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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Summary

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 characterization 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 ribosomal DNA of *T. urticae, T. turkestani*, *T. evansi*, *T. ludeni* and *P. 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 eco-region makes this DNA-sequence an excellent tool for species discrimination. ITS sequences differentiate not only species but also specimens from different geographical origin. Furthermore, PCR-RFLP analysis of the ITS2 proved adequate for a quick screening of high numbers of field samples.

Key Words: Pest management, *Citrus reticulata*, molecular species discrimination, *Panonychus citri*., *Tetranychus* spp., Internal Transcribed Spacer, phylogenetic relationships.

Introduction

Tetranychus urticae Koch (Acari: Tetranychidae) is an important pest of citrus in Spain (Aucejo-Romero et al. 2004; Ansaloni et al. 2007) 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 (39º 59'N; 00º 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). *Tetranychus 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, *T. turkestani* (Ugarov & Nikolshi), *T. ludeni* Zacher and *Panonychus citri* (McGregor) (same authors, unpublished results).

Different molecular techniques, such as microsatellites (Bailly *et al*., 2004) or the mitochondrial DNA gene coding for cytochrome oxidase I (COI) (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 to solve questions about both inter- and

intraspecific variation among populations (Navajas, 1998; Navajas *et al.,* 1998, 1999, 2000; Navajas & Fenton 2000; Hinomoto & Takafuji 2001; Tixier *et al.,* 2002a,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 ribosomal DNA (rDNA) of *T. urticae, T. turkestani*, *T. evansi*, *T. ludeni* and *P. citri,* were determined. We also investigated intraspecific variation from specimens collected from different locations. We then used 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 analyzed to determine the potential of this genetic marker as a species-specific discrimination molecular tool.

Materials and Methods

Mite Sampling for ITS sequencing

The orchards sampled were located along the Mediterranean eastern coast of Spain where citrus production is mainly located, between 39º 30' and 41º 00' N latitude. The study included six different commercial orchards located within a distance of 350 km (Figure 1), which 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. turkestani* from France (Table 1).

DNA preparation and sequencing

The ribosomal DNA ITS region of *T. urticae*, *T. ludeni*, *T. turkestani*, *T. evansi* and *P. citri* from each location were sequenced. Genomic DNA was prepared by crushing individual fresh mites with a plastic pestle in a 1.5 ml microcentrifuge tube

using a 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 the polymerase chain reaction (PCR) with the primers 5' AGAGGAAGTAAAAGTCGTAACAAG 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 10x reaction buffer Magnesium free (Promega; Madison, Wisconsin, USA), 250 µM of each dNTP, 2.5 mM of $MgCl_2$, 0.5 µM of each primer, 1 unit of *Taq* DNA polymerase in storage buffer A (Promega; Madison, Wisconsin, USA), and 2 µl of DNA template. PCR amplifications were performed with a MJ Research PTC-200 thermocycler and consisted of an initial denaturing step of 4 min at 94°C; followed by 35 cycles of 1 min at 92°C, annealing of 1 min at 50 °C, and extension of 1 min 30 sec 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, California, USA).

PCR 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 AAT TAC 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 PRSIM® 3100 Genetic Analyzer.

In order 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 72º C of 30 sec each cycle. Fragments were sequenced in both directions.

To test the homogeneity of the ribosomal DNA ITS region in *T. urticae*, specimens from Florida (Gainesville, 29º66'N 82º45'W, on citrus) and France (Alenyà, 42º63'N 2º98'E, on beans) were included. Three specimens from each location (Table 1) were sequenced. Additionally, we have sequenced *T. evansi* specimens from Portugal (Madeira, 32º44' N 17º2'W, on *Solanum nigrum*). 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 ITS-based PCR-RFLP species determination

