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Scientific Note

# Identification of Four Thrips Species (Thysanoptera: Thripidae) by Multiplex Polymerase Chain Reaction

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**Abstract:** We examined the applicability of multiplex PCR for identification of four thrips species, *Thrips palmi, T. tabaci, Frankliniella intonsa*, and *F. occidentalis* that were frequently found in Japanese quarantine inspection sites. We amplified the internal transcribed spacer 2 region (ITS2) of nuclear ribosomal DNA using 5 specific primers and used agarose gel electrophoresis to detect banding patterns. For 71 individuals of the four species examined in this study, species-specific single bands were detected. These results indicated that the method could be used to develop a simple and accurate inspection technique to supplement conventional techniques.
**Key word:** *Frankliniella, Thrips*, Discrimination, quarantine and Multiplex PCR

## Introduction

Thrips are important pests for agricultural crops such as fruits, vegetables, and flowers, and they are frequently intercepted in import quarantine sites in Japan. Although the thrips species can usually be discriminated using morphological diagnostic characters, they are sometimes impossible to differentiate because the morphological traits of immature stages, such as larvae or eggs, are few and limited. Based on morphological traits, Miyazaki and Kudo (1986) developed a key based on 2nd-instar larvae of 12 Thripidae species. However, the larvae detected in quarantine sites are not only 2nd -instar larvae but also other immature stages. Furthermore, preparing these specimens for microscopic observation is difficult and requires considerable experience and technique. In insect species that are difficult to identify due to their micro-morphology, these problems can be solved using rapid and convenient identification methods that have been developed in recent years that involve amplifying variable genes (Nakahara *et al*., 2005, Osakabe *et al*., 2008, Kox *et al*., 2005).

In particular, multiplex polymerase chain reaction (PCR) simultaneously amplifies several different fragments of various lengths in a single reaction, and under certain conditions, several species can be identified using a single PCR followed by electrophoretic separation of the amplified DNA fragments. To date, multiplex PCR methods have been described for the simultaneous detection of various insects such as white fly, armored scale, thrips, flower bug and mosquito (Tsugane *et al*., 2007, Rugman-Jones *et al*., 2006, 2009, Hinomoto *et al*., 2004, Higa *et al*., 2010). When the molecular technique is introduced into a quarantine inspection site, it provides an accurate and rapid identification tool.

In this study, we performed exploratory research for usefulness of multiplex PCR, using four thrips species frequently found at plant quarantine inspection sites to develop a more accurate and simple diagnostic method for the immature thrips.

## Materials and Methods

The materials used in this study are listed in Table 1. Most of the thrips specimens were collected from imported vegetables or cut flowers at Nagoya Seaport, Chubu International Airport, and Narita International Airport. Some of the samples for the three thrips

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Table 1. Samples used in this study

