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Tracking mite trophic interactions by multiplex PCR

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ABSTRACT

BACKGROUND: A thorough knowledge of trophic webs in agroecosystems is essential to achieve successful biological pest control. Phytoseiid mites are the most efficient natural enemies of tetranychid mites, which include several important pests worldwide. Nevertheless, phytoseiids may feed on other food sources including other microarthopods, plants, and even other phytoseiids (intraguild predation), which can interfere with biological control services. Molecular gut content analysis is avaluable tool for characterizing trophic interactions, mainly when working on microarthropods as mites. We have designed new primers for Phytoseiidae, Tetranychidae and Thysanoptera identification and they have been multiplexed in a PCR together with universal plant primers. Additionally, we have estimated prey DNA detectability success over time (DS_{50}) considering the most probable events in Spanish citrus orchards: the phytoseiid *Euseius stipulatus* as a predator*,* the phytoseiid *Phytoseiulus persimilis* as intraguild prey, and the thrips *Frankliniella occidentalis* and *Anaphothrips obscurus* as alternative prey to *Tetranychus urticae*.

RESULTS: The multiplex PCR designed allows the identification of Phytoseiidae (both predator and intraguild prey) and detects alternative food sources mentioned above in the gut of the Phytoseiidae predator. DS_{50} for *E. stipulatus* as the predator were 1.3, 2.3 and 18.7 hours post-feeding for *F. occidentalis*, *A. obscurus,* and *P. persimilis* as prey, respectively.

CONCLUSION: The tracking of the trophic relationships within the citrus acarofauna, and the unveiling of the role of alternative food sources will pave the way for enhancing *T. urticae* biological control. This multiplex PCR approach could be applicable for these purposes in similar agroecosystems.

Keywords: molecular diet analysis; prey detection; trophic links; Acari; Thysanoptera; plant

Authors' contribution statement

JAJ is the head of the Integrated Pest Management research group at UJI and was involved in the design and discussion of the assays. MAH led the molecular biology approach used in this study and designed the experiments with the support of MAG-M and TP. MAG-M and TP performed the

experiments and statistically analyzed the results. EA-F identified thrips species described in the manuscript. All authors contributed to the discussion and writing of the manuscript.

Compliance with ethical standards

Conflicts of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any study with human participants. All experiments in this study were performed in compliance with current Spanish law.

1 INTRODUCTION

Successful biological control of agricultural pests requires a thorough knowledge of the trophic relationships, especially between the second and the third trophic level (i.e., phytophagous pests and their natural enemies). However, the study and understanding of these interactions can be highly challenging, especially when generalist predators that frequently exploit multiple prey species may interact either with the target pest, alternative prey or food sources.[1] In this case, the only conclusive evidence of predation is direct observation of prey consumption by a predator and the identification of the prey remains within the predator's gut or faeces. Nevertheless, confirming trophic links under unaltered field conditions is hampered, especially when working on microarthropod species with cryptic lifestyles (i.e., nocturnal, hidden or elusive predation habits).[2] Moreover, in the case of microarthropods such as mites (Acari), microscopic analysis of the predator gut content should be discarded as they engage in extra-oral digestion and/or fluid feeding.[3] A large amount of studies have demonstrated that molecular DNA techniques can overcome these limitations by allowing the identification of both the predator and the prey present in the gut content and, therefore, favouring the unveiling of these trophic interactions.[4-8] Mitochondrial based markers (COI) are the most frequently used for this purpose, despite ribosomal ITS and 18S markers have also been proved useful.[5] These techniques have taken advantage of the growing number of sequences of important agricultural species available, including tetranychids (Acari: Prostigmata)[9,10] and thrips (Insecta: Thysanoptera).[11,12] The implementation of a multiplex PCR, with multiple target species (either prey or predator) amplifying simultaneously, is especially useful in microarthropods as mites because the amount of DNA that can be extracted from a single individual is limited and restricts the number of rounds of amplification for prey detection.[9,10,13,14]

In Spanish clementine mandarin orchards [*Citrus clementina* Hort. ex. Tan. (Rutaceae)], the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) is a key pest.[15] Many features of its biology, as rapid development, high fecundity, haplo-diploid sex determination and the plasticity of its genome, facilitate rapid evolution of pesticide resistance.[16-17] Consequently, the emphasis has been placed on implementing safer and more effective control measures including conservation biological control.[18] The implementation of a *Festuca arundinacea* Schreber (Poaceae) ground cover contributes

to better regulation of *T. urticae* populations in these orchards, due to the fact that this cover enhances the frequency and abundance of Phytoseiidae (Acari: Mesostigmata), the most important family of mite predators specialized in tetranychid prey.[18,19] Moreover, it provides alternative food sources for phytoseiids such as pollen and thrips.[20-22] Although thrips are abundant microarthropods in citrus orchards,[23-25] species composition depends on the management of the cover.[26] Whereas a wild cover, including plant species belonging to different families, may promote the appearance of potential citrus pest species such as *Frankliniella occidentalis* (Pergande) and *Thrips tabaci* Lindeman (Thysanoptera: Thripidae),[26] a ground cover of *F. arundinacea* hosts large numbers of grass-specialized thrips, where *Anaphothrips obscurus* (Müller) (Thripidae) is regularly found along the season. The most effective phytoseiids species preying on *T. urticae* in this system[19] are able to feed on thrips, at least under laboratory conditions.[20,21,27-29] Furthermore, recent studies suggest that *A. obscurus* could compete with *T. urticae* by resource exploitation and interference, and also mediate apparent competition as both are potential prey for phytoseiids.[20,21] Additionally, *F. arundinacea* and clementine mandarins could provide another food source for some phytoseiid mites. McMurtry et al.[30] proposed the possibility of considering an additional group of phytoseiid species that can pierce plant cells. This group would include phytoseiids that may complement their nutrition requirements by feeding on leaf cells without inducing any apparent damage to the plant (i.e., they cannot be considered pestiferous) but that may affect prey beyond predation through plant-mediated effects.[31] This group would mainly include species of the genera *Euseius* De Leon and *Typhlodromus* (*Anthoseius* and *Typhlodromus*) Scheuten, as *Typhlodromus* (*T.*) *pyri* Scheuten, *Typhlodromus* (*A.*) *rhenanus* (Oudemans), and *E. scutalis* (Athias-Henriot), among others.[32-35]

