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A N I N T E R N A T I O N A L J O U R N A L

Efficient detection of *Frankliniella schultzei* (Thysanoptera, Thripidae) by cytochrome oxidase I gene (mtCOI) direct sequencing and real-time PCR

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ABSTRACT

Identification of Thysanoptera is based mainly on external morphology examination that can be time-consuming and difficult for non taxonomic experts. In this work, we propose a rapid and efficient molecular method to identify Frankliniella schultzei, *an important and widespread pest thrips vector of tospoviruses in South America countries. Species-specific primers designed in the mitochondrial cytochrome oxidase I gene (mtCOI) were optimized for detection by conventional PCR and real-time PCR. The primers were tested on immature and adult thrips collected from crops and weeds found in São Paulo State. All samples collected were identified as* F. schultzei, *indicating the high prevalence of this species as vector of tospoviruses in Brazilian fields.*

Key words: molecular identification, real-time PCR, tospovirus vector.

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INTRODUCTION

Although the agricultural importance of thrips has increased considerably in the last decades, their identification, the first step for decision-making in pest management, remains as an obstacle. Currently, identification in Thysanoptera is based mainly on external morphology examination after a time-consuming slide preparation [17]. Even with alternative and faster slide mounting methods [13,16], morphological assessment of characters useful for determination of species is not trivial and requires a good level of expertise. While taxonomists are supposed to know how to access thrips morphology, it constitutes a real problem for economic entomologists that require named species for their works. For phytopathologists, this problem is even worse as thrips transmit tospoviruses during first and early second instars, and morphological identification of Thysanoptera larvae is even more limited.

The common blossom thrips or tomato thrips, *Frankliniella schultzei* (Trybom) is native to South America [18] and is one of the major pests of various ornamental and vegetable crops in tropical regions [21]. Besides the direct damages to several crops, the indirect damages are becoming increasingly important with tospovirus transmission reports in crops such as tomato (*Solanum lycopersicum* L.), sweet pepper (*Capsicum annum* L.), watermelon *Citrullus lanatus* (Thunb.) Matsum. & Nakai, cucumber (*Cucumis sativus* L.) and peanuts (*Arachis hypogaea* L.), in Brazil [5,11,14,22]. Whereas there are a few molecular identification tools for some pest thrips [1,4,6,9,24,26], only *F. occidentalis* (Pergande) and *Thrips palmi* Karny can be identified at present using real-time PCR technique [7,9]. Tools for rapid molecular identification of *F. schultzei* are absent and the determination of this major pest is mainly based on traditional methods.

In this work, we designed a primer pair for *F. schultzei* based on the mitochondrial cytochrome oxidase I gene (mtCOI), a marker well suited for species discrimination within Thysanoptera [8]. The primer pair was optimized for PCR and real-time PCR detection. *F. schultzei* was the mainly thrips species found on vegetables and weeds collected in São Paulo State, indicating the high prevalence of this species as vector of tospoviruses.

MATERIALS AND METHODS

Adult and immatures of *F. schultzei* were collected on vegetables and weeds using a hand-held aspirator from 2012 to 2014 in several sites in the state of São Paulo, Brazil (table 1). The specimens were preserved immediately in 70% ethanol and stored at -20ºC prior to morphological and molecular analysis. For molecular analysis, adults of *F. occidentalis* (from Italy and Brazil), *F. intonsa* (Trybom) and *T. tabaci* Lindeman (from Northern Italy) were equally collected and stored. For morphological identification, adults of Brazilian specimens were prepared in microscope slides and identified following Mound and Marullo [17]. The morphological features used in the identification of *F. schultzei* were the fore wing with two complete rows of setae; prothorax with five pairs of main setae; absence of companiforme sensilo in the metanotum; pedicel of the antenna is simple; abdomen showing an incomplete developed comb and head showing interocellar setae (data not shown). Italian specimens were identified under a stereomicroscope at 100× magnification using taxonomic keys Mound et al. [15] and Palmer et al. [19].

PRELIMINARY EXTRACTION AND SEQUENCING

For the first PCR assay, total DNA was extracted from each individual thrips following a modified Chelex method [2]. Briefly, adults thrips were crushed and

homogenized in a 50 µl of Chelex 5% solution in a 0.5 ml Eppendorf tube and then incubated at 94°C for 5 min. After centrifugation at 14,000 *g* for 5 min, the supernatant was collected and used as template for PCR amplification.

