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Real-time PCR assay for distinguishing *Frankliniella occidentalis* and *Thrips palmi*

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Abstract

Thrips palmi and *Frankliniella occidentalis* (order Thysanoptera) are thrips species that represent major plant pests. They are polyphagous insects capable of adversely affecting crop production. As such, in the European Union, these thrips species should be regulated as quarantine organisms. *T. palmi* and *F. occidentalis* can cause considerable damage to susceptible plants by feeding on them and transmitting several viruses responsible for serious plant diseases. Successful pest control strategies are based on an early, fast, and reliable diagnosis, which precedes the selection of appropriate steps to limit the effects of harmful organisms. We herein describe a novel diagnostic approach that enables the sensitive and species-specific detection (and differentiation) of these pests in a duplex polymerase chain reaction assay, which was adapted for both standard and real-time quantitative assays. Our method is based on the amplification of a 5.8S-internal transcribed spacer 2 ribosomal DNA fragment that is conserved between *T. palmi* and *F. occidentalis*.

Keywords: insect detection, diagnostic protocol, rDNA, quarantine pests, molecular methods, multiplex PCR, pest control

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Introduction

Thrips (order Thysanoptera) are small insects (average length: 1–2 mm) with a global distribution. More than 5500 thrips species have been identified (Mound, 1997; Morse & Hoddle, 2006). Some are considered pests that result in losses to the agriculture, horticulture, and forestry industries by directly damaging plants or through the transmission of tospoviruses (Moritz *et al*., 2000). The most harmful thrips species are *Frankliniella occidentalis* (Pergande) and *Thrips palmi* Karny (Seal *et al*., 2013), though others, including *Thrips tabaci*, are also economically important.

Melon thrips [i.e., *T. palmi* Karny (Thysanoptera, Thripidae)] are polyphagous insects that feed on Cucurbitaceae and Solanaceae plant species (Walker, 1994). Lewis (1997) compiled a list of more than 200 plants on which *T. palmi* has been detected, including eggplant, cucumber, and watermelon. Other susceptible crops include pepper, sesame, sunflower, soybean, cowpea, tobacco, and squash. *T. palmi* was discovered in Indonesia, and has since spread to other regions with tropical and subtropical climates (Smith *et al*., 1997). An outbreak of *T. palmi* destroyed almost 80% of the watermelon plantations in two regions of the Philippines in 1977. In 1978, this pest became a major threat to vegetable production in Japan, and during the subsequent decade, it spread to about 20,000 ha (Murai, 2002). *T. palmi* is also present in South America, where it was reported for the first time in Venezuela infesting bean, potato, eggplant, melon, and other crops (Cermeli & Montagne 1993). In Australia, *T. palmi* was first observed on watermelon and cucumber (Houston *et al*., 1991), while in Europe, it was detected in glasshouses in the Netherlands in 1988 and 1992 (Mound & Gillespie, 1997). In both cases, the pest was eradicated by destroying all infected plants (Cannon *et al*., 2007). *T. palmi* adults

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and larvae feed on leaves and stems, which are attacked at or near the growing tip. They also occur on fruit surfaces and on petals and developing ovaries. The estimated economic losses in the UK resulting from infestations of greenhouse-grown plants by *T. palmi* are considerable. Additionally, a benefit-to-cost ratio for one eradication campaign was calculated as 110:1 (MacLeod *et al.*, 2004). Heavy *T. palmi* infestations of plants in Florida, USA were associated with decreases in crop marketability. Moreover, commonly used insecticides alone or in combination are unable to satisfactorily control *T. palmi* during the early stages of infestation (Seal *et al.*, 2013). In Taiwan, the market value of *T. palmi*-damaged fruits was only approximately 25% of that of undamaged fruits (Yadav & Chang, 2012).