The occurrence of intraguild predation (IGP), defined as ‘‘predator-prey interactions among consumers potentially competing for limiting resources’’,[36] among Phytoseiids cannot be discarded. IGP commonly occurs within many predatory guilds,[37,38] including generalist and specialist phytoseiid mites.[39-41] IGP is a relevant issue in applied ecology, including biological control. It may have a negative impact on pest suppression depending on both the force and frequency of its occurrence and the role of the species that interact.[42-45] It is well known that the availability of alternative prey or food sources can help reduce IGP.[46] In fact, Guzmán et al.[47] observed that high availability of shared food sources entails a negligible IGP in phytoseiids. Among the predatory guild described in Spanish

clementine mandarin orchards,[19] the highly abundant generalist pollen feeder *Euseius stipulatus* (Athias-Henriot), and the generalist predator from soil/litter habitats *Neoseiulus barkeri* Hughes,[30] are seen as potential IG predators.[48-50] Whether they are involved in IGP or they exploit alternative food sources, and consequently reduce IGP, deserves further studies that could be tackled with molecular tools which have not been developed for the time being.

Pérez-Sayas et al.[14] designed a multiplex PCR to determine and quantify the extent of the trophic relationships considering the most common Phytoseiidae as predator in Spanish citrus orchards[19] and two Tetranychidae pest species (*T. urticae* and *Panonychus citri* McGregor) as prey under field conditions. However, IGP and alternative food sources including other tetranychids, thrips, or even plants, were not considered despite their potential impact on biological control. Therefore, the aim of this study has been to design a new multiplex PCR for the detection and identification of different phytoseiid predator species occurring in citrus Spanish orchards at species level (*E. stipulatus*, *N. barkeri*, *Neoseiulus californicus* (McGregor), *Phytoseiulus persimilis* Athias-Henriot and *Typhlodromus phialatus* Athias-Henriot) as both the predator and the IG prey, as well as the detection of other food sources, including tetranychids (family level), thrips (order level) and plants (kingdom level). This multiplex PCR should pave the way for a better understanding of the trophic webs involved in the regulation of tetranychid populations in field conditions.

2 MATERIALS AND METHODS

The most relevant Tetranychidae, Phytoseiidae and Thripidae species found in Spanish citrus orchards and involved in the biological control of *T. urticae*[19,26,51] (Table 1), were included in the study for the design of new primers (which identify target organism at different taxonomic levels) and the development of a multiplex PCR. Additional primers retrieved from other studies were also included in the multiplex PCR (Table 2). Phytoseiidae predators were considered representative of the third trophic level, IG Phytoseiidae, Tetranychidae and Thysanoptera of the second level, and both clementine mandarin and *F. arundinacea* of the first level (primary producers).

2.1 Arthropod species

Arthropod rearing for laboratory studies

Tetranychus urticae individuals were originally collected in clementine mandarin orchards in the region of La Plana (Castelló de la Plana, Spain). They were subsequently reared on bean plants (*Phaseolus vulgaris* L.) (Fabaceae) at room temperature and natural photoperiod. *Euseius stipulatus* individuals were collected in clementine mandarin orchards located in Montcada (Valencia, Spain) and *Phytoseiulus persimilis* Athias-Henriot in Les Alqueries (Castelló, Spain). *Euseius stipulatus* was reared on upside down placed bean leaves following the procedures described by Overmeer[52] and fed with a mixture of *T. urticae* and *Carpobrotus edulis* (L.) N. E. Br (Aizoaceae) pollen. *Phytoseiulus persimilis*was also reared following the procedures described by Overmeer[52] and fed with *T. urticae* on bean leaves. Both phytoseiid species were maintained in separate climatic chambers at 25 ± 1 °C, 70 ± 10% RH, and a photoperiod of 16:8 h (light:dark; L:D). These conditions were also used for the laboratory assays involving live mites.

Anaphothrips obscurus individuals were originally collected in *F. arundinacea* plants grown in an experimental citrus plot at Universitat Jaume I (UJI). They were later maintained on the same kind of plants (*F. arundinacea* 'Fórmula frutales y cítricos', Semillas Fitó, S.A., Barcelona, Spain) grown in a pesticide-free greenhouse at the Institut Valencià d'Investigacions Agràries (IVIA) (Montcada, Valencia, Spain). *Anaphothrips obscurus* specimens were reared following the procedures described in Gómez-Martínez et al.[20] *Frankliniella occidentalis* individuals were obtained from a colony initiated at IVIA in 2010 and originally collected at Campo de Cartagena (Murcia, Spain).[53] They were later reared following the procedures described by Debreczeni et al.[54] Both thrip species colonies were maintained in separate climatic chambers at 25 ± 1 °C, 70 ± 5% RH and 16:8 h (L:D) photoperiod.

Thysanoptera field samples

For primer specificity design and test (see section below), different citrus field samplings, including canopy and ground cover, were performed to collect Thysanoptera, which was considered as a potential food source of Phytoseiidae (Table 1). Non-starved Thysanoptera samples were isolated in 99% ethanol and frozen for further molecular analysis. Furthermore, some specimens of *Heliothrips haemorrhoidalis* (Bouché) (Thysanoptera: Thripidae) and *Thrips tabaci* obtained from persimmon [*Diospyros kaki* Thunb. (Ebenaceae)] and leek [*Allium ampeloprasum* var. *porrum* (L.) J. Gay (Amaryllidaceae)], respectively, were also collected and used for primer specificity tests (Table 1).

2.2 DNA extraction

DNA of Phytoseiidae, Tetranychidae, Thysanoptera and other potential food sources for Phytoseiidae mites in citrus orchards and those provided in the laboratory rearings was extracted following different protocols depending on the organism and the objective of the study.

