

RESEARCH ARTICLE

Sequence analysis of the ribosomal internal transcribed spacers region in spider mites (Prostigmata: Tetranychidae) occurring in citrus orchards in Eastern Spain: use for species discrimination

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Keywords

Citrus reticulata; internal transcribed spacer; molecular species discrimination; *Panonychus citri*; pest management; phylogenetic relationships; *Tetranychus* spp.

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Abstract

Tetranychus urticae is a polyphagous mite which is an important pest of citrus worldwide. This mite can be found feeding on many plant species occurring in the citrus agrosystem moving from weeds to trees. Because field samples consist of a mixture of different Tetranychidae species, as a first step necessary to further implement population characterisation of *T. urticae*, species-discriminating criteria based on molecular techniques are needed. In this study, the nucleotide variation of the internal transcribed spacers (ITS) 1 and 2 and the intergenic 5.8S fragment of nuclear rDNA of *T. urticae*, *Tetranychus turkestanii*, *Tetranychus evansi*, *Tetranychus ludeni* and *Panonychus citri* have been determined. Results demonstrate that for these species, the rDNA ITS2 regions are much more conserved than the corresponding rDNA ITS1. The high homogeneity of the ITS2 sequence observed among the specimens of *T. urticae* obtained from the same ecoregion makes this DNA sequence an excellent tool for species discrimination. ITS sequences differentiate not only species but also specimens from different geographical origin. Furthermore, polymerase chain reaction–restriction fragment length polymorphism analysis of the ITS2 proved adequate for a quick screening of high numbers of field samples.

Introduction

Tetranychus urticae Koch (Acari: Tetranychidae) is an important pest of citrus in Spain (Aucejo-Romero *et al.*, 2004; Ansaloni *et al.*, 2008) as well as in some citrus-growing areas, especially on mandarins under Mediterranean climate (Bodenheimer, 1951; Talhouk, 1975; Swirski, 1977; McMurtry, 1985; Vacante, 1986; Hmimina *et al.*, 1995; Souliotis *et al.*, 1997). This mite constitutes the key pest of clementine mandarins, *Citrus reticulata* Blanco, in the region of La Plana, the area around the city of Castelló de la Plana (39°59'N, 00°02'W), where Spanish clementine production concentrates (around 1.5 million tons from 60 000 ha). Mite infestations in clementines result in chlorotic spots on leaves but more importantly in fruit scarring, which decreases its commercial value. Moreover, severe infestations can result in massive, sudden leaf drop (Martínez-Ferrer *et al.*, 2006).

T. urticae can be found feeding on many plant species occurring in the citrus agrosystem (Aucejo *et al.*, 2003) where several other tetranychid species coexist [*Tetranychus evansi* Baker & Pritchard, *Tetranychus turkestanii* (Ugarov & Nikolshi), *Tetranychus ludeni* Zacher and *Panonychus citri* (McGregor)] (unpublished results).

Different molecular techniques such as microsatellites (Bailly *et al.*, 2004) or the mtDNA gene coding for cytochrome oxidase I (Xie *et al.*, 2006) have already been used for population genetic studies in tetranychid mites. However, before these techniques can be applied to mites occurring in citrus, reliable techniques to separate the different species, while usable material is preserved for further genetic analysis, are needed.

Although morphologically based taxonomic characters to distinguish between the five Tetranychidae species found in our citrus groves exist, these characters require the mounting of whole adult mites (around 400 µm in

size), obviously dead, which makes these specimens useless for further genetic studies. As an alternative, different biochemical and molecular techniques have been recently applied to mites not only for species diagnostics but also for solving questions about both interspecific and intraspecific variation among populations (Navajas, 1998; Navajas *et al.*, 1998, 1999, 2000; Navajas & Fenton, 2000; Hinomoto & Takafuji, 2001; Tixier *et al.*, 2002*a,b*; Xie *et al.*, 2006; Ben-David *et al.*, 2007).

In this study, the nucleotide variation of the internal transcribed spacers (ITS1 and ITS2) and the intergenic 5.8S fragment of nuclear rDNA of *T. urticae*, *T. turkestanii*, *T. evansi*, *T. ludeni* and *Pan. citri* were determined. We also investigated intraspecific variation from specimens collected from different locations. We then used polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) of the ITS rDNA as a fast and easy method to identify *T. urticae* among citrus-associated tetranychid mites. Several field mite samples were analysed to determine the potential of this genetic marker as a species-specific discrimination molecular tool.

Materials and methods

Mite sampling for internal transcribed spacer sequencing

The orchards sampled were located along the Mediterranean eastern coast of Spain where citrus production is

mainly located between 39°30'N and 41°00'N latitude. The study included six different commercial orchards located within a distance of 350 km (Fig. 1) that were sampled at different times. At each sampling date, 30 *T. urticae*-infested leaves from different trees (either clementine or lemon) were taken. Citrus leaves were kept in separate plastic bags refrigerated and transported to the laboratory where they were further processed. Additionally, we processed a few mite samples from other places: *T. urticae* from France and Florida, *T. evansi* from Madeira and *T. turkestanii* from France (Table 1).

