Discrimination of four Japanese *Tetranychus* species (Acari: Tetranychidae) using PCR-RFLP of the inter-transcribed spacer region of nuclear ribosomal DNA

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Abstract

To establish diagnostic DNA markers useful for discriminating economically important *Tetranychus* species, especially *T. kanzawai*, from other species of the *urticae* complex, we sequenced the ribosomal ITS region, including ITS1, 5.8S, and ITS2, of four *Tetranychus* species and analyzed recognition sites of restriction endonucleases. We established genetic criteria for discriminating *T. kanzawai*, *T. urticae*, *T. pueraricola* and *T. ludeni* using PCR-RFLP. We propose the establishment of a worldwide PCR-RFLP catalogue.

Key words: *Tetranychus*, ITS, PCR-RFLP, ribosomal DNA, species discrimination

INTRODUCTION

The genus *Tetranychus* (spider mites) includes serious common pests of legumes, vegetables, ornamentals, and fruit trees. Because its distribution is limited to East Asia and Australia (Ehara, 1999) and because of its development of resistance to many acaricides, *Tetranychus kanzawai* Kishida is a target pest for quarantine in international trade. Because female spider mites disperse much more than immature stages and adult males, a method for discriminating adult *Tetranychus* females is needed for quarantine, population dynamics studies, and crop damage forecastings. According to Ehara (1999), ten *Tetranychus* species live in Japan. However, only two species, *Tetranychus ludeni* Zacher and *Tetranychus okinawanus* Ehara can be identified morphologically in adult females. The remaining eight species, which are grouped into the *urticae* complex, are identified by the shape of the aedeagus (Ehara, 1999). No key for identifying adult females of the *urticae* complex has been provided, except for some tentative characterization methods (Enohara and Amano, 1996).

Goka and Takafuji (1997) revealed enzymatic differences among adult females of seven *Tetranychus* species and suggested a genetic marker, combining phosphoglucosomerase (PGI) and malate dehydrogenase (MDH) since not only interspecific but also intraspecific variation was found in PGI and MDH and some alleles were common among distinct species. Further, the position of detected bands is usually evaluated relative to the distance from the origin in comparison with standard samples. Therefore, the process of data analysis for identification would be complicated. Enohara and Amano (1996) showed differences in esterase isozymes among *Tetranychus* species. However, the activities of esterase isozymes change with host plant species (Mullin and Croft, 1983; Osakabe, 1984, 1987), and the isozymes are sometimes quite polymorphic within a species (Šula and Weyda, 1983).

Nuclear ribosomal DNA (rDNA) usually expresses low variation within a species, whereas nucleic acid substitutions are easily found among related species, especially in the rDNA's internal transcribed spacer (ITS) region. The analysis of re-

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striction fragment-length polymorphism following PCR (PCR-RFLP) is a relatively inexpensive and simple method of detecting DNA polymorphisms, and is useful in species diagnostics (Gasparich et al., 1995; Navajas and Fenton, 2000). In fact, the use of PCR-RFLP in the ITS region has already been used for species diagnostics in nematodes (Nasmith et al., 1996; Orui, 1996; Orui and Mizukubo, 1999). In spider mites, Gotoh et al. (1998) showed diagnostic RFLP in part of the ITS region, ITS2, between *Tetranychus urticae* Koch and *Tetranychus pueraricola* Ehara et Gotoh. The usefulness of ITS2 in solving *Tetranychus* species affiliations was proved by Navajas et al. (1997), and that of another ribosomal ITS region, ITS1, was suggested by Hinomoto and Takafuji (2001).

T. kanzawai, *T. urticae*, *T. pueraricola* and *T. ludeni* may all occur on the same plant together, and are sometimes misidentified. *T. urticae*, which includes two forms (green form and red form), is cosmopolitan, and has developed widespread acaricide resistance. *T. pueraricola* was recently recorded in Japan by Ehara and Gotoh (1996). The domestic and international distributions of this mite have not been sufficiently investigated. *T. ludeni* is widespread on a wide range of agricultural crops and wild plants around the world (Ehara and Masaki, 1989). This species can possibly be discriminated from the *urticae* complex by the relative position of a set of duplex setae to tactile setae on tibia I of females (Ehara, 1999). However, *Tetranychus desertorum* Banks distributed outside of Japan, resembles *T. ludeni* in these morphological characteristics (Ehara and Masaki, 1989).

