Formosan Entomologist
Journal Homepage: entsocjournal.yabee.com.tw

Multiplex PCR for the Molecular Identification of Economically Important Thrips 【Research report】

複合性PCR於經濟重要性薊馬的分子鑑定【研究報告】

Wen-Bin Yeh1*, Cindy Sin-Ying Wu1, Mei-Jung Tseng1, Niann-Tai Chang2, and Wen-Ping Chen3
葉文斌1*、吳昕縈1、曾美容1、張念台2、陳文彬3

*通訊作者E-mail : wbyeh@nchu.edu.tw

Received: 2014/11/11 Accepted: 2015/02/23 Available online: 2015/03/01

Abstract

Thrips are major pests of many agricultural crops all over the world. However, their minute size and high degree of morphological similarity often makes their identification difficult. Molecular methods, such as single-step and multiplex PCR, deserve more consideration for their identification. In this study, ITS2 sequences of 14 agronomically important thrips were analyzed. Low intraspecific distances (i.e., < 3%) in contrast with high interspecific distances (i.e., > 14%) makes ITS2 sequences an ideal marker for designing species-specific primers for thrips identification. The specificity and stability of these species-specific primers, mixed with universal paired primers as an internal positive control, were tested and verified using multiplex PCR on 23 thrips species. In conclusion, the highly variable features of ITS2 sequences among thrips species are useful in the development of species-specific primers for multiplex PCR. It provides a detection tool that allows for rapid, reliable and convenient species discrimination of the important insect pest.

摘要

薊馬是世界上許多農作的重要害蟲，常因其體形微小及形態上的高度相似而難以鑑定，單一步驟複合性PCR的分子方法遂被應用於薊馬的鑑定。本研究分析14種農作上重要性薊馬的核醣體第二區間 (ITS2) 序列，種內變異小於3% 遠低於種間14% 以上的變異，使得此ITS2序列非常適合開發專一性引子用以鑑定這些薊馬。這些專一性引子與具內控制組特性的廣效性引子混合，進行23種薊馬的複合性PCR檢測，均顯示出其專一性及穩定性。總結來說，薊馬物種核醣體第二區間的高變異特性，可開發專一性引子用於複合性PCR檢測，提供了一個可靠、方便、快速的鑑定方法，區辨這些重要薊馬害蟲。

Key words: thrips, ITS2, specific primer, internal positive control
關鍵詞: 薊馬、核醣體第二區間、專一性引子、內控制引子。

Full Text: PDF(1.65 MB)

台灣昆蟲 34: 211-225 (2014)
Formosan Entomol. 34: 211-225 (2014)

Multiplex PCR for the Molecular Identification of Economically Important Thrips

Wen-Bin Yeh[1*], Cindy Sin-Ying Wu[1], Mei-Jung Tseng[1], Niann-Tai Chang[2], and Wen-Ping Chen[3]

[1] Department of Entomology, National Chung Hsing University; 250 Kuo-Kuang Rd., South District, Taichung City 40227, Taiwan
[2] Department of Plant Medicine, National Pingtung University of Science and Technology; 1 Shuefu Rd., Neipu, Pingtung County 91201, Taiwan
[3] Yeastern Biotech Co. Ltd., 6F-3, 23 Lane169, Kang Ning St., Shijr, New Taipei City 22180, Taiwan

ABSTRACT

Thrips are major pests of many agricultural crops all over the world. However, their minute size and high degree of morphological similarity often makes their identification difficult. Molecular methods, such as single-step and multiplex PCR, deserve more consideration for their identification. In this study, ITS2 sequences of 14 agronomically important thrips were analyzed. Low intraspecific distances (*i.e.*, < 3%) in contrast with high interspecific distances (*i.e.*, > 14%) makes ITS2 sequences an ideal marker for designing species-specific primers for thrips identification. The specificity and stability of these species-specific primers, mixed with universal paired primers as an internal positive control, were tested and verified using multiplex PCR on 23 thrips species. In conclusion, the highly variable features of ITS2 sequences among thrips species are useful in the development of species-specific primers for multiplex PCR. It provides a detection tool that allows for rapid, reliable and convenient species discrimination of the important insect pest.

Key words: thrips, ITS2, specific primer, internal positive control

Introduction

Thrips are major pests of many agricultural plants all over the world. They damage the host plant by attacking its twigs, leaves, buds, flowers and fruit, resulting in stunted growth, feeding scars, distortions, and color mosaic. In addition, they are the sole vectors transmitting the plant *Tospovirus* which causes great economic loss. For example, *Thrips tabaci* Lindeman, *i.e.*, onion thrips, can also transmit the *Iris yellow spot virus* resulting in a yearly loss of more than 100

*Corresponding email: wbyeh@nchu.edu.tw

million USD (Prins and Goldbach, 1998; Gent *et al*., 2006). In order to include them in pest management programs, these tiny organisms must first be detected and correctly identified. Traditionally, the identification of thrips has been based on the morphological characters of adult specimens using slide mounting and microscopic examination. However, their minute size and high degree of morphological similarity usually makes identification difficult, and it is even more difficult to identify immature thrips to the species level. For example, a cryptic species complex such as *Scirtothrips dorsalis* Hood, *T. tabaci* and *Frankliniella occidentalis* (Pergande), can only be properly identified by their molecular characteristics, since their morphological characters are indistinguishable (Brunner *et al*., 2004; Toda and Murai 2007; Hoddle *et al*., 2008; Brunner and Frey, 2010; Rugman-Jones *et al.*, 2010).

Among the molecular markers applicable for insect identification (Hoy, 2003), mitochondrial COI has been commonly used since the development of the barcode of life (Glover *et al.*, 2010; Rugman-Jones *et al.*, 2010; Kadirvel *et al*., 2013). For example, it has been demonstrated, with the use of this marker, that *T. tabaci* includes three distinct groups (Bruner *et al*., 2004; Asokan *et al*., 2007; Toda and Murai, 2007; Tseng *et al*., 2010; Kobayashi and Hasegawa, 2012; Jacobson *et al*., 2013). The analysis of another amplicon, internal transcribed spacer 2 (ITS2), a nuclear non-coding ribosomal fragment, has shown that cryptic species of *S. dorsalis* Hood involve different lineages (Rugman-Jones *et al*., 2006). Glover *et al.* (2010) pointed out that the hypervariability of ITS may be considered an advantage for insect identification, while low interspecific distances in the COI gene will not distinguish closely related species (Chen *et al*., 2014; Lee *et al.*, 2013).

