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A Multiplex PCR Assay for Simultaneous Discrimination of Two Predominant Spider Mites of the Genus *Tetranychus* (Acari: Tetranychidae) in Greenhouses of Iran

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ABSTRACT

Molecular species identification has become more ubiquitous in diagnostics of insects, particularly in situations where morphological identification is a laborious or time-consuming process. Tetranychid mites are serious agricultural pests. Identification of tetranychid mites is usually arduous and requires a high level of taxonomic expertise because of their minute size, their close morphological similarities as well as limited number of diagnostic characters. Most species of the spider mites of the genus *Tetranychus* Dufour in Iran are morphologically similar, differing only in the diameter of the aedeagal knob in males. Because this genus contains many important pests, the unambiguous identification of species is crucial for effective pest management. In this study, a single-step multiplex Polymerase Chain Reaction (multiplex PCR) was used to discriminate two predominant spider mite species occurring in greenhouses in Iran: *Tetranychus urticae* Koch and *Tetranychus turkestani* (Ugarov and Nikolskii). The single-step multiplex PCR developed here, based on ITS regions, is rapid, reliable, sensitive and relatively simple. The entire identification protocol from DNA extraction to electrophoresis could be completed in four hours. Moreover, it is adequately simple to be implemented in any molecular laboratory.

Keywords: DNA-based Identification, Molecular acarology, Tetranychid mites, *Tetranychus turkestani*, *Tetranychus urticae*.

INTRODUCTION

Spider mites of the family Tetranychidae (Donnadieu) are polyphagous pests of great economic significance that severely damage vegetables, ornamentals, and agricultural crops throughout the world (Gotoh *et al*., 2009; Matsuda *et al*., 2013). Distinguishing tetranychid taxa is extremely tough because of their minute size, morphological similarities as well as limited number of diagnostic characters (Wauthy *et al*., 1998; Zhang and Jacobson, 2000). Moreover, for many species, males are often inevitably necessary to make precise determinations. Nevertheless, due to the female-biased sex ratio, most intercepted *Tetranychus* Dufour specimens at plant ports are adult females (Sabelis, 1991). In addition, morphological keys rely on characters of adult specimens and cannot be used to reliably identify immature stages. Hence, DNA-based methods for species identification have increasingly been used for some genera of the spider mites (Osakabe *et al*., 2002; Arimoto *et al*., 2013; Matsuda *et al*., 2014; Li *et al*., 2015).

The genus *Tetranychus* contains numerous notable pests whose unambiguous identification is crucial for efficacious pest management and procedures (Matsuda *et al*., 2013). Identification of some species in this genus is pretty strenuous as it is mainly

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based on small differences in characters expressing a range of variations (Zhang and Jacobson, 2000; Carbonnelle and Hance, 2004; Gotoh *et al*., 2009).

In Iran, the family Tetranychidae includes some of the most injurious plant feeders (Sepasgozarian, 1977). Out of 11 reported species of spider mites in Iran (Beyzavi *et al*., 2013), two of them, namely, the two-spotted spider mite, *Tetranychus urticae* Koch, and the strawberry mite, *Tetranychus turkestani* (Ugarov and Nikolskii), have been given much attention. These two species are the main and most abundant tetranychid mites damaging various plants in greenhouses throughout Iran. According to Khanjani and Haddad Irani-Nejad (2009), both green and red forms of *T.urticae* are present in Iran, but all specimens collected from the study areas consisted of only the green form. Indeed, both species are highly polyphagous, very closely related, and are morphologically extremely similar. In other words, *T. turkestani* cannot be easily distinguished from *T. urticae* by morphological methods (Navajas and Boursot, 2003; Ros and Breeuwer, 2007; Arimoto *et al*., 2013); therefore, misidentifications of these two species is not unexpected.