| Species | Number of specimens | Locality | Date | Host plant | Collection | Collector |
|---|---|---|---|---|---|---|
| *Frankliniella intonsa* | 1 | Shari, Hokkaido, Japan | 6-Sep-09 | *Trifolium pratense* (Red clover) | | S. Nakahara |
| | 1 | Abashiri, Hokkaido, Japan | 6-Sep-09 | *Cucurbita maxima* (male flower) | | S. Nakahara |
| | 2 | Narita, Chiba, Japan | 1-Sep-08 | *Trifolium pratense* (Red clover) | | M. Masumoto |
| | 2 | Shin-Hirayu, Gifu, Japan | 10-Jun-11 | *Trifolium pratense* (Red clover) | | S. Nakahara |
| | 2 | Yokohama, Kanagawa, Japan | 18-Aug-08 | *Cucurbita maxima* (male flower) | | S. Nakahara |
| | 3 | Nagoya, Aichi, Japan | 26-May-11 | *Trifolium pratense* (White clover) | | S. Nakahara |
| | 1 | China | 2-Feb-11 | *Alium cepae* (Onion) | *1 | M. Matsuda |
| | 1 | China | 20-Dec-11 | *Alium cepae* (Onion) | *1 | S. Inokuchi |
| | 1 | China | 6-Jan-12 | *Alium cepae* (Onion) | *1 | T. Kasahara |
| | 2 | Taiwan | 12-Mar-02 | *Chrysanthemum* sp. | *1 | K. Yoneyama |
| | 2 | Taiwan | 25-Oct-04 | *Gladiolus* sp. | *1 | T. Iwai |
| *Frankliniella occidentalis* | 1 | U. S. A. | 22-Apr-08 | *Asparagus officinalis* | *2 | unknown |
| | 1 | U. S. A. | 23-Apr-08 | *Asparagus officinalis* | *2 | unknown |
| | 2 | U. S. A. | 30-Apr-08 | *Rubus* sp. (Raspberry) | *2 | unknown |
| | 1 | U. S. A. | 2-May-08 | *Brassica oleracea* (Broccoli) | *2 | unknown |
| | 1 | U. S. A. | 4-May-08 | *Cichorium intybus* (Red salad) | *2 | unknown |
| | 1 | U. S. A. | 6-May-08 | *Cichorium intybus* (Red salad) | *2 | unknown |
| | 1 | U. S. A. | 6-Jun-08 | *Brassica oleracea* (Cauliflower) | *2 | unknown |
| | 1 | U. S. A. | 6-Jun-08 | *Rubus* sp. (Blackberry) | *2 | unknown |
| | 1 | China | 6-Apr-11 | *Alium cepae* (Onion) | *1 | Y. Okahara |
| | 2 | China | 13-Apr-11 | *Alium cepae* (Onion) | *1 | Y. Okahara |
| | 1 | China | 20-Apr-11 | *Alium cepae* (Onion) | *1 | Y. Okahara |
| | 1 | Ethiopia | 7-Mar-12 | *Rosa* sp. | *2 | K. Kakuta |
| | 1 | Ethiopia | 25-Mar-12 | *Rosa* sp. | *2 | H. Manago |
| | 3 | Mexico | 9-Mar-12 | *Asparagus officinalis* | *2 | H. Manago |
| | 2 | Mexico | 10-Mar-12 | *Asparagus officinalis* | *2 | H. Manago |
| *Thrips palmi* | 6 | Miyazaki, Japan | 1-Aug-08 | *Cucumis sativus* (Cucumber) | | S. Kawanobu |
| | 1 | Thailand | 9-Sep-08 | *Dendrobium* sp. | *3 | K. Minoura |
| | 1 | Thailand | 16-Sep-08 | *Dendrobium* sp. | *3 | H. Kurahashi |
| | 1 | Thailand | 21-Feb-09 | *Dendrobium* sp. | *2 | M. Fujiwara |
| | 1 | Thailand | 3-May-11 | *Dendrobium* sp. | *2 | N. Ogawa |
| | 1 | Malaysia | 1-Aug-11 | *Chrysanthemum* sp. | *3 | K. Kishimoto |
| | 2 | Malaysia | 3-Aug-11 | *Chrysanthemum* sp. | *3 | Y. Koga |
| | 1 | Malaysia | 18-Jun-12 | *Chrysanthemum* sp. | *3 | K. Kishimoto |
| | 1 | Malaysia | 27-Jun-12 | *Chrysanthemum* sp. | *3 | Y. Koga |
| *Thrips tabaci* | 1 | Ichinomiya, Aichi, Japan | 11-Jun-11 | *Alium cepae* (Onion) | | S. Nakahara |
| | 1 | Inuyama, Aichi, Japan | 21-Apr-13 | *Allium fistulosum* (Welsh onion) | | S. Nakahara |
| | 1 | China | 16-Dec-10 | *Alium cepae* (Onion) | *1 | Y. Okahara |
| | 1 | China | 22-Dec-10 | *Alium cepae* (Onion) | *1 | E. Natsume |
| | 1 | China | 23-Feb-11 | *Alium cepae* (Onion) | *1 | T. Yoshida |
| | 2 | China | 13-Apr-11 | *Alium cepae* (Onion) | *1 | I. Yamada |
| | 2 | China | 20-Apr-11 | *Alium cepae* (Onion) | *1 | M. Matsuda |
| | 1 | China | 4-Aug-11 | *Alium cepae* (Onion) | *1 | I. Yamada |
| | 1 | China | 1-Dec-11 | *Alium cepae* (Onion) | *1 | S. Inokuchi |
| | 1 | China | 19-Dec-11 | *Dianthus caryophyllus* | *1 | S. Inokuchi |
| | 1 | China | 21-Jan-13 | *Dianthus caryophyllus* | *1 | E. Natsume |
| | 2 | U. S. A. | 23-Apr-08 | *Asparagus officinalis* | *2 | unknown |
| | 1 | U. S. A. | 4-May-08 | *Cichorium intybus* (Red salad) | *2 | unknown |
| | 2 | Mexico | 23-Jan-09 | *Asparagus officinalis* | *2 | Y. Shimono |
| *Thrips alliorum* | 1 | China | 27-Jul-10 | *Allium fistulosum* (Welsh onion) | *1 | Y. Okahara |
| *Frankriniella tenuicornis* | 5 | China | 18-Oct-11 | *Brassica oleracea* (Cabegge) | *1 | A. Takahashi |
| *Chirothrips manicatus* | 5 | Nagoya, Aichi, Japan | 21-May-13 | Poaceae | | K. Minoura |
| Total | 82 | | | | | |