Seven different orchards located within the same geographical range as before (Figure 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 officinalis* D.C., *Lavatera trimestris* L.*, Lamium amplexicaule* L. and *Solanum nigrum* L.), 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 *Rsa*I was chosen to discriminate between taxa. Digestions were performed at 37º C during 1h 30 min in a final volume of 15 µl containing 10 µl of the PCR product, 1.5 µl of 10x buffer-C (Promega), 1.5 µl of acetyled bovine serum albumin10x (10 µg/µl), 10 units of

restriction enzyme *Rsa*I (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 to 500 bp, for ITS2 was 482 to 567 bp (Table 3). The 5.8 S 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. turkestani*. As expected, the nucleotide divergence between *P. citri* and the other four *Tetranychus* species was much higher: 42% for *T. urticae* and *T. ludeni*; 41% for *T. turkestani* 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 both Florida and Japan ones, this divergence did not affect the RFLP pattern.

Three restriction enzymes (*Rsa*I, *Alu*I and *Dpn*I) in combination produced restriction fragments that distinguished all species (Table 4). Restriction patterns using *Rsa*I and *Alu*I are shown in Figure 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. turkestani*. 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 are presented in Table 5.

Intraspecific variation was estimated based in the comparison of the sequences of *T. urticae* collected in 4 different countries (Spain, France, Portugal and the United States) plus already published sequences from Japan. The ITS1 region showed 2 to 3% nucleotide divergence in comparisons between specimens collected in 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 which 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. Nevertheless, comparison between the ITS2 region of *T. evansi* from Castelló and the strains obtained by Knapp *et al.* (2003) in three different locations (Brasil, 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.

PCR-RFLP was used to screen a series of field samples collected on 7 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 *Solanum nigrum* L., 143 from *Lavatera trimestris* L., 32 individuals from *Lamium amplexicaule* L.

and 22 from lemon trees. All the Tetranychidae specimens feeding on citrus analyzed in this study were identified as *T. urticae*. The proportion of *T. urticae* on *P. 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 the 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 *P. judaica*, respectively.

Discussion

Interspecific ribosomal ITS1 and ITS2 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; Knapp *et al.,* 2003; Osakabe *et al.,* 2002; Ben-David *et al*., 2007). The divergence obtained between *P. citri* and the other four *Tetranychus* species (around 40 %) was much higher than the 14% found between *P. citri* and *P. ulmi* (Hsu *et al.,* 2004; Ben-David et al., 2007), which was close to what was observed among the species belonging to the genus *Tetranychus*.

ITS 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. turkestani*, 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 ITS1 and ITS2 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 *N. 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 (39º97'º N 0º05'W) to Callosa d'en Sarrià (38º6'N 0º05'W), which present no sequence variation except for some insertions and deletions which 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 Northwestern 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. turkestani* originated from several locations in Europe and the United States (Navajas & Boursot 2003). We have only found one point mutation (one A>G transition) on 116 bp position comparing *T. turkestani* 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, *Rsa*I separates *T.*

urticae from the rest of species found in citrus orchards independently of the geographical origin of the specimen (Figure 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 *Rsa*I will be useful for broad screening of mites collected in field surveys to determine species identity.

The *Rsa*I restriction profile for the five species (Figure 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. turkestani* *Rsa*I restriction patterns appear similar on agarose gels. Therefore, additional enzymes were selected to identify these species. *Alu*I identifies *T. turkestani* (Figure 2) and *Dpn*I separates *T. evansi* from *T. ludeni*.

Although the existence of intraspecific variation could impair the use of this technique for species-discrimination, *Rsa*I restriction profiles have proved to unambiguously separate *T. urticae* from the remaining four tetranychid species found in citrus (Figure 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 *Rsa*I profile.

PCR –RFLP: 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.* (2002) for mites on the same plant species and season on previous years. In their study *T. urticae* represented 11.8 % and 50.0 % of total summer mite catches, on *P. 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 high numbers of samples to separate *T.urticae* from other mite species and quantify its relative

abundance. These results will pave the way to further studies aimed at evaluating the genetic structure of populations of *T. urticae* in citrus orchards.

