molecular methods could be a precious addition or alternative to traditional morphological methods of species recognition. PCR-based techniques have provided ideal markers for species identification (Frey and Frey, 1995; Zehner *et al*., 2004; Harper *et al*., 2006). Although species identity can readily be resolved by sequencing a variety of mitochondrial and nuclear genes (Scheffer, 2000; Scheffer and Lewis, 2001), most of these methods are relatively labor intensive and costly (Miura *et al*., 2004).

A multiplex Polymerase Chain Reaction (PCR) method, which is based on amplifying genomic DNA by species-specifically designed primers, has hitherto been successfully applied in several studies to distinguish morphologically similar species (Koekemoer *et al*., 2002; Gariepy *et al*., 2005; Hada and Sekine, 2011; Lee *et al*., 2008).

In the current study, a convenient and cost-effective multiplex polymerase chain reaction assay was used for discriminating between four economically important thrips species, namely, *Thrips tabaci*, *Thrips palmi*, *Frankliniella intonsa*, and *Frankliniella occidentalis*. The multiplex PCR uses variations in the size and number of PCR products obtained using multi primer sets to distinguish among species (Portillo *et al*., 1996; Roehrdanz, 2003). The objective of this study was to design PCR primers for four invasive thrips species in a way that unique fragment sizes were produced for each species. The designed primers should be applicable in multiplex PCR assay for rapid and easy identification and also discrimination of these thrips species, which are commonly found on various plants or plant materials.

MATERIALS AND METHODS

Samples

Thrips specimens were collected from various plants (Table 1) by shaking and beating them onto a white plastic tray. Thrips were picked up using a moistened fine brush and stored in vials of 96% ethyl alcohol. *Thrips palmi* and *Frankliniella intonsa* samples were provided by Dr. Laurence A. Mound, Australian National Insect Collection, CSIRO, Canberra, Australia and Dr. Kambiz Minaei, Shiraz University, Shiraz, Iran, respectively. For rapid and economical DNA extraction for using in multiplex PCR, only one thripid specimen was ground in 1.5-mL Eppendorf tube containing 20 µL of distilled water. This sample solution was used as a DNA source.

Primer Design

To design the specific primers for multiplex PCR, 27, 35, 18 and 30 mitochondrial gene sequences representing

Table 1. Longitude and altitude of different collection sites.

	Locality	Host plant	Latitude, Altitude
T. tabaci	Mashhad (Shirhesar)	*Allium cepa*	36° 20′ 00″ N, 59° 42′ 23″ E
	Mashhad (Agricultural research station)	*Tanacetum parthenium*	36° 16′ N, 59° 36′ E
	Neyshabour (Boojan)	*Allium cepa*	36° 24′ N, 58° 58′ E
	Neyshabour	*Beta vulgaris*	36° 12′ 48″ N, 58° 47′ 45″ E
	Torbat-e- Heidarieh	*Solanum lycopersicum*	35° 16′ 26″ N, 59° 13′ 10″ E
F. occidentalis	Mashhad (Ferdowsi University of Mashhad)	*Alcea* sp.	36° 30′ 75″ N, 59° 52′ 86″ E
	Mashhad (Agricultural research station)	*Zantedeschia aethiopica*	36° 16′ N, 59° 36′ E
	Mashhad (Mellat Park)	*Rosa* sp.	36° 19' 15" N, 59° 32' 14" E
	Neyshabour (Boojan)	*Rosa sp.*	36° 24′ N, 58° 58′ E
	Neyshabour	*Calendula persica*	36° 12′ 48″ N, 58° 47′ 45″ E

the Cytochrome Oxidase I (COI) region were retrieved from the GenBank for *T. tabaci*, *T. palmi*, *F. intonsa* and *F. occidentalis*, respectively (Table 2). The sequences were aligned using Muscle algorithm implemented in MEGA6 (Tamura *et al*., 2013) and primers were designed using Vector NTI version 10.0.1 (Invitrogen). Primer design for multiplex PCR followed a combination of criteria and prerequisites whereby: complementary between primers should be minimal, the designed primers should have at least one but no more than three G or C bases to promote specific binding at the 3′ end of the primer, not only primers should not have potential matching sequences for nonspecific target sites, but also the optimal DNA primer annealing temperature for all template primer annealing combinations should be similar (Portillo *et al*., 1996; Rugman-Jones *et al*., 2009; and Saccaggi *et al*., 2008; Daane *et al*., 2011). Furthermore, to facilitate discrimination on agarose gel, amplified fragments should be sufficiently different in size. Considering all of the above mentioned issues, four specific reverse primers and a common forward primer were designed. The sequences of the primers are given in Table 3.