As a main group of tetranychid natural enemies, most Stethorini coccinellids feed on multiple tetranychid prey species, although some are more specialized and exhibit prey preferences. *Stethorus gilvifrons* is often observed feeding on *T. turkestani* and *T. urticae* on different host crops in Iran, although its performance, probably its preference and, consequently, the effectiveness of this agent for controlling these two species might be different (Imani *et al*., 2009). Furthermore, *S. punctillum,* which has been reported from Iran (Arbabi and Singh, 2008), is reputed as an effective control predator of *T. urticae* in greenhouses (Biddinger *et al*., 2009). In a study on responses of spider mite species to propargite by Grafton-Cardwell *et al*. (2014), it was reported that all populations of *T. turkestani* were susceptible to propargite, but populations of *T. urticae* exhibited a variety of responses from highly susceptible to highly resistant. However, due to lack of knowledge in many aspects of their biology including natural enemies, susceptibility to pesticides and distribution, the impact caused by these spider mites is aggravated by imperfect management strategies applied.

According to the foregoing difficulties concerning the differences between these two species in various aspects such as suitability for natural enemies (Imani *et al*., 2009; Biddinger *et al*., 2009) and susceptibility to pesticides (Van Leeuwen *et al*., 2009; Grafton-Cardwell *et al*., 2014; Kheradmand *et al*., 2015), precise and prompt discrimination of them might be valuable in choosing the most effective control strategies against them. . Although various PCR (polymerase chain reaction) - based molecular methods such as PCR-RFLP (Arimoto *et al*., 2013; Osakabe *et al*., 2002), sequence analysis of ribosomal internal transcribed spacer region (Hurtado *et al*., 2008) and real-time PCR (Li *et al*., 2015) have hitherto been used in order to identify spider mites, an increasing trend toward developing relatively simple alternative diagnostic techniques is still continuing.

Compared to other molecular methods, using multiplex PCR has been dramatically increasing in species identification studies as it permits simultaneous amplification of several DNA fragments in a single reaction. Not only is this technique relatively quick and reliable, but also straightforward enough to be applied in any molecular laboratory. This method has already been used for simultaneous rapid identification of various arthropod pests such as pseudococcid mealybugs (Saccaggi *et al*., 2008; Daane *et al*., 2011), gall-forming aphids (Lee *et al*., 2013), eriophyid mites (Lava Kumar *et al*., 1999) and allergenic dust mites (Thet-Em *et al*., 2012). Nevertheless, there has not been any report of using this method for identification of *Tetranychus* species.

In this study, development of a multiplex PCR was undertaken for rapid discrimination of two spider mite species most commonly associated with greenhouse plants in Iran.

MATERIALS AND METHODS

Taxon Sampling

Tetranychid mite specimens collected from different greenhouses in Mashhad and vicinity or from laboratory colonies were employed throughout the multiplex PCR trials (Table 1). The common bean, *Phaseolus vulgaris* L., was used for tetranychid mite rearing under laboratory conditions (25±2°C, 60±5% RH, and a photoperiod of 16 L: 8 D). Morphological identification of samples was performed following laboratory establishment of mites. Cleared mite specimens were mounted in Hoyer's medium and identified based on their morphological characters. Tetranychid specimens were stored in absolute ethanol and kept in a -20°C freezer in order to use in subsequent DNA extractions. Both adults and immature stages of tetranychids were used in the analyses.

DNA Template Preparation

Each mite was individually put into a 1.5 mL Eppendorf tube with 20 µL of distilled water and ground with a sterilized plastic pestle. This homogenized sample solution was utilized as DNA template.

Primer Design

In order to design the specific primers for multiplex PCR, sequences of ribosomal DNA (18S-ITS1-5.8S-ITS2-28S) were retrieved from GenBank for *T. urticae*, *T. turkestani* and four other *Tetranychus* species (*T. neocaledonicus, T. kanzawai*, *T. ludeni*, *T. lambi*), all of which exist in Iran (Table 2). Using Muscle algorithm implemented in MEGA6, the sequences were aligned and two species-specific forward primers, *T. urticae* (green form) Forward primer (UrtF) and *T. turkestani* Forward primer (TurkF), were designed in regions where the *Tetranychus* sequences of various species differed. In addition to two specific forward primers, a common reverse primer, *Tetranychus* Common Reverse primer (TetCR), was designed as well. The design of common reverse primer was based

Table 1. Collection information for *Tetranychus* specimens used during testing of the multiplex PCR.