In this study, we aimed at establishing diagnostic DNA markers useful for discriminating the four species, *T. kanzawai*, *T. urticae*, *T. pueraricola* and *T. ludeni*. We sequenced and analyzed on the ribosomal ITS region of these species. We found a useful set of restriction enzymes for use in diagnostic PCR-RFLP and propose the establishment of a worldwide catalogue of DNA markers.

MATERIALS AND METHODS

Collection records and identification of mites.

Local populations of *Tetranychus* species were collected from central and western Japan in 1997 and 1998, and four laboratory strains were provided by Ibaraki University and the National Institute of

Agrobiological Science (Table 1). Twenty-four adult females of each population, except the laboratory strains, were individually reared on small detached kidney bean leaves (2×2 cm) set on wet cotton, and kept at 22°C under 16L–8D until eggs deposited by the females developed to adults. After adult emergence of the next generation, three adult male progeny were selected from each detached leaf and individually mounted on slides in Hoyer's medium. They were identified from the key described by Ehara (1996). Progeny from nine to 15 original females in each population were successfully identified and kept as sub-populations. Lines identified as the same species in each population were mixed and increased for DNA analysis.

Tetranychus parakanzawai Ehara, which is closely related to *T. kanzawai* was newly described after our identification was done (Ehara, 1999). However, morphological differences between the two species are very small. It was known that unidirectional fertilization could occur between *T. parakanzawai* and *T. kanzawai* (Gomi and Gotoh, 1996). Therefore, the populations originally identified as *T. kanzawai* on the basis of morphology had to be reidentified as *T. kanzawai* or *T. parakanzawai*. Therefore, the populations originally identified as *T. kanzawai* were reciprocally crossed with reliable laboratory strains of *T. parakanzawai* (Tp=I2) and *T. kanzawai* (Tk), supplied by Ibaraki University. The Tk strain was collected from *Camellia sinensis* (L.) O. Kuntze in Kanaya, Shizuoka Prefecture, Japan, in May 1993, and the Tp from *Pueraria lobata* (Willd.) in Ami, Ibaraki Prefecture, Japan, in Oct. 1993 (Gomi and Gotoh, 1996).

Quiescent deutonymphs of populations originally identified as *T. kanzawai* (tester) and the standard strains Tp and Tk were separately placed on detached kidney bean leaves of 5×5 cm on pieces of wet cotton. Three new adult females of one tester that emerged were then transferred to a smaller detached kidney bean leaf (2×2 cm), and three adult males of one standard strain were introduced to the leaf from the stock culture. After a couple of days at 25°C, 16L–8D, the adults were removed from the leaf. The eggs deposited on the leaf were maintained, and the sex ratio of the offspring was checked two weeks later. Crosses between standard strain females×tester males and a control reciprocal cross between the standard