DNA samples were subjected to PCR analysis with the primers mtD-7.2F and mtD-9.2R (table 1)[3]. Amplification was performed in 25 µl total reaction volumes, contained 3 µl of DNA, 12.5 µl Taq DNA Polymerase Master Mix Ampliqon III (Ampliqon ApS, Copenhagen, Denmark), and 0.25 µl of each primer. This solution was submitted in thermal cycler with the following parameters: 3 min at 94° C, 35 cycles of 30 s at 95 °C, 30 s at 55 °C, 45 s at 72 °C, and a final extension of 5 min at 72 °C. After amplification, 6 µl of the PCR products were subjected to electrophoresis on a 1 % agarose gel stained with Neotaq Brilliant Green Plus DNA Stain (Neobio, Brazil).

PCR products amplified from mtCOI of thrips were purified (QIAquick Gel Extraction Kit Qiagen) and sequenced (Macrogen, South Korea) using the primer mtD-7.2F. Sequence chromatograms were manually checked and edited in BioEdit software (version 7.1). DNA sequences were compared with the corresponding sequences of other thrips species deposited in GenBank and aligned using Clustal W in MEGA software [23]. A phylogenetic tree was constructed using the neighbour-joining method with 2000 bootstrap replications through MEGA software.

Table 1. Primers used in PCR assays.

name	sequence 5' – to – 3'	Orientation	specificity
mtD-7.2F	ATTAGGAGCHCCHGAYATAGCATT	Forward	Generic
mtD-9.2R	CAGGCAAGATTAAAATATAAACTTCTG	Reverse	Generic
FS_for	ATACCTGCTAAATGAAGGG	Forward	*F. schultzei* - specific
FS_rev	TTCCACCTTCAATAACTTTAC	Reverse	*F. schultzei* - specific

SPECIES SPECIFIC ASSAY DESIGN AND VALIDATION

In the PCR assays with species-specific primers, individuals thrips were crushed and homogenized in a 10 µl of a Milli-Q water in a 0.5 ml Eppendorf tube. The tube was incubated in boiling water for 5 min, stored at 20 °C for 10 min and centrifuged at 8,000 *g* for 5 min. Three micro liters of the supernatant were used as template for real time PCR and 5 µl were used for specific primer PCR analysis.

One set of primers targeted on the region of mtCOI gene specifically for *F. schultzei* was designed and called FS.rev and FS.for primers (Table 1). The specificity of the *F. schultzei*-specific COI primers was evaluated performing PCR assays on four thrips species (table 1). PCR assays were performed for each thrips sample with a total volume of 25 µl as follows: 5 µl of DNA, 2.5 µl of 10x Buffer, 1.0 µl of $MgCl_2$ (50 mM), 2.5 µl of dNTP's (2 mM each), 0.5 µl of each primer (final primer concentration of 10 µM each), and 0.125 µl of Polytaq (Polymed, Florence, Italy). The reaction was performed in thermal cycler using the following parameters: 3 min at 94 °C, 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 40 s at 72 °C, followed by a final extension for 5 min at 72 °C. After amplification, the PCR products were subjected to electrophoresis.

To select the optimal primer concentration, a 10-fold dilution series of positive DNA from *F. schultzei* was tested in a real-time PCR using concentrations of 50, 150, 300 or 900 nM of primers FS.rev and FS.for. After that, real-time PCR reactions were performed in a 96 well plates (Bio-Rad, California, USA) containing 3 µl DNA, 0.3 µl of primers FS.rev and FS.for (10 µM each), and 5 µl of iTaq™ Universal SYBR® Green Supermix (Bio-Rad), in a final volume of 10 µl. The SYBR Green PCR amplifications were undertaken in a CFX Connect™ Real-Time PCR detection system (Bio-Rad) with a CFX Manage Software (Bio-Rad). The reactions were run

at least in triplicate. The thermal profile for SYBR PCR was 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 sec.

RESULTS

The thrips specimens collected from watermelon, pumpkin (*Cucurbita* sp. L.), tomato, sweet pepper, lettuce (*Lactuca sativa* L.), cucumber, *Tridax procumbens* and *Emilia sonchifolia* were identified as *F. schultzei* by molecular and morphological methods (Table 2). The PCR amplification with the primers mtD7.2F and mtD-9.2R produced only a single band of 450 bp. Two sequences of *F. schultzei* mtCOI gene were deposited in GenBank (under accession numbers KJ175069 and KJ175070, respectively). BLAST results showed significant identity (95–100%) between the sequences of the mtCOI gene of *F. schutzei* with the corresponding nucleotide sequences of *F. schutzei* deposited in GenBank (accession number KF560548). The phylogenetic tree (Fig. 1) shows that the sequences of *F. schultzei* obtained in this study are close to those reported in Florida (USA) (accession number KF560548). The other thrips species, *F. occidentalis* (KC008075), *F. intonsa* (AB277215) and *T. tabaci* (FN546159) were clustered into separate branches.

