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Amplified mitochondrial DNA identify four species of *Tetranychus* mites (Acarina: Tetranychidae) in Korea

미토콘드리아 DNA 증폭을 이용한 한국의 잎응애속(*Tetranychus*; Acarina: Tetranychidae) 4종의 동정방법

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초 록 미국과 캐나다는 한국에 분포하는 잎응애속(*Tetranychus*) 중 범세계적 분포종인 점박이응애(*T. urticae* Koch)를 제외한 벚나무응애(*Tetranychus vienensis* Zacher), 차응애(*T. kanzawai* Kishida), 뽕나무응애(*T. truncatus* Ehara)를 검역대상으로 하고 있다. 잎응애속 응애들은 암컷성충으로 월동휴면에 들어가는데 기존의 수컷생식기의 형태를 위주로 한 동정방법으로는 이 휴면태의 암컷을 정확히 동정하기 어렵다. 월동을 위해 사과 과실 꼭지부에 우발적으로 부착할 가능성이 있는 것으로 우려되는 잎응애속 응애들의 월동휴면태에 대한 신속, 정확한 동정법이 수출검역현장에서 절실히 요구되는 실정이다. 사과의 주요해충인 점박이응애와 과수원 주변에서 발견되는 벚나무응애, 뽕나무응애, 차응애의 미토콘드리아 DNA(mtDNA)내 cytochrome oxidase subunit I(CO-I) 유전자를 PCR로 증폭하고 증폭된 DNA의 종간 변이를 이용하여 발육영기나 암수에 관계없이 동정할 수 있는 방법을 찾는 연구를 수행하였다. 세쌍의 primer에 의해 미토콘드리아 DNA의 CO-I 유전자 일부(680 bp)를 중복되게 증폭하였고 증폭된 유전자는 제한효소 *Alu*I, *Dde*I, *Sau*3A 대하여 응애종간 특이적 인식부위를 가지고 있었다. 제한효소에 의해 절단되는 특이적 DNA 단편은 *Tetranychus* 응애류를 동정하는데 유용한 표식인자로 사용될 수 있을 것이다. 아울러 증폭한 CO-I 유전자내의 제한효소 인식부위에 대한 이들 4종 응애의 유전자지도를 작성하였다.

검색어 *Tetranychus*, 잎응애, 미토콘드리아, DNA, cytochrome oxidase, PCR

ABSTRACT Except for a cosmopolitan and major pest of apples, *Tetranychus urticae* Koch, *Tetranychus* mites in Korea such as *T. viennensis* Zaher, *T. kanzawai* Kishida, and *T. truncatus* Ehara have been considered as quarantine pests by Canada and United States. Even though these mites are not feeders on apples, they are suspected to attach accidentally on apple fruits in autumn as females enter the diapause. The characters used to identify *Tetranychus* mites have been confined to the shape of aedeagus in adult male. To develope a fast and accurate alternative identification protocol applied to hibernating female mites on apples, their mitochondrial DNA (mtDNA) were examined to find out any polymorphisms to discriminate each species from the other ones. Three pairs of primers for polymerase chain reaction (PCR) were used to amplify cytochrome oxidase subunit I (CO-I) coding region in mitochondrial DNAs of four species of *Tetranycus* mites. The longest amplified product was estimated its size as about 680 bp. Digestion with restriction enzymes, *Alu*I, *Dde*I, and *Sau*3A, showed length polymorphisms, which will be useful as diagnostic markers to identify *Tetranychus* mites. Schematic restriction maps in amplified region were shown for each species.

KEY WORDS *Tetranychus*, mitochondrial DNA, cytochrome oxidase, mite, polymerase chain reaction

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INTRODUCTION

Apples seem to be one of the most eligible agricultural commodities in Korea to export to foreign countries such as Canada and the United States. Recently the apples produced in Korea have been exported to Canada for a trial marketing. Among *Tetranychus* mite species in Korea, *T. urticae* Koch is a cosmopolitan species but *Tetranychus viennesis* Zacher, *T. kanzawai* Kishida, and *T. truncatus* Ehara are considered as quarantine pests in Canada and the United States because they are not distributed in North America. Especially *T. viennensis* is described as a significant quarantine pest in U.S.A. (USDA, 1982).