Species	Location	Collection	Host plant
Tetranychus urticae (Green form)	Mashhad, Iran	Greenhouse, Lab colony	*Rosa* sp., *Phaseolus vulgaris*
Tetranychus urticae (Red form)	Tehran, Iran	Greenhouse	unknown
Tetranychus turkestani	Mashhad, Iran	Greenhouse, Lab colony	*Solanum melongena* *Phaseolus vulgaris*
Tetranychus kanzawai	Japan	-	*Camellia sinensis*
Tetranychus neocaledonicus	Japan	-	*Solanum melongena*

Table 2. The accession number of sequences retrieved from GenBank and used to design primers based on the ITS region.

Species name	Accession number
T. urticae (green form)	KF544955, AM408035, KP744531, HM565890
T. turkestani	AB738745, AM408032, GQ141938
T. neocladonicus	AB738752
T. kanzawai	AB304785
T. ludeni	AB738754
T. lambi	AB738743

on the region with high level of identity among *T. urticae* and *T. turkestani* sequences. Designing of primers was carried out so that the yielded products with different lengths could be separated on an agarose gel without difficulty. Primer names and sequences are mentioned in Table 3.

Detection of *T. urticae* and *T. turkestani* Using Specific Primers

In the first step, to check if the designed primers work properly, PCR reactions were performed using one specific forward primer (UrtF or TurkF) and the common reverse primer. The PCR reactions were conducted in a volume of 25 µL containing 12.5 µL of 2X PCR Master Mix® (Ampliqon, Denmark), 1 µL of one of the specific forward primers and 1 µL of the common reverse primer (stock 10 µM), 2 µL of DNA template and 8.5 µL of distilled water. The optimal annealing temperature was determined with thermal gradient PCR and the gradients consisted of 4 temperatures between 49°C and 55°C. Optimized thermocycling conditions consisted of an initial denaturation at 94°C for 1 minute, then, 30 cycles of 94°C for 30 seconds, 51°C for 45 seconds and 72°C for 90 seconds, with a final extension at 72°C for 8 minutes. PCR products were visualized under UV light on a 1% agarose gel stained with DNA Green Viewer® (Pars Tous, Iran). PCR products were stored at -20°C until needed.

Multiplex PCR

Through a single-step multiplex PCR, the two species-specific forward primers and the common reverse primer were used conjointly in a single reaction to differentially amplify DNA from each of the two tetranychid species. The PCR reactions were conducted in a volume of 25 µL containing 12.5 µL of 2X PCR Master Mix®, 1 µL of each of the specific forward primers (UrtF and TurkF) and 1 µL of the common reverse primer (stock 10 µM), 2 µL of DNA template and 7.5 µL of distilled water. Products from the multiplex PCR were visualized and imaged as above. All reactions were run with a non-template control comprising all reagents other than DNA. The species-specific primer cross-reactivities were assessed, both separately and in the multiplex reaction mix.

Detection of Immature Stages

In addition to the adults, eggs and nymphs were also assayed to inspect the method's general applicability.

Sensitivity Test

In order to evaluate the sensitivity of the primer mix in the multiplex system, different number of tetranychid adults were ground in separate Eppendorf tubes each containing 20 µL of distilled water. These solutions were

Table 3. Details and thermodynamic properties of species-specific primers designed, based on the ITS region, for use in multiplex PCR.

Specificity	Primer name	Sequence	Product length	Target region	Melting temperature
T. urticae (Forward)	UrtF	GTCTTGCTACATACTTGGTACCTGATC	739	ITS1	59
T. turkestani (Forward)	TurkF	GGTGAATGCAATTGAATTGTATTTGCAAG	950	ITS1	58
Common Reverse	TetCR	TTGCCTTTGCATTACTTCTTAGGTC		ITS2	58

then used as templates as follows: (1) Only one type of target DNA as template (either five specimens of *T. urticae* or *T. turkestani*) and (2) Different mixtures of two target species as template (4 *T. urticae*+1 *T. turkestani*, 3 *T. urticae*+2 *T. turkestani*, 2 *T. urticae*+3 *T. turkestani*, 1 *T. urticae*+4 *T. turkestani*). The PCR conditions were identical to those described above. Material from each of these reactions also was examined using agarose gel electrophoresis.