Specimens (denoted by*) were provided by Nagoya Plant Protection Station (*1), Narita International Airport Branch of Yokohama Plant Protection Station (*2) and Chubu International Airport Branch of Nagoya Plant Protection Station (*3).

species, *Thrips palmi*, *T. tabaci* and *Frankliniella intonsa* and the samples of *Chirothrips manicatus* were collected in Japan.

Template DNA for PCR was extracted from the whole bodies of individual insects using the method of Osakabe *et al.* (2008). A single insect was homogenized in 20 μl of lysis buffer (10 mM Tris-HCl, 100 mM EDTA, 0.5% NP-40 Substitute (Wako, Osaka, Japan), 10 mM NaCl, and 1 mg/ml proteinase K) in a 1.5-ml sample tube. The homogenate was incubated at 65℃ for 15 min and then at 95 ℃ for 10 min. The lysate solution was diluted with 350μl of 0.1 × TE (1 mM Tris-HCl and 0.1 mM EDTA). The primers for the multiplex PCR were designed from 75 sequences deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases (Accession Nos. AB063334, AB063335, AB063340, AB063341, AM932138-AM932141, AM932145-AM21452, AM932154, AM932156-AM932157, AM932160-AM932169, AM932171, AM932173, AM932175-AM932179, AM932181, AM932183, AM932187, AM932188, AM932192, EU315920-EU315940, CQ343258, and FM956422-FM956428). Five newly designed primers, PAL-ITS2F2(5′ -TGTGATGTACGTGCACTGGA-3′ ), TAB-ITS2F4(5′ -AACGATTHCCAGACTGCCC-3′ ), INT-ITS2F1(5′ -GACCAGACTGTTCCGAGA-3′ ), OCC-ITS2F6(5′ -TGGTCGCTTCACCGCTTCCCG-3′ ), and ThripsITS2R3 (5′ -CTCTCCTGAACWGAGGTCG-3′ ) were used for multiplex PCR amplification.

PCR amplification was performed in a total volume of 20 μl containing 5 μl template DNA, 1.6 μl of dNTPs (2.5 mM each), 0.3 μM of each primer, 2.0 μl (25 mM) of $MgCl_2$, 0.5 unit of EX-Taq DNA polymerase (Takara Bio, Otsu, Japan), and 2.0 μl 10 × PCR buffer. Amplification was performed in a Program Temp Control System PC801 (Astec, Tokyo, Japan) programmed for 40 cycles of 10 s at 98℃, 30 s at 53℃, and 60 s at 72℃.