F. occidentalis (i.e., western flower thrips) is another harmful thrips species. It has a very wide host range that spans horticultural and ornamental crops as well as trees, with hosts in 65 families (CABI, 2016). Alfalfa, chrysanthemum, corn, cotton, cucumber, eggplant, gerbera, gladiolus, grapes, impatiens, melons, peanut, pepper, plums, strawberry, tomato, and watermelon are among the plant species susceptible to *F. occidentalis* infestations (Chau & Heinz, 2006; CABI, 2016). *F. occidentalis* is one of the most important insect pests affecting glasshouse crops worldwide (Cloyd, 2009), and it is also a major pest of some outdoor crops in warm climates. It is considered one of the most invasive species in Europe and can cause yield losses of up to 70% in greenhouse-grown cucumber (Marullo, 2002). The effects are more serious when thrips populations function as virus vectors. *F. occidentalis* is endemic to North America, but it was also detected in the Netherlands in 1983, from where it spread across Europe (Kirk & Terry, 2003). It was subsequently detected in eastern Africa, New Zealand, and Australia. *F. occidentalis* occurs in many different habitats (e.g., lowland to alpine). However, because it does not undergo developmental or reproductive diapause (Ishida *et al.*, 2003), *F. occidentalis* may not survive cold winters (McDonald *et al.*, 1997). According to CABI (2016), *F. occidentalis* is currently distributed in several countries in Asia, Africa, North America, South America, the Caribbean, Europe, the Mediterranean, and Oceania. Importantly, the geographic distribution of *F. occidentalis* may overlap with that of *T. palmi*. Welter *et al.* (1990) studied mixed infestations of these two pests under experimental conditions, and observed significant decreases in total cucumber yield as well as mean and total fruit sizes.

F. occidentalis and *T. palmi* are vectors of serious plant viruses, including some of quarantine importance. *T. palmi* transmits several plant viruses, including watermelon silver mottle virus (Iwaki *et al.*, 1984; Riley *et al.*, 2011), tomato spotted wilt virus (Fujisawa, 1988), calla lily chlorotic spot virus (Chen *et al.*, 2005), groundnut bud necrosis virus (Reddy *et al.*, 1992), and melon yellow spot virus (Kato, 2000). Watermelon silver mottle virus is on the European and Mediterranean Plant Protection Organization (EPPO) A1 list of pathogens to be regulated as quarantine pests (EPPO, 2015*a*), and causes severe losses to watermelon production in Taiwan (Yeh *et al.*, 1992), Japan (Okuda *et al.*, 2002), and China (Rao *et al.*, 2011). *F. occidentalis* is a vector of tomato spotted wilt virus, tomato chlorotic spot virus, and groundnut ringspot virus or impatiens necrotic spot virus (Wang *et al.*, 2010). Worldwide crop damage caused by tospoviruses transmitted by *F. occidentalis* is likely greater than US$1 billion per year (Goldbach & Peters, 1994). Tomato spotted wilt virus is a particularly important tospovirus that is transmitted by both *T. palmi* and *F. occidentalis*. It was first reported in tomato, but has been detected in approximately 900 host plants from 80 families. It has a worldwide distribution because its thrips vectors are widespread (Boonham *et al.*, 2002). In the European Union, *T. palmi* is on the EPPO A1 list, while *F. occidentalis* is on the EPPO A2 list of pests that should be regulated as quarantine organisms (EPPO, 2015*b*).

