Disentangling mite predator-prey relationships by multiplex PCR

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Abstract

Gut content analysis using molecular techniques can help elucidate predator-prey relationships in situations in which other methodologies are not feasible, such as in the case of trophic interactions between minute species such as mites. We designed species-specific primers for a mite community occurring in Spanish citrus orchards comprising two herbivores, the Tetranychidae Tetranychus urticae and Panonychus citri, and six predatory mites belonging to the Phytoseiidae family; these predatory mites are considered to be these herbivores’ main biological control agents. These primers were successfully multiplexed in a single PCR to test the range of predators feeding on each of the two prey species. We estimated prey DNA detectability success over time (DS$_{50}$), which depended on the predator-prey combination and ranged from 0.2 to 18 h. These values were further used to weight prey detection in field samples to disentangle the predatory role played by the most abundant predators (i.e. Euseius stipulatus and Phytoseilus persimilis). The corrected predation value for E. stipulatus was significantly higher than for P. persimilis. However, because this 1.5-fold difference was less than that observed regarding their sevenfold difference in abundance, we conclude that P. persimilis is the most effective predator in the system; it preyed on tetranychids almost five times more frequently than E. stipulatus did. The present results demonstrate that molecular tools are appropriate to unravel predator-prey interactions in tiny species such as mites, which include important agricultural pests and their predators.

Keywords: corrected predation values, DS$_{50}$, molecular gut content analysis, Phytoseiidae, Tetranychidae, trophic interactions

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Introduction

DNA-based gut content analysis has become a broadly used tool to disentangle trophic interactions when direct observations of feeding events are close to impossible (Symondson 2002; Sheppard & Harwood 2005; Gariepy et al. 2007; King et al. 2008; Greenstone et al. 2014). This applies in particular to microarthropods such as mites, in which both prey and predator are minute and often cryptic, and microscopic analysis of the predator’s gut content is impossible because they ingest predigested fluids from their prey (Chant 1985). Studies on predators using primers based on specific DNA sequences of their prey have been successful in many different arthropods (e.g. Agustí et al. 1999; Zaidi et al. 1999; Monzó et al. 2010, 2011; Gomez-Polo et al. 2013). Prey DNA detection is possible, especially if genes or noncoding DNA sequences are present in multiple copies (King et al. 2008). Nuclear ribosomal DNA, such as the internal transcribed spacers (ITS) region, and mitochondrial DNA, such as cytochrome oxidase I and II (COI and COII), fulfill this criterion and have been successfully used for this purpose (Gariepy et al. 2007; King et al. 2008), specifically on mites (Fitzgerald et al. 2004; Rivera-Rivera et al. 2012; Wari et al. 2014). To avoid this method requiring the number of amplifications to be equal to the number of possible prey species, each requiring a specific primer, Harper et al. (2005) suggested using multiplex PCR. So
ELUCIDATING MITE TROPHIC RELATIONSHIPS 1331

far, the only multiplex PCR approach to assess predation in mites was used by Fitzgerald et al. (2004) on mites living in strawberries, but the results were only partially published, and relevant information (i.e. primer sequences, multiplex conditions and detectability half-life) is not available.

Spider mites of the family Tetranychidae comprise more than 1200 phytophagous species. This family includes species considered as minor pest of agricultural crops prior to World War II (Hoy 2011). However, they changed their status to key pest in many major food and ornamental crops afterwards (Helle & Sabelis 1985; Gerson et al. 2003; Zhang 2003). Nowadays, more than one hundred of them are considered to be agricultural pests, and approximately ten are key pests of economically important crops (Migeon & Dorkeld 2006-2015). One of the hypotheses developed to explain this change is based on the disruption of existing natural top-down regulation mechanisms caused by pesticide abuse (Huffaker et al. 1970). Current trends towards more environmentally friendly agriculture emphasize restoration of these trophic cascades, focusing on the conservation and enhancement of those biological control agents (BCAs) considered most effective (Polis et al. 1997; Straub & Snyder 2006; Letourneau et al. 2009). Among the BCAs of Tetranychidae, predatory mites belonging to the Phytoseiidae family are known to provide successful biological control (Helle & Sabelis 1985). To move towards increased biological control, trophic interactions occurring within the community should be appraised to take full advantage of them (Martin et al. 2013). However, the actual trophic ecology of many communities, including mites, remains poorly investigated. The composition of the predator guild occurring in each agroecosystem depends on the species/cultivar, geographical location and management practices (Helle & Sabelis 1985; McMurtry 1985, 1992; Gerson 2003; Gerson et al. 2003; Aguilar-Fenollosa et al. 2011a). Evergreen crops, such as citrus, in which pests and BCAs are present throughout the year (McMurtry 1985), can be used as a model to study trophic interactions. The two-spotted spider mite, *Tetranychus urticae* Koch, and the citrus red mite, *Panonychus citri* (McGregor), are considered key pests in different agroecosystems including citrus (Helle & Sabelis 1985; Jaques et al. 2015). In areas with a Mediterranean climate, both tetranychids are important pests, especially of clementine mandarins (*Citrus clementina* Hort. ex. Tan.) and oranges (*C. sinensis* (L.) Osbeck) (Jacas et al. 2010; Jaques et al. 2015). Both species cause serious damage to leaves and especially fruits (Pascual-Ruiz et al. 2014). In Spanish citrus orchards, tetranychids are regulated by a community of Phytoseiidae with different lifestyles, which, according to McMurtry et al. (2013), range from the specialized predator of *Tetranychus* species *Phytoseiulus persimilis* Athias-Henriot (Type I) to the pollen-feeding generalist predator *Euseius stipulatus* (Athias-Henriot) (Type IV), which has an intermediate mixed-diet lifestyle (Jaques et al. 2015).

The omnivore *E. stipulatus* is the most abundant phytoseiid in Spanish citrus agroecosystems irrespective of the cultivar and management practices used and represents approximately three-quarters of total phytoseiid counts (Abad-Moyano et al. 2009a; Aguilar-Fenollosa et al. 2011a). It is considered a key species in the regulation of *P. citri* populations (Ferragut et al. 1988; Abad-Moyano et al. 2009a) and also suppressed populations of *T. urticae* in semi-field conditions (Grafton-Cardwell et al. 1997; Pina et al. 2012). However, *E. stipulatus* behaved as a superior intraguild predator under controlled laboratory and semi-field assays (Abad-Moyano et al. 2010a,b). Moreover, field and semi-field results suggest that the availability of alternative food sources (e.g. pollen) can enhance populations of *E. stipulatus* so they can out-compete other predators of *T. urticae*, such as *Neoseiulus californicus* (McGregor) and *P. persimilis* (Aguilar-Fenollosa et al. 2011a,b; Pina et al. 2012). In these circumstances, biological control of *T. urticae* can be deficient (Aguilar-Fenollosa et al. 2011c).

