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Agronomically important thrips: development of species-specific primers in multiplex PCR and microarray assay using internal transcribed spacer 1 (ITS1) sequences for identification

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Abstract

Thrips, the sole vector of plant *Tospovirus*, are major pests of many agricultural crops throughout the world. Molecular approaches have been applied in recent decades to identify these minute and morphologically difficult to distinguish insects. In this study, sequences of internal transcribed spacer 1 (ITS1) region of 15 agronomically important thrips, including several virus transmission species, have been analyzed in order to design species-specific primers for multiplex PCR and probes for microarray assay. That the ITS1 sequence distances within species were smaller than those among species suggests that the ITS1 fragment can be used for thrips species identification. The specificity and stability of these primers, combined with universal paired primers, were tested and verified in multiplex PCR. Using these specific primers as probes, microarray assay showed that PCR products of all thrips species hybridized consistently to their corresponding probes, though some signals were weak. We have demonstrated that multiplex PCR using specific primers based on ITS1 sequences is a simple, reliable, and cost-effective diagnostic tool for thrips species identification. Moreover, the DNA microarray assay is expected to extend into a reliable high-throughput screening tool for the vast numbers of thrips.

Keywords: thrips, ITS1, multiplex PCR, species-specific probes, microarray

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Introduction

Thrips are major pests of many agricultural crops throughout the world. They directly damage the host plants by sucking fluids from buds, leaves, flowers, fruits, and twigs resulting in distortions, stunted growth, feeding scars, and color mosaicism. Moreover, thrips are the sole vectors of plant *Tospovirus*. For example, onion thrips, *Thrips tabaci* Lindeman, can transmit *Tospovirus* as well as *Iris yellow spot virus* causing more than US$100 million losses every year (Prins & Goldbach, 1998; Gent *et al.*, 2006). Traditionally, identification of thrips, which are minute in size and have a high degree of similarity in appearance, is mainly based on adult characters. Moreover, some cryptic species, such as *Frankliniella occidentalis* (Pergande), *Scirtothrips dorsalis* Hood, and *T. tabaci*, which exhibit large differences in genetic compositions,

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Table 1. Thrips species and their abbreviations in this study. Thrips used to design species-specific primers are in bold and others were included for specificity and stability examination. Arabic numerals are the representative electrophoresis lanes in Figure 2.

Abbreviations		Thrips species	Abbreviations		Thrips species
1	**Dsmi**	***Dichromothrips smithi* (Zimmermann)**	13	Rcru	*Rhipiphorothrips cruentatus* Hood
2	Teuc	*Taeniothrips eucharii* (Whetzel)	14	Bmel	*Bathrips melanicornis* (Shumsher)
3	Bgra	*Bolacothrips graminis* (Priesner)	15	Acha	*Ayyaria chaetophora* Karny
4	**Mabd**	***Microcephalothrips abdominalis* (Crawford)**	16	**Musi**	***Megalurothrips usitatus* (Bagnall)**
5	**Fwil**	***Frankliniella williamsi* Hood**	17	**Focc**	***Frankliniella occidentalis* (Pergande)**
6	Fser	*Fulmekiola serrata* (Kobus)	18	**Fint**	***Frankliniella intonsa* (Trybom)**
7	**Tflo**	***Thrips florum* Schmutz**	19	**Sbif**	***Stenchaetothrips biformis* (Bagnall)**
8	**Thaw**	***Thrips hawaiiensis* (Morgan)**	20	**Sdor**	***Scirtothrips dorsalis* Hood**
9	**Fcep**	***Frankliniella cephalica* (Crawford)**	21	**Tall**	***Thrips alliorum* (Priesner)**
10	Asud	*Anaphothrips sudanensis* Trybom	22	**Tfus**	***Thrips fuscipennis* Haliday**
11	**Tpal**	***Thrips palmi* Karny**	23	**Ttab**	***Thrips tabaci* Lindeman**
12	Srub	*Selenothrips rubrocinctus* (Giard)			

habitat preference, host plant, and *Tospovirus* transmission efficiency, are virtually indistinguishable with morphology (Brunner *et al.*, 2004; Toda & Murai, 2007; Hoddle *et al.*, 2008; Brunner & Frey, 2010; Rugman-Jones *et al.*, 2010; Jacobson *et al.*, 2013).