Testing the Specificity of the Multiplex PCR

PCR specificity was evaluated using specimens of two other polyphagous *Tetranychus* species that might exist in greenhouses, *T. kanzawai* Kishida and *T. neocaledonicus* André (Table 1). These two species were firstly evaluated as to whether they have sufficient PCR competent DNA by using LCO and HCO common primers (Folmer *et al*., 1994). To do so, the COI region of mitochondrial gene was amplified using universal primers, LCO 1490 5′-*GGTCAACAAATCATAAAGATATTGG*-3′ and HCO 2198 5′-*TAAACTTCAGGGTGACCAAAAAATCA*-3′ (Folmer *et al*., 1994). The PCR reactions were conducted in a volume of 25 µL containing 12.5 µL of 2X PCR Master Mix®, 1 µL of each primer (stock 10 µM), 2 µL of DNA template and 8.5 µL of distilled water. Thermocycling conditions consisted of an initial denaturation at 94°C for 5 minutes, then, 30 cycles of 94°C for 30 seconds, 52°C for 45 seconds, and 72°C for 90 seconds, with a final extension at 72°C for 8 minutes. In order to evaluate the applicability of the method, trials using greenhouse-collected specimens of both species were set up to assess the multiplex PCR (Table 4).

RESULTS

Detection of T. urticae and T. turkestani

The species-specific primers yielded amplicons of 739 and 950 bp from *T. urticae* (red and green forms) and *T. turkestani*, respectively, when used individually or in multiplex PCR (Figures 1 and 2). Single amplicons of the specified sizes from each species were consistently produced by using these primers and no cross-reactivity of primers was observed. In other words, the discrimination of two examined species was performed accurately using these specific primers. When each species-specific primer was trialed separately for reactivity with the other species, no amplification was observed (Figure 1). Moreover, in the multiplex reaction mix, no cross-reactivity of primers

Table 4. Collection information for *Tetranychus* specimens from various greenhouses, used as blind trials and identified through multiplex PCR.

Population#	Species	Locality	Collection	Host plants
1	*T. turkestani*	Torghabeh, Iran	Greenhouse	*Phaseolus vulgaris*
2	*T. turkestani*	Chenaran, Iran	Greenhouse	*Rosa* sp.
3	*T. urticae* (Green form)	Mashhad, Iran	Greenhouse	*Cucumis sativus*
4	*T. turkestani*	Mashhad, Iran	Greenhouse	*Convolvulus arvensis*
5	*T. turkestani*	Shandiz, Iran	Greenhouse	*Cucumis sativus*
6	*T. urticae* (Green form)	Chenaran, Iran	Greenhouse	*Gerbera jamesonii*
7	*T. urticae* (Green form)	Neyshabur, Iran	Greenhouse	unknown
8	*T. turkestani*	Torghabeh, Iran	Greenhouse	*Portulaca oleracea*