In view of the experimental time and cost, PCR-based methods such as real-time PCR (RT-PCR), restriction fragment length polymorphism (RFLP), and species-specific primers (SSP) have been applied widely in thrips identification (Liu 2004; Rugman-Jones *et al.*, 2006; Asokan *et al*., 2007; Farris *et al*., 2010; Huang *et al*., 2010). In addition, studies have demonstrated that the single-step PCR with SSP is a simple, quick, and reliable means for thrips identification (Lin *et al*., 2003; Asokan *et al.*, 2007; Farris *et al*., 2010; Kobayashi and Hasegawa, 2012). However, the use of universal primers pairs as the internal positive control (a practice routinely carried out in research on pathogens, such as viruses and bacteria, to ensure the quality of DNA templates as well as the optimum of experimental procedures) has been rarely adopted in insect studies (Li *et al*., 2010; Daane *et al*., 2011; Thet-Em *et al*., 2012; Yao *et al*., 2012; Yeh *et al*., 2012; Drali *et al.*, 2013; Nakamura *et al*., 2013).

Therefore, the aim of this study was to apply the multiplex PCR method and combine the species-specific primers with universal paired primers for thrips identification. The specific primers were designed based on the established ITS2 sequences of 14 agricultural thrips species including *Echinothrips americanus* Morgan and *Thrips vulgatissimus* Haliday. The specificity and stability of the developed species-specific primers of 23 thrips species were then examined using the previously developed universal primers as a positive control. Based on the results, we then addressed the superiority of our proposed identification method compared with other PCR-based identification methods.

Materials and Methods

Sample Collection and Identification

The thrips used in this study (Table 1) were collected from England, Italy, mainland China, The Netherlands, Taiwan, Thailand, and the USA, preserved in 95% ethanol, and stored at -20ºC. Individual specimens,

Table 1. Thrips species used to evaluate the specificity and stability of specific primers and the corresponding lanes (L) on electrophoretogram in figures

L[a]	Abb	Thrips species	L	Abb	Thrips species
1	Aobs	*Anaphothrips obscurus* (Müller)	7	Thaw	*Thrips hawaiiensis* (Morgan)
1	Aruf	*Aptinothrips rufus* (Haliday)	8	Bgra	*Bolacothrips graminis* (Priesner)
1	Asud	*Anaphothrips sudanensis* Trybom	9	Tfus	*Thrips fuspennis* (Haliday)
1	Cpha	*Caliothrips phaseoli* (Hood)	10	Teuc	*Taeniothrips eucharii* (Whetzel)
1	Eame	*Echinothrips americanus* Morgan	11	Tfla	*Thrips flavus* Schrank
1	Ffus	*Frankliniella fusca* (Hinds)	12	Fser	*Fulmekiola serrata* (Kobus)
1	Ftri	*Frankliniella tritici* (Fitch)	13	Fcep	*Frankliniella cephalica* (Crawford)
1	Nbur	*Neohydatothrips burungae* (Hood)	14	Rcru	*Rhipiphorothrips cruentatus* Hood
1	Skar	*Stenchaetothrips karnyrianus* (Priesner)	15	Fint	*Frankliniella intonsa* (Trybom)
1	Sund	*Stenchaetothrips undatus* Wang	16	Dcor	*Dichromothrips corbetti* (Zimmermann)
1	Tfor	*Thrips formosanus* Priesner	17	Acha	*Ayyaria chaetophora* Karny
1	Tmaj	*Thrips major* Uzel	18	Aauc	*Astrothrips aucubae* Kurosawa
1	Tphy	*Thrips physapus* Linneaeus	19	Asud	*Anaphothrips sudanensis* Trybom
1	Tvul	*Thrips vulgatissimus* Haliday	20	SCI	*Scirtothrips sp*
2	Focc	*Frankliniella occidentalis* (Pergande)	21	Sbif	*Stenchaetothrips biformis* (Bagnall)
3	Ttab	*Thrips tabaci* Lindeman	22	Srub	*Selenothrips rubrocinctus* (Giard)
4	Mabd	*Microcephalothrips abdominalis* (Crawford)	23	Hcom	*Helionothrips communis* Wang
5	Tpal	*Thrips palmi* Karny	24	---	Negative control
6	Musi	*Megalurothrips usitatus* (Bagnall)			

[a] Target species used for specific-primer design, i.e. Lane 1, and the thrips used to examine specific-primer stability, i.e. Lanes 2 to 23, are shown in representing panels of Figure 2 and Figure 3.

listed in Appendix I, were used for DNA extraction and then mounted on slides using Hoyer's medium for light microscopy identification. These voucher thrips were then stored in the Department of Entomology, National Chung Hsing University.

DNA Extraction

Genomic DNA was extracted using the BuccalAmp™ DNA Extraction Kit (EPICENTRE Biotechnologies, Madison, USA) with the instructions modified for thrips (Tseng *et al*., 2010). The DNA pellet was dissolved in 30 μL distilled water and stored at -20°C.

PCR and DNA Sequencing

Primers P1-2 and 28Sj2 were used to amplify and sequence the ITS2 fragment (Tseng *et al.*, 2010). A PCR assay was then carried out in a volume of 25 μL containing 5 pmoles of each primer, 0.2 mM of each dNTP, 10 mM Tris-Cl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton-X100, 1 unit of SuperTaq polymerase (Protech Technology, Taipei, Taiwan), and 1 μL of DNA template. PCR programming conditions were: 95°C for 2 min for denaturation, followed by 35 cycles of 94°C for 40 sec, 60°C for 20 sec and 72°C for 20 sec, with a final extension at 72°C for 10 min. The amplified products were stored at 4°C. The PCR product was purified directly using a Qiaquick PCR purification kit (QIAGEN, Hilden, German), or extract it using the Qiaquick gel extraction kit after having been resolved

Table 2. Species-specific paired primers, sequences (5' to 3'), and amplified approximately fragments size of fourteen thrips species. PCR amplifications of the bold paired primers are shown in Figure 2, while the others are shown in Figure 3. Abbreviations of each thrips are the same in Table 1