Table 1. Sources and collection dates of tetranychid mite populations

Species	Population	Date	Locality	Host plant	Geographic coordinates	Note
<i>T. kanzawai</i> ^a	I1	July 1997	Tsukuba, Ibaraki	<i>Pueraria lobata</i> (Willd.)	36°35'N–140°07'E	
	S1	July 1997	Kanaya, Shizuoka	<i>Camellia sinensis</i> (L.) O. Kuntze	34°49'N–138°08'E	
	N1	May 1997	Tawaramoto, Nara	<i>Citrullus lanatus</i> (Thunb.)	34°33'N–135°48'E	
	N2	May 1997	Nara, Nara	<i>Fragaria</i> × <i>ananassa</i> Duchesne	34°40'N–135°49'E	
	N3	May 1997	Gojo, Nara	<i>Zanthoxylum piperitum</i> DC.	34°21'N–135°42'E	
	N5	June 1997	Ohodo, Nara	<i>Pyrus pyrifolia</i> (Burm. F.) Nakai	34°24'N–135°48'E	
	N6B	Aug. 1997	Kashihara, Nara	<i>Solanum melongena</i> L.	34°31'N–135°48'E	
	W5	June 1997	Kishigawa, Wakayama	<i>S. melongena</i>	34°13'N–135°23'E	
	Y1	Oct. 1997	Ube, Yamaguchi	<i>Morus bombycis</i> Koids	33°57'N–131°15'E	
	W1	June 1997	Goboh, Wakayama	<i>Citrullus lanatus</i> (Thunb.)	33°51'N–135°09'E	
	W2	June 1997	Goboh, Wakayama	<i>Gypsophila paniculata</i> L.	33°51'N–135°09'E	
<i>T. urticae</i> red form	W3	June 1997	Goboh, Wakayama	<i>Eustoma grandiflorum</i> (Raf.) Shinn	33°51'N–135°09'E	
	C2	Mar. 1998	Nangoku, Kochi	<i>Lycopersicon esculentum</i> Mill.	33°34'N–133°38'E	
	C3	Mar. 1998	Nangoku, Kochi	<i>Dianthus caryophyllus</i> L.	33°34'N–133°38'E	
	L	unknown	unknown	(<i>Phaseolus vulgaris</i> L.)	unknown	Laboratory strain
	N6A	Aug. 1997	Kashihara, Nara	<i>S. melongena</i>	34°31'N–135°48'E	
<i>T. pueraricola</i>	C4	Mar. 1998	Nangoku, Kochi	<i>F.</i> × <i>ananassa</i>	33°34'N–133°38'E	
	I2	Oct. 1993	Hitaichiohta, Ibaraki	<i>P. lobata</i>	36°54'N–140°52'E	Laboratory strain
	G1	Oct. 1994	Matsumoto, Nagano	<i>P. lobata</i>	36°14'N–137°58'E	Laboratory strain
	N7	May 1996	Ikaruga, Nara	<i>P. lobata</i>	34°37'N–135°44'E	Laboratory strain
<i>T. ludeni</i>	C1	Mar. 1998	Nangoku, Kochi	<i>Perilla frutescens</i> (L.) Britt. var. <i>crispa</i> (Benth.) W. Deane	33°34'N–133°38'E	
	W4	June 1997	Kishigawa, Wakayama	<i>S. melongena</i>	34°13'N–135°23'E	
	W9B	May 1997	Ootoh, Wakayama	<i>Akebia trifoliata</i> (Thunb.) Koidzumi	33°43'N–135°30'E	

^a Species identification was done before the description of *T. parakanzawai* as a new species by Ehara (1999). Thus, *T. kanzawai* possibly includes *T. parakanzawai*.

strains were done in the same manner. *Tetranychus* species show arrhenotoky (Helle and Pijnacker, 1985). Because *T. parakanzawai* females crossed with *T. kanzawai* males produce no female offspring, whereas some female offspring were produced from crosses between *T. kanzawai* females and *T. parakanzawai* males, the mites were decisively identified from the sex ratios in the offspring produced by the reciprocal crosses.

Sample preparation for DNA analysis. We used the DNA preparation method of Goka et al. (2001) with modifications. A single adult female was homogenized in 20 μ l of lysis buffer (10 mM Tris-HCl [pH 8.0], 100 mM EDTA, 0.5% Igepal CA-630 [Sigma], 10 mM NaCl, 1 mg/ml Proteinase K) using a glass rod in a sample tube. The homogenate was incubated at 65°C for 15 min and then at 95°C for 10 min. After incubation, the homogenate was diluted with 380 μ l 0.1 $\times$ TE buffer (1 mM Tris-HCl [pH 8.0], 0.1 mM EDTA) and stored at -20°C until use in a polymerase chain reaction (PCR) as a DNA template.