Tetranychus mites are feeding on leaves of various plants during summer, but if they are encountered short photoperiod in autumn, female mites stop feeding and egg laying, become yellowish orange or red, and move to sheltered place like beneath bark or in crevices for overwintering (Veerman, 1985; Park *et al.*, 1990). Even though these mite species do not occur in apple orchards except two-spotted spider mites, *T. urticae*, which is major pests on apples, the importers suspect these non-feeders of apple to attach on apple fruits accidentally and hibernate in the concave on fruits. In order to identify *Tetranychus* mites species, the aedeagus of male has been used as an essential morphological character (Lee and Lee, 1992). However, only adult females produced in early autumn enter hibernal diapause (Veerman, 1985). If these hibernating female mites are intercepted on commercial apple fruits during the exportation and importation, it will be necessary to terminate their diapause and rear them to obtain male progenies to be identified by male reproductive organs. It needs too much time and labor to be applied to quarantine examination.

In the past several years, DNA analyses have been important tools to detect high levels of genetic variability. When compared to protein-based allozyme techniques, DNA analyses provide much more genetic variants and markers. These techniques also enabled us to use any samples of different developmental stages and samples kept in ethyl alcohol, dried for long time, and alive. However, the spider mite is so tiny that its DNA is not sufficient to be used for some popular DNA techniques such as restriction fragment length polymorphisms (RFLP) mediated by southern blotting. Even with limited DNA quantity, its DNA can be analysed through the amplification from polymerase chain reaction (PCR) (Arnheim et al., 1989). The genes in mitochondria have been chosen for systematic studies because they are easy to manipulate, maternally inherited, single copy, and nonrecombinning (Brown, 1985; Moriz *et al*, 1987; Attardi and Schatz, 1988; Wolstenholme, 1992). Several animal organisms including the drosophilid fly (*Drosophila yakuba*; Clary and Wolstenholme, 1985), the bee (*Apis mellifera*; Crozier and Crozier, 1993), nematodes (*Caenorhabditis elegans* and *Ascaris suum*; Okimoto *et al*, 1992), the mammalian (*Homo sapiens*; Anderson *et al*, 1981), and the sea urchin (*Strongylocentrotus purpuratus*; Jacobs *et al*, 1988), are well known of the genome organization and complete sequences in their mitochondrial DNA (mtDNA). From finding conserved sequences in these organisms, the primers for PCR have been designed and compiled (Kocher *et al*, 1989: Simon *et al*, 1994).

To develope a fast and precise alternative identification protocol to morphological methods, we tried to amplify the region of cytochrome oxidase subunit I (CO-I) in mtDNA mediated by PCR. In this amplified region, we found several species-specific restriction endonuclease recognition sites in *Tetranychus* female species. These specific sites will be used as diagnostic DNA markers to identify *Tetranychus* female mites attached on commercial apple fruits during quarantine examination before exportation.

MATERIALS AND METHOD

Samples of mites

Adults of *T. viennensis* were collected from cherry trees in Rural Development Administration, Suwon on September 1996. Another samples of this species were obtained from the population reared in the laboratory of Dr. Kwon, Y. J., Kyungbook University. *T. urticae*

and *T. kanzawai* samples were provided by the biological control laboratory in Entomology Division, National Institute of Agricultural Science and Technology (NIAST). Another samples of *T. kanzawaii* were also provided by Dr. Kwon. *T. truncatus* samples were obtained only from Dr. Kwon. All samples were reared again on the leaves of green bean in our laboratory except *T. viennensis*, which were reared on the leaves of cherry tree. The adult progenies derived from each samples were collected by brush, and preserved in absolute ethyl alcohol kept in -20°C freezer until their DNAs were extracted.

Mite DNA extraction

DNA extraction procedures from mites described by Kaliszewski *et al.* (1992) were followed with slight modification. About ten adult mites from each samples were taken out to a microtube and dried in the vacuum desiccator for 20 minutes. The dried mites were transferred on a frozen slide glass and minced with another slide glass covering the mites. Both glasses were washed with 200 μl of STE buffer (75 mM NaCl, 10 mM Tris-HCl/pH 7.8, 10 mM EDTA), which collected in microtubes. Into this homogenate solution, 10 μl of 20% SDS (sodium dedocyl sulphate) and 0.5 μl proteinase K (20 mg/ml) were added, and incubated at 60°C for 30 minutes and at 40°C for 3~5 hours. The DNA was purified by extracting with phenol/chloroform/isoamyl alcohol (25:24:1) and, once again, with chloroform/isoamyl alcohol (24:1). The samples were ethanol precipitated, washed twice with 70% ethanol, and air dried. The purified DNAs were dissolved with 20 μl sterile water.

