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

DNA Amplification of Single False Spider Mites (Acari: Tenuipalpidae) by Nested PCR 【Research report】

應用巢式 PCR 進行擬葉蟎 (Acari: Tenuipalpidae) 單一蟎體的 DNA 複製【研究報告】

Hui-Yi Li1 , Tsen Hua2, and Wen-Bin Yeh1*
李蕙宜1、華真2、葉文斌1*

*通訊作者E-mail : wbyeh@nchu.edu.tw

Received: 2009/11/11 Accepted: 2010/05/20 Available online: 2010/06/01

Abstract

Taxonomic recognition of tiny false spider mites using morphological features is not always easy due to their small size, especially in their immature stage. In the past decade or so, polymerase chain reaction has been introduced into molecular systematics. However, a major problem with using molecular markers in mite systematics is that sometimes not enough DNA template can be acquired from a single individual for DNA amplification. To solve this problem, we developed a nested PCR of individual false spider mites with an adult body size of ca. 300 μm, for DNA amplification. A dilution of up to 105 of the DNA from a single egg, a larva, a nymph or an adult mite contains enough template for the amplification of the target DNA, i.e., 28S ribosomal DNA.

摘要

長期以來，擬葉蟎都因其體型小特徵少而難以辨識，更遑論幼蟎期的鑑定。過去十年左右，聚合酶連鎖反應技術廣泛應用在分子分類學上，但其應用在蟎蜱系統分類學尚有其限制性，即從單隻蟎體純化而來的 DNA 量，常不足以應用於標的 DNA 的複製。為克服此一問題，我們應用巢式 PCR 的方式，複製成蟎體型僅有 300 μm 的單一擬葉蟎個體 DNA；從單一的卵、幼蟎、若蟎及成蟎純化而來的 DNA，在稀釋到 105 倍的情況下，仍可順利複製得 28S rDNA 標的區段。

Key words: nested PCR, Tenuipalpidae, false spider mite, Tenuipalpus pacificus, Raoiella indica

關鍵詞: 巢式 PCR、擬葉蟎科、太平洋擬葉蟎、印度擬葉蟎。

Full Text: PDF(4.22 MB)

下載其它卷期全文 Browse all articles in archive: http://entsocjournal.yabee.com.tw

台灣昆蟲 30: 103-111 (2010)
Formosan Entomol. 30: 103-111 (2010)

DNA Amplification of Single False Spider Mites (Acari: Tenuipalpidae) by Nested PCR

Hui-Yi Li[1], Tsen Hua[2], and Wen-Bin Yeh[1*]

[1] Department of Entomology, National Chung Hsing University, 250 Kuo Kuang Rd., Taichung City 40227, Taiwan
[2] Department of Plant Medicine, National Pingtung University of Science and Technology, 1 Shuefu Rd., Neipu, Pingtung County 91201, Taiwan

ABSTRACT

Taxonomic recognition of tiny false spider mites using morphological features is not always easy due to their small size, especially in their immature stage. In the past decade or so, polymerase chain reaction has been introduced into molecular systematics. However, a major problem with using molecular markers in mite systematics is that sometimes not enough DNA template can be acquired from a single individual for DNA amplification. To solve this problem, we developed a nested PCR of individual false spider mites with an adult body size of ca. 300 μm, for DNA amplification. A dilution of up to 10^5 of the DNA from a single egg, a larva, a nymph or an adult mite contains enough template for the amplification of the target DNA, i.e., 28S ribosomal DNA.

Key words: nested PCR, Tenuipalpidae, false spider mite, *Tenuipalpus pacificus*, *Raoiella indica*

Introduction

The family Tenuipalpidae Berlese consists of more than 800 described species in 25 genera (Zhang, 2003), including some of the world's major agricultural pests (Jeppson *et al*., 1975; Bolland *et al*., 1998). Taxonomic recognition of these mites using adult morphological features is not always easy due to their small size (300 μm) and the few characters available (Welbourn *et al*., 2003; Rodrigues *et al*., 2004; Groot and Breeuwer, 2006). Moreover, mites at the larval or nymphal stages often lack obvious diagnostic characteristics for species identification. The common method is to rear nymphal mites to adult stage and then follow the established identification procedures.