Upstream primer name and sequences		Downstream primer name and sequences		size
Aobs_1U	**TGCTAAGGGCTCCTCGGAG**	**Aobs_1D**	**CGCGAGGTGATCGCTTTCC**	**399**
Aobs_4U	TCTCTGAGTGGTACTCGG	Aobs_4D	ACAAGCTTCGGACTCTCC	208
Aruf_1U	**TCACGCTTGTAAAATACTCGG**	**Aruf_4D**	**CGACCACGGATCCCAAAAC**	**169**
Asud101_2U	**TCCCAAATGCCTTACATATACG**	**Asud101_2D**	**AGTGTTTGCGGGCGATAAGG**	**310**
Asud101_3U	ATCGGATAGAGGCCCTCTC	Asud101_3D	AAGCCATCCGTGACTTGACC	243
Cpha_3U	**GGAGCTTTCGAGTTTCCAGG**	**Cpha_3D**	**ACTTCTGGCCTTCACGTTGG**	**185**
Cpha_2U	TCTGTGATAGGAGCGGTGG	Cpha_2D	ATGGCAGAATGGCGTGAAGC	159
Eame1U	**TTGTGTGCCGGGCGATGTTG**	**Eame3D**	**ACTAGCTCGGGCAAAACTGG**	**324**
Eame3U	TCGTCTCAACCGCCCAAG	Eame4D	AGCACTCTTGCCGTCAGAAC	183
Ffus_1U	**TCTTTTGCCAAAATACAGCGG**	**Ffus_1D**	**AATCGCACCGAGGCGAGAC**	**232**
FtriI_2U	**GTTCTGAAATATTTTCAGAGCG**	**Ftri_4D**	**TCTGCTGACATCCTTTATAAGA**	**162**
Nbur_1U	**TCGCCTGAATTAGCGCGAC**	**Nbur _3D**	**TGCCCTTGTTCCCTAGGGG**	**180**
Nbur _5U	CCTTCGGGTGCCAATCGA	Nbur _4D	CGGACGTTCTGTTTCTCTC	169
Skar_1U	**ATCTTACGCGTTTTGCACCC**	**Skar_2D**	**AGGTAGTCTTTAAGGTTTTCCC**	**189**
Skar_3U	TCAAACCCGTTAGTAAAGGG	Skar_3D	TTTCCCTCTCGTACCTCAACG	146
Sund_1U	**TCACTAAGAGTTAGCTTCGGC**	**Sund_3D**	**CGTTGCACCGAAATGCAAGC**	**185**
Sund_2U	GCTGTGTGTCTTACACGCAG	Sund_2D	GTCGACGGCTAGTTAGGAGG	210
Tfor_1U	**GTAGACGGCAAAAATTTACGG**	**Tfor_1D**	**CGTGTGGATGCAAAAGTCCC**	**295**
Tfor_3U	GCAAAAATTTACGGACCAGTCG	Tfor_2D	CGGCTGCATACATCTCCCTA	451
Tmaj_2U	**TCAAGGAGAACATCCATACC**	**Tmaj_1D**	**GATCGTTGGATCGTATGAGG**	**209**
Tmaj_3U	AAATCGTCAAGGAGAACATCC	Tmaj_3D	AACGGGGTTGCATCGGACC	125
Tphy_3U	**AAAGGCGGTCCTAACACTCT**	**Tphy_3D**	**ACAAACCCTTATTGTCGTCCC**	**338**
Tphy_4U	CCTCTGCGATTGCGATAAC	Tphy_4D	GTCAACCAGAAGCGAAGAGG	272
Tvul_1U	**ATGACAAGTCATGTTGCATCG**	**Tvul_1D**	**CCTTATAGCTAAAGAGCCGG**	**170**
Tvul_5U	TGCGATGACAAGTCATGTTG	Tvul_2D	GGACAGCGCCCTTTACCGA	148

on the agarose gel. The purified DNA product was sequenced by primers P1-2 and 28Sj2 using a BigDye Terminator V3.1 Cycle Sequencing Kit and an ABI 3730XL sequencer (Applied Biosystems, California, USA). PCR conditions for specific primer pairs used in the specificity and stability tests were the same as those in the ITS2 amplification. In addition, the universal paired primers of 28Sg and 28Sh (Lin *et al*., 2003), based on the conserved 3' region of 28S rDNA (Kjer *et al*., 1994), were processed in each PCR reaction. Five μL of amplification product with DNA dye and 5 μL of Bio-100^{TM} mass DNA ladder (Protech Technology, Taipei, Taiwan) were visualized on agarose gel.

Sequence Analysis and Species-specific Primers Design

Sequences were piled up and aligned using the program BioEdit (Hall, 1999) and MUSCLE (Edgar, 2004), and were checked manually. The proportional variations within and among the thrips species were estimated with MEGA6 (Tamura *et al*., 2013). A phylogenetic tree based on the Neighbor-joining method using the Kimura-2-parameter substitution was established in MEGA6 with 1,000 bootstrap replications.

The species-specific primers were developed based on the variable regions of the aligned sequences acquired in the present study and from the GenBank. Two

Table 3. Average sequence divergences between/within thrips species in percentage (%). Abbreviations of each thrips are the same in Table 1

	Aobs	Asud	Aruf	Cpha	Emae	Nbur	Sund	Skar	Tfor	Tphy	Tvul	Tmaj	Ffus	Focc	Ftri
Aobs	0.2														
Asud	19.0	0													
Aruf	42.1	45.4	0.7												
Cpha	37.1	40.5	43.0	0.5											
Eame	41.8	44.2	38.8	44.3	2.1										
Nbur	37.0	38.6	41.6	40.2	33.6	2.4									
Sund	45.3	47.0	48.1	45.8	49.3	45.3	0								
Skar	41.1	44.7	44.0	40.1	45.4	43.2	50.6	2.4							
Tfor	48.0	46.9	46.6	45.9	49.5	48.1	55.2	39.1	0						
Tphy	40.8	42.2	41.4	41.9	47.7	44.9	49.5	40.4	43.7	0.1					
Tvul	36.0	38.4	40.6	39.0	44.1	41.7	46.1	38.6	41.6	37.8	0.1				
Tmaj	33.4	37.3	41.8	34.2	39.4	35.5	44.8	40.0	43.7	38.4	32.7	0.1			
Ffus	41.7	44.2	39.5	44.0	36.6	35.9	47.5	44.0	47.8	42.4	40.6	40.4	3.2		
Focc	40.3	44.0	37.7	43.7	33.2	33.6	45.6	41.4	45.2	40.5	39.2	37.8	14.3	0.9	
Ftri	39.5	43.2	35.3	42.6	36.9	32.3	48.0	40.8	47.1	39.2	37.2	37.2	17.6	17.1	1.5

species-specific primer sets were designed for each species (Table 2). The specificity and stability of these primer sets were examined for 23 thrips species of different genera (Table 1).

Results and Discussion

ITS2 sequence variation within and among thrips species

ITS2 sequences, obtained by the universal primer sets of 119 individuals of 14 thrips species having a length ranging from 560 bp to 770 bp were deposited to the GenBank (AB972959-AB973077). The average sequence variations within species ranged from 0 to 3.2% while the sequence variations among species ranged from 14.3 to 55.2% (Table 3). The phylogenetic tree showed that the interspecific sequence divergences were much higher than the intraspecies divergences, indicating that the ITS2 amplicon would have a significant advantage in developing species specific primers for thrips identification (Fig. 1). Although there are deep phylogenetic divergences among thrips species, there is a close relationship among the *Frankliniella* species (with 14.3 to 17.6% sequence divergence) and between *Anaphothrips obscurus* and *A. sudanensis* (with 19.0%) (Fig. 1). In addition, no distinct lineages corresponding to geographical areas or ecological niches within thrips were observed in this study, although differentiated clades in ITS amplicon in *S. dorsalis* have been reported (Rugman-jones *et al.*, 2006).