Primers

To amplify the region encoding CO-I in mtDNA of *Tetranychus* mites, we selected primers for PCR after comparing the sequences in highly conserved regions provided by Simon *et al.* (1994). The sequences of the 4 primers as follows;

(1) C1J1718: 5' GGAGGATTTGGAAATTGATTAGTTCC 3'

(2) C1N2329: 5' ACTGTAAATATATGATGTGCTCA 3'

(3) C1N2191: 5' CCTGGTAAAATTAAGATATAAACTTC 3'

(4) C1J1751: 5' GGATCACCTGATATAGCATTCCC 3'.

The letter J and N refer to the major and minor strands, and the number refers to the position of the 3' base of the primer in the complete *Drosophila* mtDNA sequence (Clary and Wolstenholme, 1985; Simon *et al.*, 1994). Each primer pairs of C1J1718-C1N2329, C1J 1718-CIN2191, and C1J1751-C1N2191 could successfully amplify the same region of CO-I from four *Tetranychus* species at different sizes.

Polymerase chain reaction

PCR reactions were in 50μl of PCR buffer (50 mM KCl, 1.5m M $MgCl_2$, 10 mM Tris; pH 8.3) with 1 μl of template DNA solution, 30 pmol of each primer, each dNTP at 200 μM, and 2 units of *Taq* DNA polymerase. The PCR was performed using an MJ Research thermal controller (Model 60; Watertown, MA). The reaction profile consisted of the following five steps: (1) initial denaturation for 1 min at 95°C; (2) denaturation for 45 sec at 94°C; (3) annealing for 1 min at 55°C; (4) extension for 1 min 30 sec 72°C (35 cycles, steps 2~4); (5) final extension for 7 min at 72°C.

Enzyme digestion and electrophoresis

The restriction enzyme digestion was followed Hall and Smith (1991)'s recipe with slight modification. Total eight restriction enzymes (*Alu*I, *Dde*I, *Hae*III, *Msp*I, *Nci*I, *Rsa*I, *Sau*3A, and *Taq*I) were used to find restriction fragments polymorphisms among *Tetranychus* mites. Ten-microliter aliquots of PCR mixture were digested with either *Alu*I, *Dde*I, or *Sau3*A (BM), which showed polymorphisms. Taking the PCR buffer into consideration, an equal volume of reaction solution was added consisting of 1× the recommended buffer, 10 mM $MgCl_2$, 40 mM Tris-HCl (pH 8.0; only for *Dde*I), 100 mM NaCl (only for *Dde*I), 25 μg of bovine serum albumin, 2 mM dithiothreitol, and 3 units of enzyme. The digestion solution was incubated

for 3 hr or overnight at 37°C. The total digestion volume was loaded into 2% agarose gel, containing 0. 5 μg/m*l* of ethidium bromide, electrophoresed with tris-borate/EDTA (TBE) buffer under standard conditions, and photographed over UV light (Sambrook *et al.*, 1989).

RESULT AND DISCUSSION

The CO-I regions in mtDNA at three different sizes were successfully amplified using three pairs of primers (Fig. 1). Sizes of these amplified DNA segments were initially predicted by matching 5' position of primers to the sequence data from *D. yakuba* (Clary and Wolstenholme, 1985) as follows; 659 bp from position 1693 to 2351 in C1J1718-C1N2329, 524 bp from position 1693 to 2216 in C1J1718-C1N2191, and 488 bp from position 1729 to 2216 in C1J1751-C 1N2191. Approximate sizes from gel (Fig. 1) were close to calculations above. The longest amplified produt from C1J1718-C1N2329 primers was estimated as 680 bp at its length and the others were 500 bp and 470 bp from C1J1718-C1N2191, and C1J1751-C1N 2191, respectively. For each ampified fragment targeted CO-I region had very close size to that of *D. yakuba*, we thought that these fragments originated exactly from CO-I region. From this cosideration, we didn't take further confirmation by southern hybridization of gel with CO-I genes of other animals. We also selected and tested other ten primer pairs to amplify the genes encoding cytochrome oxidase subunit II (CO-II), NADH hydrogenase subunit 1 and 5 (ND1 and ND5), cytochrome b oxidase (Cyt b), and large-subunit rRNA (l-rRNA) coding genes but failed to amplify, possibly owing to mismatched sequences. More primers still will be tested from the search of wide range of conserved sequences published on several animals.

The longest fragment (680 bp), which must contain more informative variability than the other shorter ones, was digested with eight 4 or 5 base-recognizing restriction enzymes to find out reliable polymorphisms to distinguish each *Tetranychus* species from the others.

