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ORIGINAL ARTICLE

Development of a multi-primer metabarcoding approach to understanding trophic interactions in agroecosystems

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Abstract To understand trophic interactions and the precise ecological role of each predatory species, it is important to know which arthropod and plant resources are used by generalist predators in agroecosystems. Molecular approaches, such as the use of high-throughput sequencing (HTS), play a key role in identifying these resources. This study develops a multi-primer metabarcoding approach for screening the most common trophic interactions of two predatory arthropods with contrasting morphologies, *Rhagonycha fulva* (Coleoptera: Cantharidae) and *Anthocoris nemoralis* (Hemiptera: Anthocoridae) collected from a peach crop. To reduce the time and cost of this metabarcoding approach, we first evaluated the effect of using two different predator-pools of different size (10 and 23 individuals of the same species). We also used our system to analyze the performance of one and two primer pairs in the same library. Our results show that the analysis of 23 individuals together with the use of two primer pairs in the same library optimize the HTS analysis. Using these best-performing conditions, we then analyzed the entire bodies of field-collected predators as well as the washing solutions used to clean the insect bodies. We were able to identify both gut content (i.e., diet) and external pollen load (i.e., on the insects' bodies). This study also demonstrates the importance of washing predatory insects' bodies prior to HTS analysis when the target species have a considerable size (>10 mm) and hairy structures. This metabarcoding approach has significant potential for the study of trophic links in agriculture, revealing expected and unexpected trophic relationships.

Key words high-throughput sequencing; metabarcoding; molecular diet analysis; multi-primer approach; predatory arthropods; trophic interactions

Introduction

Managing ecosystem services in agroecosystems is key to food production. One ecosystem service is pest control using natural enemies, such as generalist insect predators. These beneficial insects usually incorporate plant resources to their diet as well as arthropods (Demestihas *et al.*, 2017). It is necessary to thoroughly understand how generalist insect predators use these food resources in an agroecosystem to utilize these predators in pest control programs.

Studying trophic interactions within an ecosystem is inherently difficult because predation is an ephemeral process that is often difficult to visualize. Different methods have been used to measure insect predation, from direct observation in the field to the molecular analyses of gut contents (Agustí *et al.*, 2003; Pumariño *et al.*, 2011; Nielsen *et al.*, 2018). Molecular approaches enable more precise descriptions of predators' diets (Nielsen *et al.*, 2018). The use of High-throughput sequencing (HTS) platforms which make it possible to detect multiple trophic interactions in the field, are a particularly precise approach. Within HTS (also called next-generation sequencing or NGS) approaches, DNA metabarcoding, which is the identification of different organisms from a sample containing DNA from more than one organism, has been used to describe trophic interactions in terrestrial and aquatic ecosystems (Kennedy *et al.*, 2020). Metabarcoding allows understanding trophic interactions between natural enemies and other organisms present in agroecosystems (Pompanon *et al.*, 2012; Piñol *et al.*, 2014; Gomez-Polo *et al.*, 2015, 2016; González-Chang *et al.*, 2016). This information can be used to identify factors that the efficiency of natural enemies, an essential prerequisite to management strategies aimed at enhancing the role of those natural enemies.

DNA metabarcoding studies usually follow a well-established workflow that includes DNA extraction, often from the whole specimens; PCR amplification with barcoded primers; high-throughput DNA sequencing; and a tailored bioinformatic analysis to determine the

taxonomic classification (Deagle *et al.*, 2018). However, recent literature highlights several factors that can affect the final result, indicating the need to improve certain technical aspects of this process (Lamb *et al.*, 2019). These factors include the need to externally wash predator specimens to remove external contamination (e.g., pollen grains) from their exoskeleton (Jones, 2012); the need to pool samples, particularly when the ingested DNA template is low; the need to use biological replicates to obtain robust estimates of diet diversity and composition (Mata *et al.*, 2019); the need to use an appropriate number of primer pairs (Gibson *et al.*, 2014); the availability of comprehensive reference databases for the taxonomic groups of interest (Bohmann *et al.*, 2011); and the need to use appropriate pipelines and data cleaning procedures during the bioinformatic analysis (Plummer *et al.*, 2017). Previous studies have recommended the use of more than one primer set in order to minimize the effect of set biases and to obtain a higher taxonomic coverage of the diet (Piñol *et al.*, 2015; Krehenwinkel *et al.*, 2017; Hajibabaei *et al.*, 2019). Therefore, we developed a new HTS metabarcoding approach that uses two arthropod and two plant universal primer pairs per library with pooled samples to describe the taxa most commonly consumed by two generalist insect predator species.

