



# A rapid field-based assay using recombinase polymerase amplification for identification of *Thrips palmi*, a vector of tospoviruses

Priti<sup>1</sup> · Sumit Jangra<sup>1</sup> · V. K. Baranwal<sup>1</sup> · Ralf G. Dietzgen<sup>2</sup> · Amalendu Ghosh<sup>1</sup>

Received: 13 March 2020 / Revised: 11 September 2020 / Accepted: 21 September 2020 / Published online: 8 October 2020  
© Springer-Verlag GmbH Germany, part of Springer Nature 2020

## Abstract

*Thrips palmi* (Thysanoptera: Thripidae) is an important pest of vegetables, ornamentals, and legumes worldwide. Besides damage caused by feeding, it transmits several tospoviruses. Identification of *T. palmi* at an early stage is crucial in implementing appropriate pest management strategies. Morpho-taxonomic identification of *T. palmi* based on the adult stage is time-consuming and needs taxonomic expertise. Here, we report a rapid, on-site, field-based assay for identification of *T. palmi* based on recombinase polymerase amplification (RPA), its first application in insects. RPA primers designed based on 3' polymorphisms of the Internal Transcribed Spacer 2 region efficiently discriminated *T. palmi* without any cross-reactivity to other predominant thrips species. RPA was performed with crude DNA, extracted from single *T. palmi* simply by crushing in sterile distilled water and could be completed within 20 min by holding the reaction tubes in the hand. The assay was further simplified by using fluorescent as well as colorimetric dyes thus eliminating the gel-electrophoresis steps. The presence of *T. palmi* was visualized by a change in color from dark blue to sky blue. The assay was validated with known thrips specimens and found to be effective in diagnosing the presence of *T. palmi* in natural vegetation. This on-site, rapid assay for diagnosis of *T. palmi* can be used by non-expert personnel in the field of quarantine and pest management.

**Keywords** RPA · Melon thrips · Insect vector · Tospovirus transmission · Thrips diagnostics

## Key message

- *Thrips palmi* an important pest of vegetables and ornamentals transmitting several tospoviruses
- Morpho-taxonomic identification stage-specific, time-consuming, and needs taxonomic expertise
- Rapid, on-site, field-based assay for identification of *T. palmi* using recombinase polymerase amplification
- Assay uses crude extract of *T. palmi*, completed within 20 min by holding the reaction tubes in hand without use of sophisticated laboratory instruments
- The presence of *T. palmi* visualized by a change in reaction color
- Useful for non-expert personnel in field-based identification, quarantine, and adopting suitable pest management strategies

Communicated by M. Traugott .

✉ Amalendu Ghosh  
amal4ento@gmail.com

<sup>1</sup> Insect Vector Laboratory, Advanced Centre for Plant Virology, ICAR—Indian Agricultural Research Institute, New Delhi 110012, India

<sup>2</sup> Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St. Lucia, QLD 4072, Australia

## Introduction

Thrips (order Thysanoptera) are important agricultural pests that cause significant losses in yield and quality of agricultural products worldwide. These small insects cause damage to leaves, flowers, and fruits by feeding and sometimes induce galls. Distinctive silvery scarring on the leaves, stems, or fruits is often visible due to their feeding (Trdan et al. 2007; Chisholm and Lewis 2009; Ghosh et al. 2009, 2017). Besides the direct damage, thrips also transmit economically damaging tospoviruses (family *Tospoviridae*, order *Bunyavirales*) (Rotenberg et al.

2015). Thrips-transmitted tospoviruses cause significant economic losses in a large number of plant families (Pappu et al. 2009).

Melon thrips (*Thrips palmi* Karny, Thysanoptera: Thripidae) is a key pest in vegetables, legumes, and ornamentals, transmitting more than 7 different tospoviruses (Rotenberg et al. 2015). The distribution of *T. palmi* was thought to be restricted to Southern Asia, but in recent decades it has been observed throughout Asia. It is considered a predominant tospovirus vector in Asia (Mandal et al. 2012). Groundnut bud necrosis virus is transmitted by *T. palmi* and causes losses of more than US \$89 million per annum across Asia (Reddy et al. 1995; Jagdale and Ghosh 2019, Ghosh et al. 2019). It has widely invaded the Pacific, Florida, the Caribbean, South America, Africa, and Australia (Bhatti 1980; Johnson 1986; Guyot 1988; Pantoja et al. 1988; Cooper 1991; Palmer 1992; Monteiro et al. 1995; EPPO 2014; Jangra et al. 2020a, Ghosh et al. 2020). *T. palmi* and the viruses it transmits are not yet established in Europe and it is listed as a quarantine pest by EPPO (2014). *T. palmi* continues to pose a significant risk to agricultural production and has the potential to cause significant economic losses to a wide range of field- as well as glasshouse-grown crops. Diagnosis of *T. palmi* at an early stage of infestation is therefore necessary for adopting suitable pest management strategies and quarantine measures. Morphological key-based identification of *T. palmi* based on the adult stage is time-consuming and demands taxonomic expertise (Mehle and Trdan 2012). Nucleic acid- and protein-based techniques such as polymerase chain reaction (PCR), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), sequence characterized amplified regions (SCAR) markers, real-time PCR, loop-mediated isothermal amplification (LAMP), and monoclonal antibodies have been used for identification of *T. palmi* (Paran and Micheltore 1993; Banks et al. 1998; Walsh et al. 2005; Yeh et al. 2014; Nakahara and Minoura 2015; Przybylska et al. 2015). PCR-based techniques are not useful for the field-based diagnosis of *T. palmi*. LAMP is simpler than PCR but it requires nucleic acid extraction and higher temperature that may not be feasible at field conditions. Thus, we have developed an on-site, field-based rapid assay for accurate identification of *T. palmi* without the need for sophisticated laboratory equipment, based on recombinase polymerase amplification (RPA). The RPA assay includes three core enzymes viz. recombinase, single-stranded DNA binding protein, and strand-displacing polymerase, and is highly efficient in the diagnosis of zoonotic diseases and plant viruses (Li et al. 2019; Xi et al. 2019; Ma et al. 2019a, b; Cha et al. 2020). This is the first experimental demonstration where RPA has been successfully used for the identification of an insect species.

## Materials and methods

### Establishment of a homogenous population of *T. palmi*

The initial population of *T. palmi* was collected from a stock population maintained at Advanced Centre for Plant Virology, Indian Agricultural Research Institute (IARI), New Delhi. The identity was confirmed by morphometric keys (Bhatti 1980; Cluever and Smith 2017) and sequencing mitochondrial cytochrome oxidase subunit I (mtCoI). To develop a homogenous population, a single adult female of *T. palmi* was released on healthy brinjal (*Solanum melongena* var. Navkiran) plants under controlled conditions at  $28 \pm 1$  °C temperature,  $60 \pm 10\%$  relative humidity, and 8 h dark. Fresh plants were provided in the rearing cages as and when required. Adults were collected from the iso-female population with a fine Camel hairbrush for further experiments.