Accurately identifying thrips species is crucial for managing these insect pests. It is also important to distinguish pests from non-pest species, and differentiate quarantine from non-quarantine insects. Identifying different thrips species based on morphological features requires entomological expertise, especially when distinguishing among larval species. However, molecular biology-based tools may enable the development of fast, easy, and sensitive diagnostic protocols for many pest species. There are methods for the molecular identification of *T. palmi* based on real-time polymerase chain reaction (PCR) detection (Kox *et al.*, 2005), sequence-characterized amplified region marker-based real-time PCR detection using a TaqMan probe (Walsh *et al.*, 2005), PCR–restriction fragment length polymorphism (RFLP) detection (Brunner *et al.*, 2002; Toda & Komazaki, 2002), and loop-mediated isothermal amplification assays (Przybylska *et al.*, 2015). There are also protocols for identifying *F. occidentalis* using a real-time PCR assay with a TaqMan probe (Huang *et al.*, 2010), PCR–RFLP (Brunner *et al.*, 2002; Toda & Komazaki, 2002; Mainali *et al.*, 2008; Przybylska *et al.*, 2016), and a cytochrome oxidase I mitochondrial DNA marker (Zhang *et al.*, 2012) with a duplex PCR (Zhang *et al.*, 2014). Methods are also available describing a multiplex PCR assay for *F. occidentalis*, *Frankliniella intonsa*, *T. tabaci*, and *T. palmi* (Nakahara & Minoura, 2015) as well as a multiplex PCR assay for *F. occidentalis*, *F. intonsa*, *T. tabaci*, and *Thrips hawaiiensis* (Yeh *et al.*, 2014). However, a protocol for detecting and differentiating between *T. palmi* and *F. occidentalis* in a single real-time PCR has not been developed.

The aim of this study was to develop a fast and sensitive method for simultaneously identifying and differentiating between *F. occidentalis* and *T. palmi* in one tube. We developed a duplex standard and real-time PCR protocol based on a melting curve analysis of the amplified 5.8S–internal transcribed spacer 2 (ITS2) ribosomal DNA (rDNA) fragment.

Materials and methods

Thrips samples

We examined ten *F. occidentalis* populations and six *T. palmi* populations. Additionally, single populations of *T. major*, *T. menyanthidis*, *T. nigropilosus*, *T. origani*, *T. physapus*, *T. roepkei*, *T. simplex*, *T. sambuci*, *T. tabaci*, *T. trehernei*, *F. intonsa*, *F. pallida*, and *F. tenuicornis* were used as negative controls. The geographical locations and host plants from which thrips specimens were collected are indicated in table 1.

Genomic DNA extraction

Total genomic DNA was extracted from one, or in the case of some populations (derived from our previous study; Przybylska *et al.*, 2015), a few specimens, using the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) in a final volume of 100 µl. Final DNA concentrations were 5–10 ng μl^{-1}. Genomic DNA from all populations which were taken as negative controls, were the same as these used

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Table 1. Thrips populations used as positive and negative controls in the study.

Species	Origin	Host plant	GenBank accession number
Frankliniella occidentalis 1	Poznań, Poland	*Cucumis sativus*	KM886242
Frankliniella occidentalis 2	Słubice, Poland	*Dendranthema indica*	–
Frankliniella occidentalis 3	Łukomin, Poland	*Cucumis sativus*	–
Frankliniella occidentalis 4	Szamotuły, Poland	*Cucumis sativus*	–
Frankliniella occidentalis 5	Gdańsk, Poland	*Cucumis sativus*	–
Frankliniella occidentalis 6	Plewiska, Poland	*Solanum lycopersicum*	–
Frankliniella occidentalis 7	Warszawa, Poland	*Cucumis sativus*	–
Frankliniella occidentalis 8	Katowice, Poland	*Eupatorium* sp.	–
Frankliniella occidentalis 9	Warszawa, Poland	*Cucumis sativus*	–
Frankliniella occidentalis 10	Poznań, Poland	*Solanum melongena*	–
Thrips palmi 1	Japan	*Cucumis sativus*	KM877305
Thrips palmi 2	Taiwan	*Cucumis sativus*	KM877306
Thrips palmi 3	Taiwan	*Citrulus lanatus*	–
Thrips palmi 4	Vietnam	*Capsicum annuum*	–
Thrips palmi 5	Thailand	*Dendrobium* sp.	–
Frankliniella intonsa	Poland	*Nemesia strumosa*	KM886243
Frankliniella pallida	Poland	*Sedum acre*	KM886244
Frankliniella tenuicornis	Poland	*Zea mays*	KM886245
Thrips major	Poland	*Sambucus nigra*	KM877309
Thrips menyanthidis	Poland	*Menyanthes trifoliata*	–
Thrips nigropilosus	Poland	*Ocimum basilicum*	–
Thrips origani	Poland	*Origanum vulgare*	–
Thrips physapus	Poland	*Ceantaurea jacea*	–
Thrips roepkei	Poland	*Solanum dulcamara*	KM877310
Thrips simplex	Poland	*Gladiolus* sp.	KM877312
Thrips sambuci	Poland	*Sambucus nigra*	KM877311
Thrips tabaci	Poland	*Inula salicifolia*	KM877307
Thrips trehernei	Poland	*Tragopogon pratensis*	KM877313