Specificity and stability of specific primers

A test of 23 thrips species for primer specificity and stability showed the expected PCR products in the target species with no cross amplifications (Fig. 2, 3). A PCR product of 520 bp generated from the universal primers of 28S rDNA, as an internal positive control, proved that the entire experimental procedures were performed under optimal conditions. It is worthwhile noting that an examination of 23 thrips confirmed the specificity and stability of the primers for the three closely related species of *Frankliniella* (Fig. 1).

Specific primers developed by Farris *et al*. (2010) for *S. dorsalis* have been

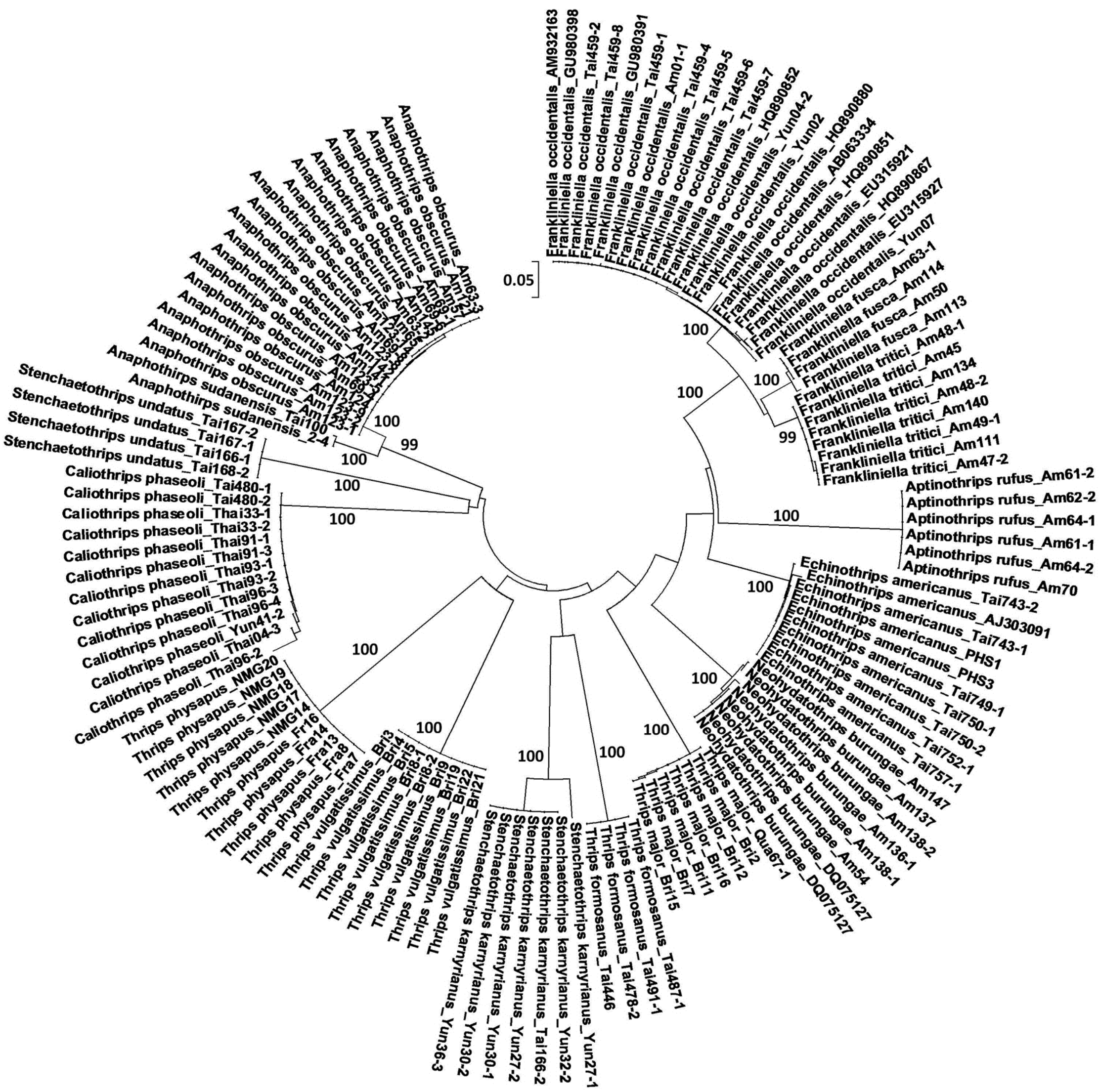

Fig. 1. Phylogenetic inference based on ITS2 sequences by the neighbor-joining clustering method using Kimura 2-parameter substitution processes with 1,000 bootstrap replications. High bootstrap values for each thrips species are shown beneath the branches. Sequences retrieved from the GenBank are labeled with their accession numbers. Scale is the relative proportion divergence.

proven satisfactory, with only rare amplification failure for this species and occasional cross amplification to other thrips species. Yeh *et al*. (2014) pointed out that a specific primer with four or fewer nucleotide mismatches to its target DNA would have little impact on the robustness of that specific primer. In the present study, the efficacy of intraspecific variation was illustrated for thrips *A. obscurus* and *E. americanus*. All these individuals generated products of the expected size (Fig. 4).

In multiplex PCR, it has been pointed out that the amplification efficiency might be associated with the competition or interference between primer pairs (Yeh *et*

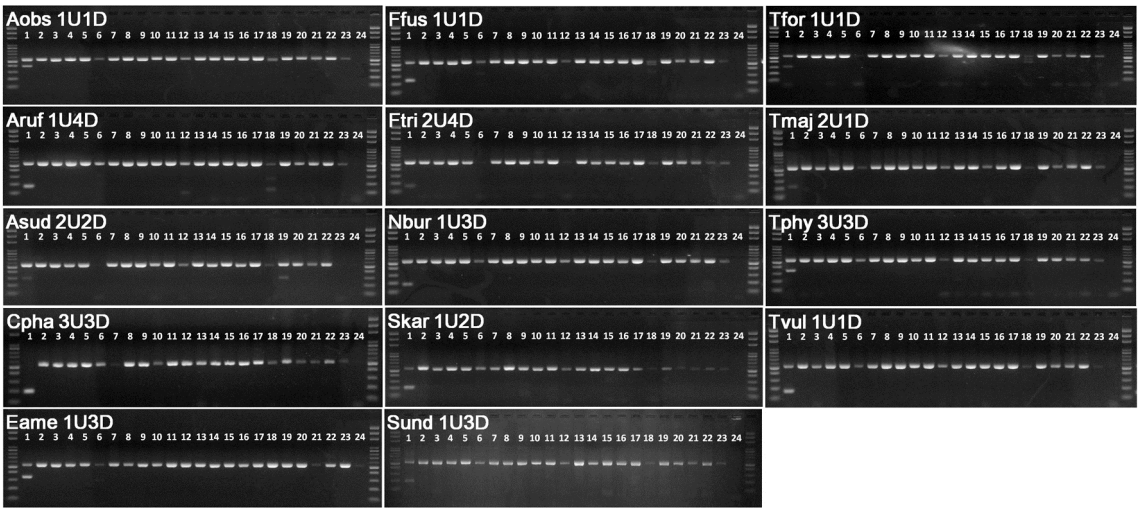

Fig. 2. Application of the multiplex PCR by one ITS2 species-specific primer set of each target thrips species with one 28S rDNA universal paired primers. The specific amplification fragment of the target thrips is visible in lane 1 with no cross amplification in the other lanes. The target and examined thrips species in the lanes are listed in Table 1 and the specific primer sequences for the target thrips are shown in Table 2. The marker is a 100 bp DNA ladder.