The specificity of the PCR with the *F. schultzei*-specific COI primers (FS.rev and FS.for) was tested by performing PCR using DNA isolated from the thrips listed in table 2. The single amplicon (145 bp) was only obtained from *F. schultzei* specimens, and the others three species were negative in our PCR assay. The PCR was checked twice to make sure that the negative results were not caused by inhibition or inefficient DNA extraction.

At 300 nM of primer concentration, cycle threshold (Ct) values were optimal and the DNA positive final dilution end was 1:100 (data not shown). All *F. schultzei* specimens were positive in the real-time PCR assay with the Ct- values ranging from 16.05 - 19.65 (Fig. 2A). Adult and immature stages were positives, demonstrating the sensitivity of the assay and validating the specificity of the primers. The DNA of *F. occidentalis, F. intonsa* and *T. tabaci* were no amplified by the primers. These species were used because they are closely related to *F. schultzei*, and are also commonly found associated with several crops in Brazil and worldwide. The corresponding dissociation curves of the samples are showed in Figure 2B.

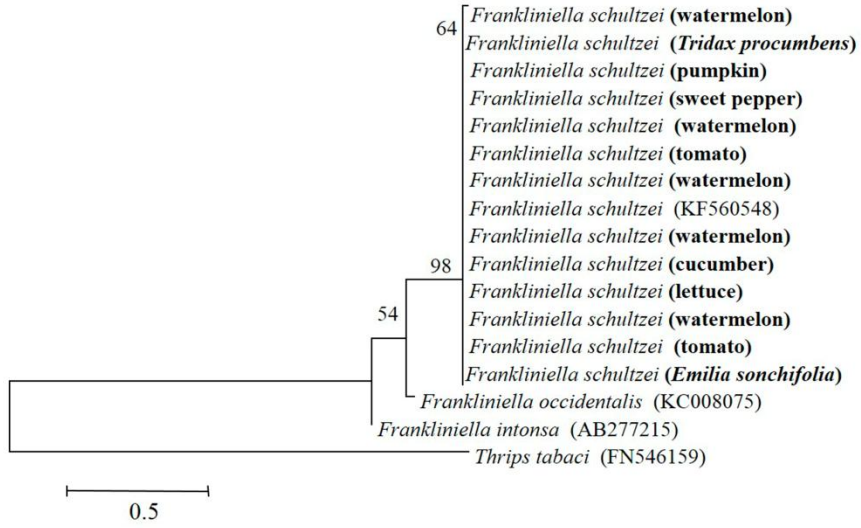

Figure 1. Phylogenetic relationships of *Frankliniella schultzei* mtCOI gene sequences (450bp) of samples (in bold) collected from different crops and weeds in São Paulo state. The tree was constructed by neighbor-joining method with the MEGA 5.0 software. Numbers at the nodes indicate the frequency of the cluster after bootstrap

analysis (2000 replicates). Only bootstrap values above 50% are shown. KF560548 - *F. schultzei* from USA; KC008075 – *F. occidentalis* from New Zealand; AB277215 – *F. intonsa* from China; FN546159 – *Thrips tabaci* from USA.