Acari and Hemiptera from the UJI-IVIA DNA collection and new Acari DNA used for feeding trials (Table 1 and 3), were previously extracted following the modified "salting out" protocol.[55] The DNA of Thysanoptera specimens reared in the laboratory was also extracted using this protocol. However, those thrip specimens obtained from field samples and isolated in 99% ethanol were handled according to Rugman-Jones et al.[12] In this case, DNA was extracted following the modified "salting out" protocol, where the grinding of the specimen was substituted by piercing one side of the abdomen using a sterilized minute pin, in 100 µl of TNES. This method allows the recovery of the remains of the individual from the original microfuge tube for species-specific identification. Adult thrips were slide mounted on Hoyer's medium for microscope observation and identified using morphological characters.[56-58] *Festuca arundinacea* leaf DNA was also extracted following the modified "salting out" protocol. Clementine mandarin (*C. clementina* Hort. ex. Tan. cvar. Clemenules) leaf DNA and *C. edulis* anther DNA were extracted using the REDExtract-N-AmpTM Plant PCR Kit (Sigma), following the manufacturer protocol. Finally, fungal DNA used for cross-reactivity test (see the section below) came from the UJI-IVIA DNA collection and it was extracted following the protocol described by Sánchez-Torres et al.[59]

2.3 Multiplex PCR design

Alignment and primer design

Multiplex PCR was designed considering Phytoseiidae mites (*E. stipulatus*, *N. barkeri*, *N. californicus, P. persimilis,* and *T. phialatus*) as predator and IG prey, and tetranychid mites, thrips, and plant as food sources. Therefore, different genes and DNA regions were selected for primer design according to the target organism.

For primer design, sequence alignment was performed with the MEGA 5.[60] For identifying every single Phytoseiidae species, different reverse primers were designed in non-conserved regions of the Internal Transcribed Spacer 1 (ITS1) (nuclear ribosomal DNA) (Table 2). For detecting Tetranychidae species, a reverse primer was designed in the conserved regions of ITS1 (Table 2). The majority of ITS1 sequences were obtained in previous work.[14] The 18S primer[61] was used as forward for both

Phytoseiidae and Tetranychidae species detection. To detect species belonging to Thysanoptera, a forward primer was designed after the alignment of COI sequences of the target species in the conserved regions (Table 2). All COI sequences used were retrieved from the GenBank (http://www.ncbi.nlm.nih.gov/genbank). The barcoding primer HCO2198[62] was used as a Thysanoptera reverse primer. For plant detection, the chloroplast region of the *trnL* gene was selected and, in this case, the universal primers designed by Taberlet et al.[63,64] were used (Table 2).

Primer specificity test

Target species (Table 1), either reared under laboratory conditions, field-collected or coming from the UJI-IVIA DNA collection, were included in our study for primer specificity test and subsequent multiplex PCR design.

All combinations of primers pairs (Table 2) were tested in at least three individuals of each target species. A single DNA template (5-10 ng/μL) of the target species was used as a positive control. Amplification reactions were performed in 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), 2.5 mM of $MgCl_2$, 0.4 μM of each primer, 1 unit of DNA Taq polymerase (Roche), and 1 μL of DNA template. Amplifications were performed in a Bio-Rad C1000™ Thermal Cycler. PCR parameters were as follows: denaturation for 4 min at 94 °C; 27 cycles of 30 s at 92 °C, 30 s at 50 °C, and 30 s at 72 °C; and a 10min final extension at 72 °C. PCR products were run on 1.5% agarose D-A low EEO (Pronadisa, Sumilab S.L., Madrid, Spain) and visualized under UV light.

Multiplex PCR design

Once the specificity of the primers had been tested by singleplex, the multiplex PCR conditions with all primers were adjusted and optimized on agarose following the steps defined in Henegariu et al.[65] Reactions were performed in a final volume of 25 μL: 1.4× Taq polymerase buffer, 200 μM of each dNTP, 2.5 mM of $MgCl_2$, 1.2 μM of the *E. stipulatus* reverse primer, forward and reverse thrips primers and 18S forward primer, 0.8 μM of Tetranychidae and *N. barkeri* reverse primers, 0.4 μM of each *P. persimilis* and *T. phialatus* reverse primers, 0.2 μM of forward and reverse chloroplast *trnL* primers and 0.1 μM of *N. californicus* reverse primer, 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 primer specificity test.

Afterwards, multiplex PCR conditions were modified and adapted to analysis with labeled primers in the ABI/PE 3730 DNA Sequencer (Applied Biosystems, Foster City, USA) at the Servei Central de Suport a la Investigació Experimental (SCSIE) (Universitat de València, Spain). An equimolar mix (5 ng/μL) of the most representative DNA target species was used as a positive control (i.e., all Phytoseiidae species, *T. urticae* for Tetranychidae, *A. obscurus* and *F. occidentalis* for Thysanoptera and *C. clementina* and *F. arundinacea* for plants). Final multiplex PCR conditions are described in the results section. Fragment length reads were carried out with Peak Scanner™ Software v.1.0 (Applied Biosystems 2006). All samples that produced peaks of the expected size were considered positive. The sensitivity of prey DNA detection was determined by assaying multiplex PCR, at nine-fold dilutions starting from 10 ng of total independently *P. persimilis*, *A. obscurus*, *F. occidentalis*, and *T. urticae* DNA.

Alternative food sources and cross-reactivity test

Species specificity of the multiplex PCR assay was tested on non-target food sources present in citrus orchards and not so frequently incorporated in phytoseiid diets[30] (Table 3). The aim of this test was to avoid false positives when predators had fed on alternative food sources not included in the multiplex PCR. We used the same positive control as in the cross-reactivity test for species-specific primers. In order to discriminate between unsuccessful amplification (i.e., absence of target DNA) and lack of DNA in the PCR (i.e., absence of both target and non-target DNA), we used the universal primer pair Univ18SrDNA and PCR conditions described in Monzó et al.[66]

2.4 Gut content detection

In order to test if multiplex PCR was able to detect possible food sources in the gut content of the target species, we performed different experiments.