PCR amplification. The primers used to amplify the ITS region were rD02 forward: 5'-GTC-GTAACAAGGTTTCCGTAGG-3' (Hinomoto and Takafuji, 2001), and HC2 reverse: 5'-ATATGCT-TAAGTTCAGCGGG-3' (Navajas et al., 1994). PCR assays were conducted with 1 μ l of each DNA template in a total reaction volume of 20 μ l buffer (2.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl [pH 8.3]) containing 0.2 mM of each dNTP, 10 pmoles of each primer, and 0.5 units of AmpliTaq DNA polymerase (PE Applied Biosystems). PCR conditions were an initial 1 min at 95°C; followed by 30 s at 92°C, 30 s at 50°C, and 1 min at 72°C for 30 cycles; and a final 10 min at 72°C (GeneAmp PCR System 2400; PE Applied Biosystems). The resulting PCR products (ca. 1,200 bp) were checked by agarose gel (2%) electrophoresis using the TAE buffer system and visualization by ethidium bromide staining under UV light, and then used for sequencing or RFLP analysis.

Sequencing and analysis of recognition sites for restriction enzymes. Amplified ITS fragments of *T. kanzawai* (S1), *T. urticae* (W1), *T. pueraricola* (I2) and *T. ludeni* (W4) were cloned into pCRII plasmids by using a TA Cloning Kit (Invitrogen) and transformed into competent *Escherichia coli* cells (INV α F'). The transformant was selected on LB plates containing kanamycin and X-gal and

cultivated in 4 ml of LB medium overnight. Plasmids were then extracted and dissolved in TE buffer (200 ng/ μ l final concentration). Sequencing was performed in an ABI Model 373S Sequencer (Applied Biosystems) using an ABI Prism Dye Terminator Kit with M13-40 forward and M13 reverse primers; specific primers for ITS: rD02 and HC2; and five inner-primers: rD03 reverse: 5'-TG-GCTGCGTTCTTCATCG-3' (Hinomoto and Takafuji, 2001), LC1 forward: 5'-CGAGTATCGAT-GAAGAACGCA-3' (Navajas et al., 1994), ITS1-378 forward: 5'-GCTTATGCTGACGGC-3', ITS1-512 forward: 5'-TAAAAGTCCCACGGT-3' and ITS2-83 reverse: 5'-TGACCTCGGAAAAGACC-C-3'. The last three primers were based on the sequence of *T. urticae* used in this study. Recognition sites for restriction endonucleases on obtained sequences were analyzed with Genetyx-Win software (Software Development). Two colonies of transformed *E. coli* were used for the sequence analysis in each species.

PCR-RFLP detection. The ITS fragments of all populations amplified as above were digested by two restriction endonucleases, *Dra*I and *Afa*I, whose recognition sites varied among species in the above sequence analysis. For the digestion, 8.5 or 9 μ l of amplified reactions was used directly. The resulting digested fragments were separated by 3.5% agarose gel electrophoresis (Agarose X, Nippon Gene) and visualized by ethidium bromide staining under UV light to compare the restriction patterns.

RESULTS

Sex ratio of offspring after crossing between local populations and standard strains of *T. kanzawai* and *T. parakanzawai*

The sex ratio (female/total) of offspring produced after the reciprocal cross between the standard *T. kanzawai* strain (Tk) and the standard *T. parakanzawai* strain (Tp) was low or 0 (Table 2). After the cross between Tk females and Tp males, a small number of F₁ adult females emerged, although the sex ratio was biased to male. No female offspring appeared after the cross between the Tp females and the Tk males.

