Running title: Elucidating mite trophic relationships

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; the predatory mites are considered to be the herbivores' main biological control agents. These primers were successfully multiplexed in a single PCR reaction 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 *Phytoseiulus 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 7-fold 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 minute species such as mites, which include important agricultural pests and their predators.

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 pre-digested 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 non-coding 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 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 significant 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 and 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 2006-13). 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 toward 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 toward 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 predatory guild occurring in each agroecosystem depends on the species/cultivar, geographical location, and management practices (McMurtry 1985, 1992; Helle & Sabelis 1985; 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 life-styles, 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 life-style (Jaques *et al*. 2015).

The omnivore *E. stipulatus* is the most abundant phytoseiid in Spanish citrus agrosystems 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. The present 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 (DS_{50}) 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 swirski* 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.) Burm f.). Both mites were maintained at room temperature and experienced a natural photoperiod. The initial individuals of *A. swirskii*, *N. californicus*, and *P. persimilis* were obtained from Koppert Biological Systems (Swirski-mite®, Spical®, Spidex®, respectively). *Neoseiulus barkeri* Hughes was collected on *Festuca arundinacea* Schreber plants in a greenhouse at Universitat Jaume I (Castelló de la Plana, Spain). *Euseius stipulatus* and *Typhlodromus phialatus* Athias-Henriot individuals were collected from the same orchards as *P. citri*. *Amblyseius swirskii*, *N. barkeri*, *N. californicus*, and *P. persimilis* were reared following the procedures described by Overmeer (1985a). Bean leaves infested with *T. urticae* were regularly added as food. *Euseius stipulatus* and *T. phialatus* were reared on upside down bean leaves and were fed with *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, Supplementary material (Table 1)). Bean leaf DNA was also extracted following this protocol. Clementine mandarin (*C. clementina* Hort. ex. Tan. cvar. Clemenules) leaf DNA and *C. edulis* anther DNA were extracted using the Sigma protocol (REDExtract-N-AmpTM 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 membrane imprinting technique (Olmos *et al*. 1996, 1999; Bertolini *et al*. 2008). This technique is a method that simplifies DNA extraction, stops digestion in the predator’s gut, reduces handling time, maximizes DNA preservation over time, and is fully compatible with field sampling (Juan-Blasco *et al*. 2013). Nylon membranes printed with field-collected phytoseiid 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 ºC for 10 min followed by 60 ºC. Then, 1 µL of fresh Proteinase-K was added and maintained at 60 ºC for 1 h. Finally, samples were heated at 95 ºC 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 ºC, samples were centrifuged for 10 min at 25 ºC, and precipitated DNA was washed in 200 µL 70% chilled ethanol. Precipitated DNA was resuspended in 15 µL of LTE and stored at -20 °C.

Multiplex PCR design

Sequencing, alignment and primer design

ITS regions and COI were selected to be tested for their applicability as specific molecular markers for mite species. All sequences used in this study are listed in Tables 1 and S2. Those not found in the Genbank (Nucleic acids research; http://www.ncbi.nlm.nih.gov/genbank) data base were obtained during this work. For sequencing both marker types, amplification reactions were performed on a final volume of 25 µL: 1× Taq polymerase buffer (Roche Applied Science, Mannheim, Germany), 200 µM of each dNTP (5 PRIME GmbH, D•22767 Hamburg), 1 mM of $MgCl_2$, 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, 35 cycles at 92 °C for 1 min, annealing at 45 or 50 °C (depending on the combination of primers; Tables 1 and S2) 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, California, USA) and visualized under UV light.

