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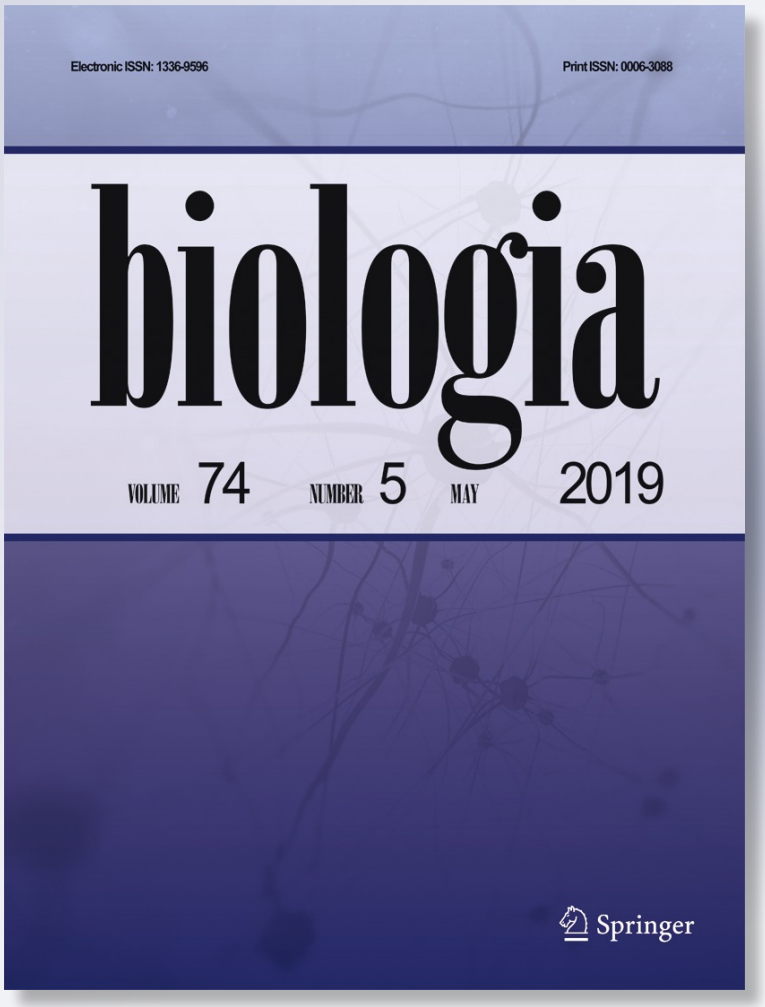

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ORIGINAL ARTICLE

Loop-mediated isothermal amplification combined with PCR for specific identification of injurious mite, *Tetranychus urticae* (Trombidiformes: Tetranychidae)

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Abstract
Identification of spider mites of the genus *Tetranychus* is strenuous due to their minute size and the restricted number of diagnostic characters. Most species of the Iranian *Tetranychus* spider mites are morphologically alike, distinguishable only based on male aedeagus; however, most of the intercepted *Tetranychus* specimens are females, leaving their identification at the genus level. *Tetranychus urticae* is a highly polyphytophagous pest species commonly found in greenhouses of Iran. As the unambiguous identification of a pest is the first crucial step toward implementing any effective pest management procedure, various DNA-based methods have formerly been used as complement to the traditional morphological methods. In the current study, a technique integrating loop-mediated isothermal amplification (LAMP) with PCR (polymerase chain reaction) was utilized for quick detection of *T. urticae* and discriminating it from other *Tetranychus* species attacking greenhouse crops. The assay was validated against various developmental stages of *T. urticae* specimens. Not only did the PCR-LAMP assay decrease the time needed for identification, but also was species-specific and sensitive enough to be applied even by non-specialists.