Detection of Thrips Species Using Specific Primers and Multiplex PCR

In the first step, to check if the designed primers work properly, PCR reactions were performed using one specific reverse primer (tabR, occiR, palR or intR, Table 3) and the common forward primer. The PCR reactions were conducted in a volume of 25 µL containing 12.5 µL of PCR Master Mix (Amplicon), 1 µL of each of the specific reverse and the common forward primers (10 PM), 2.5 µL of DNA template, 2% DSMO as a PCR additive and 7.5 µL of distilled water. DSMO, as an enhancing agent, reduces secondary structures that could inhibit the progress of the polymerase and, as a result, increases the PCR amplification yield (Sairkar *et al*., 2013). The PCR was performed on a Biometra thermal cycler (Biometra, Tpersonal combi) by using an initial denaturing step at 94°C for 5 minutes followed by 30 cycles of 94°C for 30 seconds, 50°C for 45 seconds, 72°C for 90 seconds, and a final extension step of 72°C for 8 minutes.

In the second step, multiplex PCR assays were done using five primers for detecting each of the four thrips species. PCR reactions were the same as before but 1 µL of each

Table 2. The accession number of sequences retrieved from GenBank and used to design primers.

T. tabaci	*F. occidentalis*	*T. palmi*	*F. intonsa*
AB262444	AB276376	KP871468	KF840080
AB262442	AB276374	KP871444	AB277215
KF778768	KJ592766	KP871438	AB277214
KF778764	HQ214667	KP871355	AB276377
AB554258	JX235929	KP871432	HM246175
JX403013	HQ214664	KP871413	FN546002
JX403011	HQ214658	KP871371	FN546001
JX275861	JN790698	KP871301	FN546000
AY196848	JN790696	KP871297	FN545999
AY196845	EU363490	KP871280	FN545997
AY196833	EU363486	KP871241	FN545996
GU393023	EU363483	KP871188	FN545995
FN546171	GU372403	KF840097	JX235931
FN546167	HM246176	AB277231	HQ605955
FN546162	GU372385	AB277230	HQ605953
FN546157	GU372380	AB277229	JF719594
FN546149	FN545989	FN546146	JF719593
KF036291	FN545986	FN546140	AB587606
KF036293	FN545984	FN546144	
AM932006	KF765383	FN546139	
AM932012	KF765379	FN546138	
AM932019	KC513150	KT885206	
JX438744	JQ182144	KT885205	
HQ540403	HQ605959	KJ634672	
AM932043	JF719599	KF144151	
AM932020	EF555889	KF144148	
FN546154	EF555883	KF144141	
	EF555874	KF144111	
	EF555868	KF144066	
	EF555838	KF144061	
		HQ377269	
		AB587604	
		HQ605951	
		HQ605950	
		KF015464	

Table 3. Oligonucleotide primers used in the multiplex PCR and the sizes of the PCR fragments.

Name	Specificity	Sequence	Product length
tabR	*T. tabaci*	*5′-TGTGATAGCTCCCGCTAAC-3′*	360 bp
occiR	*F. occidentalis*	*5′-GGTCCAGAGTGATAAAAAGTTGAC-3′*	163 bp
palR	*T. palmi*	*5′-TAGATGTTGRTAAAGTACWGGA-3′*	437 bp
intR	*F. intonsa*	*5′-GAGGTATTTAAGTTTCGATCTGTAAG-3′*	390 bp
Common forward		*5′-YTWGGAGCHCCHGAYATAG-3′*	

species specific reverse primer was used in the reaction. The PCR thermal conditions were identical to those described above. Finally, the PCR products were visualized on 1.5% agarose gel stained with DNA green viewer.