PCR amplicons from thrips' genomic DNA are commonly used for thrips identification and phylogenetic analysis including the nuclear ribosomal DNA and elongation factor (EF1-α) (Inoue & Sakurai, 2007; Hoddle *et al.*, 2008; Buckman *et al.*, 2013) and mitochondrial DNA, e.g., COI and 16S rDNA, (Lin *et al.*, 2003; Brunner *et al.*, 2004; Asokan *et al.*, 2007; Toda & Murai, 2007; Hoddle *et al.*, 2008). Most of the above mentioned studies have elucidated the phylogenetic relationships among species in a given genus and provided reliable tools for species identification. For example, COI and 28S rDNA sequence data showed that *S. dorsalis* consists of at least three distinct taxa (Hoddle *et al.*, 2008). The internal transcribed spacer (ITS), the non-coding fragment of the nuclear ribosomal region, has been one of the most widely used markers in thrips species identification and population delineation (Liu, 2004; Rugman-Jones *et al.*, 2006; Farris *et al.*, 2010).

PCR-based methods, such as species-specific primer assay (Liu, 2004; Asokan *et al.*, 2007; Farris *et al.*, 2010; Kobayashi & Hasegawa, 2012), restriction fragment length polymorphism (Lin *et al.*, 2003; Rugman-Jones *et al.*, 2006), and real-time PCR (Walsh *et al.*, 2005; Huang *et al.*, 2010) have been widely applied to thrips identification. However, these methods have been focused only on the identification of one or a few thrips species. It is essential to develop a more efficient method for simultaneous screening of mass samples. In the past decade, the microarray assay routinely used in pathogen investigation has been used rarely for insect pest identification (Chung *et al.*, 2011; Yeh *et al.*, 2012; de Luca *et al.*, 2013; Lee *et al.*, 2013).

Many studies have shown that sequence variation in the COI gene within thrips species is generally less than 2%, yet most of these studies did not have comparable data for ITS sequences (Brunner *et al.*, 2004; Asokan *et al.*, 2007; Rugman-Jones *et al.*, 2010; Kobayashi & Hasegawa, 2012; Kadirvel *et al.*, 2013). Glover *et al.* (2010) have pointed out that as compared to an average sequence distance of 23.1% for the COI gene, an average interspecific distance of 54% in the hypervariable ITS region would have a significant advantage for species-level identification in thrips. In this study, therefore, species-specific primers were designed based on the established ITS1 sequences of 15 agriculturally important thrips, including *T. tabaci*, *Thrips hawaiiensis* (Morgan), *Thrips palmi* Karny, *S. dorsalis*, *Frankliniella intonsa* (Trybom), and *F. occidentalis*. The specificity and stability of these primers were examined on a total of 16 thrips species. Moreover, a DNA microarray based on these verified specific sequences provides an efficient and high-throughput method for thrips identification and monitoring. This microarray assay technique using ITS sequences could be widely applied to the rapid identification of large numbers of other agricultural pests as well.

Materials and methods

Thrips specimens were collected between 2004 and 2009 from localities across Taiwan and preserved in 95% ethanol. Fifteen thrips commonly found on agricultural crops, including virus transmission species such as *T. tabaci*, *F. intonsa*, and *S. dorsalis*, were used to develop the specific primers and probes. Additionally, eight thrips were employed for primer specificity examination. Pertinent information for these thrips species is given in table 1.

DNA extraction

Total DNA was extracted from individual thrips using the BuccalAmp™ DNA Extraction Kit (EPICENTRE Biotechnologies, Madison, USA) with instructions modified for thrips (Tseng *et al.*, 2010). Individual specimens of the 23 thrips species, listed in table 1, were immersed in 50 µl DNA Extraction Solution 1.0. After shaking vigorously for 15 s, the sample was incubated at 65°C for 15–20 min, followed by an additional 15 s of shaking. After removing the specimen, the reaction mixture was incubated at 98°C for 2 min and then stored at −20°C. The specimen was subsequently mounted on slide via Hoyer's medium for identification (Han, 1997; Mound & Kibby, 1998; Wang, 2002, 2007) and these voucher specimens are stored at the Laboratory of Molecular Systematics, Department of Entomology, National Chung Hsing University.

PCR and DNA sequencing

Primer pairs used for ITS1 fragment amplification and sequencing were 18Se (5′TCCCTGCCCTTTGTACACAC3′) and 5.8SThR (5′CACAAGCCRAGGGATCCAC3′), which were designed in this study based on conserved fragments

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Table 2. Sequences of species-specific primers and probes and their amplified fragments size for 15 thrips species. Abbreviations of thrips species are the same as in Table 1. Alternative specific probes for four thrips species are shown in footnote (see Materials and methods).