The difficulty in differentiating mites morphologically has led to the search for a simple and rapid method for their identification. In the past decade or so, polymerase chain reaction (PCR) has been

*Corresponding email: wbyeh@nchu.edu.tw

introduced into molecular systematics, and has been found to be useful for species identification of a variety of economically important mites. The most popular molecular markers used in the identification and phylogenetic study of mites are the partial sequences of the mitochondrial cytochrome oxidase subunit I (COI) (Toda *et al*., 2000; Rodrigues *et al*., 2004; Osakabe *et al*., 2005; Mitani *et al*., 2007; Ros and Breeuwer, 2007; Navajas and Navia, 2009) and the internal transcribed space 2 (ITS2) region of the ribosomal DNA (Navajas *et al*., 1992, 1997; Tli-Mattila *et al*., 2000; De Rojas *et al*., 2002, 2007; Noge *et al*., 2005; Vargas *et al*., 2005; Ben-David *et al*., 2007; Navajas and Navia, 2009). Ros and Breeuwer (2007) compiled and provided an extensive database of COI sequence information for the application of barcoding in Tetranychiidae, thereby showing the need to include phylogenetic information to delineate species groupings, instead of simply relying on sequence divergences. In addition, Noge *et al*., (2005), using the ITS2 region to infer the phylogeny of 73 specimens of Astigmata, concluded that this region is useful for studying lower level phylogenetic relationships, i.e., intraspecies or species of close affinity.

A major problem of using molecular markers in mite systematics is that sometimes not enough DNA template can be acquired for PCR due to the small body size. DNA content from a single mite, especially in its immature stage, is limited. However, it is important to get DNA from a single individual, instead of multiple individuals, to avoid the possibility of intermingling specimens from distinct genotypes or different species, particularly if there is any necessity for the identification of the mites (Cruickshank, 2002). Recently, Konakandla *et al*. (2006) used a random hexamer priming method for whole genome amplification using a single mite, while Desloire *et al*. (2006) compared four extraction methods of DNA from mites to evaluate the amplification efficiency of PCR. However, Gunn *et al*. (2007) pointed out that WGA is only an effective method of enhancing template DNA quantity when the initial sample is from high-yield material.

In this study, we used a nested PCR for amplifying 28S ribosomal DNA of individual false spider mites (body size ca. 300 μm). Its detection sensitivity was assessed by sequential dilution of the template DNA.

Materials and Methods

False spider mites of *Tenuipalpus pacificus* Baker and *Raoiella indica* Hirst were collected and reared individually on host plants in a greenhouse at 25°C for several generations in order to acquire individuals of each developmental stage. The mounting method of the mites for light microscopy was modified from Gutierrez (1985), and the method used in scanning electron microscopy was modified from Crooker (1985). Taxonomic characteristics of false spider mites were described by Tseng (1990), and Krantz (1978).

DNA extraction and primer design

Single and five individual false spider mites were homogenized in a 1.5 mL microtube with the Nuclei Lysis Solution. Then the genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI) as described in Hsu *et al*. (2005) and Hua *et al*. (2007). The recovered DNA pellet was dissolved in 30 μL distilled water and stored at -20°C.

In this study, primers for nested PCR of partial 28S rDNA were synthesized from sequences of several phytophagous mites (Hua *et al*., 2007). For the first PCR, conserved primers 28SA1 and 28SB1 designed from false spider mites, bulb mites, spider mites and eriophyoid mites were used to amplify a 700 bp fragment of 28S rDNA. For the second PCR, 28SA2

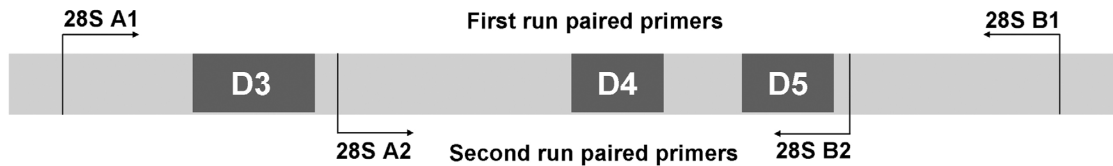

Fig. 1. Designed paired primers of nested PCR in the 28S rDNA region. Relative position of expansion segments from D3 to D5 of 28S rDNA is shown.