DNA preparation and sequencing

The rDNA ITS region of *T. urticae*, *T. ludeni*, *T. turkestanii*, *T. evansi* and *Pan. citri* from each location was sequenced. Genomic DNA was prepared by crushing individual fresh mites with a plastic pestle in a 1.5-mL microcentrifuge tube using a Cethyl Trimethyl Ammonium Bromide (CTAB)-based extraction method (Navajas *et al.*, 1999). The DNA pellet was resuspended in 20 µL of Mili-Q water.

The region including the ITS 1, 5.8S and ITS 2 was amplified using PCR with the primers 5' AGAGGAAG-TAAAAGTCGTAACAAG 3' (annealing 18S) and 5' ATATGCTTAAATTCAGGGGG 3' (annealing 28S). In a final volume of 25 µL, PCR conditions were as follows: 2.5 µL of 10× reaction buffer, magnesium-free (Promega,

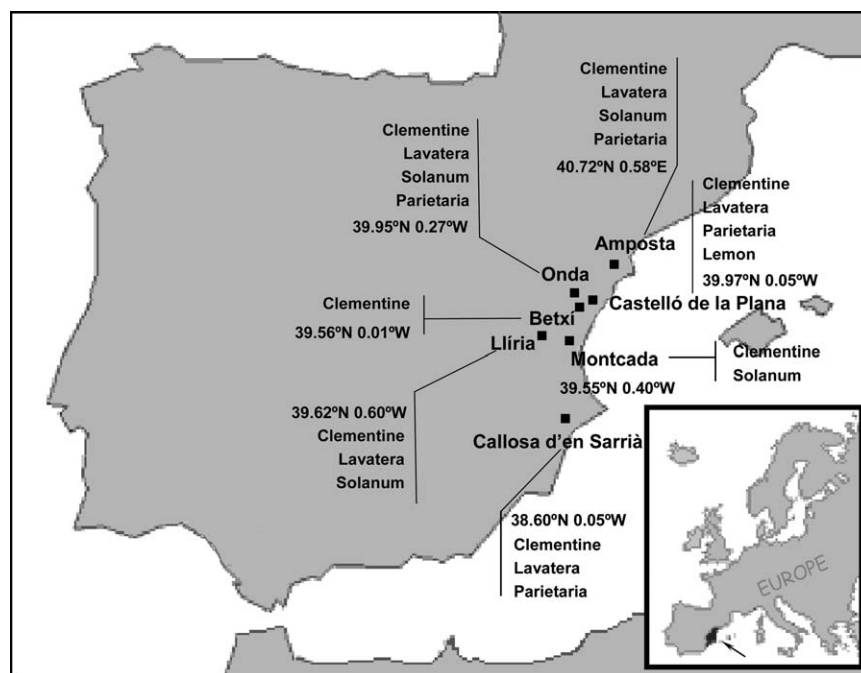


Figure 1 Location map of the study area. For each locality the host plant sampled and sampling date are indicated.

Table 1 Collection sites of Tetranychidae mites considered in this study^a

Species	Sample Abbreviation	Location	Host Plant	ITS1	ITS2	Accession Number and Total Length (bp)
<i>Tetranychus urticae</i>	Tu Cs	Spain (Castelló)	<i>Citrus reticulata</i>	×	×	AM408030, 1223
	Tu Am	Spain (Amposta)	<i>C. reticulata</i>		×	AM408042, 484
	Tu Be	Spain (Betxi)	<i>C. reticulata</i>		×	AM408043, 482
	Tu Ca	Spain (Callosa)	<i>C. reticulata</i>		×	AM408044, 481
	Tu Mo	Spain (Moncofar)	<i>C. reticulata</i>		×	AM408045, 484
	Tu On	Spain (Onda)	<i>C. reticulata</i>		×	AM408046, 481
	Tu Fr	France (Alenyà)	<i>Solanum lycopersicum</i> L. ^b	×	×	AM408031, 1220
	Tu Jp ^c	Japan	<i>Citrullus lanatus</i> (Thunb.)	×	×	AB076369, AB257738, AB257737, AB257736, Consensus, 1205
<i>Tetranychus evansi</i>	Tu Fl	Florida (Gainesville)	<i>Citrus sinensis</i> L.	×	×	AM408035, 1163
	Te Cs	Spain (Castelló)	<i>Solanum nigrum</i>	×	×	AM408033, 1218
	Te Ma	Portugal (Madeira)	<i>S. nigrum</i>	×		AM408036, 462
	Te Br ^d	Brazil (Piracaiba)	<i>S. lycopersicum</i>		×	AM408047, 498
	Te Ky ^d	Kenya	<i>S. lycopersicum</i>		×	AJ419833, 497
<i>Tetranychus ludeni</i>	Te Zb ^d	Zimbabwe	<i>S. lycopersicum</i>		×	AJ419833, 493
	Tl Cs	Spain (Castelló)	<i>Parietaria judaica</i>	×		AM408037, 464
	Tl Jp ^c	Japan	—		×	AM408040, 488
	Tl Cs	Spain (Castelló)	<i>Parietaria judaica</i>	×	×	AB076371, 1197
<i>Tetranychus turkestanii</i>	Tt Fr	France (Codognan)	<i>Solanum melongena</i> L. ^b	×	×	AM408032, 1223
	Tt Cs	Spain (Castelló)	<i>Convolvulus arvensis</i> L.	×		AM408038, 479
<i>Panonychus citri</i>	Pc Cs	Spain (Castelló)	<i>C. reticulata</i>	×	×	AM408041, 483
	Pc Mo	Spain (Moncofa)	<i>C. reticulata</i>	×	×	AM408034, 1282
						AM408039, 434