The PCR products were electrophoresed on 3.5% (w/v) agarose gel Type Ⅱ -A (Sigma-Aldrich, Tokyo, Japan) containing in 1 × TBE buffer, and were visualized by staining with ethidium bromide or GelRedTM (Biotium Inc., CA, USA). A portion of the amplified products (6-8μl) was treated with 5 U restriction enzyme *Rsa*I (Nippon Gene, Tokyo, Japan) and 1 × reaction buffer for 3 h at 37 ℃ . The digested DNA was electrophoresed on 4.0% (w/v) agarose gel Type Ⅱ-A in 1× TBE buffer.

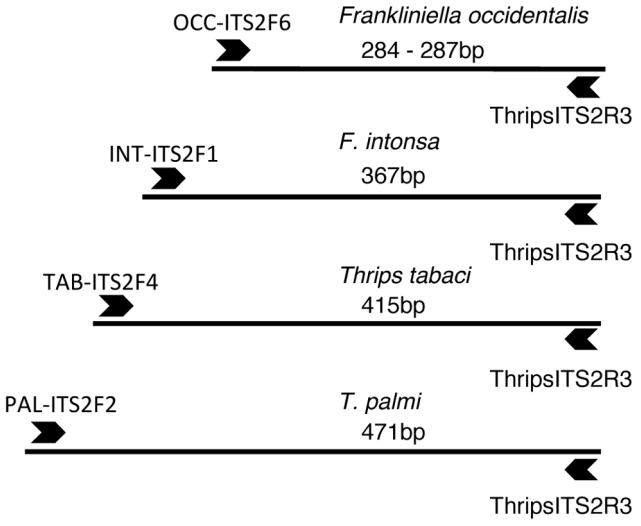


Fig. 1. PCR products size estimated from 75 sequences, 4 thrips species.

## Results and Discussion

For 71 individuals of the four species used in this study, clear bands of 0.47, 0.41, 0.37, and 0.28 kbp, corresponding to the sizes of the ITS2 fragments estimated from the consensus nucleotide sequences of each of the four species (Fig. 1), were detected by PCR, using DNA extracted from *Thrips palmi, T. tabaci, F.*

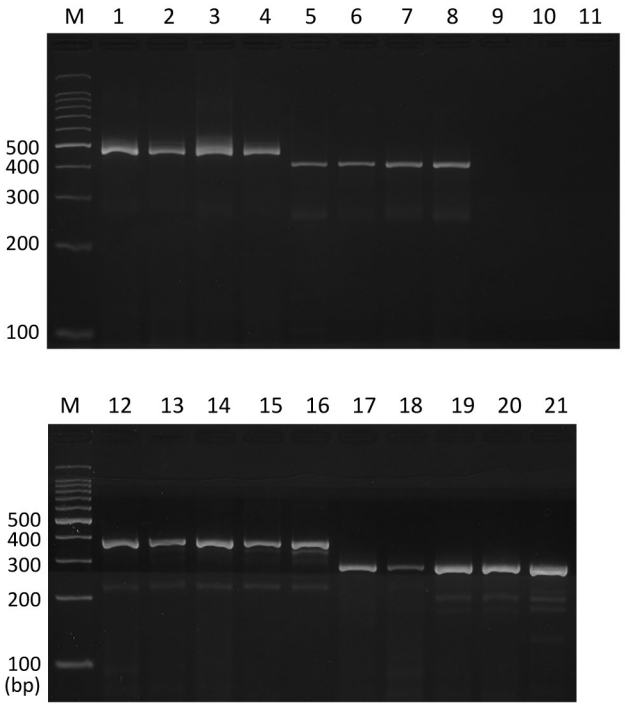


Fig. 2. Multiplex PCR products from DNA of Thrips palmi (lane 1: Miyazaki, Japan, 2: Thailand, 3, 4: Malaysia), *T. tabaci* (lane 5: Aichi, Japan, 6: China, 7: U. S. A., 8: Mexico), *Frankliniella intonsa* (lane 12: Hokkaido, Japan, 13: Narita, Japan, 14: Nagoya, Japan, 15: China, 16: Taiwan), *F. occidentalis* (lane 17, 18: China, 19: U. S. A., 20: Mexico, 21: Ethiopia), *Thrips alliorum* (lane 9), *Frankliniella tenuicornis* (lane 10), *Chirothrips manicatus* (lane 11). M: 100bp ladder size standard.