### DNA extraction from *T. palmi*

Total DNA was extracted from *T. palmi* using a cetyltrimethylammonium bromide (CTAB) method. CTAB extraction buffer was prepared in a total volume of 10 ml by adding 3.5 ml of 10% CTAB, 1 ml of 1 M Tris-HCl, 400 µl of 0.5 M EDTA (pH 8.0), 2.8 ml of 5 M NaCl, 20 µl 2-mercaptoethanol, and 2.28 ml nuclease-free water. A single adult thrips was collected in a 1.5-ml micro-centrifuge tube and crushed in 100 µl of CTAB extraction buffer with a micropestle. The lysate was vortexed and incubated at 65 °C for 30 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added to the lysate, mixed, and centrifuged at  $14,000 \times g$  for 15 min. The upper aqueous phase was transferred to a new micro-centrifuge tube. The DNA was precipitated by adding 0.7 volumes of ice-cold isopropanol and kept at  $-20$  °C for 1 h. The sample was centrifuged at  $14,000 \times g$  for 10 min and the supernatant was decanted gently. The pellet, if any, was washed with 100 µl of 70% ethanol. The ethanol was decanted and residual ethanol was removed by drying at room temperature. The DNA pellet was dissolved in 50 µl sterile distilled water and used for further experiments.

### Designing *T. palmi*-specific primers for RPA

The primers used in this study were designed following TwistAmp® assay design manual guidelines (<https://www.twistdx.co.uk>). Three pairs of RPA primers (Table 1) were designed based on the sequence polymorphism of Internal Transcribed Spacer 2 (ITS2) region of *T. palmi*. All ITS2 sequences of *T. palmi* available at the National Center for Biotechnology Information (NCBI) were utilized

**Table 1** List of RPA primers used for amplification of ITS2 region of *T. palmi*

Forward primer		Reverse primer		Observed amplicon size (bp)	Annealing temperature
Name	Sequence (5'→3')	Name	Sequence (5'→3')		
AG113F	AAAACGGGGAAAGGCCTTGCAAAG GAAAAATC	AG114R	GAATCGGAGCGAGGAGGCATTAA GAGATCCAC	280	72 °C
AG115F	TTGCGATGTGTCTCTGCACGCGCC CATCG	AG116R	CGACTACAGAGAACGGGAAACACA CAACGG	200	67.4 °C
AG117F	CTGGACTTGCTCTCACGGGCGAGTCCT CCC	AG118R	AAGCGGAAATCCACTCTCGCGTGC ACGG	290	75 °C

for primer-design. The conserved region of *T. palmi* ITS2 was chosen to cover all known intra-specific variants of *T. palmi*. Sites with a mismatch at 3'-end sequences among the congeneric thrips species were targeted to design the species-specific primers. The major characteristics such as primer length, amplicon size, GC contents, and intra-primer or inter-primer homology were taken into consideration. The site-specificity of the primers was verified by Primer-BLAST (<https://www.ncbi.nlm.nih.gov>). FastPCR (version 6.6.99) was done with available ITS2 sequences of thrips species in NCBI to analyze the in-silico specificity and cross-reactivity of the primers.

### Conventional PCR with *T. palmi* RPA primers

Conventional PCR was carried out in a 25 µl reaction mixture to validate the newly designed RPA primers. Each reaction contained 1 µl of 10 mM each forward and reverse primers (Eurofins), 260 µM dNTP mix (Thermo Fisher Scientific), 2.5 µl of 10×PCR buffer (Thermo Fisher Scientific), 20 ng DNA template, and 2U DreamTaq DNA polymerase (Thermo Fisher Scientific). PCR was done in a T100 Thermal Cycler (Bio-Rad) with one cycle at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, annealing at 63 °C–78 °C depending upon the primer pairs for 50 s, 72 °C for 50 s, and a final extension at 72 °C for 10 min. PCR products were resolved on 2% agarose gel stained with GoodView™ (BR Biochem) and visualized in a gel documentation system (MasteroGen Inc, Taiwan) with 100 bp Plus DNA ladder (Thermo Fisher Scientific).

### Recombinase polymerase amplification

RPA was done using newly designed primer pairs at a temperature range of 35–41 °C. The assay was carried out in a reaction volume of 25 µl following the protocol as outlined in the TwistAmp® Basic kit (TwistDx, Cambridge, UK) with slight modifications. The reaction mixture of 45.5 µl was prepared by adding 2.5 µl of each forward and reverse primers (10 mM), 29.5 µl rehydration buffer, and 11 µl nuclease-free water. The contents were mixed with

the freeze-dried reaction provided in TwistAmp Basic and then distributed into 2 reaction tubes. Next, 1 µl of *T. palmi* DNA template (~20 ng), and 1.25 µl of 280 mM magnesium acetate was added to the reaction and mixed gently by pipetting. The reaction mix was then incubated at a temperature gradient of 35 °C–41 °C in a heating block for 20 min. The amplified products were resolved on 2% agarose gel stained with GoodView™ and visualized in a gel documentation system with 100 bp Plus DNA ladder.

### Cross-reactivity assay for RPA primers

The specificity of the RPA primer pairs for *T. palmi* and potential cross-reactivity with other congeneric thrips species were assessed by PCR. PCR was done using primer pairs AG113F-AG114R, AG115F-AG116R, and AG117F-AG118R separately with DNA templates of *T. palmi*, *T. tabaci* Lindeman, and *Scirtothrips dorsalis* Hood. PCR was done in 25 µl reaction volumes and amplicons were resolved by 2% agarose gel electrophoresis as described above.

Cross-reactivity of the primer pairs was also assessed by RPA in 25 µl reaction volumes at 37 °C and products were resolved on 2% agarose gel as described above. Primer pairs that showed any amplification with DNA templates of other thrips species were not considered further. The primer pair that was not cross-reactive to other predominant thrips species in both PCR and RPA, and that resolved well on agarose gels, was progressed further to develop a standardized field-based assay.

### On-site crude DNA extraction

To develop an on-site rapid DNA extraction protocol, different methods for crude DNA extraction from single *T. palmi* were evaluated. Crude DNA was extracted by crushing single *T. palmi* adult in ethylenediaminetetraacetic acid (EDTA), phosphate-buffered saline (PBS), or sterile distilled water. A single *T. palmi* adult was transferred into a 1.5-ml micro-centrifuge tube with the help of a fine Camel hair-brush. Thirty µl of 0.02 M EDTA (pH 8.0) was added and the sample was crushed within the micro-centrifuge tube

using a sterile micropestle. Similarly, samples were crushed in PBS (pH 7.4) or sterile distilled water. Tubes were incubated in boiling water (100 °C) for 10 min. The quality and quantity of crude DNA extracted through these methods were determined by spectrophotometer (NanoDrop™ One, Thermo Fisher Scientific). Further, PCR was done using the DNA of *T. palmi* extracted through these methods as templates. Twenty-five µl of each PCR mixture contained 1 µl of 10 mM each forward and reverse primer (AG117F-AG118R), 260 µM dNTP mix, 2.5 µl of 10×PCR buffer, 20 ng DNA template, and 2U DreamTaq DNA polymerase. Thermal cycling was done in a T100 Thermal Cycler with one cycle of 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 75 °C for 50 s, 72 °C for 50 s, and a final extension at 72 °C for 10 min. PCR products were resolved on 2% agarose gel as described above. The crude extract that provided the best PCR amplification comparable to CTAB-extracted DNA was used in RPA assay to identify *T. palmi*.