al., 2014). In this study, the universal primers were designed based on the conserved region of 28S rDNA in insects (Lin *et al*., 2003). Therefore, the weak signals of 28S rDNA products found in some reactions, especially in lanes 6, 12, and 18 of Fig. 2, were probably due to the interaction between primer pairs or the inconsistent quality of the thrips' crude DNA, since their DNA was not extracted at the same time. An examination of the amplification efficiency for 11 thrips species, whose DNA was extracted simultaneously, shows the average amplified intensity among them (Fig. 5).

Compared to the other PCR-based identification methods such as sequencing, PCR-RFLP, AFLP, and RT-PCR, this simple, convenient, and cost-effective SSP method using only a single PCR step, with a proper internal positive control, is worthy of being promoted. The highly variable features of ITS2 sequences among thrips species are useful in the development of species-specific primers for a multiplex PCR, providing a reliable, convenient and cost-effective detection tool to discriminate species of this important insect pest.

Acknowledgments

We like to thank Mr. C. W. Chang and W. C. Tsao for collecting materials. This work was supported by the Bureau of Animal and Plant Health Inspection and Quarantine of Taiwan (99AS-9.3.1-BQ-B1), the National Chung Hsing University (NCHU-TARI 9814) and the National Science Council of Taiwan (NSC99-2324-B-005-020-CC2, NSC100-2324-B-005-013-CC2). We also like to acknowledge the technical services provided by the Sequencing Core Facility of the National Yang-Ming University Genome Research Center (YMGC), Taiwan, which is supported by the Ministry of Science and Technology of Taiwan, ROC.

References

Asokan R, Kuma NKK, Kumar V, Ranganath HR. 2007. Molecular differences in the

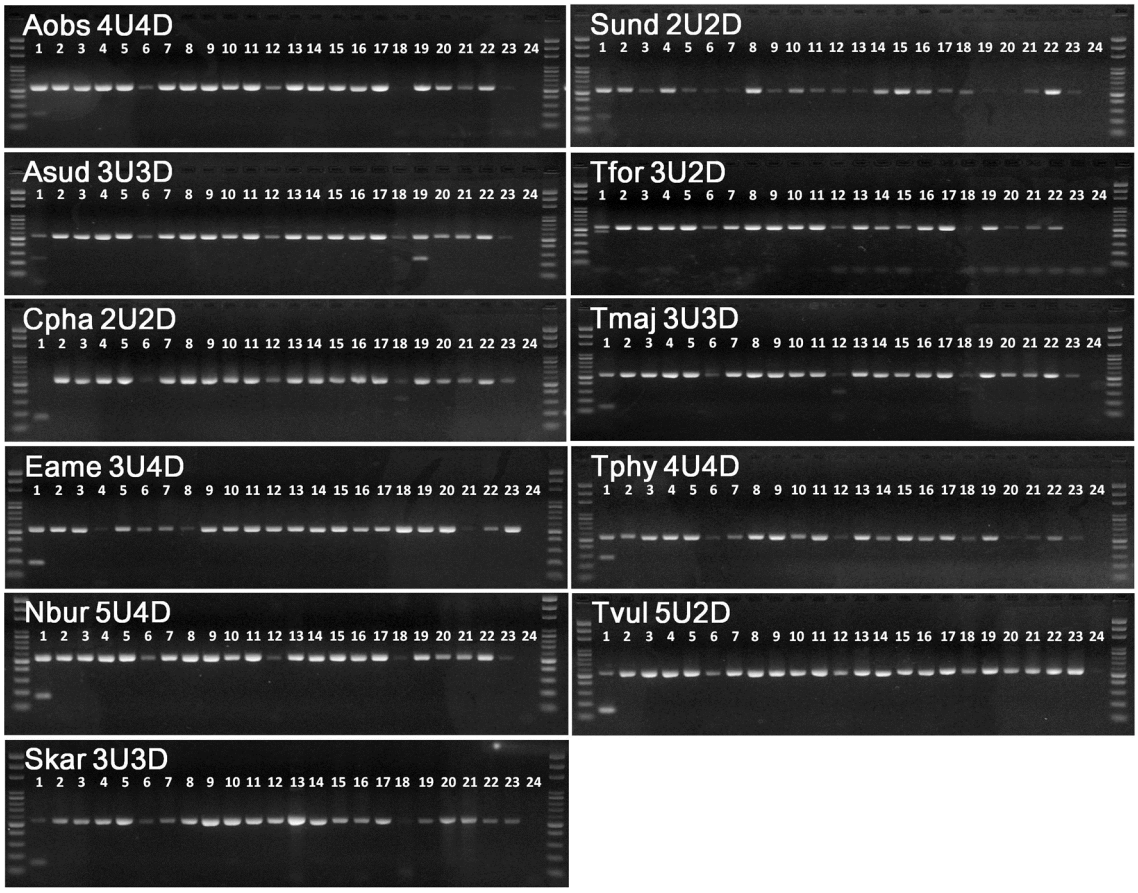

Fig. 3. Application of the multiplex PCR by one ITS2 species-specific primer set of each target thrips species with one 28S rDNA universal paired primers. The specific amplification fragment of the target thrips is visible in lane 1. The target and examined thrips species in the lanes are listed in Table 1 and the specific primer sequences for the target thrips are shown in Table 2. The marker is a 100 bp DNA ladder.

cytochrome oxidase I (mtCOI) gene and development of a species-specific marker for onion thrips, *Thrips tabaci* Lindeman, and melon thrips, *T. palmi* Karny (Thysanoptera: Thripidae), vectors of tospoviruses (Bunyaviridae). Bull Entomol Res 97: 461-470.

Brunner PC, Frey JE. 2010. Habitat-specific population structure in native western flower thrips *Frankliniella occidentalis* (Insecta, Thysanoptera). J Evol Biol 23: 797-804.

Brunner PC, Chatzivassiliou EK, Katis NI, Frey JE. 2004. Host-associated genetic differentiation in *Thrips tabaci* (Insecta: Thysanoptera), as determined from mtDNA sequence data. Heredity 93: 364-370.