P*	Upstream primer and sequences (5′–3′)		Downstream primer and sequences (5′–3′)		Size
A	Dsmi2F	TAAGAAGGTAGAGACGCTCC	Dsmi2R	GGAATGTCTCTACCTTTCCG	252
B	Dsmi3F	TAAAGGGAGGAGACCGTTTG	Dsmi4R	ACCACAGAGTGCCAAACTAC	384
C	Fcep1F	ATTTCGCGTCGAAGCAACGG	Fcep1R	ATCGGTCCGTTCCGTTCAAC	461
D	Fcep2F	TCACAACGTTCCTACGTATCC	Fcep2R	CTAGCCATACGCTCGATAAGA	238
E	Fint2F	TGCTTGAGCGGAACGAGTG	Fint3R	TCCACATAGCGGCGTGAAAG	424
F	Fint3F	TGGCTTGCTTGAGCGGAAC	Fint2R	TTGGGAGTCCACATAGCGG	436
G	Focc1F	AAATCCACTACGTTCCCGAG	Focc1R	TATGGAGAGGCTCTCGCC	381
H	Focc3F	AGACGGTTCGATTCCACTC	Focc2R	ACGCCCGCACTCTGAAATCA	225
I	Fwil1F	GTCGTACCAAATCATGAGACG	Fwil1R	GCATCGCATCATCTCTGTATG	373
J	Fwil2F	ACCCAGAGCTTTGAATGGTCG	Fwil2R	AACTCCCGTGAGAGAGTTGG	161
K	Mabd3F	GCGGTGCGTGTCATATAAGG	Mabd1R	ATTCTGGTACCGCAACTTCG	215
L	Mabd2F	CGTCTCGGTCAAAACACTC	Mabd2R	TTCCAGGGCCAACTCAAAAG	332
M	Musi1F	TTTTTCTCCGTGTGTCGTCG	Musi1R	CTGACTTTAGACCATTCCGC	376
N	Musi2F	TGCTTCGTGTCTTCTGTTCC	Musi2R	TGCATCTCTTAACTCTCCGG	179
O	Sbif1F	ACGAGATTGGATGCACTGC	Sbif1R	CACTGTTTAAAACTCGACGGC	318
P	Sbif2F	AATAATCATGCGCACCCACG	Sbif2R	TCCTTGCACTTCATTGCTTG	138
Q	Sdor1F	GGAGATGCTCTGACGAAAAGC	Sdor1R	AGAGCCGCTCATACTTAGG	305
R	Sdor2F	ACCATGAGATTTTTTCCGACC	Sdor2R	GTGAGGCGCGGATACTAG	407
S	Tall1F	AGAAGCCCGACGACTTCC	Tall1R	ATGCCATGGGACTTCAACG	253
T	Tall2F	AGTTCTCGATATGAGTGGCC	Tall2R	TTCTTAGCAAGTCTCGGAGG	325
U	Tflo1F	TCTTTCGGTTATCAGACTCGC	Tflo1R	AGAGCCGACCATTGAAAATGG	413
V	Tflo2F	ATACCCGATCTGTGCTCACG	Tflo2R	GAGGCTCCATTCCTAGAGAG	313
W	Tfus1F	TACAAGTCCCTCGTGGATCC	Tfus1R	AGTGTTGAAAACCGAGCACC	234
X	Tfus2F	GGTCCTCTTGCTCTGTCG	Tfus2R	TGGTTCGAGGAGAATCAAACC	171
Y	Thaw3F	TTGAGCACGTGCCCTGAGG	Thaw3R	ACCTGCCAAGTCCCTTTGC	266
Z	Thaw4F	TATCACCCACGTGATTCCG	Thaw4R	GCTTCATCTGTCCGTCTGG	355
a	Tpal1F	ACCAGTCGGCTTCACCACG	Tpal1R	TTCGGTTCGTTTTGGTAAGGG	148
b	Tpal2F	GGGTGCCTGTTCTCCAAAA	Tpal2R	CGCCTTCGAAGAACTTGGAA	304
c	Ttab1F	TCTAAACAGAGGGAAAGGTG	Ttab1R	AGTGTGCCAACAAGGCAATG	417
d	Ttab2F	ACTTGACTCGAAGTCACGG	Ttab2R	TAAAGGGCGAACCTCTCGAG	386