To shed light on these complex trophic relationships, a method to directly investigate the dietary choices of microarthropods in the field is urgently required. Due to the lack of tools adequate for tiny predators and the economic and ecological importance of phytoseiids, we decided to focus on the mite community occurring in Spanish citrus orchards. This study may pave the way for a better understanding of mite-mite predator-prey systems. Therefore, as a first step to disentangle these interactions, we decided (i) to design specific primers targeting the most abundant predatory and prey mites occurring in Spanish citrus orchards, (ii) to develop a multiple PCR approach for multiple prey and predatory mite identification, (iii) to assess prey DNA detectability success over time (DS50) in the most abundant predatory mite species (i.e. *E. stipulatus*, *N. californicus* and *P. persimilis*) using multiplex PCR and (iv) to assess the trophic interactions established among citrus mites under field conditions.

As far as we know, this is the first time a multiplex approach has been used to characterize a mite community; this approach could be successfully implemented in other systems for the same purpose.

Methods

Mites for laboratory feeding studies

The dominant Tetranychidae and Phytoseiidae species found in Spanish citrus orchards (Aguilar-Fenollosa et al.
2011a,b) were included in our study (Table 1). Additionally, *Amblyseius swirskii* Athias-Henriot, which we recently found in this system, was considered.

*Tetranychus urticae* individuals were originally collected in clementine mandarin orchards in the region of La Plana (Castelló de la Plana, Spain) and subsequently reared on bean plants (*Phaseolus vulgaris* L.). *Panonychus citri* adults originally came from clementine orchards in Montcada (Valencia, Spain) and were subsequently reared on lemons (*Citrus limon* L.) and *Carpobrotus edulis* (L.) N.E. Br pollen. All phytoseiid species were maintained in separate climatic chambers at 25 ± 1 °C, 70 ± 10% RH, and a photoperiod of 15:9 (light:dark; L:D) h. These conditions were also used for laboratory assays involving live mites.

**DNA extraction**

DNA was extracted following different procedures depending on the organism and the objective of the study.

The modified ‘salting out’ protocol (Monzó et al. 2010) was used for DNA extraction of phytoseiids, tetranychids and other potential food sources for Phytoseiidae mites in citrus orchards (Table 1 and Table S1, Supporting information). Bean leaf DNA was also extracted following this protocol. Clementine mandarin (*C. clementina* Hort. ex. Tan. cvar. Clemenules) leaf DNA

| Table 1 | Sequences of the internal transcribed spacers (ITS) region of the selected Acari species used in this study |
|-----------------|-----------------------------------------------------------------------------------------------------------------|
| Species (Family) | Origin | Target region* | Primer sequence (5′ → 3′)† | Size‡ | \(T_a\)§ | Sequence references | Accession number |
| Panonychus citri (Tetranychidae) | Castelló, Spain | ITS2 | GCCAAAGATACATCTCTAAAG (for)* TTCTTTCTCCTCGTTAGTATGCTTAA (rev)** | 434 | 50 | Hurtado et al. (2008) | AM408039 |
| Tetranychus urticae (Tetranychidae) | Betx, Spain | ITS2 | GCCAAAGATACATCTCTCAAG (for)* TTCTTTCTCCTCGTTAGTATGCTTAA (rev)** | 482 | 50 | Hurtado et al. (2008) | AM408043 |
| Neoseiulus barkeri (Phytoseiidae) | Castelló, Spain | ITS | AGAGGAAATGAATACGTAACAG (for)†† TTCTTTCTCCTCGTTAGTATGCTTAA (rev)** | 720 | 50 | This work KP642055 |
| Typhlodromus phialatus (Phytoseiidae) | Montcada, Spain | ITS | AGAGGAATGAGACGTCGTAACAG (for)†† TTCTTTCTCCTCGTTAGTATGCTTAA (rev)** | 770 | 50 | This work GU56315 |
| Neoseiulus californicus (Phytoseiidae) | Koppert Biol. Syst. | ITS | AGAGGAAATGAATACGTAACAG (for)†† ATATGCTTAAATTCAAGG (rev)†† | 673 | 50 | Navajas et al. (1999) | Y18269 |
| Euseius stipulatus (Phytoseiidae) | Montcada, Spain | ITS | AGAGGAATGAGACGTCGTAACAG (for)†† ATATGCTTAAATTCAAGG (rev)†† | 651 | 50 | This work GU565290 |
| Phytoseiulus persimilis (Phytoseiidae) | Koppert Biol. Syst. | ITS | AGAGGAATGAGACGTCGTAACAG (for)†† ATATGCTTAAATTCAAGG (rev)†† | 606 | 50 | Navajas et al. (1999) | Y18268 |
| Amblyseius swirskii (Phytoseiidae) | Koppert Biol. Syst. | ITS | AGAGGAATGAGACGTCGTAACAG (for)†† ATATGCTTAAATTCAAGG (rev)†† | 623 | 50 | This work GU565289 |

*ITS: DNA ribosomal ITS1, 5.8S and ITS2 region; ITS2: DNA ribosomal ITS2 region.
†For: forward primer; rev: reverse primer.
‡Size: size amplified fragment (bp).
§\(T_a\): annealing temperature (°C).
*Hurtado et al. (2008).
**Ali et al. (2003).
and C. edulis anther DNA were extracted using the Sigma protocol (REDExtract-N-Amp™ Plant PCR Kits). Fungal DNA was extracted following the protocol described by Sánchez-Torres et al. (2008).