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Table 1. Collection sites of Tetranychidae mites considered in this study. The host plant and the ITS region sequenced with the respective EMBL accession number and the total length of the amplified DNA region are indicated.

Species	Sample abbreviation	Location	Host plant	ITS1	ITS2	Accession number and total length
Tetranychus urticae	Tu Cs	Spain (Castelló)	*Citrus reticulata* Blanco	x	x	AM408030 1223 bp
	Tu Am	Spain (Amposta)	*Citrus reticulata* Blanco		x	AM408042 484 bp
	Tu Be	Spain (Betxi)	*Citrus reticulata* Blanco		x	AM408043 482 bp
	Tu Ca	Spain (Callosa)	*Citrus reticulata* Blanco		x	AM408044 481 bp
	Tu Mo	Spain (Moncofar)	*Citrus reticulata* Blanco		x	AM408045 484 bp
	Tu On	Spain (Onda)	*Citrus reticulata* Blanco		x	AM408046 481 bp
	Tu Fr	France (Alenyà)	*Solanum lycopersicum* L *	x	x	AM408031 1220 bp
	Tu Jp[1]	Japan	*Citrullus lanatus* (Thunb)	x	x	AB076369 AB257738 AB257737 AB257736 Consensus 1205 bp
	Tu Fl	Florida (Gainesville)	*Citrus sinensis* L.	x	x	AM408035 1163 bp
Tetranychus evansi	Te Cs	Spain (Castelló)	*Solanum nigrum* L.	x	x	AM408033 1218 bp
	Te Ma	Portugal (Madeira)	*Solanum nigrum* L.	x		AM408036 462 bp
					x	AM408047 498 bp
	Te Br[2]	Brazil (Piracaiba)	*S. lycopersicum* L.		x	AJ419833 497 bp
	Te Ky[2]	Kenya	*S. lycopersicum* L.		x	AJ419833 493 bp
	Te Zb[2]	Zimbabwe	*S. lycopersicum* L.		x	AJ419833 496 bp
Tetranychus ludeni	TlCs	Spain (Castelló)	*Paretaria judaica*	x		AM408037 464 bp
					x	AM408040 488bp
	TlJp[1]	Japan	-	x	x	AB076371 1197 bp
Tetranychus turkestani	Tt Fr	France (Codognan)	*Solanum melongena* L. *	x	x	AM408032 1223 bp
	Tt Cs	Spain (Castelló)	*Convolvulus arvensis* L.	x		AM408038 479 bp

					x	AM408041 483 bp
Panonychus citri	Pc Cs	Spain (Castelló)	*Citrus reticulata* Blanco	x	x	AM408034 1282 bp
	Pc Mo	Spain (Moncofa)	*Citrus reticulata* Blanco		x	AM408039 434 bp

[1]Data from Osakabe *et al.,* 2002 and 2006 [2] Data from Knapp *et al.,* 2003
* Reared on *Phaseolus vulgaris* L.

Table 3. The Internal Transcribed Spacers (ITS1 and ITS2) sizes (bp) of *Tetranychus urticae, T. turkestani, T. evansi, T. ludeni and Panonychus citri* present in citrus orchards from Castelló (Spain).

Region	*T. urticae*	*T.turkestani*	*T. evansi*	*T. ludeni*	*P. citri*
ITS1	481	483	461	463	500
ITS2	482	482	498	488	567

Table 2. Species discrimination results of PCR-RFLP analysis of the ribosomal ITS2 (650 bp) using the *Rsa*I enzyme for mites collected on several host plants and localities in Spain. For each sampling date, the number of specimens identified is presented. Tu: *Tetranychus urticae*; T sp: *Tetranychus* spp. other than *T. urticae*.