Table 1. Amplification results of single and five individuals of egg, larva, nymph, and adult of two mites; O and X mean that the products are amplified or not

Scientific name	Stage / No. / Dilution	Egg		Larva		Nymph		Adult	
		1	5	1	5	1	5	1	5
Raoiella indica	2^0	O	O	O	O	O	O	O	O
	2^1	O	O	O	O	O	O	O	O
	2^2	O	O	O	O	O	O	O	O
	2^3	O	O	O	O	O	O	O	O
	2^4	O	O	O	O	O	O	X	O
	2^5	O	O	O	O	O	O	X	O
Tenuipalpus pacificus	2^0	O	O	O	O	O	O	O	O
	2^1	O	O	O	O	O	O	O	O
	2^2	O	O	O	O	O	O	O	O
	2^3	O	O	O	X	X	O	O	O
	2^4	O	O	O	X	X	O	O	O
	2^5	O	X	O	X	X	O	O	O

and 28SB2 were used to amplify ca. 360 bp fragment of the 700 bp sequence.

Direct PCR

To determine the detection limit of direct PCR, a two-fold sequential dilution of DNA from two false spider mites, i.e., *Tenuipalpus pacificus* Baker and *Raoiella indica* Hirst, at each developmental stage was tested. The PCR was performed in a final volume of 25 μL containing 1 μL of DNA solution, 0.2 mM dNTP, 10 pmole of each primer (28SA1: 5'-CCGTCTTGAA ACACGGACCAAG-3' and 28SB1: 5'-TTC GGCAGGTGAGTTGTTACACAC-3'), 1 unit of SuperTaq polymerase (HT Biotechnology, Taiwan), and 1 unit of 1x provided the SuperTaq buffer. After a denaturation step (95°C for 2.5 min), 30 cycles of amplification (95°C for 1 min, 51°C for 1 min, 72°C for 1 min) and a final extension of 10 min at 72°C were performed. The PCR amplified product was visualized on 1% agarose gel incorporated with ethidium bromide.

Nested PCR to amplify partial 28S rDNA

DNA was extracted from two false spider mites at each of their developmental stages. This was then diluted in a 10-fold manner to 10^5, and 1 μL was used for the 1st round PCR under the same condition as in direct PCR. Then, 1 μL of the amplified product from the 1st round was used for the 2nd round PCR