In addition to the adults, larval specimens were also tested to examine the general applicability of this method. Moreover, different populations of some of the examined thrips species, i.e. *T. tabaci* and *F. occidentalis*, were collected either from

various geographical regions or from different host plants (Table 1) and assayed to evaluate the efficacy of the method for different populations of thrips species.

Resolution of Mixtures of Thrips Species

The single-step multiplex PCR was designed to allow the species-specific primers to be used together with a common forward primer in a single reaction mix to differentially amplify DNA from each of the four thrips species. The PCR reaction mix was essentially the same as that mentioned above, with the exception that 6.5 µL of DNA template containing a mix of 1, 1.5, 2 and 2 µL of *T. tabaci*, *T. palmi*, *F. intonsa*, and *F. occidentalis* DNA templates was used as a DNA source. Thermocycling conditions were identical to those described above. Products from the multiplex PCR were visualized under UV light on a 3% agarose gel stained with DNA green viewer.

RESULTS

We reported the development of new primer sets for both simultaneous diagnosis and discrimination of four economically significant thrips species in various life stages. Four primer sets, consists of a species specific revers primer and a common forward primer were designed based on COI gene and used in diagnostic multiplex PCR protocol. Figure 1 shows alignment of the

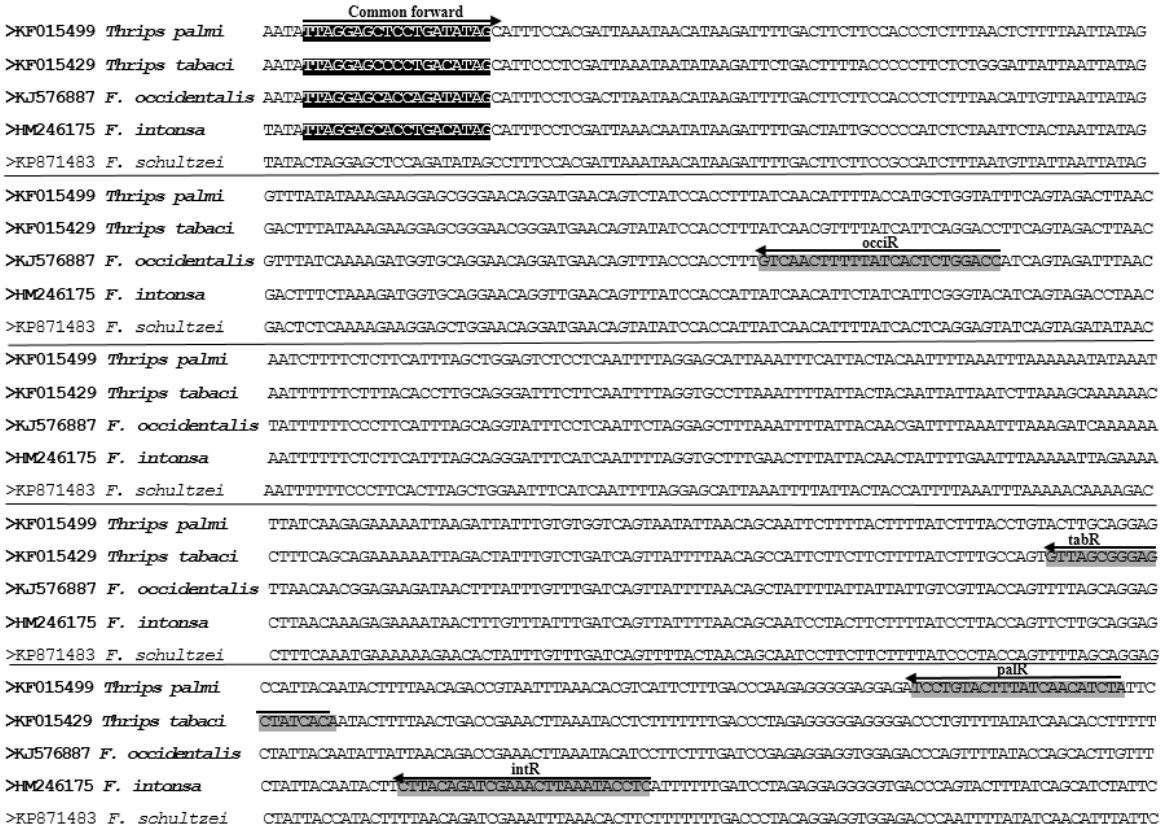


Figure 1. Diagrammatic representation of the partial *COI* region of *T. palmi* (GenBank accession number KF015499), *T. tabaci* (GenBank accession number KF015429), *F. occidentalis* (GenBank accession number KJ576887) and *F. intonsa* (GenBank accession number HM246175) and *F. schultzei* (Genbank accession number KP871483) showing location of common forward and species-specific reverse primers.