Chen YH, Liu LY, Tsai WH, Haymer DS, Lu KH. 2014. Using DNA chips for identification of tephritid pest species. Pest Manag Sci 70: 1254-1261.

Daane KM, Middleton MC, Sforza R, Cooper ML, Walton VM, Walsh DB, Zaviezo T, Almeida RPP. 2011. Development of a multiplex PCR for identification of vineyard mealybugs. Environ Entomol 40: 1595-1603.

Drali R, Boutellis A, Raoult D, Rolain JM, Brouqui PB. 2013. Distinguishing

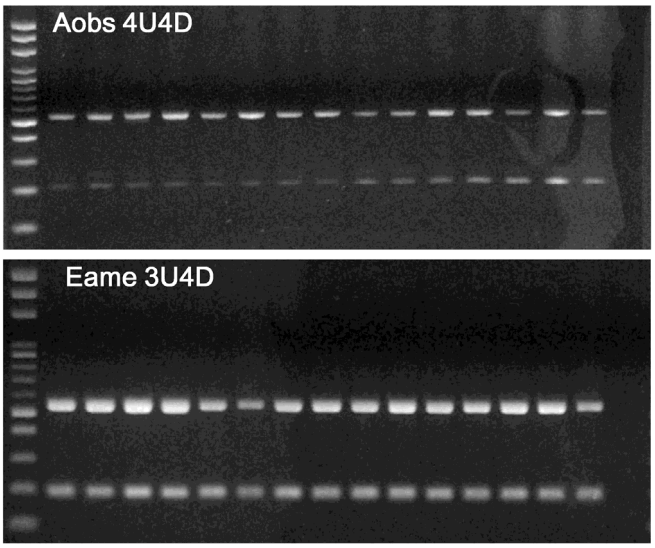

Fig. 4. Amplification efficacy for ITS2 specific primer sets with 28S rDNA universal paired primers on individuals of the thrips species of *Anaphothrips obscurus* (Aobs) and *Echinothrips americanus* (Eame). The DNA ladder is 100 bp and the last lane is the negative control.

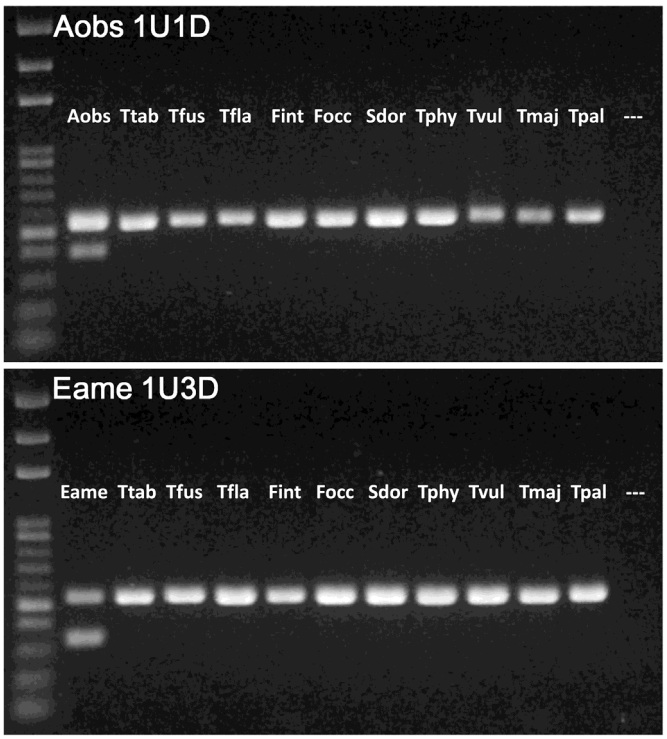

Fig. 5. Validation of ITS2 species-specific primer sets for *Anaphothrips obscurus* (Aobs) and *Echinothrips americanus* (Eame) with one 28S rDNA universal paired primers. The specific amplification fragment of the target thrips is visible in lane 1. The examined thrips species in the lanes are listed in Table 1 and the specific primer sequences are shown in Table 2. The marker is a 100 bp DNA ladder.

body lice from head lice by multiplex real-time PCR analysis of the Phum_PHUM540560 gene. PLoS One 8: e58088.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32: 1792-1797.

Farris RE, Ruiz-Arce R, Ciomperlik M, Vasquez JD, DeLeón R. 2010. Development of a ribosomal DNA ITS2 marker for the identification of the thrips, *Scirtothrips dorsalis*. J Insect Sci 10: 26.

Gent DH, du Toit LJ, Fichtner SF, Mohan SK, Pappu HR, Schwartz HF. 2006. *Iris yellow spot virus*: an emerging threat to onion bulb and seed production. Plant Disease 90: 1468-1480.

Glover H, Collins DW, Walsh K, Boonham N. 2010. Assessment of loci for DNA barcoding in the genus *Thrips* (Thysanoptera: Thripidae). Mol Ecol Res 10: 51-59.

Hall TA. 1999. Bioedit: a user-friendly biological sequences alignment [ed.], and analysis program for Windows 95/98/NT. Nucleic Acid Symp Ser 41: 95-98.

Hoddle MS, Heraty JM, Rugman-Jones PF, Mound LA, Stouthamer R. 2008. Relationships among species of *Scirtothrips* (Thysanoptera: Thripidae, Thripinae) using molecular and morphological data. Ann Entomol Soc Am 101: 491-500.

Hoy AM. 2003. Molecular systematics and evolution of arthropods. pp 350-399. In: Hoy MA (ed). Insect Molecular Genetics: an Introduction to Principles and Applications (2nd). Academic Press, California.

Huang KS, Lee E, Yeh Y, Shen GS, Mei E, Chang CM. 2010. Taqman real-time quantitative PCR for identification of western flower thrip (*Frankliniella occidentalis*) for plant quarantine. Biology Letters [Internet]. 2010 6: 555-557. Available from: doi: 10.1098/rsbl.2009.1060.

Jacobson AL, Booth W, Vargo EL, Kennedy GG. 2013. *Thrips tabaci* population genetic structure and polyploidy in relation to competency as a vector of *Tomato Spotted Wilt Virus*. PLoS One 8: e54484.

Kadirvel P, Srinivasan R, Hsu YC, Su FC, de la Peña R. 2013. Application of cytochrome oxidase I sequences for phylogenetic analysis and identification of thrips species occurring on vegetable crops. J Econ Entomol 106: 408-418.

Kjer KM, Baldridge GD, Fallon AM. 1994. Mosquito large subunit ribosomal RNA: simultaneous alignment of primary and secondary structure. Biochim Biophys Acta 1217: 147-155.

Kobayashi K, Hasegawa E. 2012. Discrimination of reproductive forms of *Thrips tabaci* (Thysanoptera: Thripidae) by PCR with sequence specific primers. J Econ Entomol 105: 555-559.