For tetranychid detection, we tested, from our DNA collection, one individual of *E. stipulatus* and *P. persimilis* per each starvation time (from 0 to 28h; n = 10 and n = 11, respectively) from the feeding event that had resulted positive for these prey in a previous study.[14]

For plant detection, we extracted DNA from *E. stipulatus* and *A. obscurus* individuals directly taken (non-starved) from the rearings. Furthermore, we also included thrips and tetranychid specimens directly taken (non-starved) from field samplings. Additionally, to ascertain if *E. stipulatus* was able to pierce surfaces and take up liquids, we performed a feeding trial using a slightly modified membrane

feeding system described in Ingwell et al.[67] The membrane feeding chamber was prepared using a modified Huffaker cell.[52] This unit consisted of a PVC plate (80 x 40 x 10 mm) with a central circular hole (diameter 2 cm). The bottom surface of the hole was closed by a microscope slide held in place with two rubber bands. One *E. stipulatus* female randomly chosen from the colony was placed into the chamber. Immediately after, the upper opening was covered by a Parafilm® (ParaFILM®; Bemis Company, Inc. Neenah, Wisconsin) stretched tightly across the hole and a drop of 5% sucrose diet dyed with blue food coloring was pipetted onto the membrane. Finally, a second layer of Parafilm® was stretched tightly to sandwich the diet in order to obtain a uniform distribution along the surface of the membrane. The Huffaker cells were maintained in a climatic chamber at 25 ± 1 °C, 70 ± 10% RH and 16:8 h (L:D) photoperiod during the whole experiment. A color change of phytoseiid gut from yellow or white into blue would imply the phytoseiid piercing the membrane and taking up liquids.

2.5 Feeding trials

We conducted specific feeding trials for thrips and IG prey detection. In this case, we chose the most probable events in the field i.e., *E. stipulatus* as a predator and *P. persimilis* as IG prey,[14] and *F. occidentalis* and *A. obscurus* -two abundant thrips in clementine mandarin orchards- as alternative prey to estimate prey DNA detectability success over time (DS_{50}). Experimental units (cells) used for the predation assays consisted of a PVC plate (80 × 35 × 3 mm) containing 2 chambers with a diameter of 15 mm. The bottom of these chambers was covered by a fine mesh glued to the plate and closed on the upper side by a microscope slide hold in place by two rubber bands.[68] Three to five days-old adult females of *E. stipulatus* were individually placed in the cells and starved for 48 h in a climatic chamber at 25 ± 1 °C, 70 ± 10% RH, and a photoperiod of 16:8 h (L:D). After starvation, each adult female was transferred to a new cell containing one prey. This prey was a first instar nymph for *A. obscurus* and *F. occidentalis*, and a protonymph in the case of *P. persimilis.* Phytoseiid activity was continuously monitored under a binocular microscope. Time after feeding was set to 0 when the phytoseiid released the dead prey. Then, *E. stipulatus* specimens were maintained individually in new cells for different time periods (0 to 20 h; Table 4) after feeding at the same abovementioned conditions. Next, they were transferred to 1.5 mL tubes, frozen at -80 °C, and processed for molecular assessment. Additionally, 48 h starved phytoseiids were used as negative controls. The number of

individuals tested for each species and time elapsed since feeding is referred between brackets in Table 4.

DNA from all phytoseiids was extracted and screened with the multiplex PCR described in the results section.

Probit analysis was used to determine the prey DNA detectability success (DS_{50}) 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".[69,70] Chi-square (χ^2) tests were used to determine the fit of the probit model. To assess whether there were significant differences between lines, when applicable, we performed a χ^2 test of parallelism and a comparison of relative median potency. Analyses were performed using SPSS v. 21 (IBM Corp. Released 2012).

3 RESULTS

3.1 Multiplex PCR design

The chosen primers and designed amplified specific bands within the expected rank length with the DNA of the target organism (Table 2). Primers used for Tetranychidae (family level), plant (kingdom level) and Thysanoptera (order level) detection were successful in most species tested (Table 1). Tetranychidae primers gave positive detection with a different fragment length amplification in *P. citri* and *T. urticae* (367 bp), *Tetranychus evansi* Baker and Pritchard (350 bp) and *T. turkestani* Ugarov and Nikoskii (371 bp) (Table 1 and 2) allowing tetranychid identification. Thysanoptera primers amplified in 13 out of 18 species tested and those for plant detection amplified in the species tested (Table 1).

The final multiplex PCR reaction was adjusted to a final volume of 25 µL: 1.4× Taq polymerase buffer, 200 µM of each dNTP, 2.5 mM of $MgCl_2$, 1 unit of DNA Taq polymerase, and 1 µL of DNA template. Primer concentration in the multiplex PCR is shown in Table 2. The multiplex PCR design was performed at the same amplification conditions as described for the primer specificity test. All the primers were successfully multiplexed in a single PCR discarding primers interference and allowing the identification of the target taxon (either species, order or higher taxonomic categories) using the previously described automated sequencer (Table 1). Positive control with single target DNA and

equimolar mixes of DNA templates from the Phytoseiidae species and five representative species of Tetranychidae, Thysanoptera, and plant amplified the expected fragment lengths (Fig. 1). Multiplex PCR sensitivity with fluorescent markers was independently estimated at 0.1 pg of total DNA for *C. clementina*, 1 pg for *P. persimilis* and *T. urticae*, 10 pg for *A. obscurus* and 1000 pg for *F. occidentalis*. Multiplex PCR exhibited no cross-amplification of the alternative food sources tested except for *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), *Aspidiotus neri* Bouché (Hemiptera: Diaspididae), *Aphis spiraecola* Patch (Hemiptera: Aphididae) and *Toxoptera aurantii* (Boyer de Fonscolombe) (Hemiptera: Aphididae) where a multi-peak pattern was observed and for the fungus *Cladosporium* sp. (Davidiellaceae), where a 290 bp peak, not coincident with any of our target organisms, was detected (Tabla 3).

3.2 Gut content detection

The designed multiplex PCR allowed the detection of degraded DNA from all food sources considered in the phytoseiid gut content after a digestive process.