DNA from field-collected phytoseiid mites was extracted with the methods described by Osman et al. (2008). This technique is a method that streamlines DNA extraction, stops digestion in the predator's gut, reduces handling time, maximizes DNA preservation over time, and is fully compatible with field sampling (Garcia-Blasco et al. 2013). Nylon membranes printed with field-collected phytophagous mites were individually transferred into 96-well multiplates and covered with 100 μL of extraction buffer (0.1 M glycine, 0.05 M NaCl, 1 mM EDTA pH 8.0) (Osman & Rowhani 2006). DNA extraction was performed in a thermocycler (Eppendorf Mastercycler gradient; Eppendorf, Hamburg, Germany) at 95 °C for 10 min followed by 60 °C. Then, 1 μL of fresh proteinase K was added and maintained at 60 °C for 1 h. Finally, samples were heated at 95 °C for 10 min. The nylon membrane was removed, and DNA precipitation was performed by adding one volume of isopropanol stored at −20 °C and 1/10 volume of 4 M ammonium acetate to each sample. After 5 min at 25 °C, samples were centrifuged for 10 min at 25 °C, and precipitated DNA was washed in 200 μL 70% chilled ethanol. Precipitated DNA was resuspended in 15 μL of LTE (10 mM Tris–HCl pH 7.5; 0.1 mM EDTA pH 8) and stored at −20 °C.

**Multiplex PCR design**

Sequencing, alignment and primer design. ITS regions and COI were selected for testing to be representative as specific molecular markers for mite species. All sequences used in this study are listed in Tables 1 and S2 (Supporting Information). Those not found in the GenBank (Nucleic acids research; http://www.ncbi.nlm.nih.gov/genbank) database were obtained during this work.

For sequencing both marker types, amplification reactions were performed on a final volume of 25 μL: 1.4 μL Taq polymerase buffer (Roche Applied Science, Mannheim, Germany), 200 μM of each dNTP (5 PRIME GmbH, D22767 Hamburg), 1 mM of MgCl₂, 0.5 μM of each primer, 1 unit of DNA Taq polymerase (Roche) and 1.5 μL of DNA template (5–10 ng/μL). Amplifications were performed in a Bio-Rad MJ Research Thermal Cycler PTC-100® and consisted of one denaturation step at 94 °C for 4 min, 25 cycles at 92 °C for 1 min, annealing at 45 or 50 °C (depending on the combination of primers; Tables 1 and S2, Supporting Information) for 1 min and 72 °C for 90 s, and a final extension at 72 °C for 10 min. PCR products were run on 2% agarose D-1 low EEO (Pronadisa, Sumilab S.L., Madrid, Spain) gel, stained with ethidium bromide using a molecular weight marker consisting of a 50 bp DNA ladder (Invitrogen, Carlsbad, CA, USA) and visualized under UV light.

Band quantification was carried out using the GeneTools program from Syngene (Cambridge, UK). For each PCR, a single band of the expected size was obtained and purified (High Pure PCR Purification Kit, Roche Applied Science) prior to sequencing. Three different individuals of each species were sequenced in both directions using an ABI/PE 3730 DNA Analyzer (Applied Biosystems, Foster City, USA) at the Servei Central de Suport a la Investigació Experimental (SCSIE) (Universitat de València, Spain). A consensus sequence was obtained using the Staden Package program (Staden 1996).

For primer design, sequence alignment was performed with the MEGA 5 program (Tamura et al. 2011). Partial mitochondrial DNA from the COI gene was rejected due to high sequence similarity between mite species, which precluded designing primers based on it. Thus, the ITS region was chosen. Forward primers were designed for each species in nonconserved nucleotide sequences to simultaneously obtain prey and predator amplification fragments of different sizes. For all species, the universal primer 5’ TTCTTTTTCCTCCGCTTAGTA-TATGCTTAA 3’ (Ji et al. 2003) was used as a reverse primer. In both prey species (T. urticae and P. citri), fragment lengths were shorter than those in the predators (Table 2). A new forward primer for P. citri that had reduced-size amplified fragment was secondarily designed to improve amplification results (Agustí et al. 1999; Zaidi et al. 1999) (Table 2).

Cross-reactivity test and multiplex PCR design. Specific primers (Table 2) were tested on all species in each combination for cross-reactivity. As a positive control, a single DNA template (5–10 ng/μL) of the multiplex PCR target species was used. Amplification reactions were performed on a final volume of 25 μL: 1 × Taq polymerase buffer, 200 μM of each dNTP, 1 mM of MgCl₂, 0.4 μM of each primer, 1 unit of DNA Taq polymerase and 1 μL of DNA template. Amplifications consisted of one denaturation step at 94 °C for 4 min, 35 cycles at 92 °C for 30 s, annealing at 55 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 10 min. PCR products were visualized on agarose gel under UV light.

Once the specificity of the primers had been tested, the multiplex PCR was adjusted on agarose with all primers according to Henegariu et al. (1997) to reach a final volume of 25 μL: 1.4× Taq polymerase buffer, 200 μM of each dNTP, 1 mM of MgCl₂, 0.2 μM of each A. swirskii, E. stipulatus, N. barkeri, and P. persimilis forward primers, 0.4 μM of each N. californicus, T. phialatus,
Table 2 Primer sequences and expected product size in the designed multiplex PCR

| Species               | Primer sequence (5'→3')* | Size (bp) |
|-----------------------|--------------------------|-----------|
| Prey                  |                          |           |
| *T. urticae*          | GGTTAGTACCCGATGTATCCCG (for) GGTCTTCGTTGTGTAATGTTA (rev) | 243       |
|                       | TCTTTTTCTCGGCTTAGGATGATTAA (rev)† | 176       |
| *P. citri*            | TCTTTTTCTCCGTTAGGATGATTAA (rev) | 121       |
| *T. urticae*          | CACCTCATCAGGTGAAGAATG (for) GAGTATCAGCTAGACATTACGT (rev) | 605       |
|                       | TCTTTTTCTCGGCTTAGGATGATTAA (rev) | 570       |
| *N. barkeri*          | GGCTGCTGCGCGGTTAAGCAG (for) GGCAGTACCCGCTTAGGATGATTAA (rev) | 540       |
|                       | TCTTTTTCTCGGCTTAGGATGATTAA (rev) | 475       |
| *E. stipulatus*       | CCGAGTGCGTACCAACCAG (for) | 409       |
| *P. persimilis*       | TTTTTCCTCGGCTTAGGATGATTAA (rev) | 369       |
| *A. swirskii*         | CTTGGAAGTAGTGGTCG (for) | 369       |
|                       | TCTTTTTCTCGGCTTAGGATGATTAA (rev) |           |

*for, forward primer; rev, reverse primer.
†Second forward primer designed for *P. citri*.

Monzó et al. (2010) were used to discriminate between unsuccessful multiplex PCR amplification (i.e. absence of target DNA) and lack of DNA in the PCR (i.e. absence of both target and nontarget DNA).