Keywords Two-spotted spider mite · Tetranychinae · Major pest · Molecular diagnosis · LAMP

Introduction

Tetranychus urticae Koch, 1836 (Trombidiformes: Tetranychidae) also known as the two spotted spider mite, is a highly polyphytophagous species which can feed on over 1100 plant species from more than 140 different plant families and is a crucial pest of greenhouse and field crops (Grbić et al. 2011). Causing outbreaks in many crops all around the world, it is assuredly one of the most studied pest species (De Mendonça et al. 2011). Despite long-running immense studies on the systematics of the genus *Tetranychus*, the minuscule size of these mites along with the finite number of diagnostic characters make the morphological identification arduous in some cases and necessitate the help of taxonomic experts for accurate diagnosis (Wauthy et al. 1998; Zhang and Jacobson 2000; Gotoh et al. 2009; De Mendonça et al. 2011). Spider mite species are identified based on characteristics of the aedeagus (Ehara 1999), whereas due to the highly biased sex ratio of the mites in favour of females, most specimens collected in the fields are female adults (Sabelis 1991). Furthermore, morphological keys for reliable identification of immature stages of spider mites are unfeasible because of their reliance on characters of the adult specimens (Matsuda et al. 2013). Therefore, molecular methods for species identification have increasingly been developed for the genus *Tetranychus* (Matsuda et al. 2013). Diverse molecular methods have hitherto been used as complement to the morphological identification of many *Tetranychus* species (Osakabe et al. 2002; Hinomoto et al. 2007; Osakabe et al. 2008; Arimoto et al. 2013; Li et al. 2015). For discriminating *Tetranychus* species, ITS and COI sequences have been extensively used as DNA barcodes (Fournier et al. 1994; Navajas and Boursot 2003; Ben-David et al. 2007; Matsuda et al. 2013; Matsuda et al. 2014). Targeting the ITS region, PCR-RFLP is another commonly used diagnostic

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method which has been utilized in *Tetranychus* species identification (Osakabe et al. 2008; Arimoto et al. 2013). However, most of the currently used identification procedures are costly, time consuming and depend on specialized equipment and technicians (Hsieh et al. 2012). Hence, developing a quick method for prompt diagnosis of *Tetranychus* species is of substantial importance in order to provide expeditious identification for the intercepted material while avoiding the time-consuming currently used available methods.

Loop-mediated isothermal amplification (LAMP) is an efficient and relatively rapid technique for DNA amplification under isothermal conditions. As this method uses a set of four specifically designed primers, forward and backward inner primers (FIP and BIP, respectively) and forward and backward outer primers (F3 and B3, respectively), six distinct regions on the target DNA are recognized (Notomi et al. 2000); making LAMP more specified compared to some other methods such as classical and nested PCR, which respectively target two and four regions of the DNA (Ravindran et al. 2012; Faggion et al. 2013). Furthermore, adding $MnCl_2$ and calcein to the LAMP reaction mix can result in the formation of a fluorescent yellowish-green color, leading to the direct visualization of reaction products in the reaction tubes (Tomita et al. 2008). Not only does this method require no specialized equipment or specifically trained technicians, but also it is cost effective and appropriate for large-scale field surveys which could provide advantageous information for pest control strategies (Hsieh et al. 2012).

As a well-liked method, LAMP has been used in a broad range of applications, from human disease pathogen identification (Iwamoto et al. 2003; Thai et al. 2004; Parida et al. 2005; Poon et al. 2006; Ohtsuki et al. 2008), embryo sex determination (Hirayama et al. 2004), plant disease pathogen identification (Fukuta et al. 2003, 2004; Kuan et al. 2010; Da Silva Gonçalves et al. 2014) to insect identification (Itakura et al. 2006; Bonizzoni et al. 2009; Huang et al. 2009; Fekrat et al. 2015; Sabahi et al. 2017).

In the current study, PCR and LAMP were integrated (PCR-LAMP) in order to develop a highly sensitive method for *T. urticae* identification in any developmental stage. In other words, because of some false negative results and in order to improve the sensitivity of LAMP assay, PCR was performed before the LAMP reaction, a method similar to nested PCR which leads to enhancement of the sensitivity of LAMP reaction. This method has benefits over nested PCR due to permitting researchers to skip the time-consuming secondary PCR and for the reason that amplicons can be identified straightforwardly by simply observing turbidity (Kitano et al. 2016).