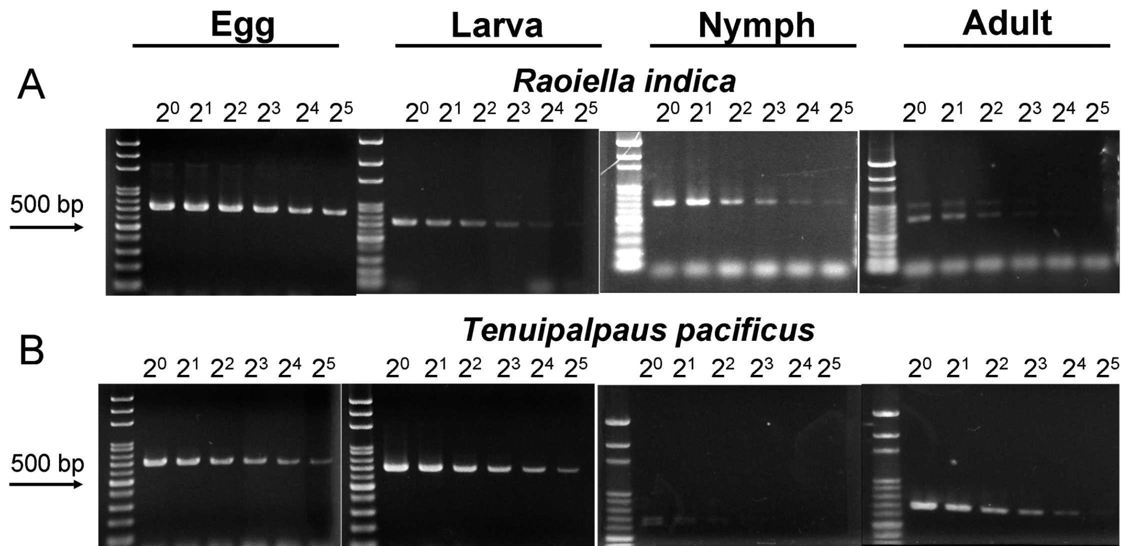

Fig. 2. Amplification results in a two-fold sequential dilution of DNA of a single individual egg, larva, nymph, and adult. (A) *Raoiella indica*; (B) *Tenuipalpus pacificus*; M: 100 bp marker.

under the same condition as in the direct PCR, except that the primers used were 28SA2 (5'-ACCCGAAAGATGGTGAACTAT-3') and 28SB2 (5'-CGCCAGTTCTGCTTACCAAA-3'), and the annealing temperature was increased to 53°C. In addition, approximate cycles of amplification were compared. The related primers and their amplified target are shown in Figure 1.

Sequencing of PCR products and DNA analysis

The nested PCR product was purified directly with the Qiaquick PCR Purification Kit or Qiaquick Gel Extraction Kit (QIAGEN, Germany) from agarose gel according to the manufacturer's instructions. Amplified fragments of 28S rDNA region from each species were sequenced directly using a *Taq* Dye Terminator Cycle Sequencing Kit with ABI 377A sequencer (Applied Biosystems, Foster City, CA).

Sequences of 28S rDNA for each species were used as query sequences to search for their homogeneous sequences under the Blastn algorithm in NCBI website (http://www.ncbi.nlm.nih.gov).

Results

Amplification of individual mites by standard PCR

The amplified 28S rDNA D3 to D5 region is about 700 bp (Fig. 1). Figure 2 shows the PCR products for a two-fold sequential dilution of genomic DNA from an individual (including egg, larva, nymph and adult) of two species, i.e., *Raoiella indica*, and *Tenuipalpus pacificus*. In general, the expected 700 bp fragment amplified by primers 28SA1 and 28SB1 is clearly observed when 2^{-2} of 1 μL DNA from each individual is used in direct PCR. In several reactions, this fragment can be amplified by up to a 2^5 dilution of the DNA template. However the amplification signals were only slightly stronger when the DNA extracted from five individuals was diluted for some reactions (Table 1).