The main aim of this study was to test whether it was possible to reduce the time and resource costs of HTS metabarcoding without significantly reducing the quality of the obtained data. We did this by pooling samples and combining two set of primers for both plants and arthropods. We focused on two predator species: the minute pirate bug *Anthocoris nemoralis* (Fabricius) (Hemiptera: Anthocoridae) and the common red soldier beetle *Rhagonycha fulva* (Scopoli) (Coleoptera: Cantharidae). Both insects are present in peach crops in the Lleida region (NE Spain), as well as in other crops in the same region, including fruit orchards, maize, and alfalfa (Pons & Eizaguirre, 2000; Jauset *et al.*, 2007). *Anthocoris nemoralis* is one of the most important biocontrol agents for pear psyllids *Cacopsylla*

pyricola (Foerster) and *Cacopsylla pyri* L. (Hemiptera: Psyllidae) (Agustí *et al.*, 2003). However, this predatory species has also been reported to feed on pollen (Naranjo & Gibson, 1996). *Rhagonycha fulva* is mainly present in wooded agricultural landscapes and arable land (Meek *et al.*, 2002; Rodwell *et al.*, 2018). Although this species is known to feed primarily on pollen and nectar from umbellifers (Apiaceae) (Meek *et al.*, 2002), it has also been cited as a predator of some insect species (Pons & Eizaguirre, 2000; Rodwell *et al.*, 2018). However, its role as a biocontrol agent is not well established. This is also the case for *A. nemoralis* in fruit crops other than pears, such as peaches. The selected predator species differ morphologically in their ability to retain pollen grains on the body. *Rhagonycha fulva* is large (10–15 mm) and pubescent, particularly on the head and ventral side, while *A. nemoralis* is much smaller (3 mm) and glabrous. These different morphological characteristics make these two species good candidates to study pollen retention on the bodies of predators, which determines the need to wash samples before HTS analysis.

In this study, we first investigated the effect of variable sample-pool sizes on the range of prey taxa detected (taxonomic coverage). We also measured the effect of using one or two primer pairs in the same library to identify ways to reduce the time required for and cost of HTS analysis. We then used the best-performing conditions to validate the developed methodology by conducting HTS analyses of the arthropod and plant diets of two small populations of *A. nemoralis* and *R. fulva*, two omnivorous insects with contrasting morphologies. The plant and other arthropod DNA content in the washing solutions was also analyzed to identify the pollen collected on the predators' bodies during foraging on diverse plants in the landscape.

Materials and methods

Predator collection, cleaning, and DNA extraction

Anthocoris nemoralis (n = 42) were collected in June and July 2016 and *R. fulva* (n = 78) in May 2017 by beating branches in a peach orchard in Vilanova de Segrià (Lleida), Spain (UTM 10 × 10: 31TCGO1). Each specimen was placed in a DNA-free tube and stored in a portable freezer to avoid DNA degradation. The specimens were then morphologically identified and stored at –20ºC until the metabarcoding analysis was conducted.

Before DNA extraction, all collected specimens were individually washed to remove contaminants from the cuticle. Each insect was submerged in a 10 mL tube containing a DNA-free water solution with Tween® 20 (0.1%), and the tube was manually shaken for 1 min. The washing solution was then stored at –20ºC for further HTS analysis (see *Analysis of field-collected predators* below). Next, the insect was submerged in another 10 mL tube with a DNA-free water solution containing sodium hypochlorite (0.5%) and Tween® 20 (1%), and the tube was shaken manually for another 1 min. The second washing solution was discarded. Finally, each insect was rinsed with DNA-free water for 30 seconds and dried on filter paper.

DNA was extracted from each insect specimen and washing solution using the Speedtools Tissue DNA Extraction Kit (Biotools, Germany; protocol for animal tissues). DNA was extracted from the washing solutions via an additional disruption step using 0.15 g of 500–750 μm diameter glass beads (ACROS Organics™). The samples were vortexed for 15 min at 50 Hz in a Gene2 vortex (MoBio Laboratories) to break any pollen grains. Whole insects were then homogenized using plastic pestles. After DNA was extracted, all DNA was eluted in 100 μL of AE buffer (provided by the manufacturer) and stored at −20 °C. A negative control with no insect or plant DNA (DNA-free water) was added to each DNA extraction set. The concentration of each DNA extraction was measured on a Qubit® 2.0 Fluorometer

using the dsDNA HS Assay kit (Invitrogen, Carlsbad, CA, USA). Equimolar amounts of each individual DNA extraction (5 ng/μL) were finally pooled by species in sample-pools, as shown in Table 1.