Lee WS, Choi H, Kang J, Kim JH, Lee SH, Lee S, Hwang SY. 2013. Development of a DNA microarray for species identification of quarantine aphids. Pest Manag Sci [Internet]. 2013 69: 1399-14061. Available from: doi: 10.1002/ps.3520.

Li HY, Hua T, Yeh WB. 2010. Amplification of single bulb mites by nested PCR: Species-specific primers to detect *Rhizoglyphus robini* and *R. setosus* (Acari: Acaridae). J Asia-Pacific Entomol 13: 267-271.

Lin JS, Wang CL, Yeh WB. 2003. Molecular identification of multiplex-PCR and PCR-RFLP for the quarantine pest, *Frankliniella occidentalis* (Pergande). Formosan Entomol 23: 353-366.

Liu YC. 2004. Molecular identification of a plant quarantine pest (*Frankliniella occidentalis*) by one-tube nested PCR targeting ribosomal DNA internal transcribed spacer regions. Plant Prot Bull 46: 27-46.

Nakamura S, Masuda T, Mochizuki A, Konishi K, Tokumaru S, Ueno K. 2013. Primer design for identifying economically important *Liriomyza* species (Diptera: Agromyzidae) by multiplex PCR. Mol Ecol Res 13: 96-102.

Prins M, Goldbach R. 1998. The emerging problem of tospovirus infection and nonconventional methods of control. Trends Microbiol 6: 31-35.

Rugman-Jones PF, Hoddle MS, Stouthamer R. 2010. Nuclear-mitochondrial barcoding exposes the global pest western flower thrips (Thysanoptera: Thripidae) as two sympatric cryptic species in its native California. J Econ Entomol 103: 877-886.

Rugman-Jones PF, Hoddle MS, Mound LA, Stouthamer R. 2006. Molecular identification key for pest species of *Scirtothrips* (Thysanoptera: Thripidae). J Econ Entomol 99: 1813-1819.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30: 2725-2729.

Thet-Em T, Tungtrongchitr A, Tiewcharoen S, Malainual N. 2012. Multiplex PCR for identifying common dust mites species (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* and *Blomia tropicalis*). Asian Pac J Allergy Immunol 30: 224-230.

Toda S, Murai T. 2007. Phylogenetic analysis based on mitochondrial COI gene sequences in *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) in relation to reproductive forms and geographic distribution. App Entomol Zool 42: 309-316.

Tseng LY, Chang NT, Tseng MJ, Yeh WB. 2010. Genetic variation of *Thrips tabaci* Lindeman (Thysanoptera: Thripidae) in the Pacific Rim. Formosan Entomol 30: 219-234.

Yao MC, Chang SC, Lee CY, Lu KH. 2012. A SCAR-based method for rapid identification of four major lepidopterous stored-product pests. J Econ Entomol 105: 1100-1106.

Yeh WB, Lee HC, Tseng MJ, Chang NT, Wu SY. 2012. Molecular identification and genetic variations of importantly agricultural thrips. In: Chang NT (eds). Proceedings of the symposium on the management of thrips pests of agriculture and quarantine; 2012 Oct 25-26; Pingtung, Taiwan: Formosan Entomologist, Special Publication No. 15. pp 33-50.

Yeh WB, Tseng MJ, Chang NT, Wu SY, Tsai YS. 2014. Development of species-specific primers for agronomical thrips and multiplex assay for quarantine identification of western flower thrips. J Eco Entomol 107: 1728-1735.

Received: November 11, 2014
Accepted: February 23, 2015

Appendix I. Sampling location, species name, abbreviation, and the sequence accession

Taxon	Abbreviation	Collecting Locality	Accession No.
Anaphothrips obscurus	Am57	USA	AB973067
Anaphothrips obscurus	Am63-2	USA	AB973068
Anaphothrips obscurus	Am63-3	USA	AB973069
Anaphothrips obscurus	Am65	USA	AB973070
Anaphothrips obscurus	Am69-1	USA	AB973071
Anaphothrips obscurus	Am69-4	USA	AB973072
Anaphothrips obscurus	Am69-6	USA	AB973073
Anaphothrips obscurus	Am69-8	USA	AB973074
Anaphothrips obscurus	Am121	USA	AB973075
Anaphothrips obscurus	Am123-1	USA	AB973076
Anaphothrips obscurus	Am123-2	USA	AB973077
Anaphothrips obscurus	Am123-5	USA	AB972959
Anaphothrips obscurus	Am123-8	USA	AB972960
Anaphothrips obscurus	Am123-9	USA	AB972961
Anaphothrips obscurus	Am123-14	USA	AB972962
Anaphothrips obscurus	Am124	USA	AB972963
Anaphothrips obscurus	Am141	USA	AB972964
Anaphothrips obscurus	Am142	USA	AB972965
Anaphothrips obscurus	Am145	USA	AB972966
Aptinothrips rufus	Am61-1	USA	AB972967
Aptinothrips rufus	Am61-2	USA	AB972968
Aptinothrips rufus	Am62-2	USA	AB972969
Aptinothrips rufus	Am64-1	USA	AB972970
Aptinothrips rufus	Am64-2	USA	AB972971
Aptinothrips rufus	Am70	USA	AB972972
Anaphothirps_sudanensis	Asud2-4	Taiwan	AB973065
Anaphothirps_sudanensis	Tai100	Taiwan	AB973066
Caliothrips phaseoli	Tai480-1	Taiwan	AB972973
Caliothrips phaseoli	Tai480-2	Taiwan	AB972974
Caliothrips phaseoli	Thai04-3	Thailand	AB972975
Caliothrips phaseoli	Thai33-1	Thailand	AB972976
Caliothrips phaseoli	Thai33-2	Thailand	AB972977
Caliothrips phaseoli	Thai91-1	Thailand	AB972978
Caliothrips phaseoli	Thai91-3	Thailand	AB972979
Caliothrips phaseoli	Thai93-1	Thailand	AB972980
Caliothrips phaseoli	Thai93-2	Thailand	AB972981
Caliothrips phaseoli	Thai96-2	Thailand	AB972982
Caliothrips phaseoli	Thai96-3	Thailand	AB972983
Caliothrips phaseoli	Thai96-4	Thailand	AB972984
Caliothrips phaseoli	Yun41-2	China: Yunnan Province	AB972985
Echinothrips americanus	PHS1	Taiwan	AB972986
Echinothrips americanus	PHS3	Taiwan	AB972987
Echinothrips americanus	Tai743-1	Taiwan	AB972988
Echinothrips americanus	Tai743-2	Taiwan	AB972989
Echinothrips americanus	Tai749-1	Taiwan	AB972990

Appendix I. (continued)