Amplification of a single mite by nested PCR

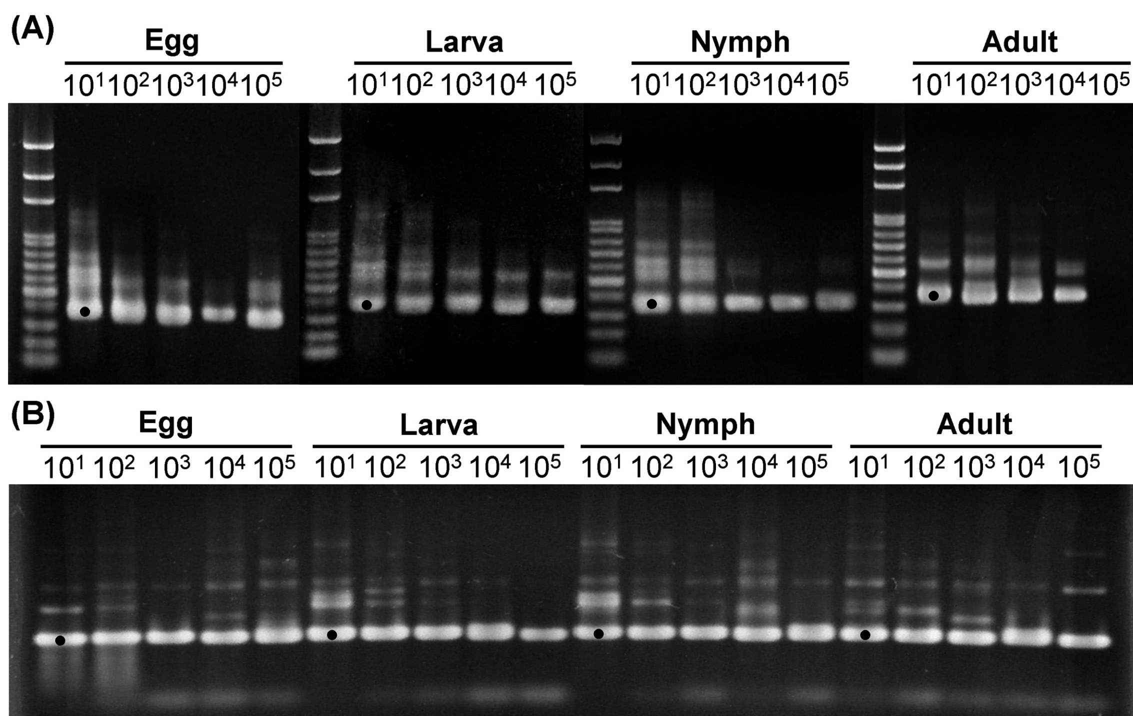

Fig. 3. Amplification results in a ten-fold sequential dilution of a single individual egg, larva, nymph, and adult by nested PCR. (A) *Raoiella indica*; (B) *Tenuipalpus pacificus*. The marked target bands were excised, purified, and sequenced. M: 100 bp marker.

Figure 3 shows the amplification products of nested PCR using a ten-fold sequential dilution of genomic DNA, up to 10^5, from a single individual of *Raoiella indica*, and *Tenuipalpus pacificus*. The expected 360 bp fragments using the second paired primers were efficiently amplified, although some non-targeted DNA fragments were also present.

One amplified fragment (with signals in Fig. 3), using paired primers of 28SA2 and 28SB2 in nested PCR, was purified and sequenced for each developmental stage of the two mites. The amplified products from *R. indica* and *T. pacificus* were identical in sequence to that of the original one.

After 15 PCR cycles, an ample amount of the expected 360 bp product was obtained when DNA from each individual was diluted up to 10^3 and used as the template for two mite species. Twenty five PCR cycles allowed the amplification of the target fragment at 10^5 dilution of DNA from each mite, but it also produced substantial non-target fragments (Fig. 4). PCR with 20 replication cycles appeared to be optimal, with clearly discernible target DNA at 10^4 and 10^5 dilutions of mite DNA and negligible non-target products.

Discussion

Lately, molecular markers have been widely used to promote the identification of mites (Noge *et al.*, 2005; Hinomoto *et al.*, 2007; Ros and Breeuwer, 2007; Navajas and Navia, 2009). Because of the small body size of mites, however, the problem of yielding enough of an amount of DNA template from a single individual for molecular identification remained to be