Materials and methods

Sample collection and DNA template preparation

Tetranychus specimens were collected from various host plants in greenhouses of Mashhad and vicinity, Razavi Khorasan province, Iran by observing mite infested leaves under a binocular microscope and removing the specimens using a fine brush. Single adult female mites from each collected population were reared on separate *Phaseolus vulgaris* L. plants in separate growth chambers (27 ± 2 °C, 70 ± 5% RH and a photoperiod of 16:8 L:D). After the establishment of colonies, individuals from each colony were identified morphologically (via optical microscope after slide preparation). Identification results showed that all collected *Tetranychus* mites belonged to two species, *T. urticae* and *Tetranychus turkestani* (Ugarov and Nikolskii 1937). Approximately 50 specimens of each life stage (eggs, larvae and adults) from each species, obtained from the colonies mentioned above, were stored in separate Eppendorf tubes containing absolute ethanol and kept in a − 20 °C freezer. DNA template preparation was carried out by grinding mite specimens in a 1.5 ml Eppendorf tube with a sterilized plastic pestle and adding 20 μl distilled water and using the homogenized sample solution as a DNA source.

Primer designing for LAMP reaction

Two pairs of primers were designed by Primer Explorer software (https://primerexplorer.jp/e/), including forward and backward inner primers (FIP and BIP) and forward and backward outer primers (F3 and B3), using sequences of ribosomal DNA (ITS1–5.8S-ITS2) of *T. urticae*. To ensure the specificity of the designed primers, 18 *T. urticae* sequences and 28 sequences from 17 other *Tetranychus* species were retrieved from GenBank and used in the alignment and designing of the primers (Online resource 1). Primer sequences are shown in Table 1 and the targeting regions of primers are depicted in Fig. 1.

PCR-LAMP reaction

Using *T. urticae* DNA samples with direct LAMP resulted in negative results in some cases, which might be related to the quality/quantity of DNA. So, in order to enhance the sensitivity of the experiment and also to avoid any false negative results, PCR was performed before the LAMP reaction (PCR-LAMP). Performing PCR before LAMP has also previously been used in order to improve the sensitivity of the LAMP assay (Kitano et al. 2016).

DNA template was prepared by grinding 5 adult mite specimens in an Eppendorf tube following the method previously mentioned. The PCR was carried out using 1 μl of each of the

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Table 1 LAMP assay primers designed using ribosomal DNA sequences (ITS1–5.8S-ITS2) of *Tetranychus urticae*

Primer	Sequence
F3	TAATGAAAAGCCTGTCTT
B3	GATGTATCTTTGCCACCG
FIP (F1c & F2)	CCTTCTTTAAACCTTGCCGTC-GCTACATACTTGGTACCTGATC
BIP (B1c & B2)	AGACAACTATGGTTTTTATAAACTGC-CTTTTAATAAGTATCCTAAATCTG

F3 and B3 primers (stock 10 μM), 12.5 μl Master Mix (Ampliqon, Denmark), 2 μl DNA and 8.5 μl distilled water. The temperature profile was the initial denaturation for 4 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at 49 °C, and 1 min at 72 °C and final extension at 72 °C for 5 min. The PCR product containing the LAMP target sequence was used as template for LAMP assay.

LAMP reactions were performed with 0.5 μl of PCR product as template, 3 μl of the 10× BSM reaction buffer (Thermo Scientific), 1.3 μM of primers FIP (forward inner primer) and BIP (backward inner primer) and 0.33 μM of primers F3 (forward outer primer) and B3 (backward outer primer), 1 M betaine and 1.6 m M dNTPs. Distilled water was added to bring the total volume to 30 μl.

The reaction was initiated by incubating the mixture at 95 °C for 5 min, followed by cooling on ice for 1 min, adding 8 U of Bsm DNA polymerase (Thermo scientific-Fermentas, Vilnus, Lithuania) and incubating at 60 °C for 15, 30, 60 and 120 min. The reaction was terminated by raising the temperature up to 80 °C for 10 min. In addition, four different temperatures (50, 55, 60 and 65 °C) and four betaine concentrations (0, 0.5, 1 and 1.5 M) were evaluated for LAMP optimization. Indeed, to eliminate the possibility of reagent contamination, a control with no DNA template was included in each assay. LAMP products were then examined using 1% agarose gel electrophoresis.