* Panel in fig. 2
Dsmi1F: 5′TCTGTGGTTCGAATAAGTCCC3′; Dsmi3R: 5′ATTTTTGTTTGCCCGACTCCC3′; Focc2F: 5′TCAGAGACGGTTCGATTCC3′; Sbif3F: 5′TGGGGCCTGAACTCGAATC3′; Sbif3R: 5′TTTCGGCGCGTTATAAACGC3′; Tpal3F: 5′ACGAACCGAAAGACGAGAAAC3′; Tpal4F: 5′TGCTTCCAAGTTCTTCGAAGG3′.

of 18S rDNA and 5.8S rDNA (Tautz *et al.*, 1988; Kjer *et al.*, 1994). PCR assay was carried out in a volume of 25 μl, with the following programming conditions: 95°C for 2 min for the first denaturation, followed by 35 cycles of 94°C for 40 s, 50°C for 50 s and 72°C for 1 min, with a final extension at 72°C for 10 min. The amplified product was purified directly using a PCR purification kit (Quiagen, Hilden, German), or after resolving on agarose gel, excised and extracted with the Qiaquick gel extraction kit. The resulting DNA product was sequenced in both directions using BigDye Terminator V3.1 Cycle Sequencing Kit and an ABI 3730XL sequencer (Applied Biosystems, California, USA).

Sequence analysis

Forty-three ITS1 sequences of the 15 target thrips species were aligned with 28 sequences of 21 thrips retrieved from GenBank, including *Echinothrips americanus* Morgan, *F. intonsa*, *F. occidentalis*, *Frankliniella schultzei* (Trybom), *Neohydatothrips geminus* (Hood), *Neohydatothrips burungae* (Hood), *Haplothrips chinensis* Priesner, and 14 *Scirtothrips* species, using the program MAFFT (Katoh *et al.*, 2005) or MUSCLE (Edgar, 2004) and manual editing. Pairwise distance was estimated using uncorrected proportional divergence with MEGA5 (Tamura *et al.*, 2011).

Design of species-specific primers and multiplex PCR

Species-specific primers were developed from the variable regions, i.e., sequences that could not be adjusted to be conserved among thrips species, including those acquired in this study and those from GenBank. Two species-specific primer pairs with Tm around 60°C and a size of 20 to 30 bp were designed for each of the target thrips (table 2), and their specificity and stability were examined on 16 thrips species of different genera (table 1). A multiplex PCR method was adopted using the species-specific primers combined with one universal primer pair. PCR conditions for testing the specificity and stability of these primer sets were the same as those employed in ITS1 amplification, except that the extension time (at 72°C) was shortened from 50 to 30 s. Moreover, the universal primer pair 28Sg and 28Sh (Lin *et al.*, 2003) were used in each multiplex reaction. Products were visualized on agarose gel.

Probe design

Based on the verified specific primers in multiplex PCR, species-specific oligonucleotide probes with 5′ biotin labeling were synthesized. One control probe, i.e., Thrips-I1-1U, from the DR Thrips™ C8 Kit (DR. Chip Biotechnology Inc., Taiwan) was used to confirm normal hybridization, and two universal