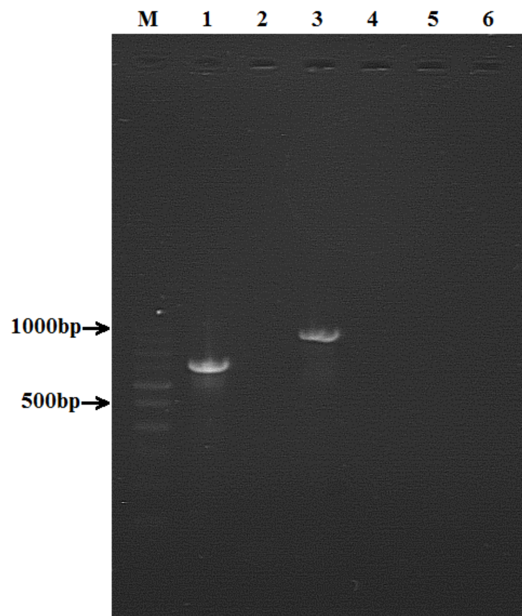

Figure 1. Diagnostic PCR using species-specific primers. Lane M: DNA size marker; Lane 1: *T. urticae* DNA+UrtF primer; Lane 2: *T. urticae* DNA+TurkF primer, Lane 3: *T. turkestani* DNA+TurkF primer; Lane 4: *T. turkestani* DNA+UrtF primer; Lane 5: C[-] (with UrtF primer), Lane 6: C[-] (with TurkF primer).

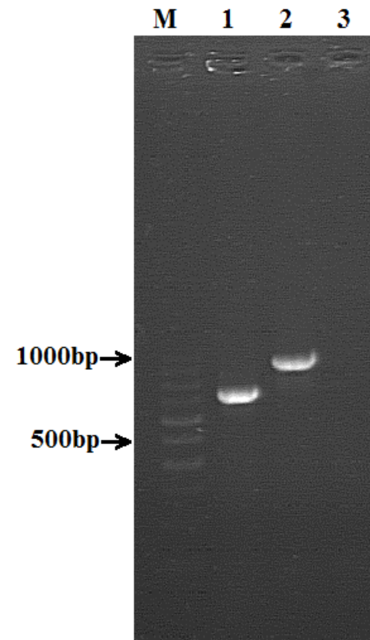

Figure 2. Diagnostic multiplex PCR using a combination of 3 primers in each reaction. Lane M: DNA size marker; Lane 1: *T. urticae*; Lane 2: *T. turkestani*, Lane 3: C[-].

was observed (Figure 2).

Detection of Immature Stages

When the various developmental stages (eggs, nymphs, and adults) of two species were tested, the banding pattern indicated that the premature stages could be identified at the same level of stringency as the adults (Figure 3).

Sensitivity to Different Ratios of *T. urticae* and *T. turkestani*

Using one type species as template (5*T. urticae* or 5*T. turkestani*), one band was produced; 739 bp for *T. urticae*, or 950 bp for *T. turkestani*. Using mix DNA from both species as template, two separate fragments were revealed (Figure 4). The results also showed that the multiplex PCR could detect one individual mite successfully (Lane 5 for *T. urticae*; Lane 2 for *T. turkestani*, Figure 4). These data suggest that this method was quite sensitive in detecting and distinguishing *T. urticae* and *T. Turkestani* using the designed primers.

Testing the Specificity of the Multiplex PCR

Multiplex PCR was tested using two other *Tetranychus* species (*T. kanzawai* and *T. neocaledonicus*), which are closely related to *T. urticae* and *T. turkestani*. DNAs extracted from the two species were successfully amplified in conventional PCR (Figure 5), indicating that the DNA extracts are PCR competent. No bands were observed when using the DNA of either *T. kanzawai* or *T. neocaledonicus* in the multiplex PCR reaction, an indication of no cross-reactivity among the tested species. (Figure 6, Lanes 4 and 5).

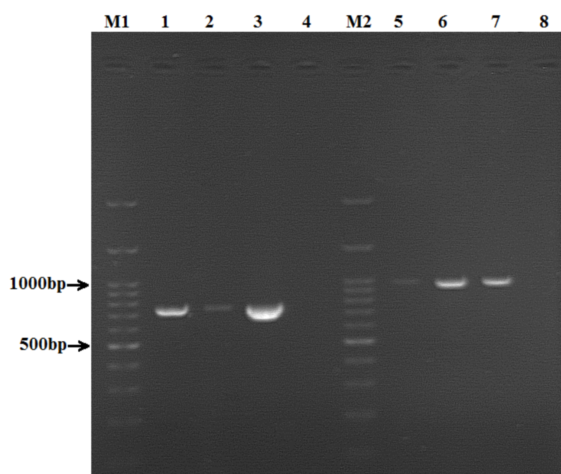

Figure 3. Visualization of multiplex-PCR products from various developmental stages of *Tetranychus* spp. Lane M1: DNA size marker; Lanes 1-3: *T. urticae* DNA; (Lane 1: Egg; Lane 2: Nymph, Lane 3: Adult); Lane 4: C-; Lane M2: DNA size marker, Lanes 5-7: *T. turkestani* DNA; (Lane 5: Egg; Lane 6: Nymph, Lane 7: Adult), Lane 8: C-.