Direct analysis of LAMP products

Addition of $MnCl_2$ and calcein (Sigma) to the reaction mix results in formation of a fluorescent yellowish-green color under UV light and pinkish-orange color under natural light (Tomita et al. 2008), leading to the visualization of reaction products in the reaction tubes by naked eye. In order to directly detect LAMP amplicons in the reaction tubes, 0, 1, 1.5, 2 and 4 μM of a calcein solution (stock 1 mM $MnCl_2$ and 50 μM calcein) were added to LAMP reactions with the same composition and using the same PCR product previously mentioned. The florescence intensities were compared under UV light. Resulting in a yellowish-green fluorescence is an indication of positive reactions (Tomita et al. 2008). Moreover, naked eye detection of reaction products in the reaction tubes was possible under visible light. Obtained results were further confirmed via gel electrophoresis on a 1% agarose gel.

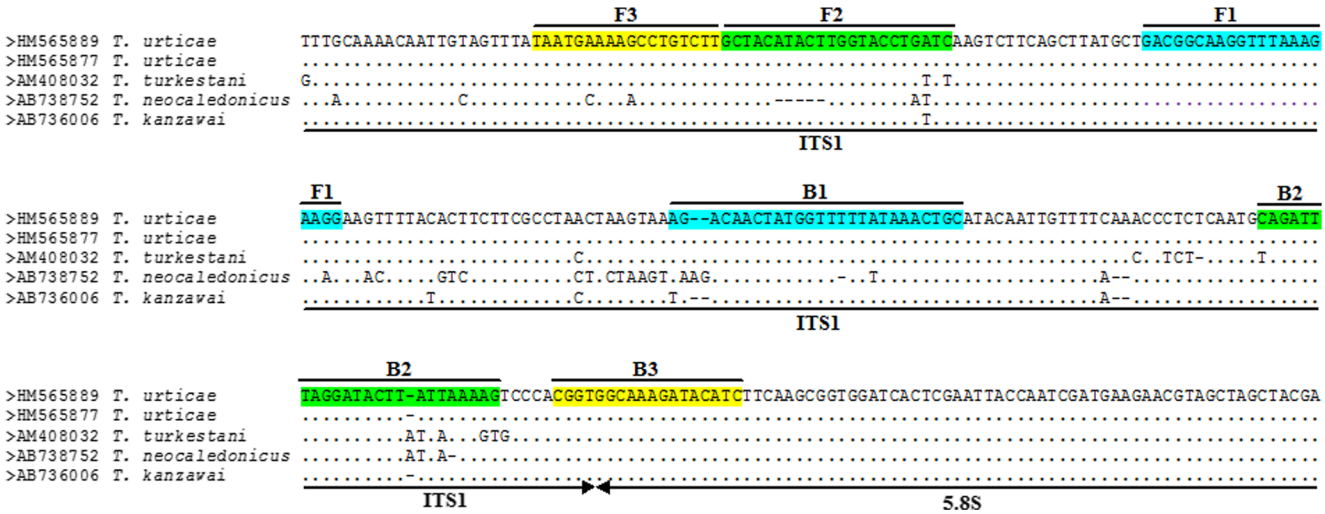

Fig. 1 Diagram depicting location of LAMP primer binding sites within partial ribosomal DNA sequences (ITS1–5.8S) of *Tetranychus urticae* and three other control species (*T. turkestani*, *T. neocaledonicus* and *T. kanzawai*). The primers include F3 (18 bp), B3 (18 bp), FIP consisting of F1c (21 bp) and F2 (22 bp), BIP consisting of B1c (26 bp) and B2 (24 bp). The lines (without arrowheads) show the location of used primers

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Detection of *T. urticae* in various life stages

DNA template preparation of different life stages of *T. urticae* was performed by grinding eggs (10 eggs), larvae (5 specimens) and adults (5 specimens) in separate Eppendorf tubes following the same method previously mentioned. The PCR-LAMP efficiency in detecting different life stages of *T. urticae* was evaluated by examining the products using 1% gel electrophoresis.

Sensitivity of PCR-LAMP

Evaluation of PCR-LAMP sensitivity was conducted by grinding 1, 3, 6, and 10 *T. urticae* adult specimens in separate Eppendorf tubes each containing 20 μl of distilled water and using them as DNA source for PCR-LAMP reaction. Results from each reaction were analyzed via gel electrophoresis.