^aThe host plant and the internal transcribed spacer (ITS) region sequenced with the respective European Molecular Biology Laboratory (EMBL) accession number and the total length of the amplified DNA region are indicated.

^bReared on *Phaseolus vulgaris* L.

^cData from Osakabe *et al.* (2002, 2006).

^dData from Knapp *et al.* (2003).

Madison, WI, USA), 250 µM of each deoxyribonucleotide triphosphate, 2.5 mM of MgCl₂, 0.5 µM of each primer, 1 unit of *Taq* DNA polymerase in storage buffer A (Promega) and 2 µL of DNA template. PCR amplifications were performed with a MJ Research PTC-200 thermocycler and consisted of an initial denaturing step for 4 min at 94°C, followed by 35 cycles for 1 min at 92°C, annealing for 1 min at 50°C and extension for 1 min 30 s at 72°C, with a final extension at 72°C for 10 min. The length of PCR products was estimated by electrophoresis on 1% agarose gel stained with ethidium bromide using a molecular weight marker of 50 bp DNA Ladder (Invitrogen, Carlsbad, CA, USA).

Polymerase chain reaction fragments of the appropriated size were excised from agarose gels, and fragments were recovered using the QIAquick gel extraction kit (Qiagen, Venlo, the Netherlands) and sequenced. To obtain the complete sequence spanning both ITS 1 and ITS 2 regions, in addition to the PCR primers defined in the 18S and the 28S, we used two internal ones: 5' GAT CAC TCG AATTAC CAA TCG 3' and 5' CGA TTG GTA ATT

CGA GTG ATC 3'. All primers were published in Navia *et al.* (2005). Fragments were sequenced in both directions using the ABI PRISM[®] 3100 Genetic Analyzer.

To obtain only the ITS 1 fragment for sequencing, we used the combination of primers 5' AGA GGA AGT AAA AGT CGT AAC AAG 3' (annealing 18S) and 5' CGT TCT TCA TCG ATT GGT A 3' (annealing in 5.8S) following the same PCR conditions described above with a time reduction in elongation at 72°C for 30 s each cycle. Fragments were sequenced in both directions.

To test the homogeneity of the rDNA ITS region in *T. urticae*, specimens from Florida (Gainesville, 29°66'N, 82°45'W, on citrus) and France (Alenyà, 42°63'N, 2°98'E, on beans) were included. Three specimens from each location (Table 1) were sequenced. Additionally, we have sequenced *T. evansi* specimens from Portugal (Madeira, 32°44'N; 17°2'W, on *Solanum nigrum* L.). Already published sequences of *T. urticae* and *T. ludeni* from Japan (Osakabe *et al.*, 2002, 2006) and sequences of *T. evansi* from Africa and South America (Knapp *et al.*, 2003) were included for comparison.

Mite sampling for internal transcribed spacer-based polymerase chain reaction–restriction fragment length polymorphism species determination

Seven different orchards located within the same geographical range as before (Fig. 1) were sampled at different times (Table 2). At each sampling date, 30 *T. urticae*-infested leaves from different trees (either clementine or lemon) were taken. Simultaneously, mites on any of the weeds selected for this study (*Parietaria judacia* L., *Lavatera trimestris* L., *Lamium amplexicaule* L. and *S. nigrum*), if present, were also sampled. Each weed sample consisted of 30 different plants taken randomly from the orchard. Samples were further processed as described by Navajas *et al.* (1999).

Restriction profiles

The ITS2 region chosen for the PCR–RFLP analysis of field samples was amplified with primers: 5' TAC CAA TCG ATG AAG AAC GTA GC 3' (annealing in the 5.8S) and 5' ATA TGC TTA AAT TCA GGG GG 3' (annealing in the 28S). The PCR conditions were as described for ITS1. Based on the restriction map of the ITS2 sequences of the different species, the restriction enzyme *RsaI* was chosen to discriminate between taxa. Digestions were performed at 37°C for 1.5 h in a final volume of 15 µL containing 10 µL of the PCR product, 1.5 µL of 10× buffer-C (Promega), 1.5 µL of acetylated bovine serum albumin 10× (10 µg µL⁻¹), 10 units of restriction enzyme *RsaI* (Promega). The restriction fragments were separated by electrophoresis on a 2% agarose gel.