Band quantification was carried out using the GeneTools program from Syngene (Cambridge, UK). For each PCR reaction, 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 non-conserved nucleotide sequences in order to simultaneously obtain prey and predator amplification fragments of different sizes. For all species, the universal primer 5' TTCTTTTCCTCCGCTTAGTGATATGCTTAA 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 a 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 $MgCl_2$, 0.4 µM of each primer, 1 unit of DNA Taq polymerase, and 1µL 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 50 °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 $MgCl_2$, 0.2 µM of each *A. swirskii, E. stipulatus*, *N. barkeri*, *P. citri*, and *P. persimilis* forward primers, 0.4 µM of each *N. californicus, T. phialatus*, and *T. urticae* forward primers, 0.4 µM of reverse primer (Table 2), 1 unit of DNA Taq polymerase, and 1 µL of DNA template. Assessment of amplification conditions and PCR products was performed as described for the cross-reactivity test.

Multiplex PCR conditions were checked on agarose gel, modified, and adapted to analysis with marked primers in the aforementioned automated sequencer. As a positive control, an equimolar mix (5 ng/µL) of the eight DNA target species was used. Final multiplex PCR conditions are described in the results section. Fragment length reads were carried out with Peak Scanner™ Software v1.0 (Applied Biosystems 2006). All samples that produced peaks of the expected size (i.e., >100 relative fluorescent light units) were considered positive. Sensitivity of prey DNA detection was determined by assaying

multiplex PCR, at nine-fold dilutions starting with 10 ng of total independently *T. urticae* and *P. citri* DNA till $1:10^{10}$.

Alternative food sources cross-reactivity test

Species specificity of the multiplex PCR assay was tested on different potential food sources available in citrus orchards, those used in the rearings, and other Phytoseiidae and Tetranychidae mites of economic importance (i.e., 2-5 samples tested for each species) (Table S1). This test aids in avoiding false positives when predators can feed on alternative food sources. We used the same positive control as in the cross-reactivity test for species specific primers, The universal primer pair Univ18SrDNA and PCR conditions described in 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 reaction (i.e., absence of both target and non-target DNA).

Feeding trials

Modified Huffaker cells (Abad-Moyano *et al*. 2009b) were used as arenas. Three to 5 d-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 to 28 h; see Table S3). Next, they were transferred to 1.5 mL tubes, frozen at -80 ºC, 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 (DS_{50}) 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 DS_{50}. Chi-square (χ^2) tests were used to determine how well a probit model fit the data. When applicable, a χ^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 χ^2 test with a Yates' correction. Analyses were performed using SPSS (v. 21).

To test if we would be able to detect both tetranychid prey species in the phytoseiid gut in a single PCR reaction, 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 non-preferred 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 ºC 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 QuantiTectTM 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 qPCR reactions containing in a final volume of 25 µL: 12.5 µL QuantiTectTM SYBR Green PCR, 0.3 µM of each primer, and 2 µL of DNA. Reaction conditions were an initial step of 95 ºC for 10 min followed by 40 cycles at 92 ºC for 30 s, 55 ºC for 30 s, and 72 ºC 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 ºC to 95 ºC, 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 four-fold dilutions of total *P. persimilis* and *T. urticae* DNA, starting with 298.3 ng and 655.4 ng, respectively till $1:10^5$, 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 (Rorabacher 1991).

Field sampling

Nine commercial citrus orchards in the region of Valencia with high densities of either or both *T. urticae* and *P. citri* were chosen (Table S4). 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 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 cm^2 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), DS_{50}values for each predator and prey combination were weighted to obtain $DS_{50}^{weighted}$ as follows: “the shortest DS_{50} was assigned a value of 1.0 and all other DS_{50} values were obtained by placing this benchmark DS_{50} in the numerator and each other DS_{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 $DS_{50}^{weighted}$. 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 non-parametric 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 criterion was carried out. Field data used in this model were previously corrected using the specific $DS_{50}^{weighted}$ 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 (Table 1 and S2). 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 reaction was adjusted to a final volume of 12.5 µL: 1.4× Taq polymerase buffer, 200 µM of each dNTP, 1 mM of $MgCl_2$, 0.2 µM of forward primer in *T. phialatus*, 0.16 µM of forward primer in *N. barkeri*, *P. citri*, and *T. urticae*, 0.1 µM of forward primer in *A. swirskii*, *E. stipulatus*, *N. californicus*, and *P. persimilis*, 0.08 µM of unlabeled reverse primer, and 0.08 µM of the same