Date	Location	Plant species											
		Clementine		Lemon		*Parietaria judaica*		*Solanum nigrum*		*Lamium amplexicaule*		*Lavatera trimestris*	
		Tu	T sp	Tu	T sp	Tu	T sp	Tu	T sp	Tu	T sp	Tu	T sp
Jul/03	Onda	22	0	-	-	9	26	8	26	-	-	-	-
Jul/03	Castelló	44	0	-	-	38	24	21	52	-	-	-	-
Sep/03	Betxí	17	0	-	-	8	6	10	35	-	-	-	-
May/04	Llíria	47	0	-	-	-	-	25	1	32	0	35	0
May/04	Amposta	9	0	-	-	5	0	6	0	-	-	16	0
May/04	Castelló	28	0	22	0	47	1	-	-	-	-	32	0
Jun/04	Onda	45	0	-	-	20	0	-	-	-	-	23	0
Jun/04	Moncofa	13	0	-	-	7	0	-	-	-	-	-	-
Sep/04	Callosa d'En Sarrià	43	0	-	-	46	0	-	-	-	-	37	0

Table 4. ITS1 and ITS2 restriction fragment length differences between *Tetranychus urticae*, *T. turkestani*, *T. evansi*, *T. ludeni* and *Panonychus citri* from Castelló (Spain) using different enzymes. For ITS2 (the only fragment used for species discrimination), within a line, same color cells indicate species not differentiated using the corresponding enzyme. Total length corresponds to the sizes of the PCR product including primers.

Fragment / enzyme	*T. urticae*	*T. turkestani*	*T. evansi*	*T. ludeni*	*P. citri*
ITS1 / *Rsa* I	367 bp	371 bp	349 bp	355 bp	320 bp
	225 bp	223 bp	225 bp	219 bp	180 bp
					80bp
ITS1/*Alu* I	392 bp	391 bp	574 bp	376 bp	400 bp
	200 bp	203 bp		198 bp	180 bp
Total length	592 bp	594 bp	574 bp	574 bp	580 bp
ITS2 / *Rsa* I	296bp	296 bp	306 bp	308bp	660 bp
	186bp	322 bp	328 bp	298 bp	
	159 bp			43 bp	
ITS2 / *Alu* I	469 bp	469 bp	634 bp	649 bp	660 bp
	149 bp	149 bp			
ITS2 / *Dpn*I	385bp	385 bp	395 bp	649bp	465 bp
	233 bp	233 bp	239 bp		195 bp
Total length	618 bp	618 bp	634 bp	649 bp	660 bp

Table 5. Pairwise distances using the Kimura 2 parameter between Tetranychidae mites based on differences in nucleotide sequences of the ribosomal ITS regions (first line corresponds to the 28S-ITS1-5,8S-ITS2-18S nucleotide distance comparison). Tetranychidae mites are from different origins: Castelló (Cs), France (Fr), Florida (Fl), Japan (Jp, Osakabe *et al.,* 2002 and 2006), Madeira (Ma) and Brasil, Kenya and Zimbabwe (Br, Ky and Zb, respectively, Knapp *et al.,* 2003). Grayed areas indicate intraspecific comparisons.

		Tu (Cs, Fr, Fl, Jp)	**Tt** (Cs, Fr)	**Te** (Cs, Ma, Br, Ky, Zb)	**Tl** (Cs, Jp)	**Pc** (Cs)
Tu (Cs, Fr, Fl, 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, 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, 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, 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

Figure Captions

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

Figure 2. Ribosomal ITS2 restriction fragment length differences in the species *T. urticae* (Tu), *T. ludeni* (Tl), *T. turkestani* (Tt), *T. evansi* (Te) and *Panonychus citri* (Pc) digested with *Rsa*I (R) and *Alu*I (A). Molecular weight marker: 50 bp DNA Ladder (Invitrogen).

Figure 1.