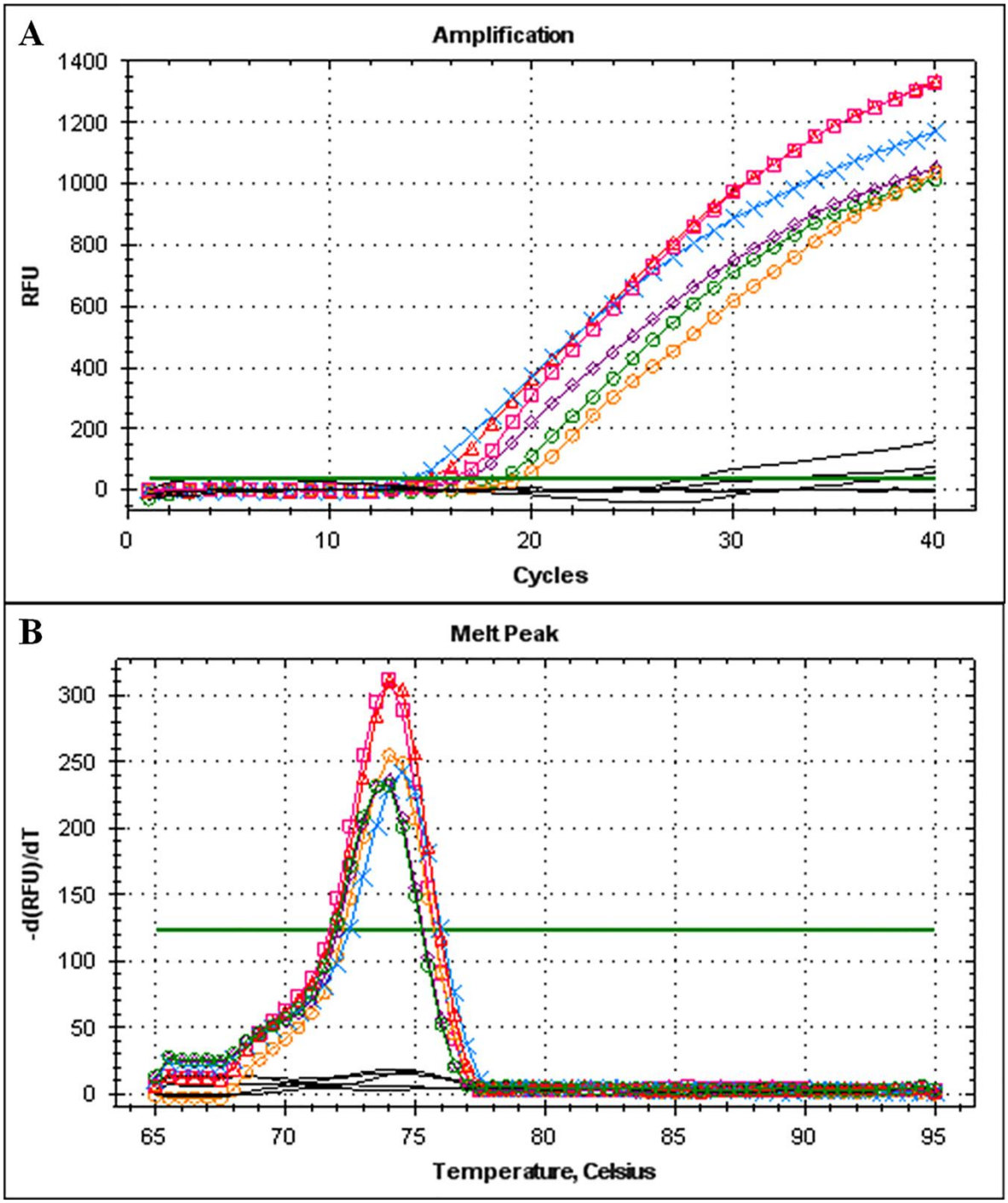

Figure 2. (A) Specific amplification curve of *Frankliniella schultzei*, collected at young stage (larvae - red line) and adult in differents crops (watermelon – pink, purple and green lines; pumpkin – blue; cucumber – orange line). Other four thrips species including *F. occidentalis* (Brazil), *F. occidentalis* (Italy), *F. intonsa* and *Thrips tabaci* did not show any positive signal (black lines). The mean Ct-values for each samples are given in a Table. (B) The corresponding dissociation curves of *F. schultzei*. The melting temperature (Tm) of each amplicon is shown alongside its dissociation curve.

DISCUSSION

The small size and cryptic behaviour of thrips make their monitoring and the identification processes difficult. In such a huge and biodiverse country as Brazil, where the Thysanoptera diversity is high, the correct thrips identification can be even more challenging. Our PCR assay using a *F. schultzei*-specific COI primer was accurate, demonstrating that can be used in other studies of molecular identification. The development of species-specific primers from PCR assays is an important tool to assist and speed up the correct identification of thrips species. Species-specific primers require only conventional PCR, which is readily available, rapid and inexpensive. The species-specific primers that have been identified in this study will enable even a non-specialist to identify *F. schultzei*. Apart from this, molecular identification has the obvious advantage of making possible the identification when only immature stages are available. However, these primers should be applied cautiously among populations of *F. schultzei* outside South and North America, as there may be the existence of other variants that may misidentify other thrips species. At present, pale and dark coloured specimens of *F. schultzei* are considered conspecific, but we did not include pale individuals in our study.