Feeding trials

Modified Huffaker cells (Abad-Moyano et al. 2009b) were used as arenas. Three- to 5-day-old adult females of *P. persimilis*, *N. californicus* and *E. stipulatus* were individually placed in the cells and starved for 48 h in a climatic chamber with a water supply only. After starvation, each adult female was transferred to a new cell containing one adult female of either *T. urticae* or *P. citri*. Phytoseiid activity was continuously monitored under a binocular microscope. For those phytoseiids feeding on the offered prey, time was set to 0 when they released the dead prey. Then, single phytoseiid specimens were maintained in cells with water supplies for various periods of time (0–28 h; Table S3, Supporting information). Next, they were transferred to 1.5-mL tubes, frozen at −80 °C and processed for molecular assessment. Additional phytoseiids were starved for 48 h and used as negative controls.

According to Greenstone et al. (2007) and Gagnon et al. (2011a), prey DNA detectability success (DS50) is defined as ‘the time after which half of the predators of a cohort that fed at the same time test positive for the presence of a species of prey, considering that the rate of prey decay is usually exponential’. Probit analysis was used to determine the DS50. Chi-square ($\chi^2$) tests were used to
determine how well a probit model fit the data. When applicable, a $\chi^2$ test of parallelism and a comparison of relative median potency were performed to assess whether there were significant differences between lines. The effect of the $P.\ citri$ primer pair on prey detection was tested using a $\chi^2$ test with a Yates’ correction. Analyses were performed using SPSS, v. 21 (IBM Corp. Released, 2012).

To test whether we would be able to detect both tetranychid prey species in the phytoseiid gut in a single PCR, a second assay in which both prey were consecutively offered to the predator was performed. We selected $E.\ stipulatus$ and $P.\ persimilis$, and we considered a worst case scenario, in which the predator first preyed on the nonpreferred prey (Ferragut et al. 1987, 1992; McMurtry et al. 2013). In the case of $E.\ stipulatus$, the first prey offered was $T.\ urticae$, and for $P.\ persimilis$, $P.\ citri$. We proceeded as in the one-prey experiment until the first prey was killed and released. Then, the phytoseiid was immediately moved to another cell, one with the alternative prey, and again we proceeded as before. When the second prey was released, the phytoseiid was starved in a new cell for 0, 2, 4 or 16 h and processed for molecular assessment. The time spent feeding on each prey, the time elapsed between the first and second successful encounters (i.e. resulting in prey death) and the rate of successful encounters were recorded for both predators.

DNA from all phytoseiids was extracted and screened with the multiplex PCR assay described in the results section. For $P.\ citri$, both primers designed were tested.

**Relative estimation of Phytoseiulus persimilis predigestion on Tetranychus urticae by real-time PCR (or qPCR) analysis**

In the preying assays, we observed that $P.\ persimilis$ released $T.\ urticae$ approximately 40 min after an attack. This phytoseiid had the shortest $DS_{50}$. To assess whether this short $DS_{50}$ was the consequence of effective pre-oral digestion, we used qPCR to detect both pre-oral (i.e. predator inside the prey) and post-oral (i.e. prey inside the predator) processes. Relative quantification was performed using standard curves for $P.\ persimilis$ as predator and $T.\ urticae$ as prey.

After 48 h of starving, one $P.\ persimilis$ adult female was transferred to a cell with one $T.\ urticae$ adult female and observed until prey attack. Each pair was separated by a gentle touch with a fine brush 0, 5, 10, 20 or 30 min after the attack, individually transferred to a 1.5-mL tube, and frozen at $-80^\circ C$ for subsequent molecular determinations. Ten predator-prey pairs per time point were used. DNA extraction of each predatory and prey individual was performed following the modified ‘salting out’ protocol (Monzó et al. 2010). Real-time PCR was conducted using the QuantiTect™ SYBR Green PCR Kit (QIAGEN) and the SmartCycler II instrument (Cepheid) to detect pre-oral and post-oral processes. Prey and predator DNA were amplified using the species-specific designed primers (Table 2) in qPCRs containing in a final volume of 25 µL: 12.5 µL QuantiTect™ SYBR Green PCR, 0.3 µM of each primer and 2 µL of DNA. Reaction conditions were an initial step of 95 °C for 10 min followed by 40 cycles at 92 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. A dissociation curve was created for each reaction, and the melting temperature for each positive reaction was measured. To do this, samples were heated from 60 °C to 95 °C, and fluorescence was continuously measured. We extracted DNA from a pool of 100 samples of both prey and predator to generate standard curves and linear equations via serial dilutions; we performed fourfold dilutions of total $P.\ persimilis$ and $T.\ urticae$ DNA, starting with 298.3 and 655.4 ng, respectively, till 1:10^6, using the same flanking primers for each species (Table 2). Real-time PCR was performed for each sample as previously described to detect DNA from the predator and prey. The amount of DNA was estimated by interpolating from the specific standard curves. To normalize the data, the ratio of [prey DNA]/[predator DNA] and [predator DNA]/[prey DNA] was calculated. The corresponding qPCR efficiencies were calculated according to the equation $E = 10^{(−1/slope)}$. PCR efficiencies were 1.98 and 2.04 for predator and prey, respectively. Ideally, $E$ values would be in the range of 1.8–2.2 (Schmittgen & Livak 2008). The Q-Dixon test at a 95% of confidence level was used for identification and rejection of outliers (Roabacher 1991).

**Field sampling**

Nine commercial citrus orchards in the region of Valen- cia with high densities of either or both $T.\ urticae$ and $P.\ citri$ were chosen (Table S4, Supporting information). Two different sampling methods were used in each orchard: a molecular technique (i.e. the membrane imprinting method) and a classical taxonomical approach (i.e. extraction using Berlese funnels). In each orchard, 10 trees were selected and 10 phytoseiids per tree were individually hand-collected with a fine brush to avoid external DNA contamination (King et al. 2008, 2010; Greenstone et al. 2011). Mites were immediately squashed on a 0.25 cm² nylon membrane (Nylon membranes, positively charged; Roche) with the rounded end of a sterile pipette tip cone previously sealed in 1.5-mL tubes. Tubes containing the membranes were stored at room temperature.

To compare the mite composition estimated with the molecular technique described above with that estimated
using classical taxonomical tools, 100 leaves from the same 10 sampled trees (10 leaves per tree) were randomly taken and transported to the laboratory in a refrigerated plastic bag for microarthropod extraction using Berlese funnels. Tetranychid and phytoseiid mite separation, mounting and identification followed standard procedures (Gutierrez 1985; Ferragut & Santonja 1989; Ferragut et al. 2010).