### Sensitivity of RPA assay

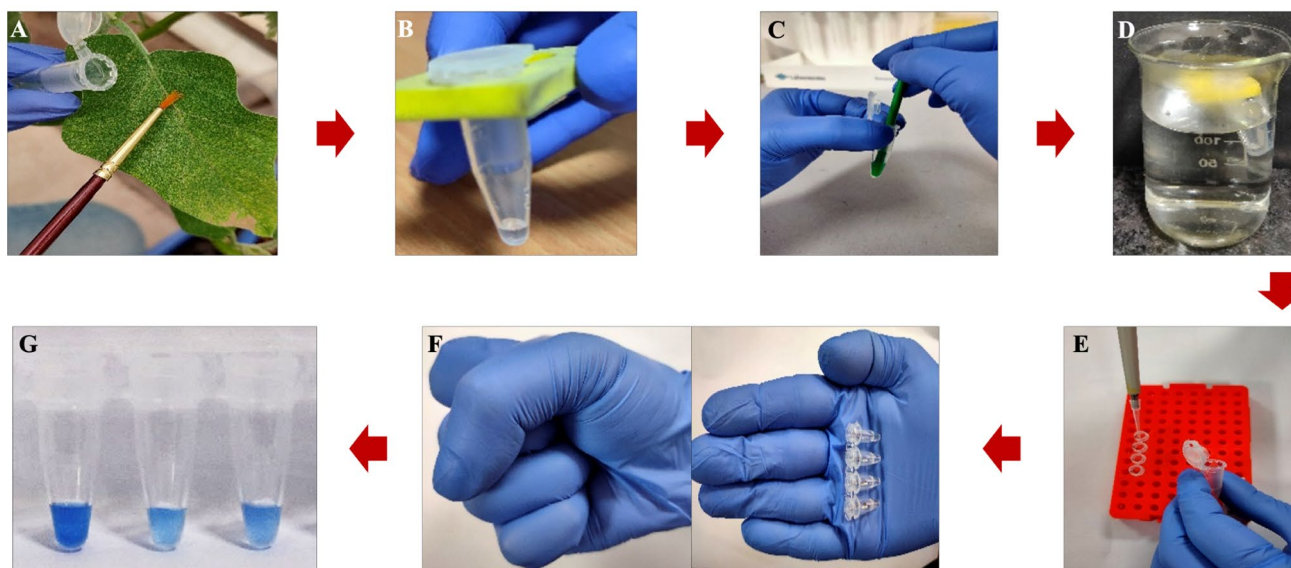
The sensitivity of RPA was determined with primer pair AG117F-AG118R using ten-fold serially diluted ( $2 \times 10^2$ – $2 \times 10^{-12}$  ng) crude DNA of *T. palmi*. RPA was done as described above. Based on the results of RPA using a temperature gradient, the reaction was incubated at 37 °C. The amplified products were resolved on 2% agarose gel as described above. The sensitivity of RPA using crude DNA extracts of *T. palmi* was compared with that of PCR using

serially-diluted crude DNA of *T. palmi* in 25 µl reaction volume and resolved on 2% agarose gel as described above.

### Field-based end-point detection of RPA

To eliminate the gel electrophoresis step used to visualize RPA results in the laboratory, different fluorescence, and colorimetric methods were evaluated. RPA was done as described above using crude DNA of *T. palmi* as positive control along with a non-template control. The reaction was incubated at body temperature (~37 °C) by holding the tubes in the hand (Fig. 1). The fluorescent nucleic acid dye SYBR Green I (Thermo Fisher Scientific) was added after completion of reaction at 0.1 µl per tube. Keeping in mind the cost of SYBR Green dye, the RPA assay was also evaluated by adding 0.5 µl of GoodView™ stain after completion of the reaction. The appearance of fluorescence under UV light was noted to determine the presence of *T. palmi*.

Both these fluorescence-based end-point detection assays required use of a UV torch or transilluminator which may not be feasible in the field. Therefore, we used a colorimetric detection of RPA using hydroxynaphthol blue (HNB, Sisco Research Laboratories Pvt. Ltd. India) dye. RPA reaction was done in 25 µl reaction volume as described above and 1 µl of 3 mM HNB was added before amplification and mixed well by pipetting. The reaction was incubated at 37 °C by holding the tubes in hand. A change in color was seen after 20 min of incubation indicative of the presence of *T. palmi*.



**Fig. 1** Workflow of the RPA assay for on-site rapid identification of *T. palmi*. **a** Collection of single thrips into a micro-centrifuge tube, **b** addition of 30 µl sterile distilled water, **c** manually crushing of thrips with a micropestle, **d** placing in boiling water for 10 min, **e** addition

of 1 µl extract to 25 µl TwistAmp® basic reaction mixture in mini strip tubes, **f** holding the reaction tubes in fist for 20 min, **g** visual assessment of results. Change in reaction color from dark blue to sky blue indicates presence of *T. palmi*



## Validation of RPA assay

The standardized RPA described above was validated with a large number of known thrips specimens. Further, the assay was used to identify *T. palmi* in natural vegetation at IARI experimental farm. Thrips DNA from unknown specimens was extracted in sterile distilled water on-site. The RPA reaction was carried out using HNB by holding the tubes in hand for 20 min. The presence of *T. palmi* was identified based on the change in reaction mix color from dark blue (negative) to sky blue (positive). MtCOI region of representative positive and negative samples was amplified by PCR with primer pair LCO-1490, and HCO-2198 (Folmer et al. 1994) and directly sequenced for further confirmation.

## Results

### Standardization of primers and RPA conditions

Three primer pairs viz. AG113F-AG114R, AG115F-AG116R, and AG117F-AG118R were designed based on sequence polymorphisms of the ITS2 region; primers were 30–35 nucleotides long (Table 1). The GC content in all these primers was moderate (43.75–66.67%). The primer pairs showed a low probability to form secondary structures and primer-primer interactions or hairpins in Primer-BLAST analysis. Primer-BLAST also ensured the specificity of the primers for ITS2 region of *T. palmi* and could amplify the intra-specific variants of *T. palmi*. The output of FastPCR with the primer pairs AG113F-AG114R, AG115F-AG116R, and AG117F-AG118R showed amplicons of calculated sizes 283 bp, 209 bp, and 282 bp for *T. palmi* at a predicted annealing temperature of 70 °C, 70 °C, and 72 °C, respectively, without any cross-reactivity to other congeneric thrips species.

In gradient PCR, all primer pairs amplified products of ITS2 of *T. palmi*. Sharp bands of ~280 bp and 200 bp were obtained in the PCR with primer pairs AG113F-AG114R and AG115F-AG116R at annealing temperatures of 73 °C and 67.4 °C, respectively. The primer pair AG117F-AG118R showed the strongest amplification of 290 bp at 75 °C annealing temperature.

RPA assay using the three primer pairs showed amplification of about 280 bp, 200 bp, and 290 bp at a temperature range of 35 °C–41 °C. Although amplification was satisfactory across this temperature range, incubation at 37 °C was considered best to proceed with the field-based assay. As the normal human body temperature is about 37 °C, standardizing the RPA assay at this temperature enabled performing the reaction by holding the tubes in one's hand and eliminated the requirement of a heating block.