Taxon	Abbreviation	Collecting Locality	Accession No.
Echinothrips americanus	Tai750-1	Taiwan	AB972991
Echinothrips americanus	Tai750-2	Taiwan	AB972992
Echinothrips americanus	Tai752-1	Taiwan	AB972993
Echinothrips americanus	Tai757-1	Taiwan	AB972994
Frankliniella fusca	Am50	USA	AB972995
Frankliniella_fusca	Am63-1	USA	AB972996
Frankliniella_fusca	Am113	USA	AB972997
Frankliniella_fusca	Am114	USA	AB972998
Frankliniella occidentalis	Am01-1	USA	AB972999
Frankliniella occidentalis	Tai459-1	Taiwan	AB973000
Frankliniella occidentalis	Tai459-2	Taiwan	AB973001
Frankliniella occidentalis	Tai459-4	Taiwan	AB973002
Frankliniella occidentalis	Tai459-5	Taiwan	AB973003
Frankliniella occidentalis	Tai459-6	Taiwan	AB973004
Frankliniella occidentalis	Tai459-7	Taiwan	AB973005
Frankliniella occidentalis	Tai459-8	Taiwan	AB973006
Frankliniella occidentalis	Yun02	China: Yunnan Province	AB973007
Frankliniella occidentalis	Yun04-2	China: Yunnan Province	AB973008
Frankliniella occidentalis	Yun07	China: Yunnan Province	AB973009
Frankliniella tritici	Am45	USA	AB973010
Frankliniella_tritici	Am47-2	USA	AB973011
Frankliniella_tritici	Am48-1	USA	AB973012
Frankliniella_tritici	Am48-2	USA	AB973013
Frankliniella_tritici	Am49-1	USA	AB973014
Frankliniella_tritici	Am111	USA	AB973015
Frankliniella_tritici	Am134	USA	AB973016
Frankliniella_tritici	Am140	USA	AB973017
Neohydatothrips burungae	Am54	USA	AB973018
Neohydatothrips burungae	Am136-1	USA	AB973019
Neohydatothrips burungae	Am137	USA	AB973020
Neohydatothrips burungae	Am138-1	USA	AB973021
Neohydatothrips burungae	Am138-2	USA	AB973022
Neohydatothrips burungae	Am147	USA	AB973023
Stenchaetothrips karnyrianus	Tai166-2	Taiwan	AB973024
Stenchaetothrips karnyrianus	Yun27-1	China: Yunnan Province	AB973025
Stenchaetothrips karnyrianus	Yun27-2	China: Yunnan Province	AB973026
Stenchaetothrips karnyrianus	Yun30-1	China: Yunnan Province	AB973027
Stenchaetothrips karnyrianus	Yun30-2	China: Yunnan Province	AB973028
Stenchaetothrips karnyrianus	Yun32-2	China: Yunnan Province	AB973029
Stenchaetothrips karnyrianus	Yun36-3	China: Yunnan Province	AB973030
Stenchaetothrips undatus	Tai166-1	Taiwan	AB973031
Stenchaetothrips undatus	Tai167-1	Taiwan	AB973032
Stenchaetothrips undatus	Tai167-2	Taiwan	AB973033
Stenchaetothrips undatus	Tai168-2	Taiwan	AB973034
Thrips formosanus	Tai446	Taiwan	AB973035

Appendix I. (continued)

Taxon	Abbreviation	Collecting Locality	Accession No.
Thrips formosanus	Tai478-2	Taiwan	AB973036
Thrips formosanus	Tai487-1	Taiwan	AB973037
Thrips formosanus	Tai491-1	Taiwan	AB973038
Thrips major	Bri2	United Kingdom: Warwickshire	AB973039
Thrips major	Bri7	United Kingdom: Warwickshire	AB973040
Thrips major	Bri11	United Kingdom: Alnwick Castle	AB973041
Thrips major	Bri12	United Kingdom: Alnwick Castle	AB973042
Thrips major	Bri15	United Kingdom: Alnwick Castle	AB973043
Thrips major	Bri16	United Kingdom: Alnwick Castle	AB973044
Thrips major	Qua67-1	Netherlands	AB973045
Thrips physapus	Fra7	Italy: Milan	AB973046
Thrips physapus	Fra8	Italy: Milan	AB973047
Thrips physapus	Fra13	Italy: Florence	AB973048
Thrips physapus	Fra14	Italy: Florence	AB973049
Thrips physapus	Fra16	Italy: Florence	AB973050
Thrips physapus	NMG14	China: Inner Mongolia	AB973051
Thrips physapus	NMG17	China: Inner Mongolia	AB973052
Thrips physapus	NMG18	China: Inner Mongolia	AB973053
Thrips physapus	NMG19	China: Inner Mongolia	AB973054
Thrips physapus	NMG20	China: Inner Mongolia	AB973055
Thrips vulgatissimus	Bri3	United Kingdom: Warwickshire	AB973056
Thrips vulgatissimus	Bri4	United Kingdom: Warwickshire	AB973057
Thrips vulgatissimus	Bri5	United Kingdom: Warwickshire	AB973058
Thrips vulgatissimus	Bri8-1	United Kingdom: Warwickshire	AB973059
Thrips vulgatissimus	Bri8-2	United Kingdom: Warwickshire	AB973060
Thrips vulgatissimus	Bri9	United Kingdom: Alnwick Castle	AB973061
Thrips vulgatissimus	Bri19	United Kingdom	AB973062
Thrips vulgatissimus	Bri21	United Kingdom	AB973063
Thrips vulgatissimus	Bri22	United Kingdom	AB973064

複合性 PCR 於經濟重要性薊馬的分子鑑定

葉文斌[1*]、吳昕縈[1]、曾美容[1]、張念台[2]、陳文彬[3]

[1] 國立中興大學昆蟲學系　台中市 40227 南區國光路 250 號
[2] 屏東科技大學植物醫學系　屏東縣 91201 內埔鄉學府路 1 號
[3] 益生生物科技有限公司　新北市 22180 汐止區康寧街 169 巷 23 號 6F-3

摘　要

薊馬是世界上許多農作的重要害蟲，常因其體形微小及形態上的高度相似而難以鑑定，單一步驟複合性 PCR 的分子方法遂被應用於薊馬的鑑定。本研究分析 14 種農作上重要性薊馬的核醣體第二區間 (ITS2) 序列，種內變異小於 3% 遠低於種間 14% 以上的變異，使得此 ITS2 序列非常適合開發專一性引子用以鑑定這些薊馬。這些專一性引子與具內控制組特性的廣效性引子混合，進行 23 種薊馬的複合性 PCR 檢測，均顯示出其專一性及穩定性。總結來說，薊馬物種核醣體第二區間的高變異特性，可開發專一性引子用於複合性 PCR 檢測，提供了一個可靠、方便、快速的鑑定方法，區辨這些重要薊馬害蟲。

關鍵詞：薊馬、核醣體第二區間、專一性引子、內控制引子。

*論文聯繫人
Corresponding email: wbyeh@nchu.edu.tw