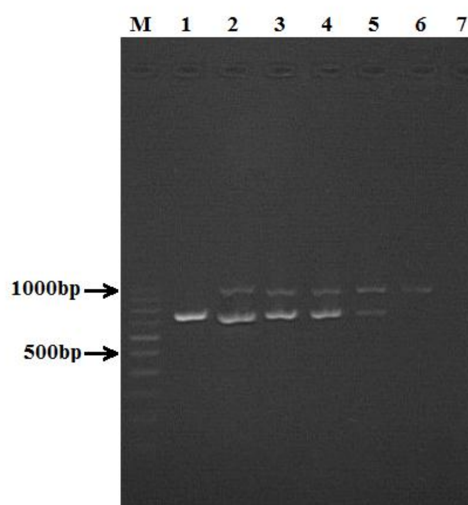

Figure 4. Resolution of mixtures of *Tetranychus* species. Lane M: DNA size marker; Lane 1: 5 *T. urticae*; Lane 2: 4 *T. urticae*+1 *T.turkestani*; Lane 3: 3 *T. urticae*+2 *T. turkestani*; Lane 4: 2 *T. urticae*+3 *T. turkestani*; Lane 5: 1 *T. urticae*+4 *T.turkestani*; Lane 6: 5 *T. turkestani*, Lane 7: C-.

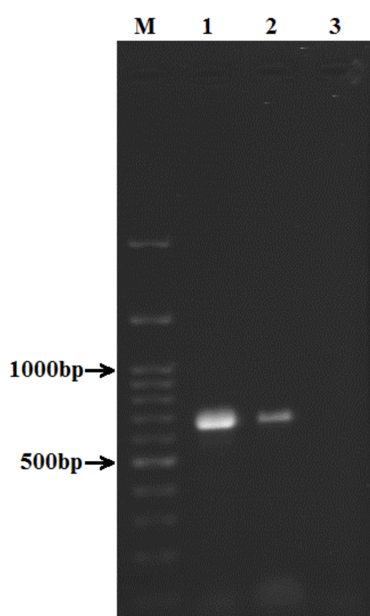

Figure 5. DNA amplification of control species using LCO and HCO common primers. Lane M: DNA size marker; Lane 1: *T. kanzawai*; Lane 2: *T. neocaledonicus*, Lane 3: C-.

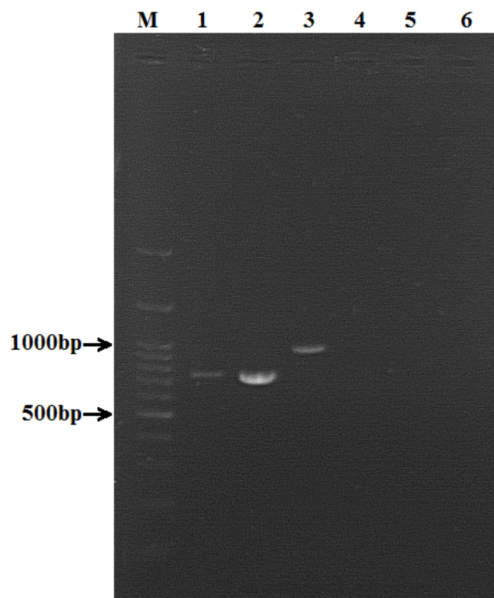

Figure 6. Specificity of the multiplex PCR. Lane M: DNA size marker; Lane 1: *T. urticae* (green form); Lane 2: *T. urticae* (red form); Lane 3: *T. turkestani*; Lane 4: *T. kanzawai*; Lane 5: *T. neocaledonicus*, lane 6: C-.