### Cross-reactivity of RPA primers with other predominant thrips species

In PCR assay, primer pair AG113F-AG114R showed cross-reactivity with *S. dorsalis*. An amplicon of ~280 bp was observed with both *T. palmi* and *S. dorsalis* DNA templates. PCR with primer pair AG115F-AG116R using DNA templates of *T. palmi*, *T. tabaci*, and *S. dorsalis* did not show any amplification for *S. dorsalis* but amplified a product of ~200 bp from both *T. palmi* and *T. tabaci* (data not shown). However, primer pair AG117F-AG118R amplified a product of ~290 bp from DNA template of *T. palmi* only and no amplification occurred from DNA templates of other thrips species tested that are predominant in India (Fig. 2a). The results of the RPA assay with primer pairs AG113F-AG114R and AG115F-AG116R mirrored those of the PCR assay. On the other hand, primer pair AG117F-AG118R did not show any cross-reactivity with DNA of other thrips species in RPA and yielded an amplicon of ~290 bp for *T. palmi* template only (Fig. 2b). Since the primer pair AG117F-AG118R was specific for *T. palmi* and did not yield an amplicon from congeneric thrips species, it was considered useful for on-site detection of *T. palmi* based on RPA.

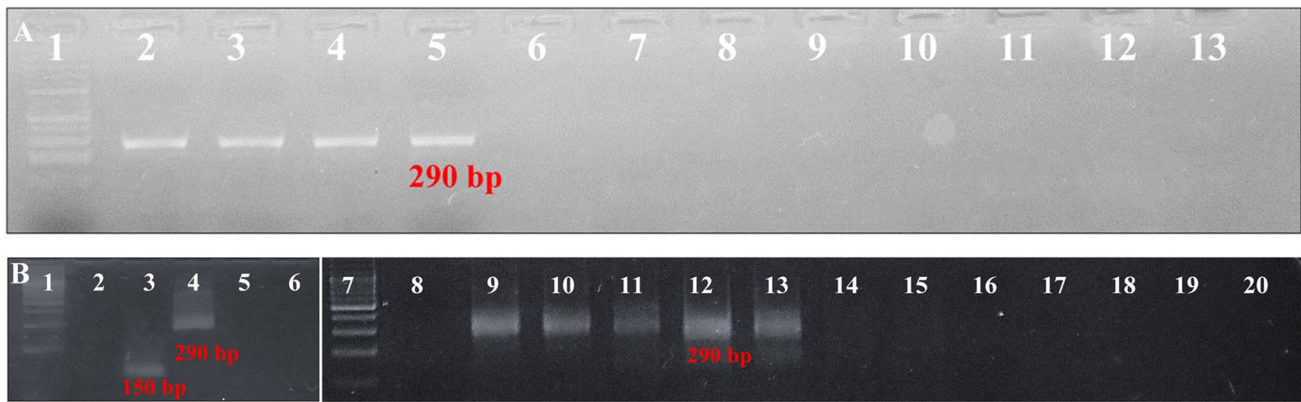
### On-site crude DNA preparation from *T. palmi*

The on-site crude DNA extraction minimized processing times and did not require sophisticated laboratory equipment otherwise essential for DNA extraction using the CTAB method. The maximum amount of total DNA from a single adult of *T. palmi* extracted in PBS was approximately 1100 ( $\pm$  120) ng. About 700 ( $\pm$  110) ng and 500 ( $\pm$  30) ng crude DNA were extracted in distilled water and EDTA, respectively. The mean absorbance 260/280 values were recorded as 1.3, 1.4, and 1.9 for the DNA extracted in PBS, distilled water, and EDTA, respectively. A 260/230 ratio of the extracted DNA extracted in EDTA (0.06) indicated the presence of contaminants.

PCR with primer pair AG117F-AG118R produced clear and sharp bands of 290 bp in 2% agarose gel for the DNA templates extracted in distilled water and PBS (Fig. 3). The quality of PCR amplification using DNA extracted in distilled water and PBS was best and comparable to PCR amplification using DNA extracted by the CTAB method. No amplicons were observed following PCR with thrips DNA extracted in EDTA. DNA extraction using distilled water did not require any buffer preparation and was therefore considered easiest for RPA assays in the field.

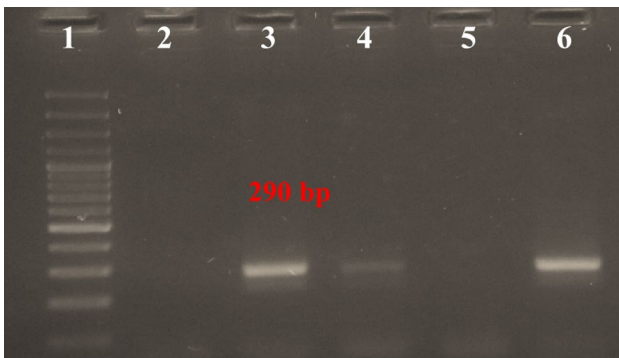
### Sensitivity of RPA assay

To determine the assay sensitivity using primer pair AG117F-AG118R, RPA was applied to a serial dilution



**Fig. 2** Assessment of potential cross-reactivity of *T. palmi*-specific primer pair AG117F and AG118R. **a** 2% agarose gel electrophoresis to resolve PCR amplicons. Lane 1: 100 bp plus DNA ladder; Lanes 2–12: PCR amplicons using DNA extracts of *T. palmi* (2–5), *T. tabaci* (6–9), and *S. dorsalis* (10–12); Lane 13: no-template control. PCR yielded a single band of 290 bp for *T. palmi* only. **b** 2% agarose gel electrophoresis showing RPA results. Lane 1 and 7: 100 bp plus DNA

ladder, Lane 2 and 8: no-template control, Lane 3: positive control provided with TwistAmp® Basic kit, Lanes 4–6 and 9–20: RPA products using DNA extracts of *T. palmi* (4, 9–13), *S. dorsalis* (5, 18–20), and *T. tabaci* (6, 14–17). RPA yielded a single 290 bp band for *T. palmi*. No amplification was observed with *T. tabaci* and *S. dorsalis* templates. The positive kit control yielded ~150 bp amplicon



**Fig. 3** PCR with DNA templates of *T. palmi* extracted by different methods. Lane 1: 100 bp plus DNA ladder, Lane 2: no-template control, Lane 3–6: PCR amplicons with *T. palmi* template extracted in sterile distilled water (3), PBS (4), EDTA (5), and CTAB (6). PCR with template extracted in EDTA did not yield any amplicons. Single amplicons of 290 bp were observed following PCR using thrips ground in sterile distilled water, PBS and CTAB

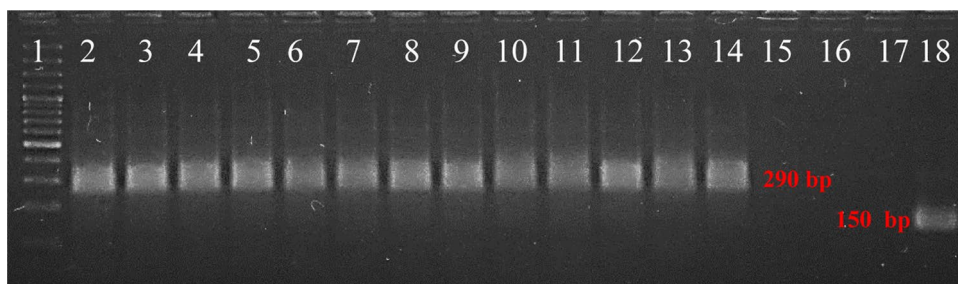
series of *T. palmi* DNA extracted in distilled water. Amplicons of ~290 bp were seen by gel electrophoresis following RPA of *T. palmi* input DNA of between 200 ng and  $2 \times 10^{-10}$  ng (Fig. 4). PCR with primer pair AG117F-AG118R amplified the corresponding product up to  $2 \times 10^{-1}$  ng of DNA template extracted in distilled water. This shows that the RPA assay for identification of *T. palmi* was  $10^9$  times more sensitive than PCR and could detect as little as 0.2 ag ( $2 \times 10^{-19}$  g) of DNA.