Species discrimination

Tetranychus turkestani, Tetranychus kanzawai Kishida, 1927 and *Tetranychus neocaledonicus* André, 1933 (Trombidiformes: Tetranychidae) [The last two species provided by Professor Tetsuo Gotoh (Laboratory of Applied Entomology and Zoology, Faculty of Agriculture, Ibaraki University, Japan)], which all are closely related to *T. urticae* were utilized as controls in order to evaluate the possibility of cross-reaction of the designed primers with some of the other common tetranychid specimens that might coexist with *T. urticae* in greenhouses. Five adult specimens each of *T. urticae*, *T. turkestani*, *T. kanzawai* and *T. neocaledonicus* were ground in separate Eppendorf tubes containing 20 μl of distilled water and the resultant homogenates were used as DNA source in a PCR reaction with F3 and B3 primers following the same protocol previously mentioned. The PCR products were used as template in the LAMP reaction and analysis of LAMP amplicons for each of the reactions was conducted via gel electrophoresis.

In silico testing for specificity

The in silico testing was performed in two steps. In the first step, the specificity testing of F3 and B3 primers was carried out by downloading ITS1–5.8S-ITS2 sequences from GenBank for 18 *Tetranychus* species. A total of 74 sequences (including 30 *T. urticae* sequences) were used to form the in silico test set in FastPCR 6.6.35 (Kalendar et al. 2017). Non-specific primer binding (allowing 1 mismatch in each) against this expanded set of species was checked (Online resource 2). In the second step, only species with positive results from the first step (species which produced amplicons with F3 and B3 primers) along with 18 sequences of *T. urticae* were included in the specificity testing using F2 and B2 primers (Online resource 3).

Results

Optimization of PCR-LAMP reaction

The PCR-LAMP reactions were successfully completed for *T. urticae* specimens. Evaluation of four amplification times in the LAMP reaction (15, 30, 60 and 120 min) disclosed that although the amplification had begun after 15 min, the efficiency of amplification differed among different times. In other words, based on the intensities of the ladder-like patterns, the amplification efficiency at 30 min was higher than that of 15 min, but 60 min and 120 min proved most efficient in amplification, although there was not any notable difference between them (Fig. 2). Evaluation of four different temperatures (50, 55, 60 and 65 °C) based on the fluorescence intensities of the ladder-like patterns depicted that 50, 55 and 60 °C were optimal amplification temperatures (Fig. 3). Betaine and calcein solution optimal concentrations were 1 and 1.5 M (Fig. 4) and 1 and 1.5 μM (Fig. 5), respectively.

Adding $MnCl_2$ and calcein during DNA synthesis produced a pinkish orange color change under natural light and a yellowish-green florescence under UV light (Fig. 5a, b). LAMP amplicons produced similar ladder-like patterns with different-sized bands in gel electrophoresis (Fig. 5c). The LAMP reaction was performed successfully in the absence of calcein, however, due to the absence of a fluorescent dye, the results were not visible in natural and fluorescent lights (Fig. 5a, b).

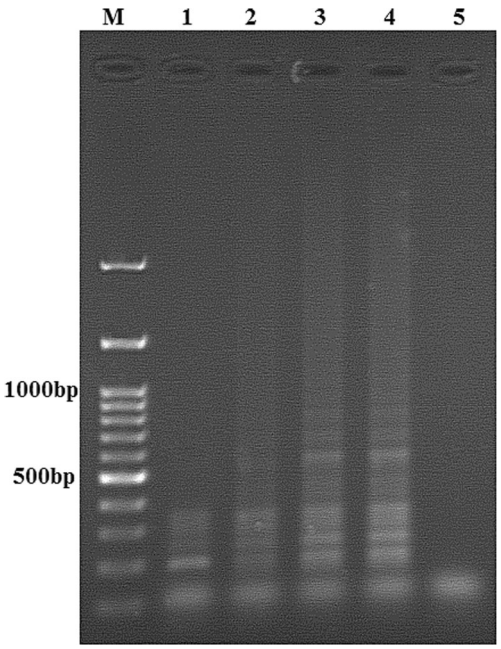

Fig. 2 Time period test for optimization of LAMP reaction in *T. urticae* samples. Lane M: DNA size marker; Lane 1: 15 min; Lane 2: 30 min; Lane 3: 60 min; Lane 4: 120 min; Lane 5: C^-