in Przybylska *et al.* (2015), and during that study, they were tested in PCR with universal primers and sequenced.

Design of species-specific primers

Sequences used to design primers were obtained from Przybylska *et al.* (2015) and from a National Center for Biotechnology Information database. Sequences were aligned with the BioEdit program (Hall, 1999), and a 5.8S rDNA fragment conserved in both species was used to design a universal forward primer (ThrUNIFw: 5′-GTGAACTGCAGGACACATG-3′). For *F. occidentalis* and *T. palmi*, regions in the ITS2 rDNA fragment lacking intraspecific and high interspecific variability were used to design species-specific reverse primers (FoRw: 5′-CGTAAACGACAGAACAG-3′ and TpRw: 5′-GCAGAGACACATCGCAAC-3′).

PCR assay

The primers were tested in PCR amplifications of *F. occidentalis* and *T. palmi* samples, in which DNA templates from both species were included in an equimolar ratio in one sample. We also completed PCR amplifications for the other thrips species (table 1), which served as negative controls. Samples with no genomic DNA were used to assess whether the reagents were contaminated. The PCR amplifications involved the following three primers sets: ThrUNIFw/FoRw to detect only *F. occidentalis* species, ThrUNIFw/TpRw to detect only *T. palmi* species, and ThrUNIFw/FoRw/TpRw to simultaneously detect both thrips species.

The singleplex PCR amplifications were completed with samples containing 10 ng template DNA, 0.5 µM forward and reverse primers, 5 µl DreamTaq master mix (Thermo Scientific, Waltham, USA), and sterile distilled water up to 10 µl. Duplex PCR amplifications were conducted with samples containing 10 ng template DNA, 0.33 µM of each of three primers, 5 µl DreamTaq master mix (Thermo Scientific), and sterile distilled water up to 10 µl. The PCR program was as follows: 95°C for 3 min; 30 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s; 72°C for 5 min. All amplified products were electrophoretically separated on a 1.5% agarose gel and visualized with Midori Green stain (Nippon) under UV light.

Duplex real-time PCR assay

A duplex real-time PCR assay was conducted for the *F. occidentalis* and *T. palmi* populations, with the other thrips species used as negative controls (table 1). A sample with no genomic DNA was used to determine whether reagents were contaminated. The results were verified based on melting curve analyses. Samples consisted of 1 µl template DNA (10 ng), 0.33 µM primers (i.e., three primers), 5 µl iTaq master mix (Bio-Rad, California, USA), and sterile distilled water up to 10 µl. The real-time PCR was conducted using a LightCycler 96 system (Roche) and the following program: 95°C for 5 min; 40 cycles of 95°C for 10 s, 54°C for 10 s, and 72°C for 10 s. The melting phase was initiated at 65°C and was completed at 95°C, with an increase of 1°C per step. To test the sensitivity of the PCR, a series of tenfold dilutions (starting from 10 ng μl^{-1}