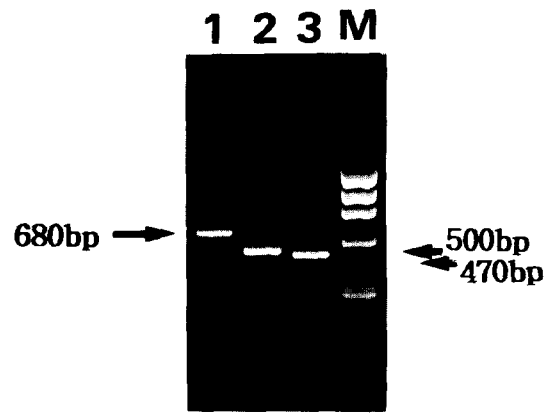

Fig. 1. PCR amplified mitochondrial DNAs from the region of encoding cytochrome oxidase subunit I (CO-I) from *Tetranychus kanzawai* Kishida. Lane 1: PCR product, 680 bp, from primer pairs of C1J1718- C1N2329, Lane 2: 500 bp from C1J1718-C1N2191, Lane 3: 470 bp from C1J1751-C1N2191, M: DNA size standard, ΦX 174 cut with *Hae*III. The approximate sizes are described by arrows and figures to each corresponding DNA bands.

Conclusively, three enzymes, *Alu*I, *Dde*I, and *Sau*3A, indicated the polymorphisms among four spider mite species as shown in Fig. 2 and 3. The other four enzymes such as *Hae*III, *Msp*I, *Nci*I, and *Rsa*I didn't cut the amplified segments, but the digestion with *Taq*I produced three fragments, which, however, were identical for all four *Tetranychus* spp.

As shown in Fig. 2, the PCR product obtained from *T. viennensis*, when it cut with *Alu*I, was separated into three fragments sized 380 bp, 170 bp, and 100 bp (two sites). That from *T. kanzawai* was cut into different 430 bp and 250 bp (one site) by *Alu*I. However, amplified products from *T. urticae* and *T. truncatus* were not cut with this *Alu*I enzyme; nevertheless, these two species were differently cut with another enzyme *Dde*I. *Dde*I produced 390 bp and 290 bp fragments (one site) from *T. urticae*, and 580 bp and 100 bp (one site) from *T. truncatus*. *Dde*I didn't cut *T. viennensis*'s and *T. kanzawai*'s DNA products. If each ten microliter of two aliquots are digested separately with *Alu*I and *Dde*I after PCR amplification with C1J1718-C1N2329, these four species should be easily identified after the followed electrophoresis. Because the amount of PCR

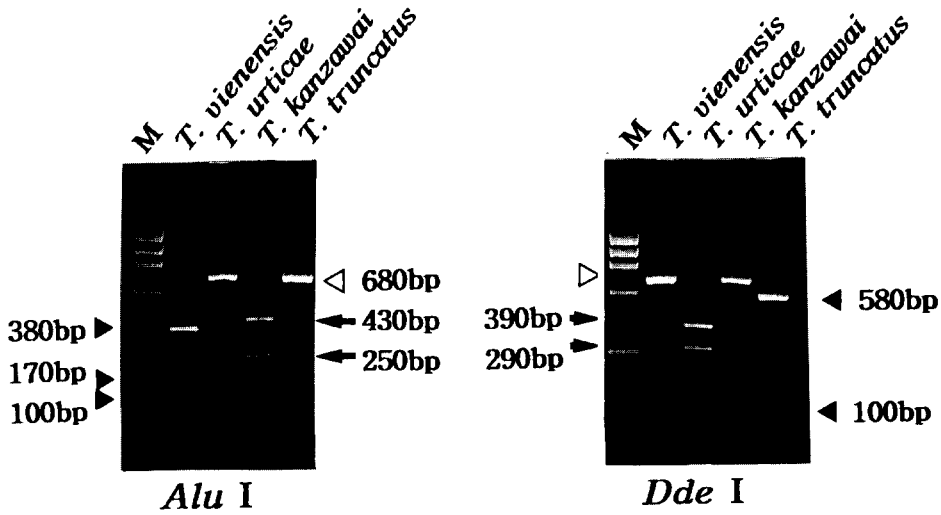

Fig. 2. Mitochondrial DNAs of four *Tetranychus* mites amplified by PCR primer pairs of C1J1718-C1N2329, and digested with restriction enzymes. M: DNA size standard, ΦX174 cut with *Hae*III. The approximate sizes are described by arrows and figures to each corresponding DNA bands. (A): digested with *Alu*I, (B): digested with *Dde*I. See text for the explanation in detail.