Plant DNA was detected within Thysanoptera, Tetranychidae, and Phytoseiidae. In Thysanoptera, plant DNA was detected in all specimens of *A. obscurus* tested (n = 3). Furthermore, plant DNA detection within field collected thrips specimens was positive for *Chirothrips manicatus* Haliday and *Aptinothrips rufus* (Haliday) (Thysanoptera: Thripidae) when feeding on *F. arundinacea*, for *Thrips angusticeps* Uzel (Thysanoptera: Thripidae) feeding on *Taraxacum* sp. (Asteraceae), for *T. tabaci* directly taken from leeks and for *H. haemorrhoidalis* when feeding on persimmon. In Tetranychidae, plant DNA was detected in *Aplonobia histricina* (Berlese) (Acari: Tetranychidae) feeding on *Oxalis pes-caprae* L. (Oxalidaceae) and in *T. urticae* on bean. Finally, in Phytoseiidae we detected plant in *Typhlodromus* (*Anthoseius*) *rhenanoides* (Athias-Henriot) (Acari: Phytoseiidae) field collected from lemon trees and later reared on bean plants but not within *E. stipulatus* directly taken from the rearings. Nevertheless, this phytoseiid was able to take up liquid by piercing the membrane feeding system (n = 3; blue colored digestive caeca) so we do not discard the possibility of plant feeding in this species (Fig. 2).

This multiplex PCR tested on DNA collection of *E. stipulatus* and *P. persimilis* that had resulted positive for tetranychid DNA in a previous study[14] allowed the detection of tetranychid DNA in the gut of both Phytoseiidae up to 28 hours after the feeding event.

3.3 Feeding trials

Positive prey DNA detection within *E. stipulatus* at time = 0 h (immediately after prey release) was 92.31%, 81.82% and 63.64% for *P. persimilis*, *A. obscurus,* and *F. occidentalis*, respectively (Table 4). Detectability of prey DNA for these prey species fitted the assumptions of the probit model for *E. stipulatus* (Fig. 3 and Table 5). DS_{50} values depended on the prey species considered. DS_{50} was 18.7 h for *P. persimilis*, 2.3 h for *A. obscurus*, and 1.3 h for *F. occidentalis*. Probit lines corresponding to these three prey species could not be successfully forced to parallelism (χ^2 = 12.853; d.f. = 2; P = 0.002), which was significant between *A. obscurus* and *F. occidentalis* probit lines only (χ^2 = 0.002; d.f. = 1; P = 0.967). Relative median potencies suggested that detection of *A. obscurus* in *E. stipulatus* was 1.93 times longer than that of *F. occidentalis* in the same predator species ($P < 0.05$).

4 DISCUSSION

The multiplex PCR designed in this study allows the simultaneous identification of the phytoseiid predator at species level and its gut content at different taxonomic categories including the three-trophic levels. Therefore, the role of arthropod species commonly found in citrus orchards and its potential food sources [plants, Tetranychidae, Thysanoptera, Phytoseiidae, and other predators (IGP events)] can be assessed. With this tool, the spectra of tetranychid species that can be detected with the multiplex PCR designed by Pérez Sayas et al.[14] has been enhanced by including *T. turkestani* and *T. evansi*, two species commonly found in the ground cover in this agrosystem as well as in other important agroecosystems.[71,72] Indeed, *T. evansi* is considered an invasive pest of solanaceous crops that can appear in citrus orchards associated with a wild cover,[73-74] where it can outcompete *T. urticae* and *T. turkestani*.[75] Tetranychidae primers were designed in the ITS1 region, which is characterized by a higher level of variability than the ITS2.[76,77] This nucleotide variability (insertions/deletions) explains the differences in length of the amplified fragments that allowed not only to detect but also to differentiate Tetranychidae prey species. *Panonychus citri* and *T. urticae* were the only exceptions, as they displayed a band of the same fragment length. If needed, these two species could be differentiated using the primers designed by Pérez-Sayas et al.[14]

The COI region was chosen to design primers for detecting all the Thysanoptera species present in our target agrosystem (Table 2), which could be a potential food source for citrus-dwelling phytoseiids. Nevertheless, the designed primers failed to amplify some of these species (Table 1). In this case, thrips were detectable as a single band, contrarily to what happened with the tetranychids. The multiplex PCR designed amplifies in the most abundant thrips species present in the *F. arundinacea* ground cover, including *A. obscurus* and *C. manicatus*, as well as the main thrips species causing fruit damage in citrus in the Mediterranean basin as *H. haemorrhoidalis*[25] and *Pezothrips kellyanus* (Bagnall) (Table 1).[24,78,79] Apart from these species, the multiplex PCR can amplify DNA of thrips species considered economically important agricultural pests worldwide, as *F. occidentalis* and *T. tabaci* (Table 1),[80-83] which could be occasional pests in citrus.[79]

The plant primers chosen for the multiplex PCR design are located in the highly conserved chloroplast *trnL* gene[63,64,84] considered indeed as a highly conserved DNA sequence region among land plants, from Angiosperms to Bryophytes, just like the reverse primer in Angiosperms and Gymnosperms.[63,64] Furthermore, the robustness of this amplification system, partly due to this region including a conserved loop, allows the amplification of highly degraded DNA.[64] This fact explains why we detected a wide range of plant DNA belonging to taxonomically distant families (Table 1 and 3) in the gut content of phytophagous thrips and mite specimens obtained from field samples and, additionally, within the generalist phytoseiid *T. (A.) rhenanoides*. In this phytoseiid species, we unexpectedly detected plant DNA when using the multiplex PCR for the cross-reactivity test. However, we did not detect plant DNA in *E. stipulatus* obtained from a laboratory colony kept on bean leaves, as it could be expected as suggested for *Euseius* spp. and proven for *E. scutalis*.[85] *Euseius stipulatus* is considered as a pollen feeding generalist predator and whether it can feed on the plant cell sap remains unclear.[31] As suggested by McMurtry et al.[30] the leaf cell piercing may be probably related to water uptake and, therefore, the uptake of nutrients could be a consequence of their presence in the imbibed liquid, extremely reducing the amount of plant DNA available for plant detection in the gut content. Furthermore, when pollen and water are abundant, as in laboratory stock colonies, the need for piercing plant cells by phytoseiids may be less frequent or even disappear. However, when we forced *E. stipulatus* to starve, we demonstrated that this species is able to obtain liquid by piercing a double parafilm membrane containing 5% sucrose (Fig. 2). Further studies should be performed considering

other methodologies to test whether *E. stipulatus* ingest cellular content allowing plant DNA detection. Whatever the case, the designed multiplex PCR may broaden the tools available to explore the ability of phytoseiid mites to feed on plants (allowing the detection of a wide range of plant species) and to study plant-feeding habits of other zoophytophagous arthropod species.