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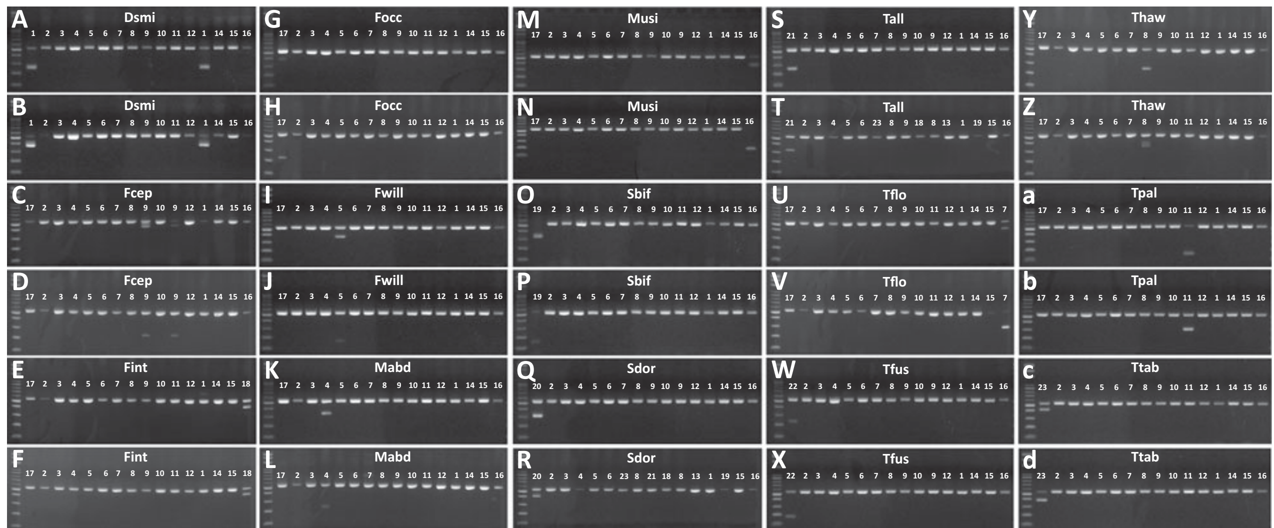

Fig. 1. Application of multiplex PCR by one ITS1 species-specific primer set of each thrips species with 28S rDNA universal paired primers. Specific amplification fragment is visible in target thrips with no cross amplification. Panels A and B: *Dichromothrips smithi*; C and D: *Frankliniella cephalica*; E and F: *F. intonsa*; G and H: *F. occidentalis*; I and J: *Frankliniella williamsi*; K and L: *M. abdominalis*; M and N: *Megalurothrips usitatus*; O and P: *Stenchaetothrips biformis*; Q and R: *S. dorsalis*; S and T: *Thrips alliorum*; U and V: *T. florum*; W and X: *T. fuscipennis*; Y and Z: *T. hawaiiensis*; a and b: *T. palmi*; c and d: *T. tabaci*. The first lane is 100 bp DNA ladder, and the examined thrips species are listed in Table 1.

thrips probes, i.e., 18Se and 5.8SThR, served as positive controls. When the specific probes yielded a weak signal or showed cross hybridization, alternative specific probes, i.e., Dsmi1F, Dsmi3R, Focc2F, Sbif3F, Sbif3R, Tpal3F and Tpal4F, were used (table 2).

Microarray chip construction

The polymer substrate and colorimetric reagents in microarray test were provided by DR Chip DIY™ Kit (DR. Chip Biotechnology Inc., Taiwan). The probe solution (20 µM), prepared by mixing the 40 µM oligonucleotide probe with 2× probe solution, was spotted on the surface of polymer membrane using the DR Fast Spotter (DR. Chip Biotechnology Inc., Taiwan); four specific probes were used for each thrips species. After the spots dried, the microarray plate was put in a UV crosslinker to immobilize the probes. With 500 µl distilled water infused into each well for 5–10 min, 95% EtOH was added and then removed. The wells were allowed to dry at 45°C.

Microarray hybridization and scanning

Microarrays were hybridized, washed and detected using the DR Chip DIY™ Kit. The spotted wells were immersed with 200 µl hybridization buffer. A 10 µl aliquot of the target PCR product generated from paired primers of 18Se and 5.8SThR for individual thrips, denatured at 94°C for 5 min and then chilled on ice, was added to the well. The microarray was then incubated at 45°C in the oven with vibration for 60 min. After removing the hybridization buffer, the well was washed by 250 µl wash buffer three times. The blocking solution, i.e., 0.2 µl Strep-AP mixed with 200 µl blocking reagent, was added in each well for 30 min, and then the well was washed with wash buffer three times. Detection solution, i.e., 4 µl NBT/BCIP mixed with 196 µl detection buffer, was added to the well for 5–10 min. The detection solution was then drawn away, and the well was washed with distilled water. The hybridized pattern was detected using DR. AIM™ reader (DR. Chip Biotechnology Inc., Taiwan).

Results

ITS1 sequence variation within and among thrips species

A total of 43 ITS1 sequences, ranging from 800 to 1250 bp, for 15 thrips species were obtained by PCR and have been deposited in GenBank (AB904169–AB904212). With ten sequences from GenBank for *F. occidentalis*, *F. intonsa*, and *S. dorsalis* included in the analysis, average sequence variation within species is less than 1%, except for those of *S. dorsalis* and *T. palmi* which are 11 and 3.5%, respectively. On the other hand, interspecific sequence distances were much higher than intraspecies distances, ranging from 15 to 56% (table 2). Deep phylogenetic divergences were found in thrips species, though there is a close relationship among *Frankliniella* species and between *Thrips fuscipennis* and *Microcephalothrips abdominalis* (data not shown).