In most of the reciprocal crosses between local populations and Tk, the sex ratios of the offspring were equal or female-biased (Table 2). However, no

Table 2. Sex ratios in offspring after copulation between local populations and standard strains of *T. kanzawai* and *T. parakanzawai*

Copulation with <i>T. kanzawai</i> (Tk)				Copulation with <i>T. parakanzawai</i> (Tp)			
♀×♂	No. eggs±SE	No. adults checked±SE	Sex ratio [♀/(♀+♂)]±SE	♀×♂	No. eggs±SE	No. adults checked±SE	Sex ratio [♀/(♀+♂)]±SE
Tk×Tp	32.0±0.5	30.0±0.7	0.185±0.435	Tp×Tk	33.0±2.7	30.3±2.2	0
I1×Tk	81.3±2.0	78.0±1.8	0	I1×Tp	87.5±1.7	85.5±1.8	0.787±0.079
Tk×I1	42.8±0.3	38.5±0.5	0.654±0.096	Tp×I1	45.0±3.5	42.8±3.5	0.818±0.043
S1×Tk	47.3±0.6	39.8±0.8	0.493±0.204	S1×Tp	49.5±1.1	44.3±1.4	0.170±0.433
Tk×S1	53.8±0.6	51.8±0.5	0.756±0.098	Tp×S1	52.3±1.3	44.0±1.7	0
N1×Tk	41.8±2.3	40.3±2.6	0.796±0.079	N1×Tp	24.5±2.4	20.0±1.8	0.478±0.617
Tk×N1	59.8±1.3	58.8±1.3	0.672±0.060	Tp×N1	18.5±1.1	16.3±1.0	0
N2×Tk	52.8±2.5	50.5±2.4	0.714±0.152	N2×Tp	39.8±0.8	34.5±0.9	0.449±0.068
Tk×N2	49.3±1.2	46.3±0.8	0.710±0.108	Tp×N2	34.3±1.0	33.0±0.9	0
N3×Tk	52.3±1.4	49.8±1.6	0.837±0.045	N3×Tp	20.0±0.4	17.3±0.1	0.592±0.292
Tk×N3	42.3±1.5	28.0±1.0	0.550±0.273	Tp×N3	52.5±2.1	52.0±2.1	0
N5×Tk	33.5±1.9	24.3±1.7	0.734±0.208	N5×Tp	26.8±0.9	24.0±1.2	0.138±0.471
Tk×N5	55.3±1.7	52.3±1.6	0.637±0.076	Tp×N5	55.0±2.2	48.5±1.7	0
N6B×Tk	66.7±0.6	62.3±1.0	0.806±0.078	N6B×Tp	51.5±0.8	49.8±0.4	0.252±0.333
Tk×N6B	81.0±0.8	63.5±0.8	0.701±0.329	Tp×N6B	51.5±1.3	48.3±1.1	0
W5×Tk	20.3±1.0	18.0±0.7	0.946±0.205	W5×Tp	16.3±1.4	15.0±1.2	0.296±0.763
Tk×W5	66.0±2.0	59.3±1.4	0.772±0.084	Tp×W5	54.5±1.8	48.8±1.8	0
Y1×Tk	30.5±3.2	27.3±2.9	0.804±0.156	Y1×Tp	34.7±2.5	34.0±1.9	0.292±0.103
Tk×Y1	48.5±1.6	46.8±1.6	0.520±0.191	Tp×Y1	49.5±2.0	39.5±1.7	0.080±0.165

female offspring were produced in the cross of I1 female×Tk male. Sex ratios of offspring were high in the reciprocal cross between I1 and Tp. On the other hand, the sex ratios of Tp females mated with males of the other local populations were very low (Tp×Y1: 0.080) or zero. In the crosses between females of local populations and Tp males, the sex ratios were 0.138–0.592.

According to Gomi and Gotoh (1996), *T. kanzawai* females mated with *T. parakanzawai* males produced female offspring, while few females were produced by *T. parakanzawai* females mated with *T. kanzawai* males. We confirmed the unidirectional fertilization of reciprocal crosses between the two species in the crosses between the standard strains Tk and Tp. The results of crossing experiments between the standard strains and the local

populations showed that I1 was *T. parakanzawai* and the others were *T. kanzawai*.