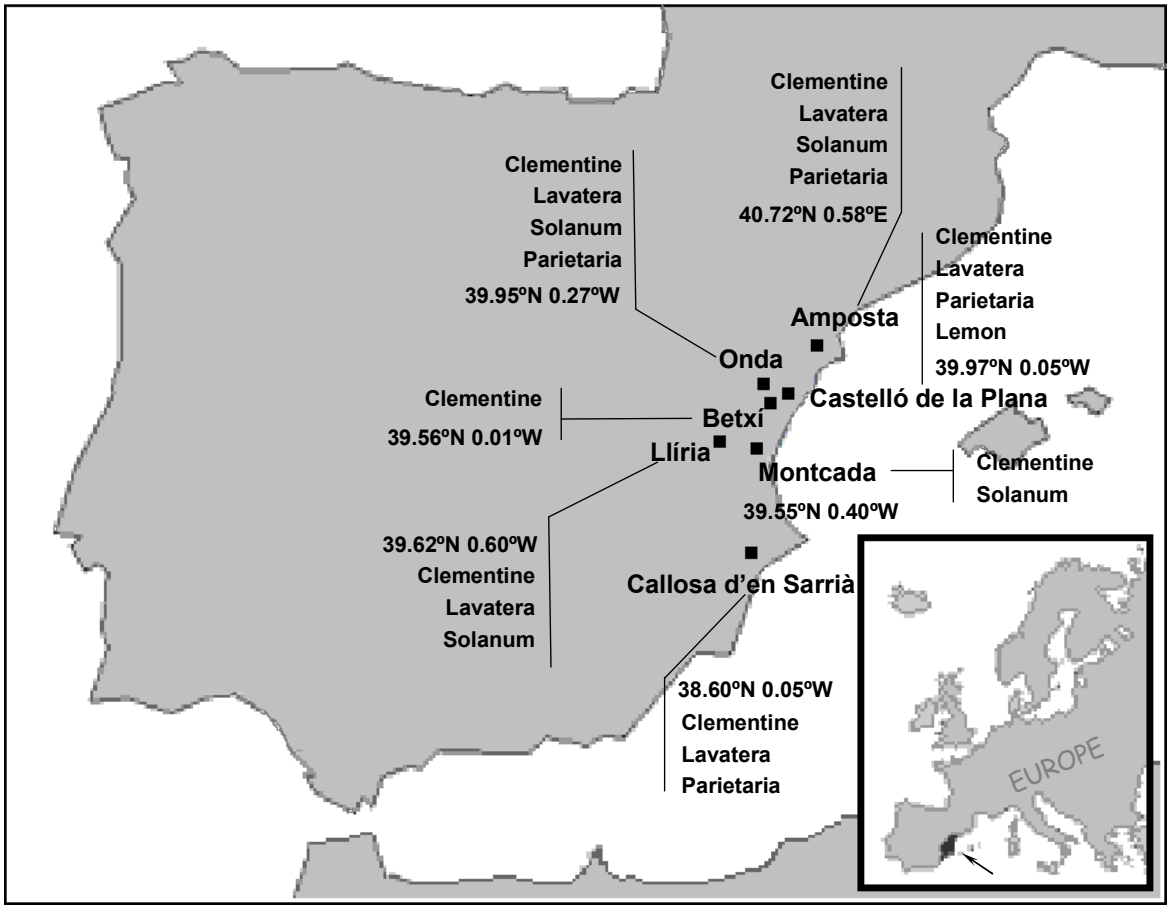
Clementine
Lavatera
Solanum
Parietaria
40.72ºN 0.58ºE
Clementine
Lavatera
Solanum
Parietaria
39.95ºN 0.27ºW
Clementine
Lavatera
Parietaria
Lemon
39.97ºN 0.05ºW
Amposta
Onda
Castelló de la Plana
Clementine
39.56ºN 0.01ºW
Betxí
Llíria
Montcada
Clementine
Solanum
39.55ºN 0.40ºW
39.62ºN 0.60ºW
Clementine
Lavatera
Solanum
Callosa d'en Sarrià
38.60ºN 0.05ºW
Clementine
Lavatera
Parietaria
EUROPE

Figure 2.