To obtain valid quantitative data to ascertain the relevance of each trophic link, it is necessary to complement assays of field-captured predators with laboratory studies that determine the detectability periods for prey DNA for each combination of predator and prey.[6] As a first step to determine the impact of each trophic link, we studied the detectability periods of the most probable field events considering *E. stipulatus* as a predator and three different prey: *P. persimilis* (IGP[14]), *A. obscurus* and *F. occidentalis*. Our results confirmed the previous observations[14,69,86,87] by which DNA-detection rates were affected by the identity of the prey. In our case, DS_{50} ranged from 1.3 to 18.7 hours post-feeding (Fig. 3 and Table 5), which corresponded to *F. occidentalis* and *P. persimilis* when preyed upon by *E. stipulatus*, respectively. This fact may be associated with the accessibility of the target DNA, the efficiency of the primer to target the prey DNA and the digestion factor that determines different detection efficiencies.[88] Some authors argued that a shorter fragment length results in longer prey DNA detectability.[7,87,89,90] The results obtained in the present study agree with this hypothesis as shorter DS_{50} corresponded to species producing longer amplification fragments (Tables 2 and 5). Gómez-Polo et al.[91] obtained an 8.6 h half-life detection of *F. occidentalis* in *Orius majusculus* (Reuter) (Hemiptera: Anthocoridae) with an amplification band length (292 bp) similar to ours. Differences in the feeding trial related to the meal size (both prey number and size) offered, may explain these contrasting results. On the one hand, Gómez-Polo et al.[91] offered second instar nymphs (N2) of *F. occidentalis*as prey instead of the two-fold smaller first instar nymphs that we used. On the other hand, Gómez-Polo et al.[91] only analyzed those specimens that had been feeding on 2 to 4 N2 *F. occidentalis*, whereas we only offered one N1 specimen. In fact, some authors have pointed out the influence of meal size in prey DNA detection within predators.[7,69,86,88] Furthermore, other non-excluding factors such as the predator size (*O. majusculus* is 6 times larger than *E. stipulatus)*, the feeding habits (generalist predator *vs* omnivore) and the specific digestive processes resulting from the distance between their respective taxonomical groups (Insecta *vs* Arachnida), could also explain these differences. In the case of *P. persimilis* as prey of *E. stipulatus*, we obtained the longest DS_{50}

(18.7 h), similar to the one observed when feeding on *T. urticae* (18.3 h).[14] These results could be associated with the non-preference of *E. stipulatus* for these types of prey. Indeed, DS_{50} for the preferred prey (*A. obscurus*)[21] is one order of magnitude shorter (2.3 h).

The occurrence of IGP is controversial. While some authors consider IGP a widespread interaction, reaching frequencies between 58.4 and 86.7%[37] occurring in a great diversity of animal taxa,[45,69] others consider that it may be not that common among phytoseiids.[60] The study of IGP occurring among predatory mites in the field is still in its infancy due to the scarcity of suitable methodologies allowing the appraisal of this phenomenon without altering predator behavior. Just a few studies have quantified IGP rates in the field,[42,69] although lately, they are receiving more attention[92-94] .The multiplex PCR designed in the present work is suitable for the study of IGP among phytoseiids. The detection of this trophic relation in clementine mandarin orchards, e.g., predation of *E. stipulatus* on *P. persimilis* nymphs, was highly efficient along time after the feeding event happened (detection efficiency of 42.83% 20 hours after feeding). Therefore, the present multiplex PCR could be used in similar systems where phytoseiid species could become prey in order to detect disruption of biological control. For example, Janssenet al.[95] observed that *F. occidentalis* preyed on eggs of *T. urticae*, *P. persimilis* and *Iphiseius degenerans* (Berlese) (Acari: Phytoseiidae) when host plants were of low quality.To sum up, this newly developed multiplex PCR could contribute to shed light on the so far cryptic trophic relationships occurring among mites. Importantly, it could allow to better answer the question debated by Fonseca et al.[96] regarding how to evaluate the potential occurrence of IGP and, therefore, establish how widespread this phenomenon is actually occurring in nature among phytoseiids.[47]

A better knowledge of the trophic relationships established within the citrus acarofauna, including *T. urticae*, a key pest of Spanish citrus, and of the role of alternative food sources to conserve and enhance predator populations, will pave the way to enhance the biological control of this worldwide pest. Now, a suitable molecular tool developed with this aim is ready to be used.

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DATA AVAILABILITY

The datasets generated during and/or analyse during the current study are available from the corresponding author on reasonable request.

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.

TABLES

Table 1 Target species tested in this work with the singleplex and multiplex PCR and DNA detection success with the new multiplex PCR.