Specificity and stability of specific primers

An examination of primer specificity and stability on 16 thrips species (table 1) shows the expected amplified products in target species with no cross amplifications (fig. 1). For each reaction, the successful generation of a PCR product of 520 bp by universal primers of 28S rDNA ensures a qualitative control for the entire experimental process. A few reactions with weak signals were likely due to competition or interference between primer pairs.

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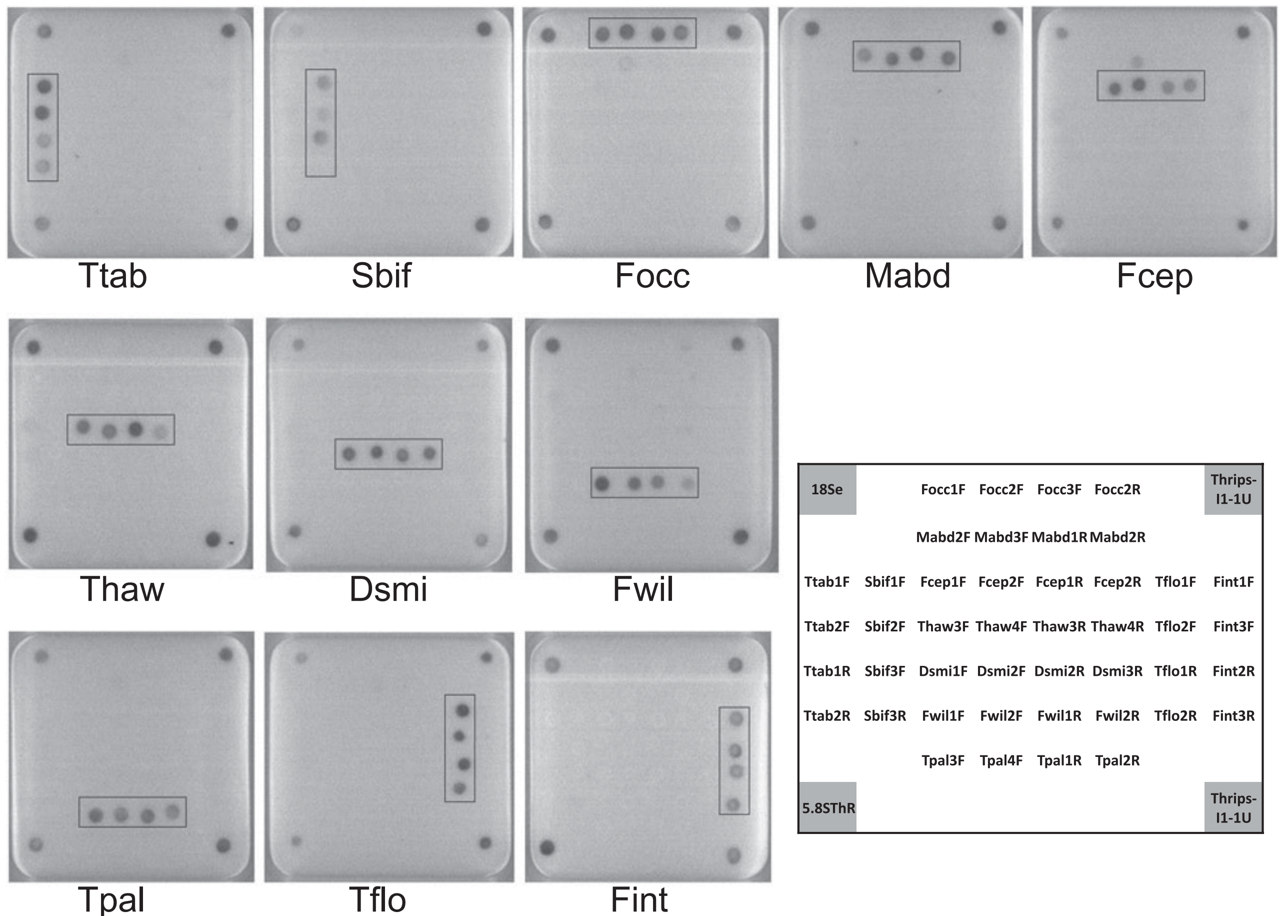

Fig. 2. Hybridization pattern of microarray for 11 thrips species. The representative probes and spotted positions are shown in the right-bottom panel. Four spots in the corner are the positive controls and the hybrid signals of each target thrips are labeled with a box. Abbreviations for thrips species and their probes are as in Tables 1 and 2.