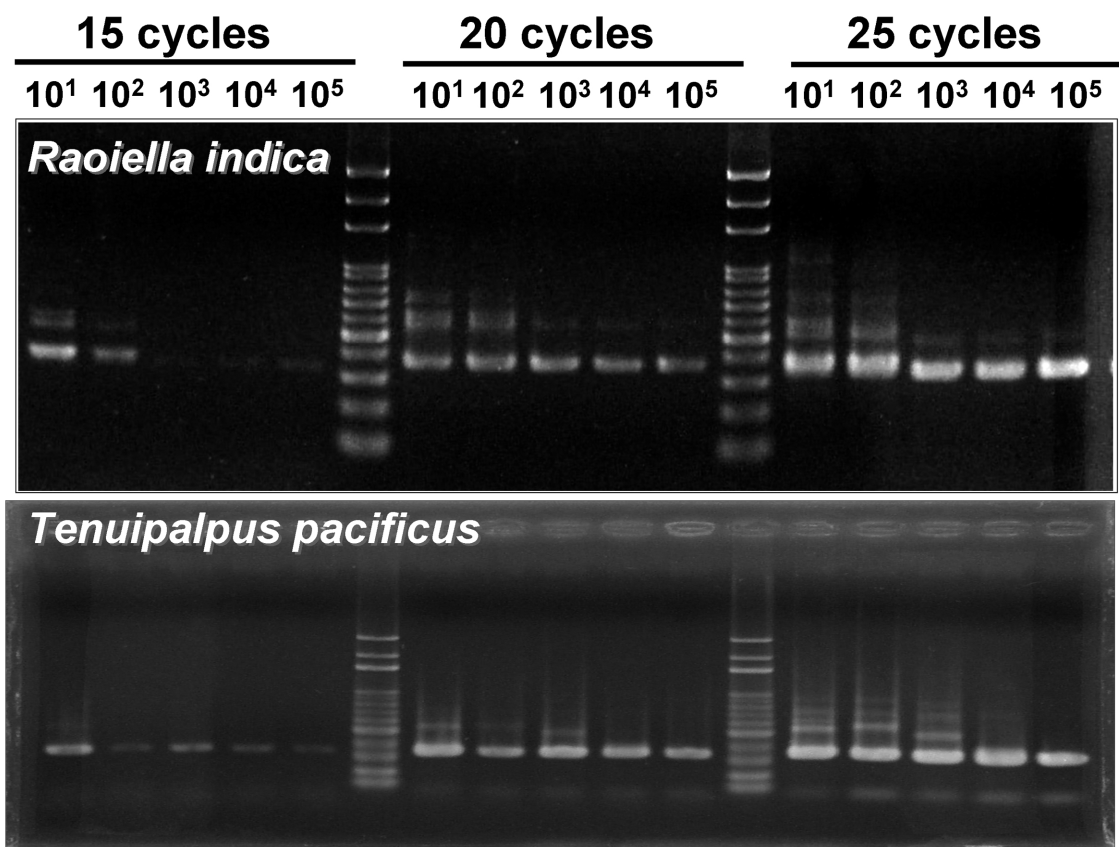

Fig. 4. Amplification efficiency of nested PCR with 15, 20, and 25 cycles for a single adult of two false spider mites. M: 100 bp marker.

solved. Taking the advantage of nested PCR which has been applied to a variety of organisms to amplify tiny amounts of DNA, we have proved herein that it is also useful for the molecular amplification of tiny mites.

This study shows that using a large amount of template DNA from tiny mites usually has a high amplification of the target DNA by PCR (see Table 1, and Figs. 2 & 4). However, in some cases the amplification possibility of using DNA from 5 individuals of *T. pacificus* larva is not necessarily higher than that of using DNA from a single individual. The amplification efficiency of target fragments was a 2^5-fold dilution for a single individual, but only a 2^2-fold dilution for 5 individuals, of *T. pacificus* larvae DNA (see Table 1). Thus, proper preservation of specimens is necessary to obtain optimal DNA quality for efficient amplification. Moreover, an optimal electrophoresis resolution is obtainable and helpful, if the electronic voltage and DNA concentration is adjusted approximately. Finally, the use of specific primers in the second PCR makes it possible to avoid generating non-target DNAs from symbiotic organisms, such as fungus, or other sources of contamination.

In this study, we applied nested PCR to successfully amplify the amplicon of a single individual (egg, larva, nymph, and adult) false spider mite, at 10^5 dilution of the extracted genomic DNA. Using DNA

from one individual for PCR provides more reliable amplification results than using DNA from multiple individuals, which may consist of more than one species.

Acknowledgments

We like to thank Dr. Yi-Hsiung Tseng for the identification of materials. This research was supported by grants from the Bureau of Animal and Plant Health Inspection and Quarantine, Council of Agriculture, Executive Yuan (94AS-13.3.1-BQ-B2, 95AS-13.3.1-BQ-B2, 96AS-13.3.1-BQ-B2), and the National Science Council of Taiwan, R.O.C. (NSC95-2317-B-005-009, NSC96-2317-B-005-004).

References

Ben-David, T., S. Melamed, U. Gersion, and S. Morin. 2007. ITS2 sequences as barcodes for identifying and analyzing spider mites (Acari: Tetranychidae). Exp. Appl. Acarol. 41: 169-181.

Bolland, H. R., J. Gutierrez, and C. H. W. Flechtmann. 1998. World Catalogue of the Spider Mite Family (Acari: Tetranychidae). Koninklijke Brill Press, Netherlands.