PCR amplification, library preparation, and sequencing

Two pairs of universal arthropod primers that partially amplify the mitochondrial Cytochrome Oxidase subunit I (COI) region were used to amplify the DNA from the field-collected insects. These two pairs of primers were selected because they amplify different amplicon sizes (ZBJ-ArtF1c/ZBJ-ArtR2c, 157 bp and mlCOIintF/HC02198, 313 bp) and do not overlap in the COI region (Table S1; Fig. S1). This was done to avoid competition for the same primer binding sites. Similarly, we used two pairs of universal plant primers that amplify different amplicon sizes (ITS-S2F/ITS4R, 350 bp and cA49325/trnL110R, 80 bp) (Table S1). In this case, primer pairs were chosen to amplify fragments different regions the first in the nuclear Internal Transcribed Spacer 2 (ITS2) and the second in the chloroplast *trnL* intron.

The sample-pools shown in Table 1 were amplified using a universal multi-primer approach with these four pairs of universal primers for arthropods and plants. One PCR was performed with both pairs of arthropod primers, and another one was performed with both pairs of plant primers. Each PCR reaction (50 μL) contained 25 μL of Multiplex Master Mix (Qiagen, Hilden, Germany), 1 μL of each primer [10 μmol/L], 8 μL of DNA-free water, and 15 μL of DNA from each sample-pool. The PCR conditions for the arthropod primers were as follows: 95 °C for 5 min for the initial denaturation, followed by 30 cycles at 95 °C for 30 s, 46 °C for 30 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. PCR conditions used with the plant primers were: 95 °C for 3 min, followed by 30 cycles at 95 °C for 30 s, 55 °C for 30 s and at 72 °C for 30 s, and a final extension at 72 °C for 5 min. The

amplifications were conducted in a 2720 thermocycler (Applied Biosystems, CA, USA). The target DNA and DNA-free water were included in each PCR run as positive and negative controls, respectively. The PCR products were purified using the QIAquick PCR Purification kit (Qiagen), and 5 μL of each PCR product was then used as a template to prepare the libraries for sequencing. The HTS analysis was conducted in two batches (Table 1), and libraries of both batches were built by mixing the PCR products of either both pairs of arthropod primers or both pairs of plant primers. Both HTS batches were processed on a MiSeq sequencing platform (Illumina, San Diego, CA, USA) at the *Servei de Genòmica i Bioinformàtica* of the Autonomous University of Barcelona, Spain. Illumina adapters were attached using the Nextera XT Index kit. The amplicons were purified with magnetic beads, and 5 μL from each library were pooled and sequenced using a paired - end approach (2 × 225 bp).

Taxonomic coverage: sample-pool size and number of arthropod primer pairs

Two different sample-pools of *A. nemoralis* were built. Sample-pool 1 contained with 10 individuals, and sample-pool 2 contained 23 individuals (Table 1, *Taxonomic coverage*). In this trial, both sample-pools were tested using either both universal arthropod primer pairs together in the same library (L3 and L6) or separated into different libraries (L1, L2, L4, and L5). The non-parametric Kruskal-Wallis rank sum test was used to compare the effects of the size of the sample-pool (sample-pool 1 vs. 2) and of the use of one or both primer pairs in the same library on the number of taxa obtained (taxonomic coverage) after HTS. The statistical analyses were performed using R version 3.5.1 (R Development Core Team, 2018).

Plant primer resolution

To test the efficacy of each pair of plant primers and to assess their levels of taxonomic resolution, we built a plant sample-pool with five plant species that are common in orchard ground covers and the field margins of the study area (Table 1, sample-pool 3) (Ibáñez-Gastón, 2018; Juarez-Escario *et al.*, 2010). In addition, to validate the parameterization of the bioinformatic pipeline (Jusino *et al.*, 2019), we included three positive controls containing only the crop plants (sample-pools 4–6). Unlike the arthropods, the plant samples (1 cm^2 leaf disc) were not washed prior to DNA extraction, which was conducted in the same way as for the arthropod samples.