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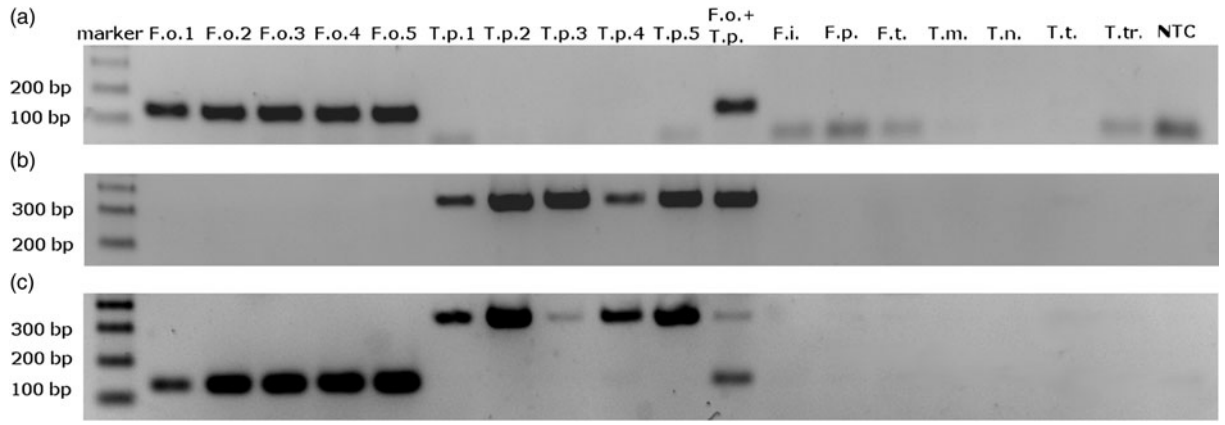

Fig. 1. Electrophoretic separation of PCR amplification products obtained with (a) ThrUNIFw/FoRw primers pair; (b) ThrUNIFw/TpRw primers pair; (c) ThrUNIFw/FoRw/TpRw primers set. DNA used to PCR were isolated from: F.o. – *Frankliniella occidentalis*, T.p. – *Thrips palmi*, F.i. – *F. intonsa*, F.p. – *F. pallida*, F.t. – *F. tenuicornis*, T.m. – *T. major*, T.n. – *T. nigropilosus*, T.t. – *T. tabaci*, T.tr. – *T. trehernei*; NTC – no template control.

genomic DNA) of *F. occidentalis* and *T. palmi* templates was examined by real-time PCR as described above.

Results

Detection of F. occidentalis *and* T. palmi *using singleplex and duplex PCR assays*

We obtained single amplification products for *F. occidentalis* and *T. palmi* samples using the ThrUNIFw/FoRw and ThrUNIFw/TpRw primer pairs, respectively. Amplifications with the ThrUNIFw/FoRw/TpRw primer set produced bands corresponding to the expected sizes of both thrips species. Amplicons were not detected for the other thrips species (table 1) and for the control sample lacking genomic DNA (fig. 1a–c). Product sizes were 128 bp for *F. occidentalis* and 333 bp for *T. palmi*.

Detection of F. occidentalis *and* T. palmi *using a real-time PCR assay*

A duplex real-time PCR assay involving the ThrUNIFw/FoRw and/or ThrUNIFw/TpRw primer sets enabled us to distinguish between *F. occidentalis* and *T. palmi* species. Amplification and derivative melt curves were generated for *F. occidentalis* and *T. palmi* (fig. 2a, b), with a melting peak temperature range of 83.5–84.0°C for *F. occidentalis* and 85.5–87.0°C for *T. palmi*. No amplification products were produced for the other thrips species (table 1) or the control sample lacking genomic DNA.

Tenfold serial dilutions of DNA isolated from *F. occidentalis* and *T. palmi* were used to evaluate the sensitivity of the detection assays. Our method was sufficiently sensitive to detect genomic DNA at a final concentration as low as 0.001 ng μl^{-1} for both species (fig. 3a, b). There were still observed derivative melt curves that matched the correct species in that concentration.