Crooker, A. R. 1985. Histological techniques. pp. 359-381. *In*: W. Helle, and M. W. Sabelis, eds. Spider Mites, Their Biology, Natural Enemies and Control, vol 1A. Elsevier, Amsterdam.

Cruickshank, R. H. 2002. Molecular markers for the phylogenetics of mites and ticks. Syst. Appl. Acarol. 7: 3-14.

De Rojas, M., J. M. Ubeda, C. Cutillas, M. D. Mora, C. Ariza, and D. Guevara. 2007. Utility of ITS1-5.8S-ITS2 and 16S mitochondrial DNA sequences for species identification and phylogenetic inference within the *Rhinonyssus coniventris* species complex (Acari: Rhinonyssidae). Parasitol. Res. 100: 1041-1046.

De Rojas, M., M. D. Mora, J. M. Ubeda, C. Cutillas, M. Navajas, and D. C. Guevara. 2002. Phylogenetic relationships in rhinonyssid mites (Acari: Rhinonyssidae) based on ribosomal DNA sequences: insights for the discrimination of closely related species. Parasitol. Res. 88: 675-681.

Desloire, S., C. V. Moro, C. Chauve, and L. Zenner. 2006. Comparison of four methods of extracting DNA from *D. gallinae* (Acari: Dermanyssidae). Vet. Res. 37: 725-32.

Groot, T. V. M., and J. A. J. Breeuwer. 2006. *Cardinium* symbionts induce haploid thelytoky in most clones of three closely related *Brevipalpus* Species. Exp. Appl. Acarol. 39: 257-271.

Gunn, M. R., K. Hartnup, S. Boutin, J. Slate, and D. W. Coltman. 2007. A test of the efficacy of whole-genome amplification on DNA obtained from low-yield samples. Mol. Ecol. Notes 7: 393-399.

Gutierrez, J. 1985. Mounting techniques. pp. 351-353. *In*: W. Helle, and M. W. Sabelis, eds. Spider Mites, Their Biology, Natural Enemies and Control, vol 1A. Elsevier, Amsterdam.

Hinomoto, N., D. P. Tran, A. T. Pham, T. B. N. Le, R. Tajima, K. Ohashi, M. Osakabe, and A. Takafuji. 2007. Identification of spider mites (Acari: Tetranychidae) by DNA sequences: a case study in Northern Vietnam. Int. J. Acarol. 33: 53-60.

Hsu, K., T. Hua, N. T. Chang, and W. B. Yeh. 2005. Molecular identification of important spider mites using multiplex PCR and PCR-RFLP. Formosan Entomol. 25: 211-223.

Hua, T., K. Hsu, H. Y. Li, N. T. Chang, and W. B. Yeh. 2007. 28S ribosomal DNA of tetranychid mites: a gene applicable to higher-level Acari phylogeny. Formosan Entomol. 27: 317-326.

Jeppson, L. R., H. H. Keifer, and E. W. Baker. 1975. Mites Injurious to Economic Plants. California University Press, California.

Konakandla, B., Y. Park, and D. Margolies. 2006. Whole genome amplification of Chelex-extracted DNA from a single mite: a method for studying genetics of the predatory mite *Phytoseiulus persimilis*. Exp. Appl. Acarol. 40: 241-247.

Krantz, G. W. 1978. A Manual of Acarology. 2nd ed. Oregon State Univerity Book Stores, Corvallis.

Mitani, H., M. Takahashi, M. Masuyama, and M. Fukunaga. 2007. *Ixodes philipi* (Acari: Ixodidae): phylogenetic status inferred from mitochondrial cytochrome oxidase subunit I gene sequence comparison. J. Parasitol. 93: 719-722.

Navajas, M., and D. Navia. 2009. DNA-based methods for eriophyoid mites studies: review, critical aspects, prospects and challenges. Exp. Appl. Acarol. 51: 257-271.

Navajas, M., D. Cotton, S. Kreiter, and J. Gutierrez. 1992. Molecular approach in spider mites (Acari: Tetranychidae): preliminary data on ribosomal DNA sequences. Exp. Appl. Acarol. 15: 211-218.