Data analysis

Sequences were edited and compared using the BioEdit software (Hall, 1999). Restriction maps were designed using the same software package. Final sequence alignment was performed using GeneDoc Version 2.6.002 (Nicholas *et al.*, 1997). A distance matrix was calculated using the Kimura 2 parameters pairwise distance analysis (Kumar *et al.*, 2004).

Results

The complete sequences of the rDNA region including ITS1, 5.8S rRNA, ITS2 and partial fragments of 18S and 28S rRNA of the five Tetranychidae species commonly found in citrus orchards in Eastern Spain were obtained. The nucleotide sequences are available from the EMBL database (Table 1).

Size range of ITS1 was 461–500 bp and of ITS2 was 482–567 bp (Table 3). The 5.8S gene length was fixed at 160 bp in all species.

Alignments of sequences of the ITS1 and ITS2 of *Tetranychus* species remained unambiguous, despite some insertions and deletions. The nucleotide divergence between *T. urticae* and the other three *Tetranychus* species was 11% for *T. evansi* and *T. ludeni* and 3% for *T. turkestanii*. As expected, the nucleotide divergence between *Pan. citri* and the other four *Tetranychus* species was much higher: 42% for *T. urticae* and *T. ludeni* and 41% for *T. turkestanii* and *T. evansi*. All samples collected in both Spain and France showed the same ITS2 sequence. Despite a low sequence divergence between these samples and samples of both Florida and Japan, this divergence did not affect the RFLP pattern.

Table 2 Species discrimination results of polymerase chain reaction–restriction fragment length polymorphism analysis of the ribosomal internal transcribed spacer 2 (650 bp) using the *RsaI* enzyme for mites collected on several host plants and localities in Spain^a

Date	Location	Plant Species											
		Clementine		Lemon		<i>Parietaria judaica</i>		<i>Solanum nigrum</i>		<i>Lamium amplexicaule</i>		<i>Lavatera trimestris</i>	
		Tu	T sp.	Tu	T sp.	Tu	T sp.	Tu	T sp.	Tu	T sp.	Tu	T sp.
July 2003	Onda	22	0	—	—	9	26	8	26	—	—	—	—
July 2003	Castelló	44	0	—	—	38	24	21	52	—	—	—	—
September 2003	Betxí	17	0	—	—	8	6	10	35	—	—	—	—
May 2004	Llíria	47	0	—	—	—	—	25	1	32	0	35	0
May 2004	Amposta	9	0	—	—	5	0	6	0	—	—	16	0
May 2004	Castelló	28	0	22	0	47	1	—	—	—	—	32	0
June 2004	Onda	45	0	—	—	20	0	—	—	—	—	23	0
June 2004	Moncofa	13	0	—	—	7	0	—	—	—	—	—	—
September 2004	Callosa d'En Sarrià	43	0	—	—	46	0	—	—	—	—	37	0

Tu, *Tetranychus urticae*; T sp., *Tetranychus* spp. other than *T. urticae*.

^aFor each sampling date, the number of specimens identified is presented.

Table 3 The internal transcribed spacers (ITS1 and ITS2) sizes (bp) of *Tetranychus urticae*, *Tetranychus turkestanii*, *Tetranychus evansi*, *Tetranychus ludeni* and *Panonychus citri* present in citrus orchards from Castelló (Spain)

Region	<i>T. urticae</i>	<i>T. turkestanii</i>	<i>T. evansi</i>	<i>T. ludeni</i>	<i>P. citri</i>
ITS1	481	483	461	463	500
ITS2	482	482	498	488	567

Three restriction enzymes (*RsaI*, *AluI* and *DpnI*) in combination produced restriction fragments that distinguished all species (Table 4). Restriction patterns using *RsaI* and *AluI* are shown in Fig. 2.

Comparison of sequences using optimal global pairwise alignment (BioEdit software) (Hall 1999) showed that the most interspecific variation appeared on the ITS1, with a sequence divergence ranging from 14% to 16% between the four *Tetranychus* species considered. ITS2 showed less variation among species: 11% between *T. urticae* and *T. evansi*, 11% between *T. urticae* and *T. ludeni* and 1–3% between *T. urticae* and *T. turkestanii*. A pairwise nucleotide distance matrix for the five mite species considered in this study was calculated. The range of sequence divergence observed within and among taxa is presented in Table 5.

Intraspecific variation was estimated based on the comparison of the sequences of *T. urticae* collected in four different countries (Spain, France, Portugal and the USA) plus already published sequences from Japan. The ITS1 region showed 2–3% nucleotide divergence in comparisons between specimens collected from Europe and from other continents (America and Asia). By contrast, intraspecific comparisons of ITS2 sequences of *T. urticae* revealed only a nucleotide substitution in position 361 in the sample from Castelló that differentiates this sequence from specimens coming from different continents: America (Florida) and Asia (Japan) (Osakabe *et al.*, 2002, 2006).