### Field-based end-point detection RPA assay

To use the RPA assay in an on-site field situation, agarose gel electrophoresis was replaced by fluorescent and colorimetric dyes. RPA assay showed distinct fluorescence by UV illumination in the reaction tubes in positive samples (*T. palmi* DNA) when SYBR Green I was added, whereas no fluorescence was observed in negative samples (Fig. 5a). Addition of GoodView™ into the reaction mix after RPA also showed fluorescence in the samples that contained *T. palmi* DNA, whereas no fluorescence was observed in the negative samples under a UV light (Fig. 5b). The RPA assay with HNB dye changed color from dark blue (negative) to sky blue (positive) for samples that contained *T. palmi* DNA (Fig. 5c), whereas no such color change was observed in negative samples.

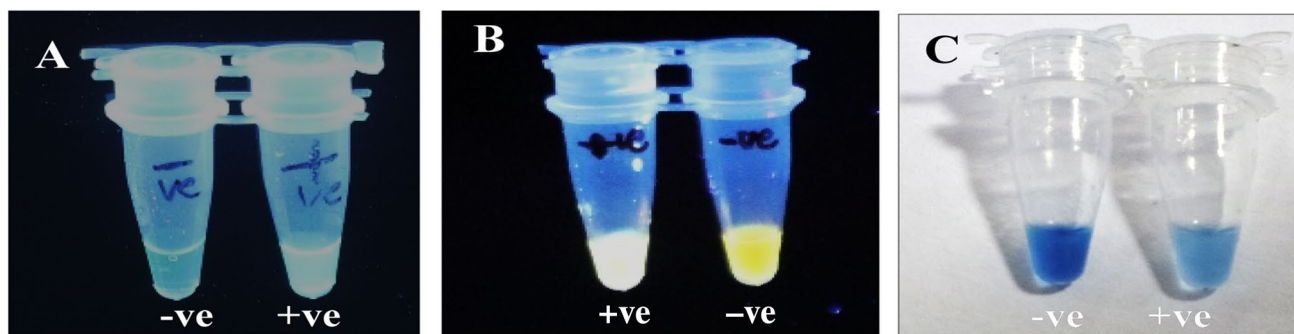
### Identification of thrips from natural vegetation

The RPA assay was validated with more than 60 specimens (~30 *T. palmi*, ~15 each of *T. tabaci*, and *S. dorsalis*) of known thrips. The results confirmed the test–retest reliability and reproducibility of the assay (Fig. 6). RPA was then used to identify *T. palmi* from natural vegetation by extracting DNA in distilled water on-site and adding HNB to the reaction mix. In more than 30 collections from natural vegetation, *T. palmi* was identified in 17 samples collected from cucumber, brinjal, cotton, lettuce, soybean, mungbean, and sponge gourd plants. A change in reaction mix color from dark blue to sky blue after holding in one's hand for 20 min indicated the presence of *T. palmi* in the test samples (Fig. 7). The reactions with thrips samples collected



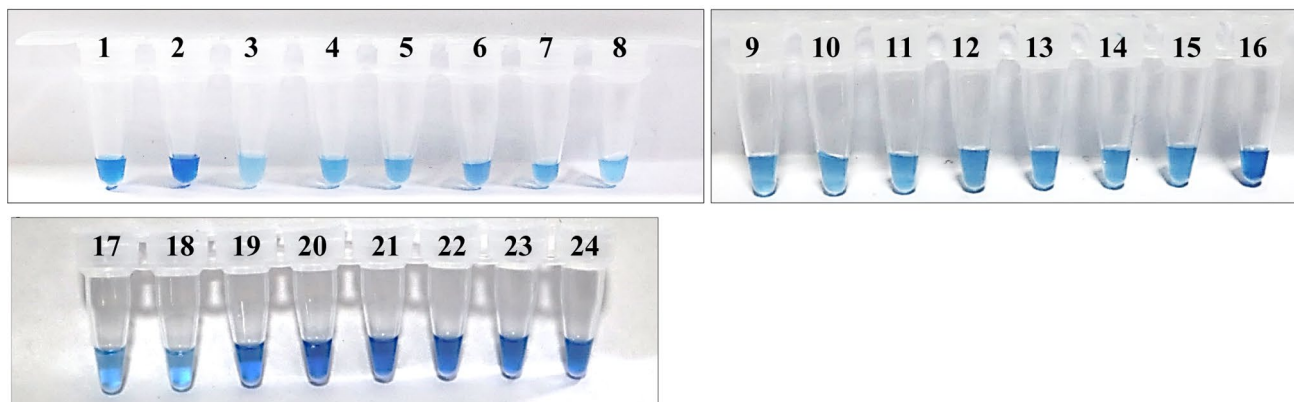
**Fig. 4** Sensitivity of the RPA assay using primer pair AG117F and AG118R. Serially ten-fold diluted *T. palmi* DNA extracted in sterile distilled water was used as template and products resolved on 2% agarose gel. Lane 1: 100 bp plus DNA ladder, Lanes 2–16: RPA product with *T. palmi* template of  $2 \times 10^2$  ng (2),  $2 \times 10^1$  ng (3), 2 ng

(4),  $2 \times 10^{-1}$  ng (5),  $2 \times 10^{-2}$  ng (6),  $2 \times 10^{-3}$  ng (7),  $2 \times 10^{-4}$  ng (8),  $2 \times 10^{-5}$  ng (9),  $2 \times 10^{-6}$  ng (10),  $2 \times 10^{-7}$  ng (11),  $2 \times 10^{-8}$  ng (12),  $2 \times 10^{-9}$  ng (13),  $2 \times 10^{-10}$  ng (14),  $2 \times 10^{-11}$  ng (15),  $2 \times 10^{-12}$  ng (16), Lane 17: no-template control, Lane 18: 150 bp product positive kit control



**Fig. 5** Visualization of RPA products using **a.** SYBR Green I, **b.** GoodView™, and **c.** Hydroxynaphthol blue (HNB). RPA was done using crude DNA of *T. palmi* as positive (+) and water as negative (–) samples in 25  $\mu$ l reactions. Addition of SYBR Green and GoodView™ at 0.1 and 0.5  $\mu$ l, respectively, after completion of the reactions showed fluorescence under UV illumination only in the posi-

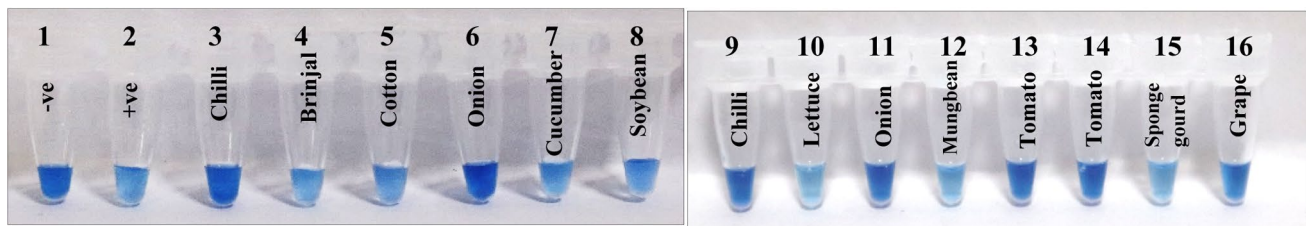
tive sample containing *T. palmi* template while no fluorescence was observed in the negative samples. The RPA mixed with 1  $\mu$ l of 3 mM HNB added prior to amplification showed a change in color from dark blue to sky blue in the case of *T. palmi* DNA, whereas no corresponding change was observed in the negative sample