Analysis of field-collected predators

The field-collected predators were analyzed using the multi-primer approach described above. The most effective sample-pool size and number of primer pairs were used, as determined by the trials described in the sections *Taxonomic coverage* and *Plant primer resolution* above. Four sample-pools were tested for *R. fulva* (Table 1, sample-pools 7–10) and two for *A. nemoralis* (sample-pools 11 and 12). In addition, five sample-pools were analyzed to identify the pollen load on the insects' bodies: four sample-pools from the *R. fulva* washing solutions (sample-pools 13–16) and one from the *A. nemoralis* washing solutions (sample-pool 17). To determine whether both predators only foraged on plants or also consumed plant resources, we compared the plant taxa obtained from the washing solutions to those obtained from their gut contents. Finally, in order to increase the number of taxa detected in the sequencing process to show the highest diet diversity consumed by the predator, each sample-pool of the same predator species was considered as a different

biological replicate. The use of biological replicates provides greater variability than technical replicates for the taxa detected (Mata *et al.*, 2019).

Bioinformatics

Raw Illumina reads were merged using the VSEARCH 2.0 algorithm (Rognes *et al.*, 2016) and then analyzed using a restrictive strategy to reduce biases. The assembled reads were filtered for quality using the FASTX-Toolkit (Gordon & Hannon, 2010) with a minimum of 75% of bases ≥ Q30. The resulting reads were then split by length according to the expected amplicon from each primer using custom Python scripts. Primer sequences were removed using Cutadapt 1.11 (Martin, 2017). The obtained reads were clustered into OTUs with a similarity threshold of 97% using VSEARCH 2.0. Chimeras were removed using the UCHIME algorithm (Edgar *et al.*, 2011). The remaining OTUs were queried against a custom database using BLAST 2.2.31+ (BLASTN, E-value 1e-10, minimum coverage of the query sequence: 97%; number of alignments: 9) (Camacho *et al.*, 2009). This custom database contained all arthropod and plant sequences present in the study area that were included in the NCBI database (http://www.ncbi.nlm.nih.gov) at the time of the analysis (October 2019). For this, we used a European and a regional biodiversity database: GBIF.org (http://www.gbif.org/) with the geographical criteria of Spain and France, as well as the *Banc de dades de biodiversitat de Catalunya* (http://biodiver.bio.ub.es/biocat/) which includes only species found in Catalonia. This database included 54 274 plant and 62 074 arthropod species. Taxonomy was assigned at ≥ 97% identity using the Last Common Ancestor algorithm (LCA) with BASTA (Kahlke & Ralph, 2019). To remove possible contaminants from the OTUs assigned to different taxa for each group of primer pairs (arthropods or plants), we only included OTUs that had more than five reads and that were detected in at least two sample-pools of the same species in the analysis (Boyer *et al.*, 2013). OTUs found

in only one sample-pool were included in the analysis only if they had more than five reads with both primer pairs or if they exceeded the 0.03% threshold of the total reads for plants or arthropods. Obtained OTUs were then categorized as predator or prey based on their taxonomy.

To minimize other biases, such as secondary predation (an important limitation of HTS when studying food webs) (da Silva *et al.*, 2019), and to identify the most important taxa, dietary data were presented using two dietary metrics, as recommended by Deagle *et al.* (2018). The first metric was the percentage of Relative Read Abundance (RRA), which was calculated using the total number of reads of each consumed resource (arthropod or plant) amplified with each primer pair and for each library, divided by the total number of reads of all resources obtained with each primer pair for each library. After that, a filter was applied to eliminate resources < 1% of the amplified taxa, as recommended by Deagle *et al.* (2018). This was applied to each primer pair in each library. After the taxa were obtained, the second metric was calculated, which was the percentage of Frequency of Occurrence (FOO), or the percentage of the total number of pools of each specimen analyzed that contained a given resource item, indicating the most commonly consumed resources.

Results

The analysis of 33 libraries (120 predators and 20 sample-pools) conducted in two HTS batches (Table 1) generated 9 047 294 raw paired-end reads, 95% of which were successfully merged (Table 2, step 1). After that, 40 582 (step 2) and 53 286 reads (step 3) that did not meet our quality and/or length requirements were discarded, along with 2 512 chimera reads (step 5). After the taxonomic assignment (step 6), 1548 arthropod and 649 plant OTUs were filtered (step 7 and step 8). From the initial raw paired-end reads, only 8 930 (0.098%) were obtained from the DNA extraction blank (sample-pool 20) and both PCR blanks (sample-

pools 18 (batch 1) and sample-pool 19 (batch 2)) (Table 1). Those reads were eliminated in step 7. After calculating the RRA and FFO percentages and eliminating taxa with reads below 1% (Table 2, step 8; Table S2), we finally obtained 299 arthropod and 206 plant OTUs (Table 2), which corresponded to 14 arthropod and 20 plant taxa (Table 3).