There was no difference between the ITS2 region from *T. evansi* specimens coming from Castelló and Madeira.

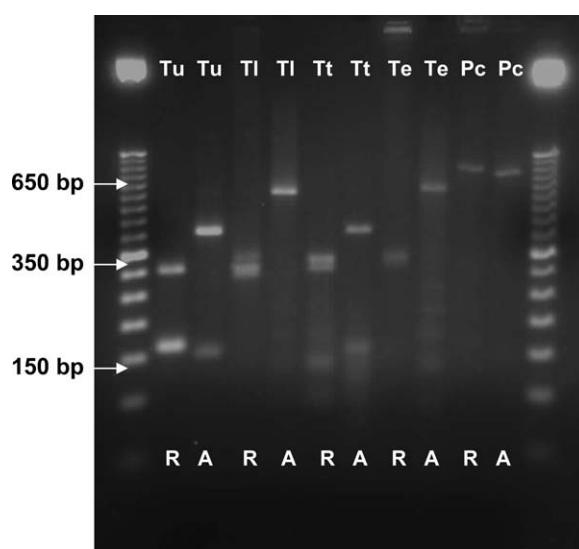


Figure 2 Ribosomal internal transcribed spacer 2 restriction fragment length differences in the species *Tetranychus urticae* (Tu), *Tetranychus ludeni* (Tl), *Tetranychus turkestanii* (Tt), *Tetranychus evansi* (Te) and *Panonychus citri* (Pc) digested with *RsaI* (R) and *AluI* (A). Molecular weight marker, 50 bp DNA Ladder (Invitrogen).

Nevertheless, comparison between the ITS2 region of *T. evansi* from Castelló and the strains obtained by Knapp *et al.* (2003) in three different locations (Brazil, Zimbabwe and Kenya) detected several point mutations between sequences of individuals originated from different locations, which represents a sequence divergence 1–2%, higher than the one observed for the *T. urticae* ITS2 region.

Polymerase chain reaction–restriction fragment length polymorphism was used to screen a series of field samples collected on seven different locations from May 2003 to December 2004 (Table 2). We processed a total of 886 individuals: 268 from clementine trees, 237 from *Parietaria judaica*, 184 from *S. nigrum*, 143 from *L. trimestris*, 32 individuals from *Lam. amplexicaule* and 22 from lemon trees. All the Tetranychidae specimens feeding on

Table 4 Internal transcribed spacers (ITS1 and ITS2) restriction fragment length differences among *Tetranychus urticae*, *Tetranychus turkestanii*, *Tetranychus evansi*, *Tetranychus ludeni* and *Panonychus citri* from Castelló (Spain) using different enzymes^a

Fragment/Enzyme	<i>T. urticae</i>	<i>T. turkestanii</i>	<i>T. evansi</i>	<i>T. ludeni</i>	<i>P. citri</i>
ITS1/ <i>RsaI</i> (bp)	367 and 225	371 and 223	349 and 225	355 and 219	320, 180 and 80
ITS1/ <i>AluI</i> (bp)	392 and 200	391 and 203	574	376 and 198	400 and 180
Total length (bp)	592	594	574	574	580
ITS2/ <i>RsaI</i> (bp)	296, 186 and 159	296 and 322	306 and 328	308, 298 and 43	660
ITS2/ <i>AluI</i> (bp)	469 and 149	469 and 149	634	649	660
ITS2/ <i>DpnI</i>	385 and 233	385 and 233	395 and 239	649	465 and 195
Total length	618	618	634	649	660

^aFor ITS2 (the only fragment used for species discrimination), within a line, same colour cells indicate species not differentiated using the corresponding enzyme. Total length corresponds to the sizes of the PCR product including primers.

Table 5 Pairwise distances using the Kimura 2 parameter between Tetranychidae mites based on differences in nucleotide sequences of the ribosomal internal transcribed spacer (ITS) regions (first line corresponds to the 28S-ITS1-5.8S-ITS2-18S nucleotide distance comparison)^a

		Tu (Cs, Fr, Fl and Jp)	Tt (Cs and Fr)	Te (Cs, Ma, Br, Ky and Zb)	Tl (Cs and Jp)	Pc (Cs)
Tu (Cs, Fr, Fl and Jp)	ITS	0.0000–0.0091	0.0103–0.0148	0.0620–0.0694	0.0603–0.0712	0.3514–0.3542
	ITS1	0.0000–0.0116	0.0186–0.0232	0.0905–0.0974	0.0766–0.0882	0.4362–0.4385
	ITS2	0.0000–0.0021	0.0083–0.0124	0.0669–0.0729	0.0818–0.0861	0.4116–0.4142
Tt (Cs and Fr)	ITS		0.0000	0.0712–0.0731	0.0686–0.0721	0.3516–0.3529
	ITS1		0.0000–0.0093	0.1021–0.1090	0.0905–0.0951	0.4362–0.4455
	ITS2		0.0000–0.0021	0.0689–0.0728	0.0798–0.0837	0.4163–0.4176
Te (Cs, Ma, Br, Ky and Zb)	ITS			0.0000–0.0009	0.0442–0.0483	0.3614–0.3624
	ITS1			0.0000–0.0023	0.0537–0.0580	0.4617–0.4640
	ITS2			0.0000–0.0061	0.0515–0.0646	0.4121–0.4182
Tl (Cs and Jp)	ITS				0.0000–0.0036	0.3673–0.3677
	ITS1				0.0000–0.0023	0.4710–0.4733
	ITS2				0.0000–0.0062	0.4195–0.4225