Discussion

The method developed in this study proved to be species-specific, fast, and sensitive. We used a wide array of thrips species closely related to *T. palmi* and *F. occidentalis* as negative controls. Przybylska *et al.* (2015) conducted a phylogenetic analysis based on rDNA fragments from many thrips species, and confirmed a close phylogenetic relationship between *T. palmi* and *T. nigropilosus*. Other closely related species are *T. simplex*, *T. tabaci*, and *T. trehernei*. Regarding *F. occidentalis*, all tested *Frankliniella* species clustered together in the phylogenetic tree. Thus, developing tests involving DNA samples isolated from several thrips species as negative controls is important because many of these species have common hosts and/or geographic distributions. However, most of the included thrips species do not affect crop production. No amplification product was observed for any of the non-target species during the singleplex PCR or real-time PCR assays. Moreover, PCR amplifications were completed with a mixture of *F. occidentalis* and *T. palmi* DNA or a mixture of their species-specific primer sets to assess reaction specificity. Our results indicated that the developed assay was species-specific, and able to distinguish between *F. occidentalis* and *T. palmi* in a single sample. Our method was sufficiently sensitive to detect genomic DNA at concentrations as low as 0.001 ng μl^{-1}. Furthermore, the protocol was completed quickly because both species were simultaneously detected in a single real-time PCR sample and differentiated based on the melting temperature of the amplification products, which eliminates the need for a gel electrophoresis step. Therefore, our method may be useful for routine analyses of insect samples collected from fields or in greenhouses. Our assay may also enable the monitoring of imported plant material to prevent the spread of *T. palmi* (i.e., quarantine species) throughout Europe.

While designing primers, we examined rDNA sequences based on experimental data and National Center for Biotechnology Information database records to identify regions conserved in *T. palmi* and *F. occidentalis* populations from various geographical locations and plant hosts. We did not detect significant differences in the 5.8S–ITS2 fragment sequences among the *F. occidentalis* and *T. palmi* populations. We analyzed 5.8S-ITS2 fragment with the length of about 460 nucleotides for *F. occidentalis* with differences in length between populations not more than seven nucleotides and 580 nucleotides for *T. palmi* with no differences in length between all analyzed populations. There were some single nucleotide