Relative phytoseiid and tetranychid abundances determined by molecular and classical taxonomical techniques were compared using Pearson’s product correlation (R Core Team 2013).

Prey detection in field-collected predators

Once factors that affect the detectability of prey DNA such as temperature, amplified fragment size, feeding protocols and meal size (Greenstone et al. 2014) are controlled and standardized in laboratory assays, we are more likely to detect prey in a ‘slowly digesting’ species combination than in a ‘rapidly digesting’ one (Gagnon et al. 2011b). To avoid this bias, a comparative weighting was performed on field-collected predators (Chen et al. 2000; King et al. 2008; Gagnon et al. 2011a,b; Greenstone et al. 2014). Following Chen et al. (2000) and Gagnon et al. (2011a,b), $D_{50}$ values for each predator and prey combination were weighted to obtain $D^{\text{weighted}}_{50}$ as follows: ‘the shortest $D_{50}$ was assigned a value of 1.0 and all other $D_{50}$ values were obtained by placing this benchmark $D_{50}$ in the numerator and each other $D_{50}$ value in the denominator’. The corrected predation value of each predator at each sampling point was then calculated by multiplying the number of field-collected predators that were positive for each prey by their specific $D^{\text{weighted}}_{50}$. Because predators were randomly collected, this final number not only compensates for species-specific differences in digestion time but also for the abundance of each predator; additionally, it allows comparison of all predator-prey combinations under field conditions. The values corresponding to the nine samplings were averaged. Corrected predation values were checked for normality (Shapiro-Wilk test) and homogeneity of variance (Levene test). When normality assumptions were fulfilled, data were compared using a $t$-test. Otherwise, the nonparametric Mann-Whitney $U$-test was used.

To reveal prey preferences, predation estimated by molecular techniques was related to tetranychid mite abundances determined by classical taxonomic techniques using a multinomial logit model (Venables & Ripley 2002). This model was fitted for the two most abundant phytoseiid species, $E$. stipulatus and $P$. persimilis, and predicts the probability that each phytoseiid will prey on either or both $T$. urticae and $P$. citri, or even other food sources, as a function of tetranychid abundances determined from Berlese funnel extraction. To define the model, four categories were considered as the dependent variable: only one prey (either $T$. urticae or $P$. citri), both prey simultaneously or no tetranychid prey. A model selection procedure using the AIC was carried out. Field data used in this model were previously corrected using the specific $D^{\text{weighted}}_{50}$ for each predator.

Results

Multiplex PCR design

Nuclear ribosomal DNA from the ITS region and partial mitochondrial DNA from the COI gene were obtained from the various mites included in this study (Tables 1 and S2, Supporting information). Because the COI genes of the target species were highly similar, COI was not used in the rest of the study. As a result, the ITS region was chosen for multiplex PCR primer design (Table 2). Cross-reactivity tests verified that each primer was amplified only in the species for which it had been designed and produced a single band of the expected size on agarose gel (Table 2).

The final multiplex PCR was adjusted to a final volume of 12.5 $\mu$L: 1.4× Taq polymerase buffer, 200 $\mu$M of each dNTP, 1 mM of MgCl$_2$, 0.2 $\mu$M of forward primer in $T$. phiaulatus, 0.16 $\mu$M of forward primer in $N$. barkeri, $P$. citri, and $T$. urticae, 0.1 $\mu$M of forward primer in $A$. swirskii, $E$. stipulatus, $N$. californicus and $P$. persimilis, 0.08 $\mu$M of unlabelled reverse primer, and 0.08 $\mu$M of the same reverse primer labelled with FAM-6 (Table 2), 1 unit of DNA Taq polymerase, and 1 $\mu$L of DNA template for feeding trials samples and 7.25 $\mu$L for field samples. The multiplex PCR design was performed at the same amplification conditions as described for the cross-reactivity test. This multiplex PCR allowed the identification of all target species using a unique PCR in the previously described automated sequencer (Fig. S1, Supporting information). Positive controls with single target DNA templates and equimolar mixes of DNA templates from the eight target species amplified the expected size fragments. The DNA template mix did not affect the sensitivity of the species-specific fragment length detection. The two primers designed for $P$. citri (176 and 243 bp fragment sizes) (Table 2) rendered the same prey DNA detectability in $E$. stipulatus and $N$. californicus ($P > 0.05$). However, in $P$. persimilis, the 176 bp primer significantly increased $P$. citri detection ($\chi^2 = 4.810$; d.f. = 1; $P = 0.028$). Therefore, this primer was selected for multiplex PCR (Table 2). Multiplex PCR sensitivity with
ELUCIDATING MITE TROPHIC RELATIONSHIPS

Fig. 1 Tetramychus urticae (A) and Panonychus citri (B) DNA detection probability curves in Phytoseiulus persimilis (black diamonds), Neoseiulus californicus (white squares) and Euseius stipulatus (black circles) samples after feeding. Lines are fitted probit model with 95% confidence intervals (dashed lines).

fluorescent markers was independently estimated at 1 and 100 pg for total T. urticae and P. citri DNA, respectively.

Multiplex PCR exhibited no cross-amplification of most of the alternative food sources tested except for aphids, which produced a peak of the same size as T. urticae.

Feeding trials

Detectability of prey DNA for T. urticae and P. citri fits the assumptions of the probit model for P. persimilis, N. californicus and E. stipulatus (Fig. 1 and Table S5, Supporting information). DS50 values depended on the phytoseiid and the prey species considered and ranged from 0.2 to 18 h postfeeding (Fig. 1 and Table S5, Supporting information), which corresponded to 0.2 to 18 h postfeeding (Fig. 1 and Table S5, Supporting information). Detection in E. stipulatus lasted 1.7 times longer than in P. persimilis (P < 0.05). Likewise, P. citri detection in E. stipulatus and N. californicus lasted 5.2 and 5.7 times longer than in P. persimilis (P < 0.05), respectively. However, when comparisons were performed for each phytoseiid, probit curves corresponding to the two prey species could not be successfully forced to parallelism (P < 0.020 in all three cases).