The real-time PCR assay as described here is rapid, specific and sensitive, easy and quick to perform for identification of *F. schultzei* in institutions research. The time necessary to obtain definitive results with this method is less than one working day. At present, only three real-time PCR assays have been developed for the identification of thrips species: two specific for *T. palmi* [9,25] and one specific to *F. occidentalis* [7]. All these assays are based on TaqMan chemistry and just in the study of Kox et al. [9] is used the mtCOI gene as target gene.

Although the equipment is expensive, the use of real-time PCR is rapid, accurate, can be applied in large-scale process of identifying species that are found in plants and / or plant material derived from trade, and is suitable with insects caught on sticky traps [12], when morphological identification is sometimes difficult due to the loss of several strucutres.

Due to *F. schultzei* prevalence in many countries [17], this method can be essential for the control of plant material to prevent further spread of this vector thrips in areas where it is not yet present or even in the entrance of new tospoviruses in Brazil. As we known *F. schultzei* also can transmitted *Capsicum chlorosis virus* (CaCV) and *Groundnut bud necrosis virus* (GBNV) [10,20], that by the time has not been reported in Brazil.

Table 2. Samples and localities of thrips specimens collected and results of DNA based methods for molecular identification.

Species	Location	Host plant	Cropping system	Year of collection	Molecular identification (PCR) [(b)]		
					DS[(c)]	SP	RT
Frankliniella schultzei	Presidente Prudente, SP (S22°7′33″/W51°23′20″)	Watermelon	Open field	2012/2013	+	+	16.05
	Marília, SP (S22°12'50"/W49º56'45")		Open field	2012/2013	+	+	18.13
	Botucatu, SP(adult) (S22º53'09"/W48º26'42")		Greenhouse[(a)]	2013/2014	+	nt	18.17
	Botucatu, SP, (larvae) (S22º53'09"/W48º26'42")		Greenhouse	2013/2014	nt	+	16.05
	São Manuel, SP (S22º43'52"/W48º34'14")		Greenhouse	2013/2014	+	+	17.98
	Borborema, SP (S21º37'11"/W49º04'25")		Open field	2014	+	+	15.90
	Presidente Prudente, SP (S22°7′33″/W51°23′20″)	Pumpkin	Open field	2013	+	+	14.58
	Botucatu, SP (S22º53'09"/W48º26'42")	Tomato	Greenhouse	2013/2014	+	+	nt
	São Manuel, SP (S22º43'52"/W48º34'14")		Greenhouse	2013/2014	+	+	19.65
	Campinas, SP (S22º54′21”/W 47º03′39”)	Sweet Pepper	Greenhouse	2014	+	nt	nt
	Campinas, SP (S22º54′21”/W 47º03′39”)	Lettuce	Greenhouse	2014	+	+	18.51
	Campinas, SP (S22º54′21”/W 47º03′39”)	Cucumber	Greenhouse	2014	+	nt	16.03
	Botucatu, SP (S22º53'09"/W48º26'42")	*Emilia sonchifolia*	Greenhouse	2012/2013	+	+	17.59
	São Manuel, SP (S22º43'52"/W48º34'14")	*Tridax procumbens*	Open field	2014	+	nt	nt
F. occidentalis	Andradas, MG (S22º04'05”/W46º34'04”)	Chrysanthemum	Greenhouse	2014	nt	-	nd
F. occidentalis	Piedmont, Italy (N45°3’/ E7°40’)	Sweet Pepper	Greenhouse	2011	nt	-	nd

F. intonsa	Piedmont, Italy (N45°3'/ E7°40')	Strawberry	Greenhouse	2011	nt	-	nd
Thrips tabaci	Piedmont, Italy (N45°3'/ E7°40')	Sweet Pepper	Greenhouse	2011	nt	-	nd

[(a)] experimental conditions; [(b)] (+) positive for *F. schultzei*; (-) negative for *F. schultzei*; nt – not tested; nd – not detected; [(c)]DS-Direct Sequencing; SP – Specific primers; RT- Real time PCR (Ct-values).

CONCLUSIONS

In this study we got sequencing several specimens of *F. schultzei* and we confirmed the presence of this species in different crops and regions in the São Paulo state. This result may lead us to believe that *F. schultzei* is the largest tospovirus vector in these regions. We also observed that in plants infected with GRSV, the presence of this insect was high.

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