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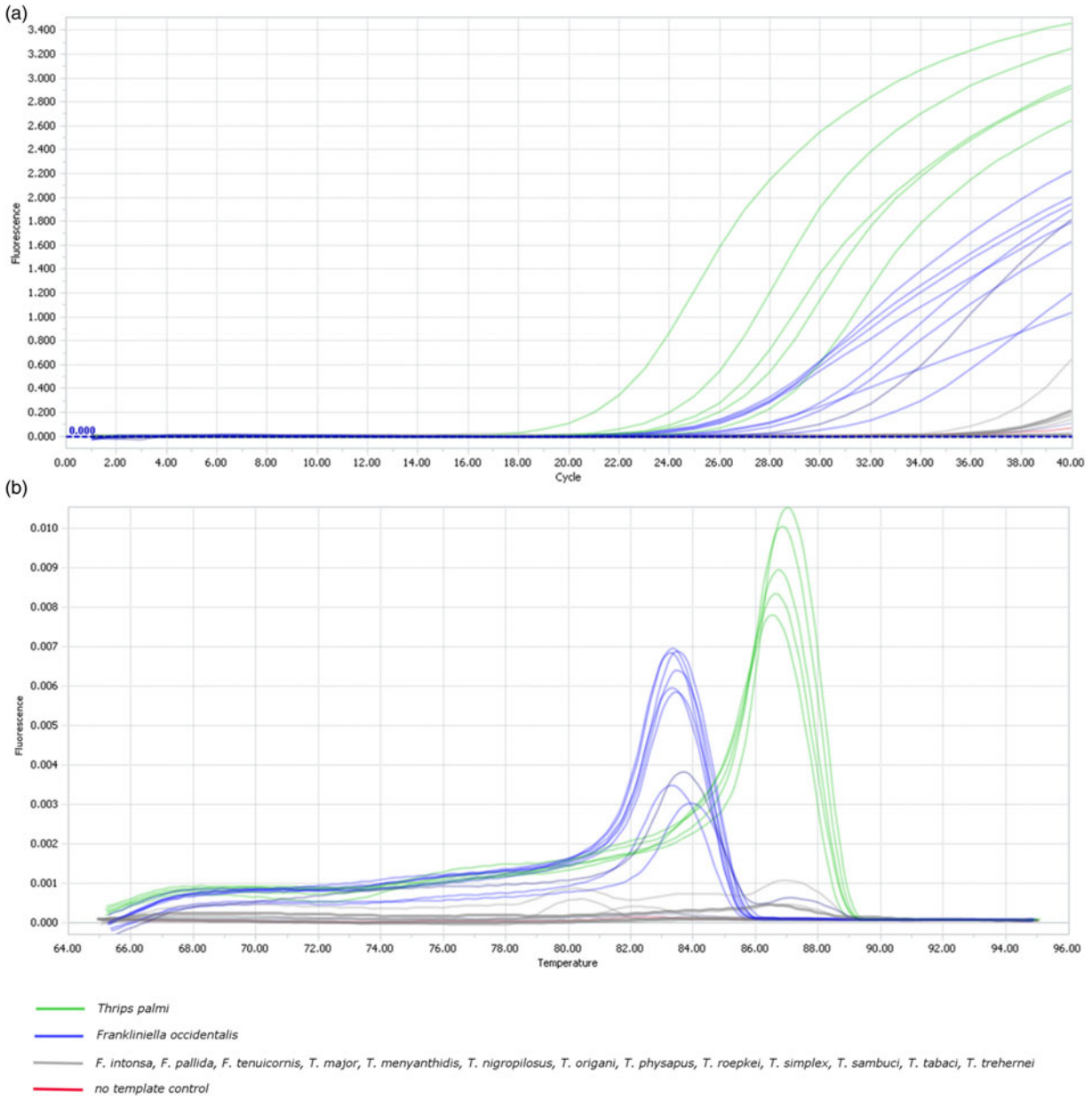

Fig. 2. Results of real-time PCR amplification for all tested species. (a) Amplification curves; (b) Dissociation curves with melting peak temperature range between 83 and 83.5°C for *F. occidentalis* populations and between 85.5 and 87°C for *T. palmi* populations.

substitutions observed within amplified region of each species (2–15 different nts between analyzed *F. occidentalis* populations and 1–21 nts for *T. palmi* populations), but it should not have influence on primer hybridizations, PCR amplification, PCR product length, or the melting temperature of real-time PCR products. Thus, we assume that our method may be applied for detecting *T. palmi* and *F. occidentalis* populations regardless of their origin.

T. palmi and *F. occidentalis* are highly polyphagous pests with overlapping host ranges. The most important common plant hosts are cucumber, melon, and watermelon. These species can occur together in greenhouses or under field conditions in relatively warm countries where *T. palmi* occurs (CABI, 2016).

There are currently two PCR–RFLP assays for detecting and differentiating between *T. palmi* and *F. occidentalis* (Brunner *et al.*, 2002; Toda & Komazaki, 2002). However, point mutations can generate false negative results in PCR–RFLP assays because restriction enzymes cut DNA at or near specific restriction sites and they are unable to recognize sites in which a nucleotide change has occurred. In contrast, PCR primers can hybridize properly even if there are single nucleotide mismatches between the primers and target sequence (Bru *et al.*, 2008; Stadhouders *et al.*, 2010; Wieczorek &