Sequence and recognition sites for restriction enzymes

The lengths of ITS regions were as follows: *T. kanzawai*, 1,203 bp; *T. urticae*, 1,205 bp; *T. pueraricola*, 1,208 bp; and *T. ludeni*, 1,197 bp, including the PCR primers. The nucleotide sequences are available from the DDBJ/EMBL/GenBank database with accession numbers of AB076369–72. At least one recognition site for 34 to 36 of 131 restriction endonucleases was found on each sequence. Differences in the position or number of recognition sites among species were found in 22 of the enzymes. Even if there is recognition site difference between species, it is not useful for

Fig. 1. RFLP banding patterns of the ITS region of rDNA expected from the sequence in each species. K: *T. kanzawai*, U: *T. urticae*, P: *T. pueraricola*, L: *T. ludeni*. Figures in parentheses show the number of recognition sites.

species discrimination if the size difference between the fragments after digestion is not big enough to be shown by electrophoresis.

The banding patterns of the fragments that would be detected by electrophoresis were expected by the fragment sizes calculated from the sequencing data and the recognition sites. Eight of the expected banding patterns are shown in Fig. 1. Many banding patterns show differences among species. However, some of the characteristic banding patterns, for example, those in *T. urticae* after digestion by *Alu*I, *Bcl*I, and *Sau*3A I and in *T. pueraricola* by *Mun*I, depend on point mutations among copies of ribosomal DNA at the recognition sites. Such point mutations cause partial digestion, increase the number of electrophoretically detectable bands, and sometimes produce characteristic banding patterns. However, partial digestion makes the fragment signal weaker and throws the judgement of the banding pattern into confusion. Additionally, very small fragments can not be detected by the agarose gel electrophoresis. Consequently, we chose two restriction endonucleases, *Dra*I and *Afa*I, as candidates for diagnostic agents.

PCR-RFLP using diagnostic restriction endonucleases

The expected patterns shown in Fig. 1 matched the actual banding patterns after the digestion of

Fig. 2. Photograph of *Dra*I-digested patterns of the PCR-amplified ITS region of rDNA from a single adult female on a 3.5% agarose gel. K: *T. kanzawai*, U: *T. urticae*, P: *T. pueraricola*, L: *T. ludeni*. The right lane shows the banding pattern of a standard size marker (λ -EcoT14I).

amplified ITS fragments by *Dra*I and *Afa*I (Figs. 2 and 3). The smallest band produced by *Dra*I in both *T. pueraricola* and *T. ludeni* was not detectable by electrophoresis. The sizes of the larger of the two detected fragments followed the order *T. kanzawai*=*T. urticae*>*T. ludeni*>*T. pueraricola*, characterizing the latter two species. The fragments produced by *Afa*I characterized *T. urticae* with a smaller band, which was expected to consist of 163 and 185 bp fragments (Fig. 1). The bands appear-

ing around 350 bp were also estimated to be complex, with two similar-sized fragments except in *T. urticae* (Fig. 1). Consequently, the banding patterns produced by *Dra*I were grouped into three types,

Fig. 3. Photograph of *Afa*I-digested patterns of the PCR-amplified ITS region of rDNA from a single adult female on a 3.5% agarose gel. K: *T. kanzawai*, U: *T. urticae*, P: *T. pueraricola*, L: *T. ludeni*. The right lane shows the banding pattern of a standard size marker (100 bp ladder).

k_D , p_D , and l_D , and those by *Afa*I into two types, k_A and u_A , on the basis of perceptible variation. This set of RFLP banding patterns should allow the *Tetranychus* species to be discriminated: k_D and k_A banding patterns indicate *T. kanzawai*, k_D and u_A indicate *T. urticae*, p_D and k_A indicate *T. pueraricola*, and l_D and k_A indicate *T. ludeni*.

The types of PCR-RFLP banding patterns detected in the local populations and laboratory strains are shown in Table 3, except for that of I1, which we identified as *T. parakanzawai*. We found no contradiction between the morphological and genetic criteria in any population. This result strongly supports the usefulness of PCR-RFLP for the discrimination of adult females of the four *Tetranychus* species. After some modification, this genetic criterion could be applied to any immature stages.