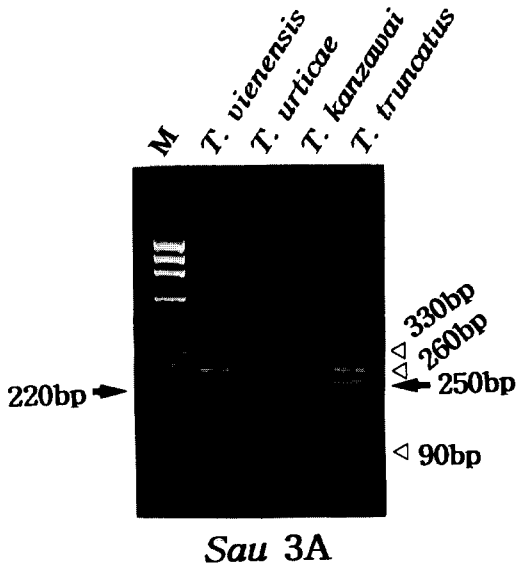

Fig. 3. *Sau*3A digested PCR products from four *Tetranychus* mite species. *T. kanzawai* has two different enzyme recognition sites from the other three species. *T. viennensis* has 220 bp medium-sized fragments but *T. urticae* and *T. truncatus* have 250 bp fragments instead of 220 bp. The size marker (M) is ΦX174 cut with *Hae*III.

product (50 μl) from each sample is more than enough to be tested with two enzymes, we don't need to spend extra time to amplify again. These length polymorphisms will be used as diagnostic DNA markers to identify *Tetranychus* mites for quarantine examination of apples.

Fig. 3 shows three fragments (two sites) of each species produced by *Sau*3A. *T. kanzawai* has different fragments from other three species. The median sized fragments, 220 bp, of *T. viennensis* is little shorter than those, 250 bp, of *T. urticae* and *T. truncatus*. To determine if there is another *Sau*3A site in the fragment of 250 bp in *T. viennesis*, we compared uncut DNAs of four species on the gel loaded partially digested PCR products for estimating distances between restriction sites of each respective restriction enzyme (figure not shown). From finding the amplified product of *T. viennensis* is still shorter than those of the other three species at its size, we concluded that *T. viennensis* loses the approximate 30 bp sequences inside of 250 bp fragment, which is conserved in *T. urticae* and *T. truncatus*.

Through the partial digestion by shortening digestion time to 30 min or 1 hr, the arrangement of fragments produced by each enzyme of *Alu*I, *Dde*I, or *Sau*3A was considered in each species. The double digestion with two different enzymes brought informations on

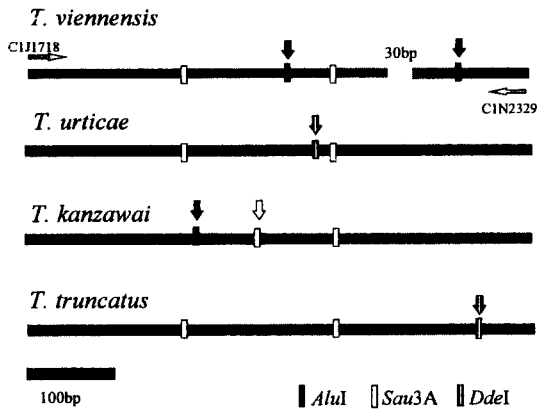

Fig. 4. Schematic restriction maps of four species of *Tetranychus* mites in the PCR amplified DNAs from CO-I encoding region of mtDNA. The distances between restriction sites were approximately estimated from Fig. 1 and 2, partial digestion, and double digestion (not shown).

the distances between enzyme recognition sites, thus schematic restriction maps in the amplified mtDNAs of four species could be drawn as Fig. 4.

These length polymorphisms of restriction fragments in mtDNA still need to be examined using more samples from populations distributed in different regions and hosts because we cannot exclude any variabilities in different populations within a species. If other genes in mitochondria or nucleus were found to be very variable and show informative polymorphism among mite species, the identification procedures can be more simplified from not treating or treating only one restriction enzyme.

To apply these techniques to the quarantine examination, the simpler and easier techniques should be developed. We are planning to modify the time-consuming phenol-mediated extraction of DNA in this study to be faster and simpler. There are alternative methods of preparing DNA from arthropods for PCR; chelating resin extraction (Edwards and Hoy, 1993; Jeyaprakash and Hoy, 1995) and boiling extraction in TE (10 mM Tris-HCl pH8, 1 mM EDTA) buffer (Field *et al.*, 1996). Even though, we extracted DNA from ten adult mites, enough template DNA could be obtained from single adult or nymph mite to react with PCR.

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