Navajas, M., J. Gutierrez, and T. Gotoh. 1997. Convergence of molecular and morphological data reveals phylogenetic information on *Tetranychus* species and allows the restoration of the genus *Amphitetranychus* (Acari: Tetranychidae). Bull. Entomol. Res. 87: 283-288.

Noge, K., N. Mori, C. Tanaka, R. Nishida, M. Tsuda, and Y. Kuwahara. 2005. Identification of astigmatid mites using the second internal transcribed spacer (ITS2) region and its application for phylogenetic study. Exp. Appl. Acarol. 35: 29-46.

Osakabe, M., K. Goka, S. Toda, T. Shintaku, and H. Amano. 2005. Significance of habitat type for the genetic population structure of *Panonychus citri* (Acari: Tetranychidae). Exp. Appl. Acarol. 36: 25-40.

Rodrigues, J. C., M. Gallo-Meagher, R. Ochoa, C. C. Childers, and B. J. Adams. 2004. Mitochondrial DNA and RAPD polymorphisms in the haploid mite *Brevipalpus phoenicis* (Acari: Tenuipalpidae). Exp. Appl. Acarol. 34: 275-290.

Ros, V. I. D., and J. A. J. Breeuwer. 2007. Spider mite (Acari: Tetranychidae) mitochondrial COI phylogeny reviewed: host plant relationships, phylogeography, reproductive parasites and barcoding. Exp. Appl. Acarol. 42: 239-262.

Tli-Mattila, Y., S. Paavanen-Huhtala, B. Fenton, and T. Tuovinen. 2000. Species and strain identification of the predatory mite *Euseius finlandicus* by RAPD-PCR and ITS sequences. Exp. Appl. Acarol. 24: 863-880.

Toda, S., M. Osakabe, and S. Komazaki. 2000. Interspecific diversity of mitochondrial COI sequences in Japanese *Panonychus* species (Acari: Tetranychidae). Exp. Appl. Acarol. 24: 821-829.

Tseng, Y. H. 1990. A Monograph of the Mite Family Tetranychidae (Acarina: Trombidiformes) from Taiwan. Special Publ. No. 9. Taiwan Museum, Taiwan.

Vargas, M., M. Garcia-Varlea, J. P. Laclette, and T. M. Perez. 2005. Application of ITS-2 sequences as markers for identification and phylogenetic inference within the genus *Geomylichus* (Acari: Listrophoridae). Exp. Appl. Acarol. 35: 223-238.

Welbourn, W. C., R. Ochoa, E. C. Kane, and E. F. Erbe. 2003. Morphological observations on *Brevipalpus phoenicis* (Acari: Tenuipalpidae) including comparisons with *B. californicus* and *B. obovatus*. Exp. Appl. Acarol. 30: 107-133.

Zhang, Z. Q. 2003. Mites of Greenhouses, Identification, Biology and Control. CABI Publishing, Wallington, UK.

Received: November 11, 2009
Accepted: May 20, 2010

應用巢式 PCR 進行擬葉蟎 (Acari: Tenuipalpidae) 單一蟎體的 DNA 複製

李蕙宜[1]、華眞[2]、葉文斌[1*]

[1] 國立中興大學昆蟲系　40227 台中市國光路 250 號
[2] 國立屏東科技大學植物醫學系　91201 屏東縣內埔鄉學府路 1 號

摘　要

長期以來，擬葉蟎都因其體型小特徵少而難以辨識，更遑論幼蟎期的鑑定。過去十年左右，聚合酶連鎖反應技術廣泛應用在分子分類學上，但其應用在蟎蜱系統分類學尚有其限制性，即從單隻蟎體純化而來的 DNA 量，常不足以應用於標的 DNA 的複製。為克服此一問題，我們應用巢式 PCR 的方式，複製成蟎體型僅有 300 μm 的單一擬葉蟎個體 DNA；從單一的卵、幼蟎、若蟎及成蟎純化而來的 DNA，在稀釋到 10^5 倍的情況下，仍可順利複製得 28S rDNA 標的區段。

關鍵詞：巢式 PCR、擬葉蟎科、太平洋擬葉蟎、印度擬葉蟎。

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