On the other hand, the Tp strain showed the same banding patterns as *T. kanzawai*: k_D and k_A

Table 3. Types of fragments digested by restriction endonucleases

Species	Population	<i>Dra</i> I		<i>Afa</i> I	
		No. ♀ tested	Type of fragment ^a	No. ♀ tested	Type of fragment ^b
<i>T. kanzawai</i>	S1	7	k_D	8	k_A
	N1	4	k_D	2	k_A
	N2	2	k_D	2	k_A
	N3	1	k_D	1	k_A
	N5	2	k_D	2	k_A
	N6B	3	k_D	3	k_A
	W5	2	k_D	2	k_A
	Y1	2	k_D	2	k_A
<i>T. urticae</i> red form	W1	4	k_D	3	u_A
	W2	4	k_D	2	u_A
	W3	3	k_D	3	u_A
	C2	2	k_D	2	u_A
	C3	6	k_D	4	u_A
<i>T. urticae</i> green form	L	2	k_D	2	u_A
	N6A	4	k_D	2	u_A
	C4	5	k_D	5	u_A
<i>T. pueraricola</i>	I2	7	p_D	6	k_A
	G1	2	p_D	2	k_A
	N7	3	p_D	3	k_A
<i>T. ludeni</i>	C1	7	l_D	5	k_A
	W4	5	l_D	3	k_A
	W9B	2	l_D	2	k_A

^a See Fig. 2. ^b See Fig. 3.

Fig. 4. Comparison of ITS fragments digested by *Dra*I and *Afa*I between *T. kanzawai* (S1 and Tk) and *T. parakanzawai* (I1 and Tp). s: standard size marker (100 bp ladder).

(Fig. 4). This indicates that *T. parakanzawai* is not discriminated from *T. kanzawai* by the PCR-RFLP analysis developed in this study. The I1 population also showed the k_D and k_A banding patterns in RFLP (Fig. 4).

DISCUSSION

We settled the problem of how to discriminate adult females of *T. kanzawai*, *T. urticae*, *T. pueraricola* and *T. ludeni* by electrophoretic separation of banding patterns of the ribosomal ITS region. During the past decade, three new species of *Tetranychus*—*T. pueraricola*, *T. parakanzawai* and *T. okinawanus* Ehara—have been recorded (Ehara, 1995, 1999; Ehara and Gotoh, 1996). Additionally, Ehara and Yamaguchi (2001) discovered *Tetranychus neocaledonicus* André in Amami-Ōshima, Japan. Consequently, the number of *Tetranychus* species in Japan has been increased to eleven. Adult females of these species are reddish except for the green form of *T. urticae*. Recent trade globalization has improved the chance of invasion by exotic pests to new areas. Several controversies in classification—for example, *T. urticae* and *Tetranychus cinnabarinus* (Boisduval) (Dupont, 1979; Gotoh et al., 1993; Goka et al., 1996), and *T. kanzawai* and *Tetranychus hydrangeae* Pritchard and Baker (Ehara and Wongsiri, 1975; Navajas et al., 2001)—are evidence of the difficulty of identifying

Tetranychus species solely by morphological characters, and of availability of molecular markers. We expect that the identification method using PCR-RFLP can be generalized to include species we have not analyzed. However, agarose gels and acrylamide gels are usually temporary, and it is difficult to exactly compare small differences between close bands on photographs of separate gels. On the other hand, sequence analysis is too expensive and too time consuming to be used in routine affairs such as quarantine.

We have developed a model of RFLP patterns based on the ITS sequence and the results of computer analysis of the recognition site for restriction endonucleases (Fig. 1). All expected banding patterns were represented by electrophoresis. Such standardized molecular data will be convenient for quarantine services, because it is easy to compare foreign species with domestic ones without importing species. If the primer set used in this study can be used for every species, then we can create a worldwide catalogue of ITS RFLPs for *Tetranychus*.

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