In the multiple prey detection experiment, in which we offered the nonpreferred prey first, we were able to detect both prey simultaneously (Table 3). Nevertheless, detectability of prey DNA depended on the predator species’ identity. In the case of E. stipulatus, 58 of 191 individuals tested attacked T. urticae successfully. Of these, only 10 individuals attacked the preferred prey (P. citri) 2 h later (Table 4). Only 22.2% of these predators tested positive for P. citri, despite the fact that the predator was frozen immediately after prey release (digestion time = 0 h). The detection rate was much higher (77.8%) for the first prey, T. urticae, which had been consumed 2 h earlier (Table 3), as observed in the digestion curves for this predator-prey combination (Fig. 1). Euseius stipulatus preyed for 20 min on both prey species before prey release. No additional digestion times were studied due to the low frequency of second prey attacks (Tables 3 and 4). In the case of P. persimilis, a shorter latency time
between first and second attacks and a longer preying time than in *E. stipulatus* were found. *Phytoseiulus persimilis* was twice as fast as *E. stipulatus* when attacking the second prey (*T. urticae*) (1 h) and spent three times longer when feeding on *T. urticae* (c. 61 min) compared with the time observed for *P. citri* (c. 23 min) (Table 4). Immediately following the release of the second prey (time 0), the first prey (*P. citri*) was detected in 12.5% of the samples. Positive detection of the second prey (*T. urticae*) was common, even 16 h later (Table 3).

Relative estimation of *Phytoseiulus persimilis* predigestion on *Tetranychus urticae* by qPCR analysis

*Phytoseiulus persimilis* spent 41.3 ± 1.3 min (time 0 on Fig. 2) feeding on prey. Real-time PCR analysis suggested that *P. persimilis* devoted the first 20 min after attack to injecting saliva into *T. urticae* in a pre-oral digestive process (i.e. predator DNA detection in prey) (Fig. 2, shaded area). During this time, very low prey DNA detection in the predator was observed. In the following 20 min, analyses revealed an increase of prey DNA detection in the predator (Fig. 2, nonshaded area).

**Field sampling: Phytoseiidae and Tetranychidae species identification**

We were able to identify the six target species of the multiplex PCR assay among the 622 phytoseiid specimens that were successfully amplified. The most frequent one was *E. stipulatus* (87.3%), followed by *P. persimilis* (11.9%). The other four species (*A. swirskii, N. barkeri, N. californicus* and *T. phialatus*) were found at similarly low percentages (<1%). Phytoseiid species composition identified by classical taxonomy applied to specimens obtained from the same orchards (*n* = 158) resulted in 86.1% *E. stipulatus*, 13.3% *P. persimilis* and 0.6% *N. barkeri*. Relative abundances of *E. stipulatus* and *P. persimilis* in each sampling estimated by molecular and classical techniques were strongly correlated (Pearson’s product correlation; *P* = 0.009, *r* = 0.801; *P* = 2.513 × 10⁻⁵, *r* = 0.965, respectively). Tetranychidae species composition identified by classical taxonomy from the same samples (*n* = 121) rendered 67.8% and 32.2% *T. urticae* and *P. citri*, respectively. For each field sampling, these proportions were not correlated with the Tetranychidae composition obtained by gut content analysis of the phytoseiids via multiplex PCR (see below) (Pearson’s product correlation; *P* = 0.117, *r* = 0.560; *P* = 0.677, *r* = 0.162, for *E. stipulatus* and *P. persimilis*, respectively).

**Prey detection in field-collected predators**

From the 543 field-collected specimens identified as *E. stipulatus* by molecular techniques, 32.8% tested positive for tetranychid prey (Fig. 3A, nonpatterned area),

| Table 3 Percentage of successful prey detection for *Euseius stipulatus* and *Phytoseiulus persimilis* depending on the order of the species offered |
|-------------------------------|-----------------|-----------------|-----------------|-----------------|
| Predator                     | Time (h) elapsed after 2nd prey attacked* | No. predators | Positive for 1st prey (%)† | Positive for 2nd prey (%) |
| *E. stipulatus*              | 0               | 9‡             | 77.8            | 22.2            |
| *P. persimilis*              | 0               | 16             | 12.5            | 100             |
|                              | 2               | 10             | 0               | 90.0            |
|                              | 4               | 10             | 0               | 80.0            |
|                              | 16              | 6              | 0               | 50.0            |

*Time elapsed until the predator is frozen for subsequent molecular analysis.
†For *E. stipulatus*, the first prey was *Tetranychus urticae* and for *P. persimilis*, *Panonychus citri*. Second prey was the alternative prey.
‡One of 10 failed in the amplification.

| Table 4 Time invested in feeding and rate of successful encounters of *Euseius stipulatus* and *Phytoseiulus persimilis* when preying on *Tetranychus urticae* and *Panonychus citri* |
|---------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| Predator                     | Successful encounter on 1st prey (%)* | Successful encounter on 2nd prey (%) | Time feeding on 1st prey†‡ (min) | Time feeding on 2nd prey†‡ (min) | Time elapsed between successful encounters (min) |
| *E. stipulatus*              | 30.4            | 17.2           | 20.48 ± 3.01    | 20.36 ± 5.40    | 120.02 ± 26.02 |
|                              | *n* = 191§      |                |                |                |                |
| *P. persimilis*              | 39.9            | 71.2           | 22.53 ± 1.53   | 61.48 ± 3.51   | 61.24 ± 8.13   |
|                              | *n* = 148       |                |                |                |                |

*An encounter was considered successful when resulting in prey death.
†For *E. stipulatus* the first prey was *T. urticae* and for *P. persimilis* is *P. citri*. Second prey was the opposite combination.
‡Time was considered only in those cases where the predator preyped on both tetranychid species.
§Number of individuals tested.
whereas 77.0% of *P. persimilis* tested positive for tetranychid prey (*n* = 74) (Fig. 3B, nonpatterned area), resulting in a 2.3 times higher detection rate in the latter species. In *E. stipulatus*, the detection rate was 81.5% for *T. urticae*, 13.5% for *P. citri* and 5.1% for both simultaneously. In *P. persimilis*, 94.7% tested positive for *T. urticae*, 1.8% for *P. citri* and 3.5% for both. Two *A. swirskii*, two *N. barkeri* and one *N. californicus* specimens were identified via the molecular approach. Of these, only a single specimen per species tested positive for *T. urticae*. These DNA detection rates were corrected for their specific DSₜ₉₀ for each predator and prey species (Table 5). According to these corrected estimations, prey-specific predation was similar for each phytoseiid, and phytoseiid-specific predation was significantly higher in *E. stipulatus* (0.157 ± 0.027 and 0.107 ± 0.058 for *E. stipulatus* and *P. persimilis*, respectively) (Mann-Whitney *U*-test = 79.0, *n*₁ = *n*₂ = 18, *P* = 0.008). As *E. stipulatus* densities in the orchards were on average seven times higher than those of *P. persimilis*, the difference in the corrected predation values observed (1.5×) was much lower than would have been forecasted (7×).