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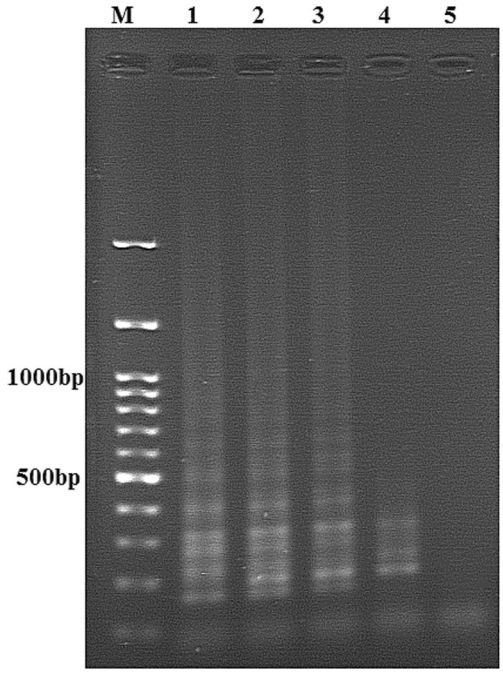

Fig. 3 Temperature test for optimization of LAMP reaction in *T. urticae* samples. Lane M: DNA size marker; Lane 1: 50 °C; Lane 2: 55 °C; Lane 3: 60 °C; Lane 4: 65 °C; Lane 5: C^{-}

Detection of different life stages of *T. urticae*

Different developmental stages of *T. urticae*, including eggs, larvae and adults were successfully detected by performing PCR-LAMP. In other words, *T. urticae* can be detected successfully by this method using material from all life cycle stages (Fig. 6).

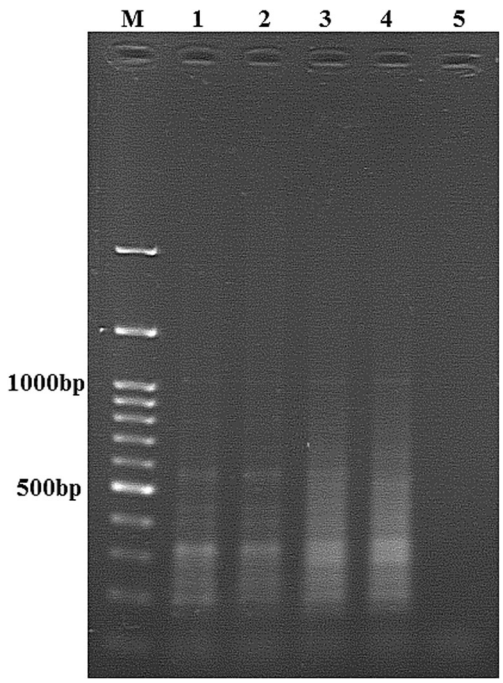

Fig. 4 Betaine concentration test for optimization of LAMP reaction in *T. urticae* samples. Lane M: DNA size marker; Lane 1: 0 M; Lane 2: 0.5 M; Lane 3: 1 M; Lane 4: 1.5 M; Lane 5: C^{-}

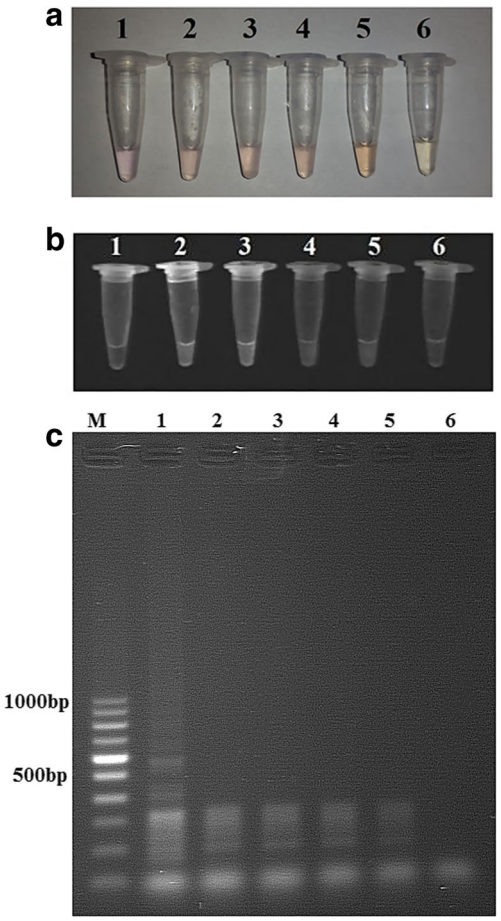

Fig. 5 Calcein concentration test (**a**) under normal light, (**b**) under UV light (black and white image) and (**c**) on electrophoresis gel. Lane M: DNA size marker; Lane 1: 0 μM; Lane 2: 1 μM; Lane 3: 1.5 μM; Lane 4: 2 μM; Lane 5: 4 μM; Lane 6: C^{-}