partial COI gene sequences and location of each primer used for the multiplex PCR. Each primer set selectively amplified the DNA of each species and yielded the expected product size. For each species, a single band resulted from amplification with a set of two primers; *i.e.* tabR, occiR, palR or intR and the common forward primer. As expected, four bands corresponding to a 360 bp, a 163, a 437, and a 390 bp fragment from the amplification of target region in *T. tabaci*, *F. occidentalis*, *T. palmi* and *F. intonsa* were observed, respectively (Figure 2). Moreover, to confirm our results, the PCR products were sent to Macrogen Company (Seoul, Korea) for purification and bidirectional sequencing. The consensus sequence of each PCR product was queried to the GenBank sequence database by using nucleotide BLAST. In every case, the thrips species identified by the multiplex PCR assay corresponded with the correct species defined by GenBank BLAST. When each species-specific primer was tested separately for reactivity with the other examined species, none occurred.

Combining the four species-specific reverse primers (tabR, occiR, palR and intR) and the common forward primer in a multiplex PCR protocol distinguished each of the four species, and amplified the appropriate species-specific fragment; 360 bp for *T. tabaci*, 163 bp for *F. occidentalis*, 437 for *T. palmi* and 390 for *F. intonsa* (Figure 3: Lanes 1-4). These results were consistent with those obtained using the species specific primer sets individually (Figure 2.). No bands were produced when either *T. meridionalis* or *F. schultzei* DNA were used as templates. (Figure 3: Lanes 5

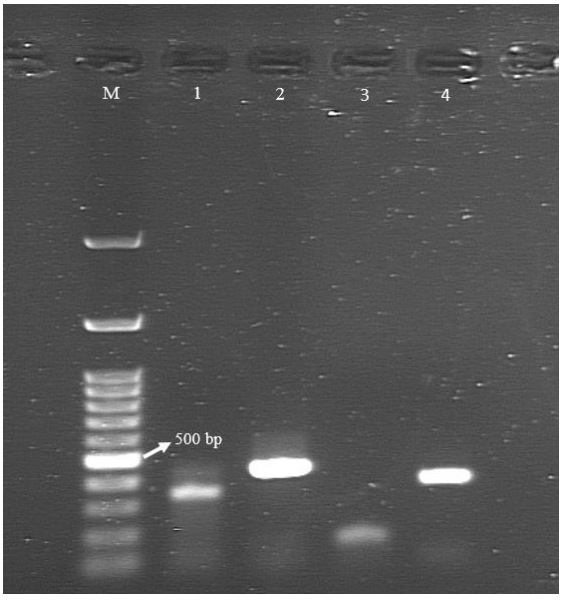


Figure 2. Diagnostic PCR using species-specific primers for *T. tabaci*, *T. palmi*, *F. occidentalis*, and *F. intonsa*. M: Marker; Lane 1: *T. tabaci* template with tabR specific primer, Lane 2: *T. palmi* template with palR specific primer; Lane 3: *F. occidentalis* template with occiR specific primer, Lane 4: *F. intonsa* template with intR specific primer.