*intonsa*, and *F. occidentalis*, respectively (Fig. 2). An additional weak band appeared occasionally, but did not affect identification of the four species (Fig. 1, Fig. 2).

To substantiate amplification of the target DNA, the PCR products from 8 specimens were treated with the restriction enzyme *Rsa*I. These results corresponded to the banding patterns, *Thrips palmi*: 365, 98, and 8bp, *T. tabaci*: 215, 111, and 87bp, *Frankliniella intonsa*: 143, 98, 62, 54, and 10bp, *F. occidentalis*: 234 - 237 and 50bp predicted from the nucleotide sequences on the DNA databases (Fig. 3). The same PCR amplification was also performed using DNA extracted from other thrips species, such as *T. alliorum, F. tenuicornis*, and *C. manicatus* that were occasionally detected in the quarantine inspection site. However, no PCR products were amplified from these specimens.

These results suggest the possibility that the multiplex PCR primers designed in this study are useful for confirmation and identification of the four thrips species. Based on the high sensitivity of the multiplex PCR system, we speculated that it has the potential to be used for species identification and discrimination of thrips larvae, the life stages of particularly small size found at plant quarantine inspection sites.

Brunner *et al*., (2002) and Toda and Komasaki (2002) reported useful approaches for identification of thrips species using PCR-RFLP of the mitochondrial DNA cytochrome oxidase subunitI (COI) region and the internal transcribed spacer (ITS2) region of nuclear ribosomal DNA, respectively. Furthermore, a useful identification tool using the LAMP (loop-mediated isothermal amplification) method, which allows gene amplification without heat denaturation, has also been developed in recent years (Fukuda *et al*., 2008). However, the former technique requires additional time to process the restriction enzyme treatment, and the latter method requires a complicated procedure for primer design and is difficult to modify to include additional species of thrips. Therefore, the multiplex PCR method may be the most useful tool for easy identification and rapid diagnosis at plant quarantine inspection sites. To facilitate the development of this method for introduction at quarantine inspection sites, it will be necessary to further evaluate the reliability of species determination. Therefore, a greater number of thrips specimens at each stage and another thrips species that may be found with the four species should be examined to determine the validity of the markers and the presence or absence of intraspecific variation.

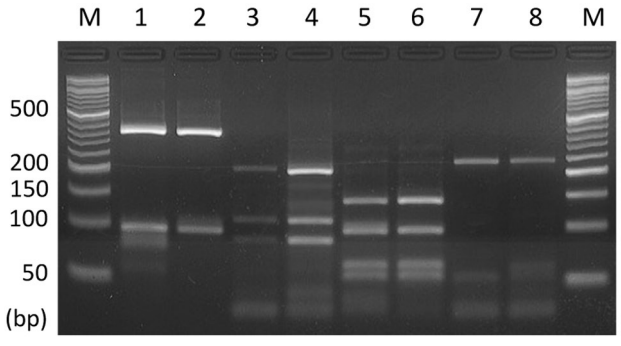


Fig. 3. Banding patterns detected from PCR products of the 4 thrips species using *Rsa*I restriction enzyme. The samples were electrophoresed in 4.0% agarose gel. Lane1, 2: *Thrips palmi* (Thailand), Lane3, 4: *T. tabaci* (Mexico), Lane5, 6: *Frankliniella intonsa* (Taiwan) , Lane7, 8: *F. occidentalis* (Ethiopia, Mexico), Lane M: 50bp ladder size standard. Restriction fragments shorter than 0.05kbp were ignored.

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**References**

Brunner, P. C., C. Flemming, and J. E. Frey (2002) A molecular identification key for economically important thrips species (Thysanoptera: Thripidae) using direct sequencing and a PCR-RFLP-based approach. *Agricultural and Forest Entomology*. **4**: 127-136.