Apart from predation on tetranychids, we also observed intraguild predation among phytoseiids: one *N. barkeri* specimen tested positive for *T. phialatus* and six *E. stipulatus* individuals tested positive for a combination of *T. urticae* and *N. californicus* or *P. persimilis*. Three additional *E. stipulatus* specimens were positive for phytoseiids, each having one of the following species present: *N. barkeri*, *N. californicus* and *P. persimilis*.

When models relating orchard prey abundance (Berlese funnels) and gut prey detection in *E. stipulatus* and
P. persimilis were fit, a completely different pattern regarding feeding behaviour was identified for the species tested. In the case of E. stipulatus, non-tetranychid food sources turned out to be significantly preferred over both T. urticae (P < 0.001) and P. citri (P < 0.001), independently of their respective densities. However, these densities affected the prey choice of E. stipulatus, which means that it fed most frequently on the most abundant prey species (P ≤ 0.001). In contrast, P. persimilis always preferred to feed on T. urticae (P = 0.029) irrespective of the relative densities of tetranychids in the orchard.

Discussion

Multiplex PCR design

A multiplex PCR assay for the simultaneous specific identification of eight mite species, six predators and two prey, using the ITS region has been developed and successfully tested with laboratory and field samples. This is, to our knowledge, the first fully described multiplex system for Acari. Previous approaches (Fitzgerald et al. 2004) published partial information only. Furthermore, this is the first time that field data and molecular gut content information have been integrated to estimate predation rates of field-caught predatory mites and these values have been adjusted to correctly interpret field results.

The ITS region has previously been used to differentiate mite species (Yli-Mattila et al. 2000; Hurtado et al. 2008) and has proved suitable for studies of predation in various arthropod classes (Hoogendoorn & Heimpel 2001; Monzó et al. 2010, 2011; Gomez-Polo et al. 2013). Most molecular analyses of predation in terrestrial ecosystems have considered the COI region as the target (see the revision of Gariepy et al. 2007; King et al. 2008; Furlong 2015); this is true in studies of mites as well (Rivera-Rivera et al. 2012). For the species included in our study system, the COI turned out to be inappropriate for species differentiation.

There is no clear consensus on whether detectability of prey DNA remains the same (Chen et al. 2000; Sheppard et al. 2005; King et al. 2008; Monzó et al. 2010) or increases (Agusti et al. 1999; Zaidi et al. 1999; Juen & Traugott 2005; Waldner et al. 2013) when using primers that amplify shorter DNA fragments. The present results suggest no overall enhancement of prey detection with shorter fragments of P. citri. Both P. citri primer combinations rendered the same results for E. stipulatus and N. californicus but not for P. persimilis. In P. persimilis, the second primer, resulting in shorter amplification fragments (Table 2), improved detection.

Multiplex PCR revealed cross-amplification of aphid DNA. However, the likelihood of these insects being preyed upon by phytoseiids seems quite remote due to phytoseiid feeding habits (Overmeer 1985b; McMurtry & Croft 1997; McMurtry et al. 2013) and differences in body size (i.e. aphids are four to nine times larger). In spite of this positive cross-amplification, our multiplex system could be applied to generalist arthropod predators preying on tetranychids and aphids (e.g. coccinellids, chrysopids, anthuricids) with an additional singleplex that uses general aphid primers (Chapman et al. 2010).

Feeding trials

Several authors have described that predators often differ in their detectability half-lives with prey species and that prey may have different half-lives in different predators (Gagnon et al. 2011a; Greenstone et al. 2014). In the present study, we observed both. We found differences in DS50 values for the same prey species in the three phytoseiid species considered (Fig. 1 and Table S5, Supporting information). The differences could be related to various nonexclusive traits, such as morphophysiological features of the digestive system (Akimov & Starovir 1978) and different pre- and post-oral digestive processes (Chant 1985; Greenstone et al. 2014). Akimov & Starovir (1978), comparing the digestive systems of one specialist (P. persimilis) and two facultative

| Predator          | Prey             | DS50 (h) | Total positive prey detection (N) | DS50 weighted (A) | Positive prey detection (n) (B) | Corrected predation value (A*B) |
|-------------------|------------------|----------|----------------------------------|-------------------|---------------------------------|---------------------------------|
| Euseius stipulatus| Tetranychus urticae | 18.33   | 145                              | 0.010             | 3-28                            | 0.154 (0.030-0.280)              |
|                   | Panonychus citri  | 2.99     | 24                               | 0.060             | 0-8                             | 0.160 (0.048-0.480)              |
| Neoseiulus californicus | T. urticae         | 14.99   | 1                                | 0.012             | 0-1                             | 0.001 (0.0-0.12)                 |
|                   | P. citri          | 3.16     | 0                                | 0.057             | 0                               | 0                               |
| Phytoseiulus persimilis | T. urticae        | 10.67   | 54                               | 0.017             | 0-19                            | 0.102 (0-0.323)                  |
|                   | P. citri          | 0.18     | 1                                | 0.01               | 0-1                             | 0.111 (0-1-0.000)                |
generalist predators (both belonging to the genus Amblyseius), realized that morphological differences (i.e. different numbers of caeca) were related to functional modifications in their epithelial cells favouring relatively faster digestion in the specialist. Although there are no similar studies for E. stipulatus and N. californicus currently available, our observations suggest the number of caeca present is similar to that in Amblyseius spp. In relation to pre- and post-oral digestive processes, we cannot dismiss differences in digestive enzyme composition (Greenstone et al. 2014), which is not yet studied for phytoseiids, that are related to different food regimes of strictly entomophagous vs. omnivorous species; such differences could explain the longer postfeeding detection time in omnivores (Waldner et al. 2013). In this study, we investigated P. persimilis preying on T. urticae. This predator is a Tetranychus specialist that spends more time (c. 40 min) feeding on T. urticae than the omnivorous E. stipulatus or the tetranychid specialist N. californicus (c. 18 and 30 min, respectively). We demonstrated that half of the time P. persimilis spends feeding on prey is devoted to a pre-oral digestive process and the other half to extracting prey contents, suggesting the occurrence of a predator-prey-predator flux process. This long food processing could be related to the shorter prey DNA detection time observed in P. persimilis. Future research to elucidate the situation in the other two phytoseiids considered in this study is needed.