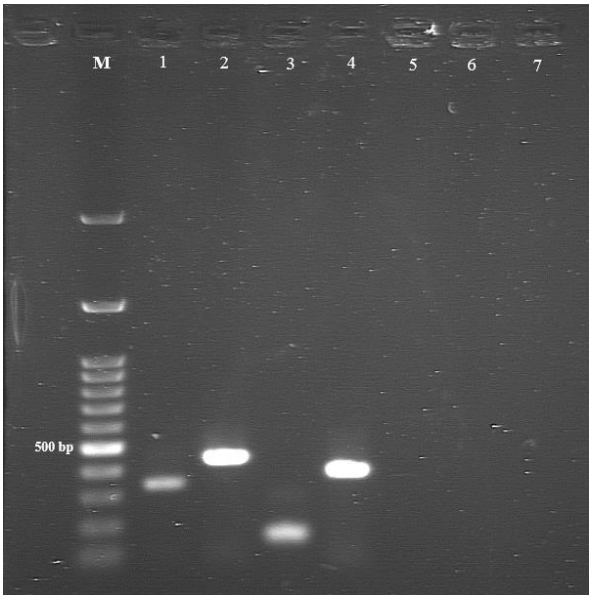


Figure 3. Diagnostic multiplex PCR of *T. tabaci, T. palmi, F. occidentalis* and *F. intonsa* DNA using a combination of tabR, palR, occiR and intR primers and the common forward primer in each reaction. M: Marker (Invitrogen); Lane 1: *T. tabaci* template with five primers; Lane 2: *T. palmi* template with five primers; Lane 3: *F. occidentalis* template with five primers; Lane 4: *F. intonsa* template with five primers; Lane 5: *F. schultzei* template with five primers; Lane 6: *Thrips meridionalis* template with five primers, Lane 7: Negative control (no DNA).

and 6). We confirmed that this protocol enables rapid and robust identification and also distinct differentiation of the four species.

When *T. tabaci* larval stages were used in order to test the applicability of the method for identifying the immature stages of thrips species, the banding patterns revealed that the immature could be identified at the same level of stringency as the adults (Figure 4-A: Lanes 1-2). Moreover, in order to evaluate the efficacy of the method for different populations of thrips species, specimens from geographically different populations of *T. tabaci* and *F. occidentalis* were collected from different host plants. Similar consistent results were obtained for different populations of the examined species; *i.e*. a 360 bp band for *T. tabaci* and a 163 bp band for *F. occidentalis* (Figure 4-A: Lanes 3-7, Figure 4-B).

To determine whether primers produce different banding patterns in samples containing four species, a mixed template composed of DNA from four species was assayed. The resulting banding patterns revealed differences among the species tested (Figure 5). The species-specific primers yielded amplicons of 360, 163, 437 and 390 bp for *T. tabaci*, *F.*

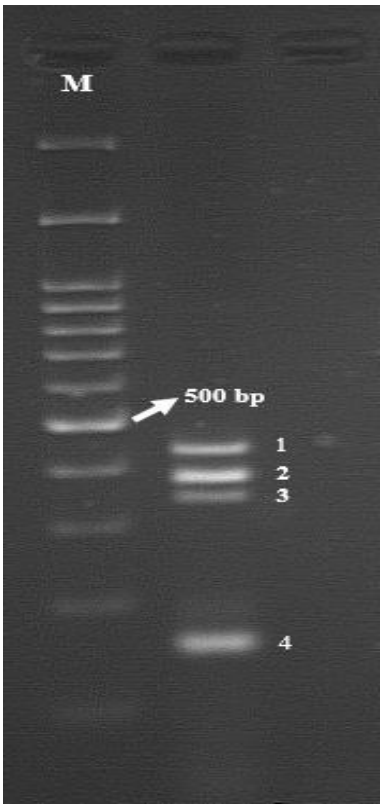
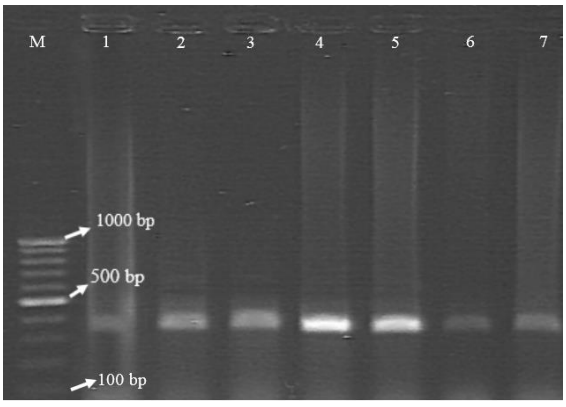


Figure 5. Electrophoretic patterns of multiplex PCR products by grounding and using mix of four thripid species as templates. M: Marker; 1: *T. palmi*; 2: *F. intonsa*; 3: *T. tabaci*, and 4: *F. occidentalis*.