Sensitivity of PCR-LAMP

Using 1, 3, 6, and 10 *T. urticae* specimens in PCR-LAMP reaction showed that just one specimen resulted in visible ladder-like bands (Fig. 7 lane 2); so, it can be concluded that the method is sensitive enough for detecting just one specimen of *T. urticae*.

Specificity of detection

PCR-LAMP specificity was assessed by using three other *Tetranychus* species (*T. turkestani, T. kanzawai* and *T. neocaledonicus*) in reactions. Results showed that only material obtained from *T. urticae* was specifically detected by the procedure (Fig. 8). In other words, the set of designed primers amplified material from *T. urticae*, but not from the other three control species.

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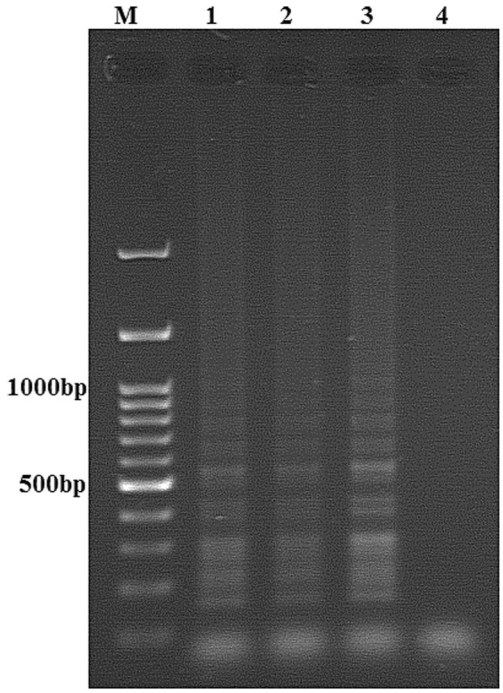

Fig. 6 Detection of different life stages of *T. urticae* samples. Lane M: DNA size marker; Lane 1: egg; Lane 2: larvae; Lane 3: adult; Lane 4: C⁻

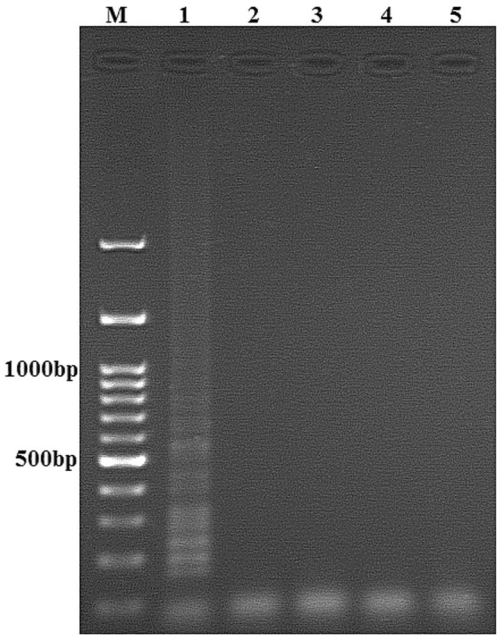

Fig. 8 PCR-LAMP specificity test. Lane M: DNA size marker; Lane 1: *Tetranychus urticae*; Lane 2: *Tetranychus turkestani*; Lane 3: *Tetranychus kanzawai*; Lane 4: *Tetranychus neocaledonicus*; Lane 5: C⁻

In silico testing for specificity

Using the "*In silico* PCR" tool in FastPCR 6.6.35, successful amplification with the primers was evaluated. Using the primers F3 and B3, all *T. urticae* sequences were amplified along with the sequences of: *T. turkestani*; *Tetranychus lambi* Pritchard and Baker, 1955; *Tetranychus ezoensis* Ehara, 1962; *Tetranychus puerariicola* Ehara and Gotoh, 1996; *T. kanzawai*; *Tetranychus parakanzawai* Ehara, 1999 (Online resource 2). In the second step and using the primers F2 and B2, only *T. urticae* sequences were amplified successfully (Online resource 3).