In parallel with results from other authors (Harwood et al. 2007; Gagnon et al. 2011a; Waldner et al. 2013), this study confirms that the identity of the prey affects prey DNA detection rates. Closely related prey species such as T. urticae and P. citri (Tetranychini tribe) did not result in similar digestion rates in the same phytoseiid species (i.e. 10.67 and 0.18 h for P. persimilis preying on T. urticae and P. citri, respectively). In some species investigated, meal size in terms of food availability (immature vs. adult size) was positively related with detection time (King et al. 2010; Gagnon et al. 2011a), whereas this was not the case for other species (Juen & Traugott 2005; Waldner et al. 2013). In the present case, individuals of both species were adults of approximately the same size (~0.4 mm long). Because we did not weigh the prey before and after the attack, differences in effective exploitation cannot be excluded. Furthermore, differences in prey composition (e.g. lipid content) could also affect prey DNA detection (e.g. Thomas et al. 2014). Further research is needed to understand these differences. Interestingly, multiple prey detection assays demonstrated that detection of more than one prey in the same phytoseiid is possible; this was also observed in field-collected specimens. However, the probability of detecting a second prey depended on the species identity of both the predator and the second prey.

Field sampling: Phytoseiidae and Tetranychidae species identification

Phytoseiid abundances were similar in a comparison of the two different assessment methods (i.e. molecular vs. classical approach). Euseius stipulatus was the most abundant species followed by P. persimilis, which was seven times less abundant. These results coincide with previous studies highlighting the predominance of E. stipulatus in Spanish citrus orchards (Abad-Moyano et al. 2009a; Aguilar-Fenollosa et al. 2011a). However, P. persimilis abundance has been described as ranging from rare (Garcia-Mari et al. 1986) to abundant, is always associated with the presence of T. urticae (Abad-Moyano et al. 2009a; Aguilar-Fenollosa et al. 2011a) and occurs mainly in orchards with low pesticide pressure (Argolo et al. 2014; Pascual-Ruiz et al. 2014). The selected orchards fulfil the two last conditions, and P. persimilis was present in all the orchards sampled but one. Neoseiulus californicus was rarely found (i.e. only one individual was identified via molecular techniques). This result does not coincide with previous surveys that described a higher prevalence of this phytoseiid in Spanish citrus orchards (Abad-Moyano et al. 2009a; Aguilar-Fenollosa et al. 2011a).

Prey detection in field-collected predators

In our study, the analyses of the gut contents of the two most abundant predator species (E. stipulatus and P. persimilis) revealed for the first time under field conditions that both species prey on T. urticae and P. citri (Fig. 3 and Table 5). Corrected predation values used to quantify the relative importance of each predator (Greenstone et al. 2014) revealed the higher importance of E. stipulatus in our system when compared with P. persimilis. However, this importance should be attributed to its higher abundance given that E. stipulatus was far less effective than expected. In summary, our results demonstrate that high functional and numerical responses do not always co-occur in the most effective predator, in accordance with the suggestion of Lester & Harmsen (2002). In the present case, most P. persimilis specimens analysed preyed on tetranychids, whereas the highly abundant E. stipulatus preferred to feed on nontetranychid food sources.

Euseius stipulatus is a generalist that also feeds on pollen (McMurry & Croft 1997; McMurry et al. 2013) and as such, it is expected to switch its feeding preferences in response to the relative availability of alternative food types (Murdoch 1969; Murdoch & Oaten 1975). Therefore, its prevalence in citrus orchards is likely linked to the presence of other food sources, such as pollen, fungi, sugary secretions and other microarthropods not
targeted in our multiplex PCR (Ferragut et al. 1987; Pina et al. 2012), including other phytoseiids (Abad-Moyano et al. 2010a,b). As demonstrated in this study under field conditions, intraguild predation is a probable event, and it should be considered in future research dealing with tetranychid biological control. Indeed, the results of the models adjusted for prey preference in the present study could be explained by this feeding behaviour; even when tetranychids are abundant, *E. stipulatus* is expected to feed on alternative food sources. This result may be taken as an indication that the common assumption that *E. stipulatus* is an effective predator of *P. citri* (Ferragut et al. 1987, 1988; Grafton-Cardwell et al. 1997) should be attributed to a numerical rather than a functional response. Because we have demonstrated that *P. persimilis* can feed on *P. citri* in the field, it would be interesting to determine whether this predator is able to survive in citrus orchards by feeding on only *P. citri*.

It is widely acknowledged that it is crucial for the success of biological control to enhance the relative abundance of the most effective predators within the community (Straub & Snyder 2006). In our system, this predator is the specialist *P. persimilis*, which is usually present in low numbers but, in contrast to the dominant generalist *E. stipulatus*, preferentially feeds on *T. urticae* even when it is scarce, as expected for specialized predators of *Tetranychus* species (Type I, McMurtry et al. 2013). Therefore, all cultural practices, including selective pesticide treatments, that favour and enhance the presence of this phytoseiid (Aguilar-Fenollosa et al. 2011a; Argolo et al. 2014; Pascual-Ruiz et al. 2014) are expected to contribute to the restoration of effective natural regulation of *T. urticae* on citrus plants.

**Conclusions**

One of our goals was the development of a multiplex PCR method that could be used to disentangle trophic relationships among mites. This technique has been successfully developed both as a valid alternative to classic taxonomy for determining mite species composition and as a tool to appraise the trophic ecology of phytoseiids in real field conditions. Awareness of these relationships should help us understand how mite communities such as the one considered in this study work, and contribute to enhancing the sustainability of our agroecosystems.

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Data accessibility

DNA sequences: GenBank accessions AM408039, AM408043, GU565289, GU565290, GU565315, GU565317, GU565320, GU565321, GU565324, KP642055, KP642058, KP642059, KP642063, Y18268, Y18269.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Example of amplification of multiplex PCR with all species considered together in the same electropherogram. Individual electropherograms are detailed below for each species

Table S1 Non-target organisms screened for cross-reactivity in the multiplex PCR

Table S2 Sequences of the mitochondrial cytochrome oxidase I (COI) gene of the selected Acari species used in this study

Table S3 Number of positive detections for each predator and prey combination at different time intervals since feeding

Table S4 Description of sampling sites

Table S5 Probit curves adjusted for positive detections and prey detection success over time (DS50) from a single *T. urticae* or *P. citri* specimen