^aTetranychidae mites are from different origins: Castelló (Cs), France (Fr), Florida (Fl), Japan (Jp); Osakabe *et al.*, 2002, 2006), Madeira (Ma) and Brazil, Kenya and Zimbabwe (Br, Ky and Zb, respectively; Knapp *et al.*, 2003). Greyed areas indicate intraspecific comparisons.

citrus analysed in this study were identified as *T. urticae*. The proportion of *T. urticae* on *Par. judaica* was highly variable depending on both the sampling location and the date. By contrast, less than 30% of the specimens collected on *S. nigrum* during summer were *T. urticae*. The results obtained by RFLP analysis during summer showed that *T. urticae* represented 25.8% and 49.5% on *S. nigrum* and *Par. judaica*, respectively.

Discussion

Interspecific ribosomal internal transcribed spacers 1 and 2 sequence variation

The sizes of the ITS1 and ITS2 regions of the mites considered in this study (Table 3) are in the range of other tetranychid mites reported in the literature (Navajas *et al.*, 1999; Osakabe *et al.*, 2002; Knapp *et al.*, 2003; Ben-David *et al.*, 2007). The divergence obtained between *Pan. citri* and the other four *Tetranychus* species (around 40%) was much higher than the 14% found between *Pan. citri* and *Panonychus ulmi* (Hsu *et al.*, 2004; Ben-David *et al.*, 2007) that was close to what was observed among the species belonging to the genus *Tetranychus*.

Internal transcribed spacers sequence variation was further investigated based on comparisons between present and already published Tetranychidae sequences (Navajas *et al.*, 1998; Osakabe *et al.*, 2002; Knapp *et al.*, 2003; Ben-David *et al.*, 2007). Pairwise distance matrices (Table 5) show that the ITS1 had more interspecific variation than ITS2. As expected, ITS2 variation between *T. urticae* and *T. turkestanii*, which are very closely related species (Navajas & Boursot, 2003; Ben-David *et al.*, 2007), was very low. Therefore, the low sequence divergence in ITS2 between species allows us to use these small differences as species-specific markers.

Intraspecific ribosomal internal transcribed spacers 1 and 2 sequence variation

The 2–3% intraspecific variation found for ITS1 of *T. urticae* from different locations exceeds the 2% variation threshold in use for spider mite species diagnosis (Ben-David *et al.*, 2007) and is of the same magnitude as that found between two distinct species of phytoseiid mites, *Neoseiulus fallacis* (Garman) and *Neoseiulus californicus* (McGregor) (Navajas *et al.*, 1999). Therefore, the variability of this region is too high to be consistently used for species discrimination.

Navajas & Boursot (2003) showed that ITS2 sequences from European specimens of *T. urticae* were perfectly homogeneous, as did Ben-David *et al.* (2007) for Israeli mites. These results are in agreement with the ITS2 *T. urticae* sequences from specimens collected along the 350 km in the Mediterranean eastern coast of Spain from Amposta (39°97'N; 0°05'W) to Callosa d'en Sarrià (38°6'N, 0°05'W), which present no sequence variation except for some insertions and deletions that resulted in different fragment sizes (Table 1). However, these variations never affected the enzyme restriction sites. Interestingly, previous studies had revealed that this homogeneity extended worldwide (Navajas *et al.*, 1998). Our results provide confirmation of lack of variation in ITS2 among north-western Mediterranean specimens (France and Spain). However, the intercontinental variation found (Japan and Florida versus Europe) deserves further investigation.

Intraspecific polymorphism has been reported in the ITS2 region from *T. turkestanii* originated from several locations in Europe and the USA (Navajas & Boursot, 2003). We have only found one point mutation (one A>G transition) on 116-bp position comparing *T. turkestanii* from Castelló and France, but intraspecific ITS1

variation was more important. It consisted of one deletion, two transitions (C>T) and two transversions (G>T and A>T).

The observed ITS2 intraspecific variation differentiates between geographically long distant localities, whereas specimens from the same area showed homogenization of sequences. This makes ITS2 PCR–RFLP tests useful for species discrimination of Tetranychidae specimens, as also suggested by Ben-David *et al.* (2007).