**Fig. 6** RPA assay for field-based identification of *T. palmi*. RPA was done using primer pair AG117F and AG118R with extracts of known thrips species. Hydroxynaphthol blue was added prior to amplification. Tube 1, 19–21: *T. tabaci*; Tube 2, 22–24: *S. dorsalis*; Tube 3–15,

17–18: *T. palmi*, Tube 16: no-template control. RPA clearly discriminated *T. palmi* from other tested thrips species by a change in reaction color from dark blue to sky blue





**Fig. 7** Identification of *T. palmi* collected from different crop species using the RPA assay. DNA was extracted in sterile distilled water on-site. RPA assay was performed with primer pair AG117F and AG118R. 1: no-template control, 2: *T. palmi* DNA positive control. 3–8: unknown thrips collected from chilli (3, 9), brinjal (4), cotton (5), onion (6, 11), cucumber (7), soybean (8), lettuce (10), mungbean (12), tomato (13, 14), sponge gourd (15), and grape (16). The RPA

from chilli, onion, tomato, and grape did not show any color change indicating that the collected thrips were not *T. palmi*. The sequences of mtCOI further confirmed the identity of the positive samples as *T. palmi* and negative samples as *T. tabaci* (four samples), *S. dorsalis* (six samples), and *F. schultzei* (three samples). The sequences are available in NCBI with the accession numbers given in the data availability section.

## Discussion

RPA is an isothermal alternative to PCR. RPA assay utilizes three components viz. recombinase, single-strand DNA binding protein, and a strand-displacing polymerase. Recombinase forms a complex with the RPA primers and assists in binding the homologous sequence of DNA. Single strand binding protein binds to a displaced strand and stabilizes the subsequently formed D loop of the duplex. Strand-displacing polymerase begins the extension from the primers only if the target sequence is present. This technique has been successfully used for diagnosis of animal and human diseases including porcine coronavirus in piglets, *Pasteurella multocida* in cattle, *Schistosoma haematobium* in humans, and zoonotic diseases caused by *Mycobacterium avium* subsp. *paratuberculosis* (Rosser et al. 2015; Hansen et al. 2016; Zhao et al. 2019; Ma et al. 2019a, b). RPA has also been used in the diagnosis of plant pathogens including banana bunchy top virus, little cherry virus 2, and citrus greening bacterium (Mekuria et al. 2014; Londono et al. 2016; Kapoor et al. 2017; Ghosh et al. 2018). However, RPA has not been exploited so far to identify any insect species. This is the first report of a field-based assay for the detection of *T. palmi* based on RPA.

*Thrips palmi* is a major insect pest of vegetable and ornamental crops worldwide and a major vector for transmitting several tospoviruses (Ghosh et al. 2019; Jangra et al. 2020a). It is a predominant tospovirus vector in Asia and

also invaded Africa, America, and Australia (Houston et al. 1991; Cermeli and Montagne 1993; Murai 2002; Yadav and Chang 2012; Seal et al. 2013). *T. palmi* was detected in glasshouses in the Netherlands in 1988 and 1992 (Mound and Gillespie 1997) and subsequently eradicated by destroying all affected plants (Cannon et al. 2007). *T. palmi* is on the EPPO A1 list and regulated as a quarantine pest in the European Union (EPPO 2015). Rapid identification of *T. palmi* is crucial in pest and disease management and quarantine. Several methods have been reported to date for the molecular identification of *T. palmi* including PCR, RFLP, SCAR marker, multiplex PCR, real-time PCR, LAMP, and monoclonal antibodies (Paran and Micheltore 1993; Banks et al. 1998; Walsh et al. 2005; Yeh et al. 2014; Nakahara and Minoura 2015; Przybylska et al. 2015, 2017; Jangra et al. 2020b). Most of these techniques cannot be done in a farmer's field without access to a laboratory. LAMP can be done in the field but it has limitations for complex primer designing and by non-specific amplification with crude extract. Moreover, LAMP requires either a real-time PCR platform or a heating block for detection, and incubation is required at a higher temperature than that feasible on-site. In the present report, an RPA assay has been standardized for field-based identification of *T. palmi*—a first for detection of an insect. The assay does not require any sophisticated laboratory equipment and can be completed in 20 min by holding the reaction tubes in one's hand. A LAMP assay reported for identification of *T. palmi* took at least 30 min to complete at 63 °C incubation and analyzed in a real-time PCR machine and/or visualized under UV light (Przybylska et al. 2015). Using RPA, the presence of *T. palmi* can be quickly determined by the naked eye based on the change in reaction color from dark blue to sky blue.

assay showed the change in color from dark blue to sky blue with DNA of thrips collected from brinjal, cotton, cucumber, soybean, lettuce, mungbean, and sponge gourd plants indicating infestation with *T. palmi*. No change in color with DNA of thrips collected from chilli, onion, tomato, and grape plants confirmed that different thrips species were present

Out of three primer pairs designed for the assay based on 3' polymorphism at ITS2 region of *T. palmi*, only one pair (AG117F-AG118R) was shown to be specific to *T. palmi* without cross-reactivity to congeneric thrips species. ITS region refers to the chromosomal spacer DNA situated



between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes. This hypervariable region has been preferred by several workers for discrimination of thrips at the species level because of a larger interspecific genetic distance than mtCoI (Glover et al. 2010; Przybylska et al. 2015, 2017; Ghosh et al. 2020). Recent reports of several variants of mtCoI in *T. palmi* (Iftikhar et al. 2016; Tyagi et al. 2017; Ghosh et al. 2020) were another reason to choose ITS2 over mtCoI in designing the RPA primers.

To use the RPA assay in the farmer's field, an on-site crude DNA extraction protocol from single adult thrips was standardized. Crude DNA was extracted from single thrips by simply crushing in distilled water. This method minimized the time required for insect DNA extraction and could be easily done in the field. The RPA assay reported here was sensitive enough to amplify as little as 0.2 ag of template DNA,  $10^9$ -fold more sensitive than PCR using the same primers. The previously reported sensitivity of real-time PCR for discrimination of *T. palmi* was 1 pg (Przybylska et al. 2017). The detection threshold for LAMP assay was  $2 \times 10^{-10}$  parts of an adult *T. palmi* (Przybylska et al. 2015), whereas the RPA assay could detect as low as  $2.9 \times 10^{-13}$  parts of an adult considering 700 ng total DNA extracted from a single adult *T. palmi*. In summary, the RPA assay developed here is  $2 \times 10^7$ -fold and  $> 10^3$ -fold more sensitive than real-time PCR and LAMP, respectively.