DNA microarray for thrips identification

With two microarrays spotted with 11 and 15 thrips and four specific probes tested for each thrips species, all representative PCR products hybridized consistently to their corresponding probes. Probe Sbif3R, however, failed to detect Sbif PCR products (fig. 2), However, it showed no cross amplification in multiplex PCR (fig. 1). Probe Mabd3F showed weak signal in false hybridization to Fcep PCR products, and cross hybridization was observed for Tfus PCR product to probe Mabd (fig. 3).

Discussion

The high variability of ITS1 suggests that it can be used for developing species-specific primers and probes for thrips identification. However, the sometimes low sequence divergence, e.g., between Mabd and Tfus and among *Frankliniella* species, may hamper species-specific primers designation. Fortunately, this examination of 16 thrips species confirms the specificity and stability of these primers (fig. 1). PCR-based identification for thrips species has been applied in a number of studies (Lin *et al.*, 2003; Liu, 2004; Rugman-Jones *et al.*, 2006; Asokan *et al.*, 2007; Farris *et al.*, 2010; Huang *et al.*, 2010; Kobayashi & Hasegawa, 2012); however, none of them has introduced the internal control using universal primers pairs as we have done in this study, i.e., 28S rDNA, to ensure the DNA-template quality and optimal experimental procedures.

In the microarray assay, the weak signals shown by several hybridized spots might have resulted from an irregular manipulation of the spotted needles when spotting probes onto the polymer membrane. In the same microarray, some positive spots showed strong signal while others were weak or nearly invisible (fig. 3). Inconsistent signal intensity may have been due to the ΔT_m values of probes (table 2). High sequence similarity between species might have increased the possibility of cross hybridization, as observed for target DNA of *T. fuscipennis* on the probes of *M. abdominalis* (fig. 3). Both phylogenetic relationships and sequence divergence (table 3) have revealed the close affinity between Mabd and Tfus. Regarding the possible mis-identification based on weak signal or cross hybridization, this study has adopted multiple probes for each thrips species in order to improve the accuracy of identification. In conclusion, we have demonstrated that multiplex PCR using universal primers with species-specific primers based on ITS1 sequences is a reliable, convenient and cost-effective diagnostic method to discriminate thrips species.

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Table 3. Average sequence divergences between thrips species.

	Dsmi	Fcep	Fint	Focc	Fwill	Mabd	Musi	Sbif	Sdor	Tall	Tflo	Tfus	Thaw	Tpal	Ttab
Dsmi															
Fcep	0.497														
Fint	0.497	0.219													
Focc	0.540	0.204	0.181												
Fwil	0.488	0.197	0.188	0.152											
Mabd	0.482	0.470	0.446	0.501	0.419										
Musi	0.495	0.473	0.474	0.544	0.459	0.497									
Sbif	0.542	0.513	0.501	0.545	0.493	0.498	0.565								
Sdor	0.524	0.444	0.434	0.413	0.396	0.530	0.532	0.495							
Tall	0.491	0.451	0.432	0.496	0.409	0.416	0.488	0.511	0.502						
Tflo	0.501	0.429	0.411	0.484	0.391	0.362	0.494	0.455	0.480	0.392					
Tfus	0.409	0.418	0.403	0.461	0.332	0.148	0.378	0.443	0.413	0.335	0.300				
Thaw	0.461	0.409	0.393	0.457	0.374	0.352	0.476	0.442	0.475	0.354	0.181	0.291			
Tpal	0.529	0.510	0.483	0.510	0.475	0.447	0.537	0.475	0.526	0.436	0.421	0.388	0.428		
Ttab	0.548	0.484	0.481	0.519	0.466	0.440	0.532	0.516	0.530	0.465	0.421	0.373	0.429	0.499	