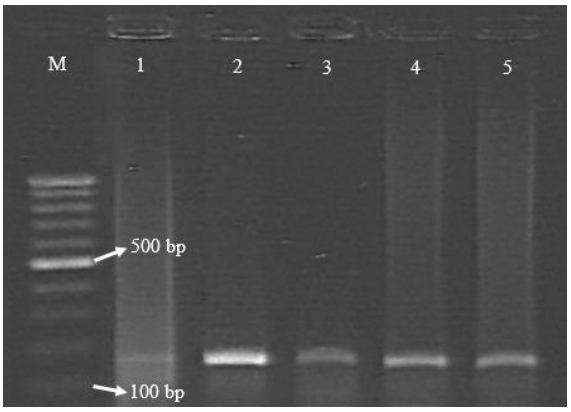


Figure 4. Electrophoretic patterns of PCR products from: (A) Various developmental stages (lanes 1-2) and different populations (lanes 3-7) of *T. tabaci*, (B) Different populations (lanes 1-5) of *F. occidentalis*. M: 100 bp marker; (A) Lane 1: First instar larvae; Lane 2: Second instar larvae; Lane 3: (Mashhad, onion), Lane 4: (Neyshabour, onion); Lane 5: (Neyshabour, *Beta vulgaris*); Lane 6: (Torbat-e-Heidarieh, *Solanum lycopersicum*), Lane 7: (Mashhad, *Tanacetum parthenium*). (B) Lane 1: (Mashhad, *Alcea* sp.); Lane 2: (Mashhad, *Zantedeschia aethiopica*); Lane 3: (Mashhad, *Rosa* sp.); Lane 4: (Neyshabour, *Rosa* sp.), Lane5: (Neyshabour, *Calendula persica*).

occidentalis, *T. palmi*, and *F. intonsa*, respectively (Figure 5). The differences in the size of PCR products among species meant that the multiplex PCR method was capable of differentiating among the thrips species on an agarose gel.

DISCUSSION

Precise identification of potential invasive species is the cornerstone of effective plant quarantine as well as potent biological control; so utilizing methods that can definitively identify the species seems to be essential, particularly when morphological variation among species is slight (Haung *et al*., 2009; Fekrat *et al*., 2015). The speed, accuracy and reliability of molecular techniques have made them a valuable standard tool for monitoring agricultural pests and assisting in researches (Armstrong and Ball, 2005). Identification of species using molecular methods has become ubiquitous in diagnostics and ecological studies, especially with regard to insects for which morphological identification is difficult or time-consuming. Identification of thrips species based on morphology is usually tedious and laborious, requires a high level of taxonomic expertise and usually only adults can be identified. So, lots of research efforts using different molecular methods have hitherto been dedicated to this group of insects, especially to those with economic importance, in order to solve their morphological identification problems (Toda and Komazaki, 2002; Asokan *et al*., 2007; Mainali *et al*., 2008; Huang *et al*., 2010; Zhang *et al*., 2012; Fekrat *et al*., 2014, Toda *et al*., 2014; Fekrat *et al*., 2015; Przybylska *et al*., 2016).

Among various molecular methods, using multiplex PCR has dramatically increased in species identification studies as it permits simultaneous amplification of several DNA fragments in a single reaction (Saccaggi *et al*., 2008). Compared to other methods that often need several procedures to complete, this method only requires a single PCR followed by a single electrophoresis to identify several species simultaneously (Gariepy *et al*., 2005; Saccaggi *et al*., 2008). Reduction of the number of reactions needed to test a sample for different targets leads to saving time and money and makes multiplex PCR significantly efficient, especially when a large number of samples have to be screened in a short period of time (Staudacher *et al*., 2011; Sint *et al*., 2012).