Kingdom	Phylum	Class	Order	Family	Species	Origin	DNA detection
Animalia	Arthropoda	Arachnida	Acari	Phytoseiidae	*Euseius stipulatus* (Athias-Henriot)	Montcada, Spain	+ †
					Neoseiulus barkeri Hughes	Castelló de la Plana, Spain	+
					Neoseiulus californicus (McGregor)	Koppert Biol. Syst.	+
					Phytoseiulus persimilis Athias-Henriot	Les Alqueríes, Spain	+
					Typhlodromus phialatus Athias-Henriot	Montcada	+
				Tetranychidae	*Aplonobia histricina* (Berlese)	Montcada	- ‡
					Eutetranychus banksi (McGregor)	Huelva, Spain	-
					Eutetranychus orientalis (Klein)	Málaga, Spain	-
					Panonychus citri (McGregor)	Montcada	+
					Tetranychus evansi Baker and Pritchard	Valencia, Spain	+
					Tetranychus turkestani Ugarov and Nikolskii	Castelló de la Plana	+
					Tetranychus urticae Koch	Betxí, Spain	+
		Insecta	Thysanoptera	Aeolothripidae (S.O. Terebrantia)	*Aeolothrips* sp.	Montcada	+
					Rhipidothrips brunneus Williams	Montcada	+
				Melanthripidae (S.O. Terebrantia)	*Melanothrips fuscus* Sulzer	Montcada	+
				Thripidae (S.O. Terebrantia)	*Anaphothrips obscurus* (Muller)	Castelló de la Plana	+
					Anaphothrips sudanensis Trybom	Montcada	-
					Aptinothrips rufus (Haliday)	Montcada	+
					Chirothrips manicatus Haliday	Montcada	+
					Frankliniella occidentalis (Pergande)	Montcada	+
					Frankliniella tenuicornis (Uzel)	Montcada	-
					Heliothrips haemorrhoidalis (Bouché)	Castelló de la	+

					Limothrips cerealium Haliday	Plana Montcada	+
					Pezothrips kellyanus (Bagnall)	Montcada	+
					Stenothrips graminum Uzel	Montcada	-
					Tenothrips frici (Uzel)	Montcada	-
					Thrips angusticeps Uzel	Montcada	+
					Thrips tabaci Lindeman	Viena (Boku lab. strain)	+
					Thrips vulgatissimus Haliday	Montcada	+
				Phlaeothripidae (S.O. Tubulifera)	*Haplothrips tritici* Kurdjumov	Montcada	-
	Plantae	Streptophyta	Eudicotyledoneae	Caryophyllales	Aizoaceae	*Carpobrotus edulis* (L.) N. E. Br	Montcada	+
			Liliopsida	Poales	Poaceae	*Festuca arundinacea* Schreb.	Castelló de la Plana	+
			Magnoliopsida	Fabales	Fabaceae	*Phaseolus vulgaris* L.	Montcada	+
				Sapindales	Rutaceae	*Citrus clementina* Hort. ex. Tan.	Montcada	+

740 †+: Positive DNA detection
741 ‡ -: Negative DNA detection

Table 2 Primer sequences, concentration in final multiplex PCR and amplified fragment length of the target organisms.

Target group	Target region	Primer name	Primer sequence (5' -> 3') †		Primer references	Primer concentration (µM)	Length (bp)
Phytoseiidae; Tetranychidae	ITS1	18S	AGA GGA AGT AAA AGT CGT AAC AAG ‡	F	Navajas et al., 1999	1.2	
		18S FAM-6			Navajas et al., 1999	1.2	
Phytoseiidae	ITS1						
Euseius stipulatus		Esdep2	CGC GTC TGT GGA CGG TAA CG	R	This work	1.2	247; 257
Neoseiulus barkeri		Abpr	CAT TCT TCC ATG TGAT GGA GTG	R	This work	0.8	93
Neoseiulus californicus		Ncpr2	ACG TAC GAC GGC CAG CAG GC	R	This work	0.05	155
Phytoseiulus persimilis		Pppr2	CTG GTT GGT ACC GAC TCG CG	R	This work	0.3	277
Typhlodromus phialatus		Tppr2	CGA GCA GTA GGA CTG ACC TC	R	This work	0.2	234
Tetranychidae	ITS1	TeUniITS1	CCA AGT ATG TAG CAA GAC AGG C	R	This work	0.8	350-371‡
Thysanoptera	COI	TripUniCOI	TCA ACA TTT TTT CAT TCT GG	F	This work	1.2	
		TripUniCOI FAM-6		F	This work	1.2	
		HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA §	R	Folmer et al., 1994	1.2	330
Plants	*trn*L	trnL_a	CGA AAT CGG TAG ACG CTA CG	F	Taberlet et al., 1991	0.1	
		trnL_a FAM-6		F	Taberlet et al., 1991	0.1	
		trnL_h	CCA TTG AGT CTC TGC ACC TAT C #	R	Taberlet et al., 2007	0.1	190

† F: forward primer; R: reverse primer.

‡Amplified fragment length for Tetranychidae species are: *T. evansi* (350 bp), *P. citri* and *T. urticae* (367 bp) and *T. turkestani* (371 bp).

748 **Table 3** Non-target organisms and species DNA detection screened with the multiplex PCR for cross-reactivity test.
749

Kingdom	Phylum	Class	Order	Family	Species	Origin	DNA detection
Animalia	Arthropoda	Arachnida	Acari	Acaridiidae	-	Montcada	-†
				Phytoseiidae	*Amblyseius andersoni* (Chant)	Syngenta	-
					Amblyseius cucumeris (Oudemans)	Syngenta	-
					Amblyseius swirskii (Athias-Henriot)	Syngenta	-
					Typhlodromus (*Anthoseius*) *rhenanoides* Athias-Henriot	Montcada	-
				Tydeidae	-	Montcada	-
		Insecta	Hemiptera	Aleyrodidae	*Aleurothrixus floccosus* (Maskell)	Les Alqueries,	-
					Aphis (*Aphis*) *gossypii* Glover	Montcada	-
				Aphididae	*Aphis* (*Aphis*) *spiraecola* Patch	Montcada	MPP‡
					Toxoptera aurantii (Boyer de Fonscolombe)	Montcada	MPP
					Aonidiella aurantii (Maskell)	Carcaixent, Spain	MPP
				Diaspididae	*Aspidiotus nerii* Bouché	Montcada	MPP
					Parlatoria pergandii Comstock	Montcada	-
				Coccidae	*Saissetia oleae* (Olivier)	Bétera, Spain	-
				Margarodidae	*Icerya purchasi* Maskell	Montcada	-
				Pseudococcidae	*Planococcus citri* (Risso)	Bétera	-
Plantae	Streptophyta	Liliopsida	Asparagales	Amaryllidaceae	*Allium ampeloprasum* var. *porrum* (L.) J. Gay	Montcada	+§
		Magnoliopsida	Ericales	Ebenaceae	*Diospyros kaki* Thunb.	L'Alcúdia, Spain	+
		Magnoliopsida	Asterales	Asteraceae	*Taraxacum* sp.	Montcada	+
Fungi	Ascomycota	Dothideomycetes	Capnodiales	Davidiellaceae	*Cladosporium* sp.	Montcada	NCP¶
			Pleosporales	Pleosporaceae	*Alternaria* sp.	Montcada	-
		Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium* sp.	Montcada	-

750 † -: Negative DNA detection
751 ‡MPP: Multi-peak pattern
752 §+: Positive DNA detection
753 ¶NCP: Not coincident peak

Table 4 Number of positive detections for each predator and prey combination at different time intervals since feeding.