Taxonomic coverage: sample-pool size and number of arthropod primer pairs

The six libraries analyzed in this trial (Table 1, L1–L6) yielded 10 arthropod taxa (Table S3; Table 3). Besides the predator itself (*A. nemoralis*), we detected other anthocorids (*Orius* and *O. laevigatus* Fieber), another potential predator (Cecidomyiinae), and some pest (Aphididae, *Grapholita molesta* Busck*, Myzus persicae* Sulzer (Aphididae)*, Thrips fuscipennis* Haliday) and non-pest prey (*Diaphorina lycii* Loginova).

The sample pool size (n = 10 and n = 23 predators) did not significantly affect the number of arthropod taxa identified (Kruskal-Wallis chi-squared = 0.78, df = 1, p-value = 0.37) (Table S4). Similarly, when the number of arthropod taxa obtained using two pairs of primers in two different libraries or two pairs of primers in the same library were compared, no significant differences were observed (Kruskal-Wallis chi-squared = 0.16, df = 1, P-value = 0.68) (Table S4). Therefore, to reduce the time and cost in the following *Analysis of field-collected predators* trial, we decided to pool up to 26 predators together, and to use both pairs of arthropod primers together in the same library.

Plant primer resolution

The four plant libraries analyzed in this trial (Table 1, L7–L10) yielded 11 plant taxa (Table S3; Table 3). Most of these taxa were expected because they were present in sample-pools 3–6 (Table 1) (*Medicago sativa* L. [alfalfa], *Prunus persica* (L.) [peach], *Convulvulus arvensis* L.*, Picris echioides* L.*, Setaria* sp.), which were used as positive controls. Other

plant taxa were also detected, including Streptophyta, which corresponds to a clade that shows only plant DNA amplified without additional taxonomic information. The families Fabaceae, Rosaceae, Convolvulaceae and Asteraceae were also detected; these families correspond to those of the plant species in sample-pools 3–6 (Table S3; Table 3). The genus *Trifolium* (Fabaceae) was also detected in library 7 (L7) (Table S3). However, as it represented only 0.026% of the total reads obtained, it was not included in further analyses.

Analysis of field-collected predators

The 17 libraries analyzed in this trial (Table 1, L11–L27) yielded 28 taxa (14 arthropod taxa and 14 plant taxa) (Table S3; Fig. 1). In the diet of *R. fulva* (Cantharidae), besides *R. fulva*, we detected three other arthropod taxa: *Nysius graminicola* Kolenati (Lygaeidae), *Cantharis livida* L. (Cantharidae) and Coccinellidae; as well as five plant taxa: Streptophyta, Convolvulaceae, Solanaceae, Fabaceae and Poaceae (Table S3; Fig. 1; Table 3). In the diet of *A. nemoralis* (Anthocoridae), besides *A. nemoralis*, we detected nine other arthropod taxa: *Orius* and *O. laevigatus* (Anthocoridae), Aphididae, *M. persicae* (Aphididae), *D. lycii* (Liviidae), *Oenopia conglobata* L. (Coccinellidae), Cecidomyiinae, *G. molesta* (Tortricidae) and *T. fuscipennis* (Thripidae). No plant taxa were obtained in this HTS analysis from whole specimens of *A. nemoralis* (Table S3; Fig. 1).

We had amplification in two of the four libraries from the washing solutions of *R. fulva* analyzed (Table 1, L23–L26; Table S3). The 11 plant taxa detected were: Streptophyta, Asteraceae, *Sonchus* (Asteraceae), *M. sativa* (Fabaceae), *Olea europea* L. (Oleaceae), *Pinus sp* (Pinaceae), Poaceae, and *Dactylis glomerata* L., and *Poa annua* L. (Poaceae), Caryophyllales, *Beta vulgaris* L. (Amaranthaceae) (Table S3; Fig. 1). No plant taxa were detected in the *A. nemoralis* washing solutions (Table 1, L27; Table S3).