In the current study, we described the development and application of a single-step multiplex PCR for the identification of four pest thrips species. Although some other studies used multiplex PCR method for identification of thrips species (Yeh *et al*., 2014; 2015), these studies used ITS region for this purpose. Moreover, in the above mentioned studies, separate forward and reverse primers were designed for each of the examined species, but we used one common forward primer for all of the examined species. The multiplex PCR developed here, based on the mitochondrial Cytochrome *c* Oxidase subunit 1 (COI) gene, is expeditious, reliable, highly sensitive, accurate, and also straightforward. Indeed, it needs no procedures other than PCR and subsequent electrophoresis. The PCR primers developed in this study for *T. tabaci*, *F. occidentalis*, *T. palmi*, and *F. intonsa* are specific and sensitive when used individually or in multiplex with DNA extracted from thrips adults or from immature stages. The primers yield unique fragment size for each species when used individually or in multiplex, allowing simultaneous identification of thrips species based on the size of the PCR fragments generated. Furthermore, the multiplex PCR system amplified all targets at approximately the same efficiency, resulting in an even signal strength for all amplicons. This method is advantageous not only for distinguishing between species, but also for identifying both adults and immature stages of thrips. It allows rapid and accurate identification of multiple thrips species simultaneously. Our results supported the use of grounding specimens for DNA

extraction because it is very easy, fast, and also inexpensive. The entire identification protocol, including DNA extraction, PCR and electrophoresis, can be completed in a few hours. The technique developed is simple enough to be implemented in any molecular laboratory and does not require extensive taxonomic or molecular experience. Moreover, the use of a multiplex PCR assay for thrips species improves upon the conventional and molecular techniques currently used to identify Thysanoptera. It also allows rapid and accurate identification of multiple thrips species. It is envisioned that the principles described here will be extended to any organism for which rapid and reliable identification is needed.

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روش مولکولی ساده و سریع برای شناسایی همزمان چهار گونه تریپس با اهمیت اقتصادی

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چکیده

شناسایی سریع و صحیح تاکسون‌های غیربومی و بالقوه خسارت‌زا با توانایی ایجاد خسارات اقتصادی قابل توجه از اولویت‌های اصلی در برنامه‌های قرنطینه گیاهی و کنترل بیولوژیک به شمار می‌رود. راسته بالریشکداران مشتمل بر تعدادی از گونه‌های آفت کشاورزی می‌باشد که نه تنها به دلیل اندازه کوچکشان، بلکه به خاطر رفتارهای استتاری‌شان معمولا در قرنطینه‌های گیاهی کشف نشده باقی می‌مانند. شناسایی تریپس‌ها، به خصوص در سطح گونه، عموما کاری دشوار بوده و نیازمند دارا بودن دانش و تخصص در این زمینه است. علاوه بر این، در اکثر موارد شناسایی افراد نابالغ در سطح گونه بدون حضور افراد بالغ تقریبا غیرممکن است. در مطالعه حاضر برای چهار گونه از تریپس‌های حائز اهمیت اقتصادی آغازگرهای اختصاصی طراحی شده و از روش multiplex PCR برای تفکیک و تمایز بین این گونه‌ها استفاده شده است. پنج آغازگر به طور همزمان نواحی خاصی از دی‌ان‌ا میتوکندریایی را تکثیر نمونه و قطعات تکثیر شده خاصی را برای هر گونه تولید می‌کنند. نتایج حاصل

بیانگر این است که آغازگرهای مورد استفاده قادر به تفکیک این چهار گونه بوده و قطعاتی خاص با اندازه منحصر به فرد را برای هر گونه تولید می‌کنند. علاوه بر این، با استفاده از این روش، تفکیک و شناسایی این چهار گونه تنها در یک واکنش امکان‌پذیر است. کارایی این روش برای مراحل مختلف زیستی نیز ارزیابی شده و نتایج قابل قبول برای تمامی نمونه‌های مورد بررسی به دست آمد. این روش روشی ساده بوده و می‌تواند براحتی توسط افراد غیرمتخصص به کار برده شده و همچنین می‌تواند برای هر ارگانیسمی که شناسایی سریع و قابل اطمینان در مورد آن مورد نیاز است، تعمیم داده شود.