All the examined specimens collected from different greenhouses (Table 4) were successfully amplified in the multiplex PCR assay and correctly identified (Figure 7). In all cases, not only were the specimen identities correctly determined using multiplex PCR, but all diagnostics also were in agreement with sub-samples used for morphological identifications.

DISCUSSION

Accurate species identification is the first step in any biological study. Identification of tetranychid species through traditional taxonomic keys often demands a high level of expertise and can be tedious and time consuming, especially where there is no specialist. Moreover, morphological determination is not applicable for immature stages of tetranychid mites. On the other hand, differentiation of *T. urticae* and *T. turkestani* from each other and also from other *Tetranychus* species is necessary for successful pest management. To avoid any misidentification and serious confusion in future studies, a combination of morphological and molecular identification is suggested. In this study, a multiplex PCR was utilized for identification and discrimination of two morphologically similar species of spider mites associated with greenhouse plants in Iran. In comparison to morphological keys, the entire protocol of the molecular approach used in this study took less than four hours to complete. Moreover, by using this method, the overall processing time was shortened as a large number of samples can be assessed concurrently.

The utility of designed primers for the discrimination between the target tetranychid species was evaluated by collecting samples from various greenhouses, using them as blind trials and confirming their identification through multiplex PCR. All identities were in agreement with sub-samples used for morphological identifications. The outcome

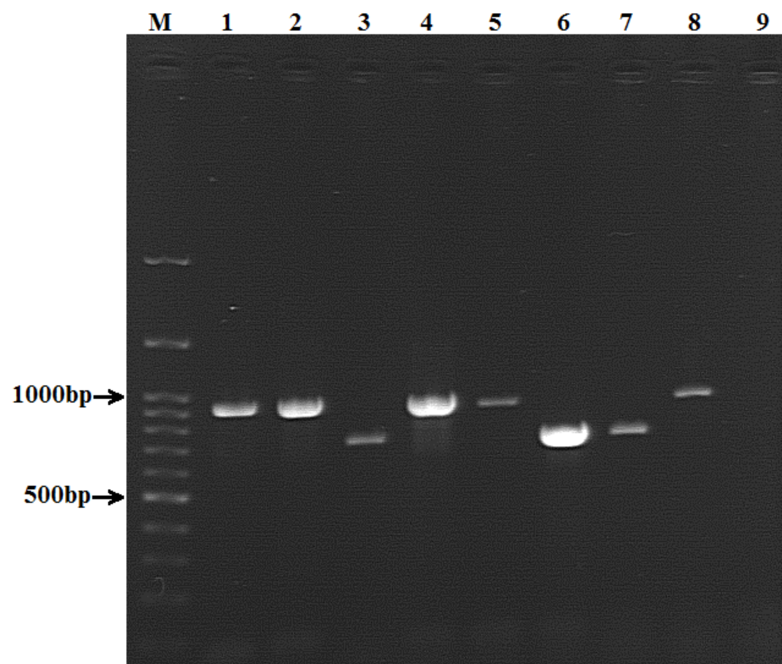

Figure 7. Blind trial identification of various greenhouse *Tetranychus* specimens through multiplex PCR for populations #1 to #8 mentioned in Table 4 (*T. urticae*: 739 bp; *T. turkestani*: 950bp). Lane M: DNA size marker; Lane 1: #1; Lane 2: #2; Lane 3: #3; Lane 4: #4; Lane 5: #5; Lane 6: #6; Lane 7: #7; Lane 8: #8, Lane 9: C^-.

of the study showed that the multiplex PCR approach accurately identified the targeted species. The method not only has the capability of reliable amplification of DNA from damaged samples but also provides the means by which various life stages of any of two target species can be accurately and expeditiously identified. Although *T. urticae*, *T. turkestani*, *T. kanzawai* and *T. neocaledonicus* are very closely related, the multiplex PCR tested with these different tetranychid species showed no cross-reactivity. No other *Tetranychus* species were obtained for testing in the multiplex PCR protocol, such as *T. truncates*. However, mismatches of the species-specific primers were detected (2 mismatches with Urtf and 7 mismatches with Turkf), therefore, the possibility of cross-reactions are low. Also, as there was no sequence data for some species (*T. frater, T. schoenei* and *T. tumidellus*) in GenBank, it should be considered that testing the cross reactivity of the designed primers for these species was not possible. However, according to available literatures, the possibility of presence of these species in Iranian greenhouses is very low.