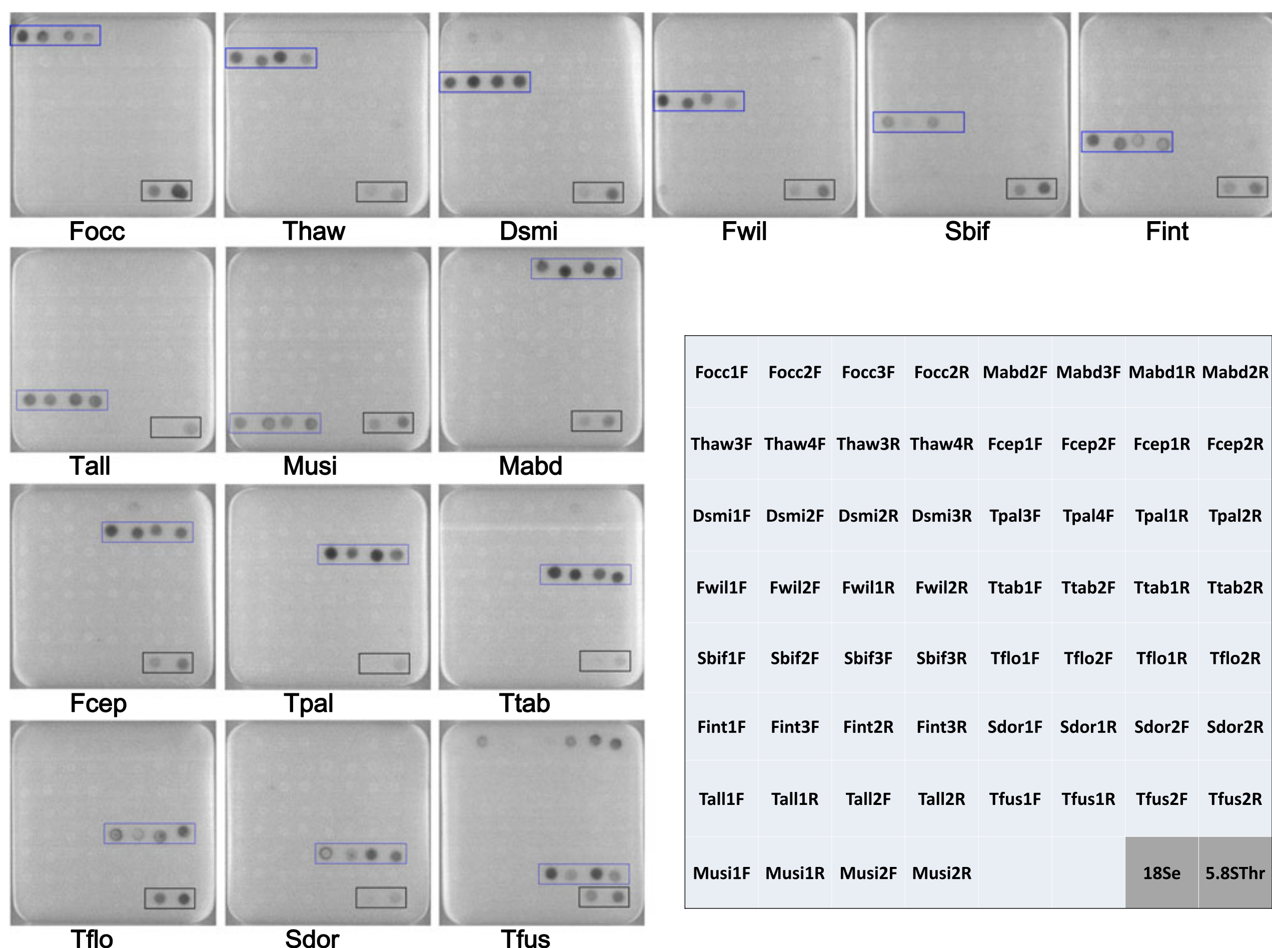

Fig. 3. Hybridization pattern of microarray for 15 thrips species. The representative probes and spotted positions are shown in the right-bottom panel. Two spots in the bottom-right corner box are the positive controls and hybrid signals of each target thrips are labeled with a box. Abbreviations for thrips species and their probes are as in Tables 1 and 2.

Moreover, the microarray assay appears to be a comprehensive tool for the simultaneous identification of a number of thrips species. Of the ca. 6000 described species of thrips throughout the world, approximately 2% have been considered as crop pests (Inoue & Sakurai, 2007; Mound & Morris, 2007), hence developing a high-throughput detection system with probes for the vast number of thrips pest in a single microarray is a worthwhile pursuit.

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