Fukuda, S, K. Ichikawa and T. Ohya (2005) Identification of the thrips by the LAMP method. *Kanto Tokai Hokuriku agricultural research information*. 2004 edition **2**: 192-193. (In Japanese)

Higa, Y., T. Toma, Y. Tsuda and I. Miyagi (2010) A multiplex PCR-based molecular identification of five morphologically related, medically important subgenus stegomyia mosquitoes from the genus *Aedes* (Diptera: Culicidae) found in the Ryukyu archipelago, Japan. *Japanese Journal of Infectious Diseases*. **63**: 312-316.

Hinomoto, N., M. Muraji, T. Noda, T. Shimizu and K. Kawasaki (2004), Identification of five *Orius* species in Japan by multiplex polymerase chain reaction, *Biological control*. **31**(3): 276-279.

Kox, L. F. F., H. E. Van Den Beld, B. I. Lindhout and L. J. W. De Goffau (2005) Identification of economically

important *Liriomyza* species by PCR-RFLP analysis. *Bulettin OEPP/EPPO Bulletin*. **35**: 79-85.

Miyazaki, M. and I. Kudo (1986) Descriptions of Thrips Larvae which are Noteworthy on Cultivated Plants (Thysanoptera: Thripidae). I. Species Occurring on Solanaceous and Cucurbitaceous Crops. *Akitu* (New Series) **79**: 1-26.

Nakahara. S, T. Ishida, T. Dohino, S. Mizuniwa, M. Kaneda and M. Muraji (2005) Phylogenetic Relationships and Discrimination among 12 *Bactrocera* Species (Diptera: Tephritidae) Based on Sequences of Mitochondrial CO Ⅱ. *Res. Bull. Pl. Prot. Japan*. **41**: 15-25.

Osakabe, M., Y. Kotsubo, R. Tajima and N. Hinomoto (2008) Restriction Fragment Length Polymorphism Catalog for Molecular Identification of Japanese *Tetranychus* Spider Mites (Acari: Tetranychidae). *J. Econ. Entomol*. **101**(4): 1167-1175.

Rugman-Jones, P. F., J. G. Morse and S. Richard (2009) Rapid Molecular identification of Armored scale insects (Hemiptera: Diaspididae) on Mexican 'Hass' Abocado. *J. Econ. Entomol*. **102**(5): 1948-1953.

Rugman-Jones, P. F., M. S. Hoddle, L. A. Mound and S. Richard (2006) Molecular identification key for pest species of *Scirtothrips* (Thysanoptera: Thripidae). *J. Econ. Entomol*. **99**(5): 1813-1819.

Toda, S. and S. Komasaki (2002) Identification of thrips species (Thysanoptera: Thripidae) on Japanese fruit trees by polymerase chain reaction and restriction fragment length polymorphism of the ribosomal ITS2 region. *Bull. Entomol. Res*. **92**: 359-363.

Tsugane, T., H. Oida, C. Kubo and K. Shimizu (2007) Development of a Multiplex PCR Assay for Identification of *Bemicia tabaci* Biotype B, Biotype Q and *Trialeurodes vaporariorum*, *Annual Report of the Kanto-Tozan Plant Protection Society*. **54**: 159-164 (in Japanese).

## 和 文 摘 要

# マルチプレックスPCRによるアザミウマ４種の識別法の検討（英文）

中原　重仁・箕浦　和重

名古屋植物防疫所

輸入植物検疫で発見される４種アザミウマ(ネギアザミウマ、ミカンキイロアザミウマ、ヒラズハナアザミウマ、ミナミキイロアザミウマ)を迅速かつ簡易に識別するため、rRNA遺伝子のスペーサー領域であるITS2を対象としてマルチプレックスPCRの適用性を検討した。インターネット上のDNAデータベースから入手した４種アザミウマのITS2の塩基配列を比較し、284-471塩基の長さのDNA断片を増幅するための５種類の種特異的プライマーを設計した。日本国内で採集した個体及び輸入検疫で発見された個体について、これらのプライマーを用いたPCRを行ったところ、各種ごとに塩基配列から予想された長さのDNA断片が増幅され、それら断片長の比較により４種を識別することが可能であった。今後再現性や種内変異に関するデータをさらに蓄積して問題点等を精査することにより、特に未成熟ステージにおいて現状の形態による識別を補完する迅速な識別技術となりうる可能性が示された。