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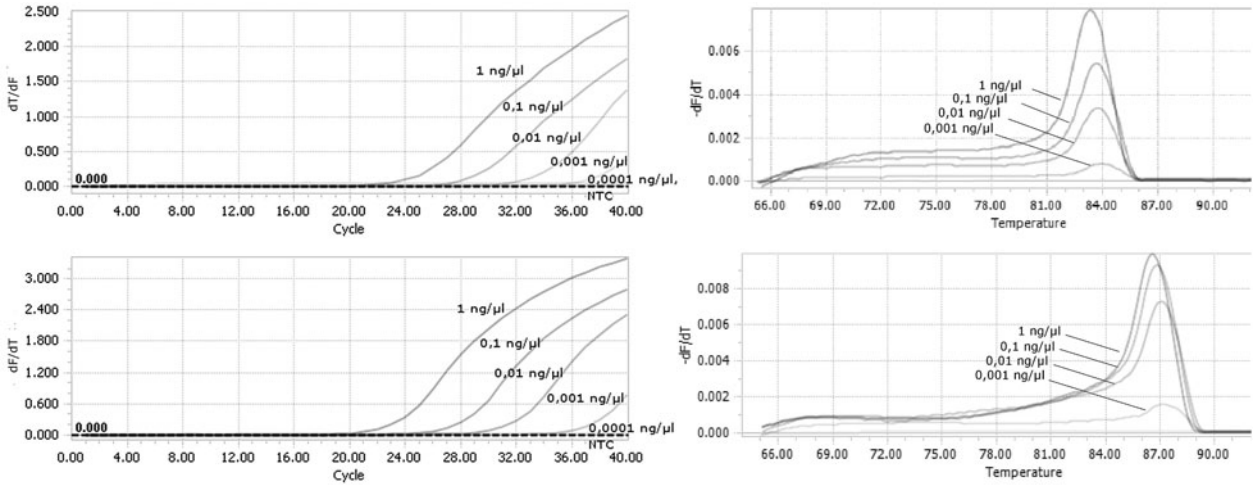

Fig. 3. Analysis of the sensitivity of real-time PCR detection. Genomic DNA from (a) *F. occidentalis*; (b) *T. palmi* were used as a template starting from the concentration of 1 ng μl^{-1} in final reaction mixture.

Obrępalska-Stęplowska, 2013). Moreover, a PCR–RFLP assay cannot be completed as quickly as duplex PCR or duplex real-time PCR assays because it involves two enzymatic reactions (i.e., PCR and RFLP) followed by an electrophoretic analysis. A multiplex PCR protocol is available for differentiating among *F. occidentalis*, *F. intonsa*, *T. tabaci*, and *T. palmi* (Nakahara & Minoura, 2015), but this method is not as fast as our real-time PCR protocol because it requires an additional step involving electrophoretic separation and visualization of amplification products. Furthermore, many previously published diagnostic protocols required the amplification of the cytochrome oxidase I mitochondrial DNA fragment. However, high intraspecific variability in this region has been reported for *T. palmi* and other thrips species (Rebijith *et al*., 2012). Therefore, we focused on a 5.8S–ITS2 rDNA fragment because Przybylska *et al*. (2015) revealed this region was relatively stable among examined populations. Moreover, part of the 5.8S rDNA fragment is conserved among many thrips species, enabling the use of a common forward primer for detecting *F. occidentalis* and *T. palmi*. In contrast, the ITS2 fragment is characterized by high interspecific sequence variability and thus, this region was ideal for designing species-specific reverse primers. In the majority of previously published protocols, the sensitivity was not tested or was assessed only for diluted larval or adult thrips samples. Thus, it is difficult to compare the sensitivity of our method with previously published procedures. Only Huang *et al*. (2010) used tenfold dilutions of DNA samples starting from 10 ng μl^{-1}. Their detection limit was 0.1 pg μl^{-1}, suggesting their method is ten times more sensitive than our assay, but their protocol was designed to detect only *F. occidentalis* species.

The method described herein may be useful as a rapid test for the early and reliable detection of *F. occidentalis* and *T. palmi*, which may be relevant for increasing the quality and yield of agriculturally important crops.

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