Recognition sites and patterns

Restriction enzymes were selected that isolated species-specific nucleotide differences (Table 4). Among all potential restriction enzymes, *RsaI* separates *T. urticae* from the rest of species found in citrus orchards independent of the geographical origin of the specimen (Fig. 2). Because *T. urticae* is the main pest in citrus and it will be the target for further population genetic studies, ITS2 PCR–RFLP using *RsaI* will be useful for broad screening of mites collected in field surveys to determine species identity.

The *RsaI* restriction profile for the five species (Fig. 2) shows the existence of two restriction sites for both *T. urticae* and *T. ludeni*, whereas only one restriction site exists for the remaining species. The size of the restriction fragment unambiguously identifies these two species (Table 4). However, *T. evansi*, *T. ludeni* and *T. turkestanii* *RsaI* restriction patterns appear similar on agarose gels. Therefore, additional enzymes were selected to identify these species. *AluI* identifies *T. turkestanii* (Fig. 2), and *DpnI* separates *T. evansi* from *T. ludeni*.

Although the existence of intraspecific variation could impair the use of this technique for species discrimination, *RsaI* restriction profiles have proved to unambiguously separate *T. urticae* from the remaining four tetranychid species found in citrus (Fig. 2). Furthermore, this study did not detect any ITS2 sequence variation for *T. urticae* specimens collected along the Spanish Mediterranean coast, whereas the differences with Florida and Japan samples does not interfere with *RsaI* profile.

Polymerase chain reaction–restriction fragment length polymorphism: as a quick species diagnostic of field samples

The results obtained from the PCR–RFLP analyses (Table 2) are consistent with those obtained by Aucejo *et al.* (2003) for mites on the same plant species and season on previous years using a classical taxonomic approach. In their study, *T. urticae* represented 11.8% and 50.0% of total summer mite catches on *Par. judaica* and *S. nigrum*, respectively, whereas in spring, *T. urticae* clearly

predominated and represented 94.1% and 98.6% on RFLP samples of *S. nigrum* and *P. judaica*, respectively.

The PCR–RFLP approach is a promising method to screen large numbers of samples to separate *T. urticae* from other mite species and quantify its relative abundance. These results will pave the way for further studies aimed at evaluating the genetic structure of populations of *T. urticae* in citrus orchards.

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References

- Ansalmi T., Pascual-Ruiz S., Hurtado M.A., Jacas J.A. (2008) Can summer and fall vegetative growth regulate the incidence of *Tetranychus urticae* Koch on clementine fruit? *Crop Protection*, **27**, 459–464.
- Aucejo S., Foó M., Gimeno E., Gómez-Cadenas A., Monfort R., Obiol F., Prades E., Ramis M., Ripollés J.L., Tirado V., Zaragoza L., Jacas J.A., Martínez-Ferrer M.T. (2003) Management of *Tetranychus urticae* in citrus in Spain: acarofauna associated to weeds. *Integrated Control in Citrus Fruit Crops. IOBC/wprs Bulletin*, **26**, 213–220.
- Aucejo-Romero S., Gómez-Cadenas A., Jacas-Miret J.A. (2004) Effects of NaCl-stressed citrus plants on life-history parameters of *Tetranychus urticae* (Acari: Tetranychidae). *Experimental and Applied Acarology*, **33**, 1–2, 55–67.
- Bailly X., Migeon A., Navajas M. (2004) Analysis of micro-satellite variation in the spider mite *Tetranychus turkestanii* (Acari: Tetranychidae) reveals population genetic structure and raises questions about related ecological factors. *Biological Journal of the Linnean Society*, **82**, 69–78.
- Ben-David T., Melamed S., Gerson U., Morin S. (2007) ITS2 sequences as barcodes for identifying and analyzing spider mites (Acari: Tetranychidae). *Experimental and Applied Acarology*, **41**, 169–181.
- Bodenheimer F.S. (1951) *Citrus Entomology*. The Hague, the Netherlands: Dr. W Junk Publishers.
- Hall T.A. (1999) Bioedit, a user-friendly biological sequences alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95–98.
- Hinomoto N., Takafuji A. (2001) Genetic diversity and phylogeny of the Kanzawa spider mite, *Tetranychus*