Moreover, simultaneous detection of both tetranychid species was feasible using specific primers in multiplex PCR, indicating that the capability of the method for detection of one or both targeted species in the resulting mixture was not affected by existence of multiple tetranychids at once, which in its turn resulted in reducing per individual cost of tetranychid identification. In addition, the multiplex PCR was fully optimized for diagnostics in greenhouses of Iran. Pre-deployment testing is suggested in locations outside Iran, to ensure no false-negative or positives are detected, although they would be highly unlikely.

Our results corroborate preceding studies on the application of multiplex PCR as a reliable method for both simultaneous and quick identification of insects or mites (Daane *et al*., 2011; Lava Kumar *et al*., 1999; Lee *et al*., 2013; Saccaggi *et al*., 2008; Sint *et al*., 2014; Thet-Em *et al*., 2012; Pérez-Sayas *et al*., 2015). In conclusion, because of the simplicity of the procedure, it can be implemented even in less-equipped laboratories and does not necessitate extensive taxonomic or molecular experience. This identification protocol aids in discriminating *T. urticae* and *T. turkestani* accurately and swiftly, which would be valuable for management of these pest species by natural enemies and pesticides. However, a combination of different methods including DNA-based methods in conjunction with morphological characters and ecological information will provide more reliable identification of *Tetranychus* species (Matsuda *et al*., 2013).

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استفاده از روش Multiplex PCR برای تشخیص همزمان دو گونه غالب از کنه‌های تارتن جنس تترانیخوس (Acari: Tetranychidae) در گلخانه‌های ایران

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چکیده

به‌کارگیری روش‌های شناسایی مولکولی در تشخیص حشرات، به‌خصوص در شرایطی که شناسایی مورفولوژیکی بسیار پر زحمت و زمان‌بر است، متداول‌تر شده است. کنه‌های تترانیخید از آفات بسیار مهم در کشاورزی هستند. شناسایی کنه‌های تترانیخید معمولاً دشوار است و به‌دلیل اندازه بسیار کوچک آنها، شباهت‌های مورفولوژیکی گونه‌ها و تعداد محدود صفات تشخیصی، نیازمند افرادی بسیار متخصص می‌باشد. اغلب گونه‌های کنه‌های تارتن جنس *Tetranychus* در ایران از نظر مورفولوژیکی

مشابه هستند و تنها وجه تمایز آنها تفاوت در ضخامت گره اِدیاگوس در نرها است. به‌دلیل اینکه این جنس دربرگیرنده بسیاری از آفات مهم می‌باشد، شناسایی دقیق گونه‌ها به‌منظور مدیریت آنها و از اهمیت زیادی برخوردار است. در این تحقیق، از روش تک مرحله‌ای multiplex PCR برای تفکیک دو گونه غالب از کنه‌های تارتنِ فعال در گلخانه‌های ایران، *Tetranychus urticae* و *Tetranychus turkestani*، استفاده شد. روش Multiplex PCR به‌کار گرفته شده در اینجا یک روش تک‌مرحله‌ای مبتنی بر DNA ریبوزومی است که سریع، قابل اطمینان، حساس و نسبتاً ساده می‌باشد. تمام مراحل انجام کار، از استخراج DNA تا راندن روی ژل الکتروفورز، در چند ساعت قابل تکمیل است. علاوه بر آن، به اندازه کافی ساده است تا در آزمایشگاه‌های تحقیقاتی مولکولی قابل اجراء باشد.