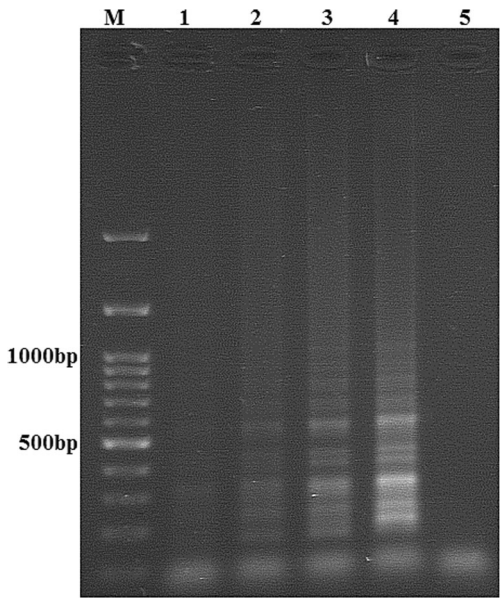

Fig. 7 PCR-LAMP sensitivity test. Lane M: DNA size marker; Lane 1: 1 specimen; Lane 2: 3 specimens; Lane 3: 6 specimens; Lane 4: 10 specimens; Lane 5: C⁻

Discussion

Not only is *T. urticae* a polyphytophagous pest, which could cause significant economic loss to field crops and ornamentals, but it is also detrimental in greenhouses where conditions could be optimal for its rapid multiplication which could lead to substantial devastation to greenhouse crops or ornamentals. The accurate identification of any pest species is considered the first basic step toward implementing any successful management strategies against it. Several molecular methods have already been developed to tackle the problems related to the diagnosis of *Tetranychus* species including PCR-RFLP (Osakabe et al. 2008; Arimoto et al. 2013), DNA sequencing (Matsuda et al. 2012, 2013) and real-time PCR (Li et al. 2015). These methods not only mostly require specialized equipment and well-trained technicians, but are also expensive and time consuming (Hsieh et al. 2012).

The development of a PCR-LAMP assay was described in this paper. The method was found to be sensitive and specific for the detection of *T. urticae*. LAMP, as a superb nucleic acid amplification method, does not require high level of expertise

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and the entire PCR-LAMP procedure described in this study, including DNA template preparation, could be completed in approximately four hours in any moderately equipped molecular laboratory. Moreover, by adding a fluorescent dye, such as calcein, into the reaction mixture, the PCR-LAMP results can be straightforwardly determined visually via unaided eye without requiring time-consuming electrophoretic analysis (Guan et al. 2010; Xu et al. 2013; Li et al. 2014). Although this method is rather similar to the nested PCR, it is more advantageous because of skipping the time consuming secondary PCR and also the possibility of direct visualization of amplified products with naked eye.

In this study, a set of four specifically designed primers targeting and amplifying six regions of the DNA were used for rapid identification of *T. urticae* specimens in all developmental stages. Following the optimization of reagent concentrations and the assay conditions, the efficiency of the method for detecting *T. urticae* was assessed. The optimal reaction time for efficient DNA amplification was determined to be 60 min. Regarding the temperature, it seems that 50, 55 and 60 °C were all optimal amplification temperatures because there were not distinct differences in intensity of ladder-like patterns among these temperatures. The optimal concentration of betaine was 1 M.

The results depicted that LAMP was capable of successful identification of *T. urticae* using material from various developmental stages. Moreover, a minimum number of one mite was sufficient to obtain a visible ladder-like banding pattern on agarose gel. In addition, the specificity of the primers was put to test and as expected, no bands were formed on the electrophoresis gel for any of the non-target examined species. It was also demonstrated that by adding 1 μM of calcein dye, reaction products were distinctly visible under both natural and UV lights.

In conclusion, PCR-LAMP was proven to be a quick and reliable technique in the detection of *T. urticae* material. The method could be a great advantage in greenhouses, fields and quarantine sites. Since a relatively short time is needed for the entire identification process, developing and extending this method to other economic or quarantine main insect pests would be precious, particularly for maintaining pest free areas as well as preventing the introduction and spread of specific pest groups.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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