reverse primer labeled with FAM-6 (Table 2), 1 unit of DNA Taq polymerase, and 1 µL of DNA template for feeding trials samples and 7.5 µL 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 reaction in the previously described automated sequencer (Fig. S1, Supplementary material (Fig. 1)). 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 ($\chi^2 = 4.810$; df = 1; $P = 0.028$). Therefore, this primer was selected for multiplex PCR (Table 2). Multiplex PCR sensitivity with 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* fit the assumptions of the probit model for *P. persimilis*, *N. californicus*, and *E. stipulatus* (Fig. 1 and Table S5). DS_{50} values depended on the phytoseiid and the prey species considered

and ranged from 0.2 to 18 h post-feeding (Fig. 1 and Table S5), which corresponded to *P. citri* and *T. urticae* when preyed upon by *P. persimilis* and *E. stipulatus*, respectively. For *T. urticae*, there was still some detection 28 h after prey release for all three predators (20% detection for *P. persimilis* and *E. stipulatus* and 40% for *N. californicus*) (Table S3). However when *P. citri* was the prey, no detection was observed after 4 h in *P. persimilis* and 8 h in *E. stipulatus* and *N. californicus* (Table S3).

Probit curves for each phytoseiid species when preying on either *T. urticae* or *P. citri* were successfully forced to parallelism (*T. urticae*: $\chi^2 = 0.545$; df = 2; $P = 0.761$; *P. citri*: $\chi^2 = 0.347$; df = 2; $P = 0.841$). Relative median potencies for *T. urticae* suggested that detection in *E. stipulatus* lasted 1.7 times longer that 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 non-preferred 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 out of 191 individuals tested attacked *T. urticae* successfully. Of these, only ten 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. *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 (Table 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* (ca. 61 min) compared with the time observed for *P. citri* (ca. 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, non-shaded 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' 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' 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, non-patterned area), whereas 77.0% of *P. persimilis* tested positive for tetranychid prey (n = 74) (Fig. 3B, non-patterned 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. Out of these, only a single specimen per species tested positive for *T. urticae*. These DNA detection rates were corrected for their specific $DS_{50}^{weighted}$ 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 = 79.0, n1 = n2 = 18, $P = 0.008$). As *E. stipulatus* densities in the orchards were on average 7-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 behavior 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 ($P \leq 0.001$). 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 (Agustí *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 4-9 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, anthocorids) 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 DS_{50} values for the same prey species in the three phytoseiid species considered (Fig. 1 and Table S5). The differences could be related to various non-exclusive traits, such as morpho-

physiological 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 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 favoring 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 post-feeding detection 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 (ca. 40 min) feeding on *T. urticae* than the omnivorous *E. stipulatus* or the tetranychid specialist *N. californicus* (ca. 18 min 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 h 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 (ca. 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 7 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-Marí *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 fulfill 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* (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 analyzed preyed on tetranychids, whereas the highly abundant *E. stipulatus* preferred to feed on other food sources. *Euseius stipulatus* is a generalist that also feeds on pollen (McMurtry & Croft 1997; McMurtry *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 *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 behavior; 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 pesticide treatments, that favor 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.

General 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 accesibility

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

Data files from all figures and tables can be found on dryad: http://datadryad.org/review?doi=doi:10.5061/dryad.5k927"

Voucher especimens have been deposited in the Entomology Collection of the Departament de Ciències Agràries i del Medi Natural, Universitat Jaume I, Castelló de la Plana, Spain.

Authors' contribution

J. A. Jaques is the head of the Integrated Pest Management research group at UJI and was involved in the design and discussion of the assays. M. A. Hurtado led the molecular biology approach used in this study and designed all the experiments except the qPCR, which was developed by G. Camañes. C. Pérez-Sayas and T. Pina performed all the experiments with support from M. A. Gómez-Martínez. M. V. Ibáñez-Gual and T. Pina statistically analyzed the results, which were discussed by all authors. All authors contributed to writing of the manuscript.

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.