		Time since feeding (h)					
Predator	**Prey**	**0**	**2**	**4**	**6**	**16**	**20**
Euseius stipulatus	*Anaphothrips obscurus*	9(11)†	5(11)	4(11)	1(12)	0(12)	-
	Frankliniella occidentalis	7(11)	5(11)	2(11)	0(12)	-	-
	Phytoseiulus persimilis	12(13)	12(13)	10(11)	10(12)	7(11)	6(14)

† Number in parenthesis represents the number of individuals tested.

Table 5 Probit curves adjusted for positive detections and prey DNA detectability success (DS_{50}) from a single prey specimen.

Predator	Prey	n	Slope	Intercept	d.f.	χ^2	*P*	DS_{50} (h)	95% f.l. †
Euseius stipulatus	*Anaphothrips obscurus*	57	-0.344 ± 0.101	0.802 ± 0.352	3	0.829	0.843	2.33	0.59 - 3.61
	Frankliniella occidentalis	45	-0.392 ± 0.118	0.504 ± 0.340	2	0.889	0.641	1.29	0 - 2.42
	Phytoseiulus persimilis	74	-0.082 ± 0.023	1.541 ± 0.298	4	0.302	0.990	18.72	14.11 -30.03

†f.l.: fiducial limits.

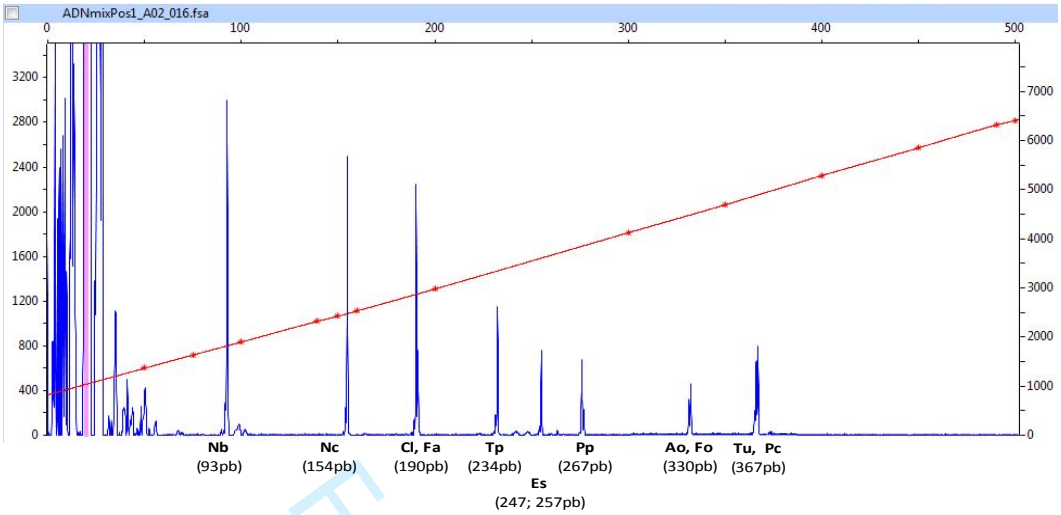

Figure 1. Example of amplification of multiplex PCR with all species considered together in the same electropherogram. Nb: *N. barkeri*; Nc: *N. californicus*; Tp: *T. phialatus*; Es: *E. stipulatus*; Pp: *P. persimilis* (Phytoseiidae species included in PCR multiplex); Cl: *C. clementina* and Fa: *F. arundinacea* as representative species of plant detection; Ao: *A. obscurus* and Fo: *F. occidentalis* as representative species of thrips detection; Tu: *T. urticae* and Pc: *P. citri* as representative species of tetranychid detection.

Figure 2. Colored digestive caeca of *Euseius stipulatus* after taking up blue colored liquid by piercing the feeding membrane.

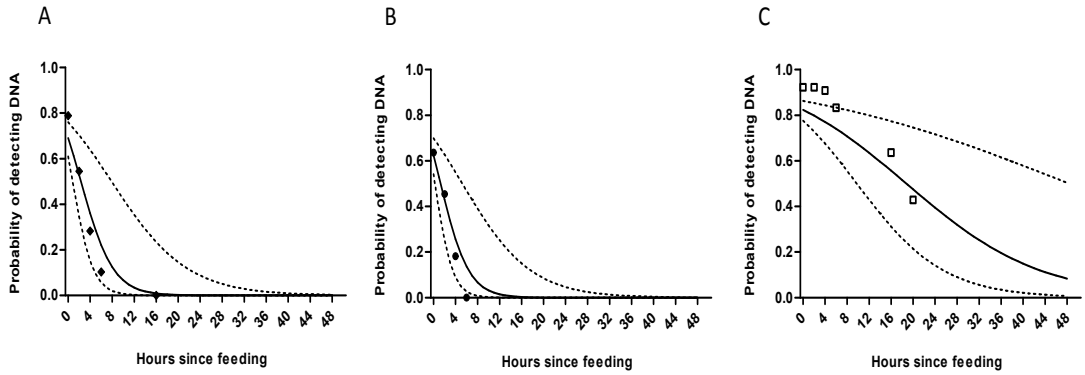

Figure 3. *Anaphothrips obscurus* (A), *Frankliniella occidentalis* (B) and *Phytoseiulus persimilis* (C) DNA detection probability curves in *Euseius stipulatus* samples after feeding. Lines are fitted with probit model with 95% confidence intervals (dashed lines)