The RPA assay was further simplified by using a colorimetric dye to facilitate reaction end-point detection in the field. This negated the requirement of gel electrophoresis. HNB is a metal ion indicator, reported to be useful as a colorimetric indicator for  $Mg^{2+}$  ions (Ito and Ueno 1970). It has been successfully used in LAMP reactions (Goto et al. 2009). HNB was included in the present assay as a novel, inexpensive indicator of the RPA reaction result. The color change from dark blue (negative) to sky blue (positive) is induced by the chelation of  $Mg^{2+}$  ions by dNTPs (Gelfand 1989). The RPA assay using HNB has several advantages over other diagnostic assays including that opening the reaction tubes was not required to read the results, and that results could be easily judged by the naked eye dispensing the use of a UV torch. The on-site assay has been validated and used to identify *T. palmi* from natural vegetation. Crude DNA extractions could be completed within 15 min, preparing the RPA reaction mixture took less than 5 min followed by 20 min incubation in the fist. The whole process was completed in 40 min. Hence, this on-site, rapid, and sensitive method for field-based identification of *T. palmi* can be performed by non-expert personnel without any sophisticated laboratory equipment and will be helpful in quarantine and adopting suitable pest management strategies quickly.

## Author contributions

AG conceived and designed research. PS designed, validated primers, standardized RPA assay. SJ performed cross-reactivity assay, validated the RPA assay, tested field samples. VKB, RGD suggested improvements in the methods. AG analyzed data. PS and SJ wrote the draft manuscript. VKB, RGD critically reviewed the manuscript. AG wrote and edited the final manuscript. RGD redrafted the manuscript, assisted with English language editing. All authors read and approved the manuscript.

**Acknowledgements** The authors thank Dr. Sunil Kumar Mukherjee (IARI) for providing critical suggestions during the study. The support received from IARI is thankfully acknowledged. This research was also jointly supported by the Queensland Department of Agriculture and Fisheries and the University of Queensland through the Queensland Alliance for Agriculture and Food Innovation.

**Funding** IARI, Department of Biotechnology (BT/PR26136/AGIII/103/1005/2018), and Science and Engineering Research Board (EMR/2017/000590).

## Compliance with ethical standards

**Conflict of interests** The authors declare that they have no conflict of interests.

**Ethical approval and consent to participate** The samples were collected by the authors from the experimental farm of IARI, New Delhi with permission.

**Availability of data and material** The datasets generated and/or analyzed during the current study are available in NCBI database with accession numbers MT984265, MT984266, MT984268, MT984269, MT991561, MT992018, MT992019, MT992026, MT992047, MW020346, MW020349, MW020350, and MW020351.

## References

- Banks JN, Collins DW, Rizvi RH, Northway BJ, Danks C (1998) Production and characterization of monoclonal antibodies against the EU-listed pest *Thrips palmi*. Food Agric Immunol 10:281–290
- Bhatti JS (1980) Species of the genus Thrips from India (Thysanoptera). Syst Entomol 5(2):109–166
- Cannon R, Matthews L, Collins D (2007) A review of the pest status and control options for Thrips palmi. Crop Prot 26:1089–1098
- Cermeli M, Montagne A (1993) Present situation of *Thrips palmi* Karny (Thysanoptera: Thripidae) in Venezuela. Manejo Integrado de plagas 28:22–23
- Cha D, Kim D, Choi W, Park S, Han H (2020) Point of care diagnostic (POCD) method for detecting *Bursaphelenchus xylophilus* in pinewood using recombinase polymerase amplification (RPA) with the portable optical isothermal device (POID). PLoS ONE 15(1):e0227476
- Chisholm IF, Lewis T (2009) A new look at thrips (Thysanoptera) mouthparts, their action and effects of feeding on plant tissue. Bull Entomol Res 74:663–675