- kanzawai*, in Japan. *Experimental and Applied Acarology*, **25**, 355–370.
- Hmimina M., Allam L., Ougass Y., Marmouche A. (1995) Circonstances des pullulations de *Tetranychus urticae* Koch (Tetranychidae: Acarina) en verger d'agrumes. *IOBC/wprs Bulletin*, **18**, 28–35.
- Hsu K., Hua T., Chang N.T., Yeh W.B. (2004) Nuclear ribosomal DNA sequence in tetranychid mites: a conflict phylogeny to mitochondrial cytochrome oxidase I. GenBank accession # AY750708.
- Knapp M., Wagener B., Navajas M. (2003) Molecular discrimination between the spider mite *Tetranychus evansi* Baker & Pritchard, an important pest of tomatoes in southern Africa, and the closely related species *T. urticae* Koch (Acarina: Tetranychidae). *African Entomology*, **11**, 300–304.
- Kumar S., Tamura K., Nei M. (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, **5**, 150–163.
- Martínez-Ferrer M.T., Jacas J.A., Ripollés J.L., Aucejo S. (2006) Approaches for sampling the two spotted spider mite *Tetranychus urticae* on clementines in Spain. *Journal of Economic Entomology*, **99**, 1490–1499.
- McMurtry J.A. (1985) Citrus. In *Spider Mites. Their Biology, Natural Enemies and Control*, Vol IB, pp. 339–347. Eds. W. Helle and M.W. Sabelis. Amsterdam, The Netherlands: Elsevier.
- Navajas M. (1998) Host plant associations in the spider mite *Tetranychus urticae* (Acari: Tetranychidae): insights from molecular phylogeography. *Experimental and Applied Acarology*, **22**, 201–214.
- Navajas M., Boursot P. (2003) Nuclear ribosomal DNA monophyly versus mitochondrial DNA polyphyly in two closely related mite species: the influence of life history and molecular drive. *Proceedings of the Royal Society of London Series B, Biological Sciences*, **270**, S124–S127.
- Navajas M., Fenton B. (2000) The application of molecular markers in the study of diversity in acarology: a review. *Experimental and Applied Acarology*, **24**, 751–774.
- Navajas M., Lagnel J., Gutierrez J., Boursot P. (1998) Species-wide homogeneity of nuclear ribosomal ITS2 sequences in the spider mite *Tetranychus urticae* contrasts with extensive mitochondrial COI polymorphism. *Heredity*, **80**, 742–752.
- Navajas M., Lagnel J., Fauvel G., De Moraes G. (1999) Sequence variation of ribosomal internal transcribed spacers (ITS) in commercially important Phytoseiidae mites. *Experimental and Applied Acarology*, **23**, 851–859.
- Navajas M., Tsagkarakov A., Lagnel J., Perrot-Minnot M.J. (2000) Genetic differentiation in *Tetranychus urticae* (Acari: Tetranychidae): polymorphism, host races or sibling species? *Experimental and Applied Acarology*, **24**, 365–376.
- Navia D., de Moraes G., Roderick G.K., Navajas M. (2005) The invasive coconut mite, *Aceria guerreronis* (Acari: Eriophyidae): origin and invasion sources inferred from mitochondrial (16S) and ribosomal (ITS) sequences. *Bulletin of Entomological Research*, **95**, 505–516.
- Nicholas K.B., Nicholas H.B. Jr, Deerfield D.W. II (1997) GeneDoc: analysis and visualization of genetic variation. *EMBNEW NEWS*, **4**, 14.
- Osakabe M., Hirose T., Sato M. (2002) Discrimination of four Japanese *Tetranychus* species (Acari: Tetranychidae) using PCR-RFLP of the inter-transcribed spacer region of nuclear ribosomal DNA. *Applied Entomology and Zoology*, **37**, 399–407.
- Osakabe M., Kotsubo Y., Tajima R., Hinomoto N. (2006) Phylogenetic relationship and RFLP catalogue for molecular identification of *Tetranychus* Spider mites (Acari: Tetranychidae) in Japan. GenBank.
- Souliotis P., Tsagkarakou A., Nomikou M. (1997) Field observations on some eco-ethological aspects of Phytoseiid mites in Greek citrus groves. *Acarologia* **XXXVIII**, 29–37.
- Swirski E. (1977) Integrated control of mites in Israel. In *I Congreso Mundial de Citricultura*, Vol. 2, pp. 477–480. Ed. O. Carpens. Murcia, Spain and Valencia, Spain: CEBAS.
- Talhok A.S. (1975) Citrus pests throughout the world. Technical Monograph No. 4. Ciba-Geigy Agrochemicals, Basel, Switzerland.
- Tixier M.S., Kreiter S., Auger P. (2002a) How can molecular data contribute to analyse the colonisation of vineyards by *Kampimodromus aberrans*? Acari: phylogeny and evolution. In *Acarid Phylogeny and Evolution: Adaptation in Mites and Ticks: Proceedings of the IV Symposium of the European Association Siena 2000*. Amsterdam, The Netherlands, Kluwer Academic Publications. pp. 331–340.
- Tixier M.S., Kreiter S., Croft B.A., Auger P. (2002b) Colonization of vineyards by *Kampimodromus aberrans*: dispersal from surrounding plants as indicated by random amplified polymorphism DNA typing. *Agricultural and Forest Entomology*, **4**, 255–264.
- Vacante V. (1986) Influence of white mineral oil treatments on Eastern Sicily. CEC Experts Meeting. Acireale 1985. In *Integrated Pest Control in Citrus Groves*, pp. 423–431. Rotterdam, the Netherlands: Balkema.
- Xie L., Hong X.-Y., Xue Y.-F. (2006) Population genetic structure of the two-spotted spider mite (Acari: Tetranychidae) from China. *Annals of the Entomological Society of America*, **99**, 959–965.