- Cluever JD, Smith HA (2017) A photo-based key of thrips (Thysanoptera) associated with horticultural crops in Florida. *Fla Entomol* 100:454–467
- Cooper B (1991) Infestation of the southern yellow thrips *Thrips palmi*, in vegetables. *J Agric Soc Trinidad Tobago* 88:37–38
- EPPO (2014) PQR database. Paris, France: European and Mediterranean plant protection organization. Available online at <https://www.eppo.int/DATABASES/pqr/pqr.htm>
- EPPO (2015) A2 list of pests recommended for regulation as quarantine pests EPPO (version 2015–09) Available online at <https://www.eppo.org/QUARANTINE/listA2.html>
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol* 3:294–299
- Gelfand DH (1989) Taq DNA polymerase. In: Erlich HA (ed) *PCR technology: principles and applications for DNA amplification*. Stockton Press, New York, pp 17–22
- Ghosh A, Chatterjee ML, Chakraborti K, Samanta A (2009) Field evaluation of insecticides against *chilli thrips* (*Scirtothrips dorsalis* Hood). *Ann Plant Prot Sci* 17:69–71
- Ghosh A, Dey D, Timmanna B, Mandal B, Jain RK (2017) Thrips as the vectors of tospoviruses in Indian agriculture. In: Mandal B, Rao GP, Baranwal VK, Jain RK (eds) *A century of plant virology in India*. Springer, Singapore, pp 537–556
- Ghosh A, Basavaraj YB, Jangra S, Das A (2019) Exposure to watermelon bud necrosis virus and groundnut bud necrosis virus alters the life history traits of their vector, *Thrips palmi* (Thysanoptera: Thripidae). *Arch Virol* 164(11):2799–2804
- Ghosh A, Jagdale SS, Basavaraj, Dietzgen RG, Jain RK (2020) Genetics of *Thrips palmi* (Thysanoptera: Thripidae). *J Pest Sci* 93:27–39
- Ghosh DK, Kokane SB, Kokane AD, Warghane AJ, Motghare MR, Bhose S et al (2018) Development of a recombinase polymerase based isothermal amplification combined with lateral flow assay (HLB-RPA-LFA) for rapid detection of *Candidatus Liberibacter asiaticus*. *PLoS ONE* 13(12):e0208530
- Glover RH, Collins DW, Walsh K, Boonham N (2010) Assessment of loci for DNA barcoding in the genus *Thrips* (Thysanoptera: Thripidae). *Mol Ecol Resour* 10:51–59
- Goto M, Honda E, Ogura A, Nomoto A, Hanaki K (2009) Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. *Biotechniques* 46:167–172
- Guyot J (1988) Review and first observations in Guadeloupe on *Thrips palmi* Karny. *Agronomie* 8(7):565–575
- Hansen S, SchaEfer J, Fechner K, Czerny C-P, Wahed AAE (2016) Development of a recombinase polymerase amplification assay for rapid detection of the *Mycobacterium avium* subsp. *Paratuberculosis*. *PLoS ONE* 11(12):e0168733
- Houston K, Mound L, Palmer J (1991) Two pest thrips (Thysanoptera) new to Australia, with notes on the distribution and structural variation of other species. *Aust J Entomol* 30:231–232
- Iftikhar R, Ashfaq M, Rasool A, Hebert PDN (2016) DNA barcode analysis of thrips (Thysanoptera) diversity in Pakistan reveals cryptic species complexes. *PLoS ONE* 11:e0146014
- Ito A, Ueno K (1970) Successive chelatometric titration of calcium and magnesium using hydroxy naphthol blue (HNB) indicator. *Jpn Anal* 19:393–397
- Jagdale SS, Ghosh A (2019) In silico analyses of molecular interactions between groundnut bud necrosis virus and its vector, *Thrips palmi*. *Virus Dis* 30(2):245–251
- Jangra S, Dhall H, Aggarwal S, Mandal B, Jain RK, Ghosh A (2020a) An observation on the embryonic development in *Thrips palmi* (Thysanoptera: Thripidae) eggs obtained by an artificial oviposition setup. *J Asia-Pac Entomol* 23(2):492–497
- Jangra S, Mittal A, Dhall H, Jain RK, Ghosh A (2020b) A multiplex PCR assay for rapid identification of major tospovirus vectors reported in India. *BMC Genom* 21:170
- Johnson MW (1986) Population trends of a newly introduced species, *Thrips palmi* (Thysanoptera: Thripidae), on commercial watermelon plantings in Hawaii. *J Econ Entomol* 79(3):718–720
- Kapoor R, Srivastva N, Kumari S, Sarithal RK, Sharma SK, Jain RK, Baranwal VK (2017) Development of a recombinase polymerase amplification assay for the diagnosis of banana bunchy top virus in different banana cultivars. *Arch Virol* 162:2791–2796
- Li T-T, Wang J-L, Zhang N-Z, Li W-H, Yan H-B, Li L, Jia W-Z, Fu B-Q (2019) Rapid and visual detection of *Trichinella* spp. using a lateral flow strip-based recombinase polymerase amplification (LF-RPA) assay. *Front Cell Infect Microbiol* 9:1
- Londono MA, Harmon CL, Polston JE (2016) Evaluation of recombinase polymerase amplification for detection of begomoviruses by plant diagnostic clinics. *Virol J* 13:48
- Ma B, Fang J, Lin W, Yu X, Sun C, Zhang M (2019a) A simple and efficient method for potential point of care diagnosis of human papillomavirus genotypes: combination of isothermal recombinase polymerase amplification with lateral flow dipstick and reverse dot blot. *Anal Bioanal Chem* 411:7451–7460
- Ma L, Zeng F, Huang B, Zhu Y, Wu M, Xu F, Li X, Huang R, Ma J, Cong F, Guo P (2019b) Point of care diagnostic assay for rapid detection of porcine delta coronavirus using the recombinase polymerase amplification method. *Transbound Emerg Dis* 66:1324–1331
- Mandal B, Jain RK, Krishnareddy M, Kumar NKK, Ravi KS, Pappu HR (2012) Emerging problems of tospoviruses (Bunyaviridae) and their management in the Indian subcontinent. *Plant Dis* 96:468–479
- Mehle N, Trdan S (2012) Traditional and modern methods for the identification of *thrips* (Thysanoptera) species. *J Pest Sci* 85:179–190
- Mekuria TA, Zhang S, Eastwell KC (2014) Rapid and sensitive detection of little cherry virus 2 using isothermal reverse transcription-recombinase polymerase amplification. *J Virol Methods* 205:24–30
- Monteiro RC, Zucchi Ra, Mound LA (1995) Record of *Thrips palmi* Karny, 1925 (Thysanoptera, Thripidae) in the state of Sao Paulo, Brazil. *Revista de Agricultura (Piracicaba)* 70(1): 53–55
- Mound LA, Gillespie PS (1997) Identification guide to thrips associated with crops in Australia. Orange NSW and Canberra: NSW agriculture and CSIRO <https://hdl.handle.net/102.100.100/220816?index=1>
- Murai T (2002) The pest and vector from east: *Thrips palmi*. In: *Proceeding of the Thrips and Tospoviruses—PROCEEDINGS of the 7th international symposium in Thysanoptera*. Canberra, Australia national insect collection 19–32
- Nakahara S, Minoura K (2015) Identification of four thrips species (Thysanoptera: Thripidae) by multiplex polymerase chain reaction. *Res Bull Plant Prot Jpn* 42:37–42
- Palmer JM (1992) Thrips (Thysanoptera) from Pakistan to the Pacific: a review. *Bull Br Mus (Nat Hist) Entomol Ser* 61(1):76
- Pantoja A, Segarra A, Ruiz H, Medina-Gaud S (1988) *Thrips palmi* (Thysanoptera: Thripidae): a new insect pest for Puerto Rico. *J Agric Univ Puerto Rico* 72(2):327
- Pappu HR, Jones RAC, Jain RK (2009) Global status of tospovirus epidemics in diverse cropping systems: successes achieved and challenges ahead. *Virus Res* 141:219–236
- Paran I, Michelmore RW (1993) Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theor Appl Genet* 85:985–993
- Przybylska A, Fiedler Ż, Frąckowiak P, Obreńska-Stęplowska A (2017) Real-time PCR assay for distinguishing *Frankliniella occidentalis* and *Thrips palmi*. *Bull Entomol Res* 108:413–420

- Przybylska A, Fiedler Ż, Kucharczyk H, Obrępańska-Stęplowska A (2015) Detection of the quarantine species *Thrips palmi* by loop-mediated isothermal amplification. PLoS ONE 10:e0122033
- Reddy DVR, Buiel AAM, Satyanarayana T, Dwivedi SL, Reddy AS, Ratna AS, Vijayalakshmi K, Ranga Rao GV, Naidu RA, and Wightman JA (1995) Peanut bud necrosis virus disease: an overview. In: Recent studies on peanut bud necrosis disease—Proceedings of a Meeting, 20 March. In: Buiel AAM, Parevliet JE, Lenne JM (eds) International crop research institute for semi-arid tropics, Patancheru, Andhra Pradesh, India pp 3–7
- Rosser A, Rollinson D, Forrest M, Webster BL (2015) Isothermal recombinase polymerase amplification (RPA) of *Schistosoma haematobium* DNA and oligochromatographic lateral flow detection. Parasites Vector 8:446
- Rotenberg D, Jacobson AL, Schneweis DJ, Whitefield AE (2015) Thrips transmission of tospoviruses. Curr Opin Virol 15:80–89
- Seal DR, Kumar V, Kakkar G, Mello SC (2013) Abundance of adventive *Thrips palmi* (Thysanoptera: Thripidae) population in Florida during the first sixteen years. Fla Entomol 96:789–796
- Trdan S, Valič N, Žnidarčič D (2007) Field efficacy of deltamethrin in reducing damage caused by *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) on early white cabbage. J Pest Sci 80:217
- Tyagi K, Kumar V, Singha D, Chandra K, Laskar BA, Kundu S et al (2017) DNA Barcoding studies on Thrips in India: Cryptic species and Species complexes. Sci Rep 7:1–14
- Walsh K, Boonham N, Barker I, Collins DW (2005) Development of a sequence specific real-time PCR to the melon thrips *Thrips palmi* (Thysan., Thripidae). J Appl Entomol 129:272–279
- Xi Y, Xu CZ, Xie ZZ, Zhu DL, Dong JM (2019) Rapid and visual detection of dengue virus using recombinase polymerase amplification method combined with lateral flow dipstick. Mol Cell Probes 46:101413
- Yadav R, Chang NT (2012) Temperature dependent development and life table parameters of *Thrips palmi* on eggplant. Appl Entomol Zool 47(4):301–310
- Yeh WB, Tseng MJ, Chang NT, Wu SY, Tsai YS (2014) Agronomically important thrips: development of species-specific primers in multiplex PCR and microarray assay using internal transcribed spacer 1 (ITS1) sequences for identification. Bull Entomol Res 105:52–59
- Zhao G, He H, Wang H (2019) Use of a recombinase polymerase amplification commercial kit for rapid visual detection of *Pasteurella multocida*. BMC Vet Res 15:154

**Publisher's Note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.