Discussion

Methodological issues

The present study seeks to develop a multi-primer approach for using DNA metabarcoding analysis to identify the most common plant and arthropod resources ingested by field-collected omnivorous predators. The digestion process makes it difficult to detect ingested DNA from gut or whole specimens. One way to improve the usefulness of PCR for insect diet analyses is to increase the amount of DNA template by pooling individual specimens of the same species. This kind of pooling has been used in previous metabarcoding studies to estimate predator diets in bats (*Chalinolobus gouldii* Gray) and birds (*Sialia mexicana* Swainson) (Burgar *et al.*, 2014; Jedlicka *et al.*, 2017). This approach leads to the detection of the most commonly ingested taxa (Mata *et al.*, 2019). This strategy is less expensive and less time consuming than other strategies, such as nested tagging, that have also been used in insect predation studies (Kitson *et al.*, 2019). However, nested tagging can be highly sensitive to cross-contamination between the analyzed samples and the control, introducing other biases that can be avoided with our approach.

Our first objective was to determine the effect of a variable sample-pool size (10 or 23 *A. nemoralis*) on the taxonomic coverage. The number of taxa detected did not differ significantly between the two sample-pool sizes (Table S3, *Taxonomic coverage* trial). The most likely explanation for this is that the number of individuals included in each pool did not differ enough to include a statistically different range of taxa. Therefore, to reduce the time and cost of the HTS run we pooled up to 26 individuals together in the same library for our *Analysis of field-collected predators*.

Our second objective was to compare the impact of using either one or two pairs of primers in the same library. Most previous HTS studies of arthropods (Burgar *et al.*, 2014) and plants (Richardson *et al.*, 2015) have used one pair of primers per library. However, some studies

have found that a multi-primer approach makes it possible to characterize the full diet of omnivorous species, and the choice of the primer pairs is key as it determines the richness of the taxa obtained (Hajibabaei *et al.*, 2019). Some aspects of each primer pair used, such as taxonomic coverage, taxonomic resolution and complementarity, are not well known, despite their potential impact on the final results (Deagle *et al.*, 2018; Corse *et al.*, 2019). Here, we demonstrated the benefits of using two pairs of arthropod primers together in the same library. No significant differences were observed in the number of taxa obtained in the *Taxonomic coverage* trial. Furthermore, the use of two primer pairs in the same library reduced the number of libraries by half, decreasing the time and cost of preparing the libraries. Therefore, both arthropod and both plant pairs of primers were used in one library in the *Analysis of field-collected predators* trial.

The present study included three trials. We observed that the arthropod primer pair mlCOIintF/HC02198 amplified around 20% more taxa than ZBJ-ArtF1c/ZBJ-ArtR2c in both batches (Fig. S2A). Both arthropod primer pairs amplified a short fragment within the multicopy COI region, improving the detection of DNA degraded by the digestion process (Agustí *et al.*, 2003). However, although they amplify fragments in the same region, the two primer pairs have different primer binding sites (Fig. S1), which increases the likelihood that they will amplify different taxa (Table S3). As suggested by Piñol *et al.* (2015), this is probably due to the different number of templates mismatches between each arthropod primer pair and each amplified taxon. High rates of template mismatches negatively impact the amplification efficiency by reducing the number of amplified taxa. Seven arthropod taxa were detected when using ZBJ-ArtF1c/ZBJ-ArtR2c and 11 with mlCOIintF/HC02198 (Fig. S3A). However, when both pairs of primers were used together, we were able to increase the detection rate to 14 different arthropod taxa (only four of which were amplified by both

primer pairs), demonstrating that the use of both primer pairs resulted in a higher taxonomic coverage.

The plant primer pairs were also selected for different primer binding sites. The primer pair ITS-S2F/ITS4R amplifies a fragment of the nuclear ITS region, and the pair cA49325/trnL110R amplifies a fragment of the chloroplast *trnL* region. The first pair was chosen because it is the most common region for mixed pollen loads identification from insects (Suchan *et al.*, 2019). The second was chosen because it is recommended for analyzing degraded DNA (Taberlet *et al.*, 2007). Our results confirmed this, as a higher percentage of taxa was amplified with cA49325/trnL110R than with ITS-S2F/ITS4R in both batches (Fig. S2B); this was especially true for the second batch, which contained mostly ingested DNA (Table 1). In both trials together, a total of 11 plant taxa were detected using each primer pair (Fig. S3B). However, when using both primer pairs together, we detected 20 different plant taxa, resulting in a higher taxonomic coverage, as only three taxa were detected by both pairs of primers.

The use of two arthropod primer pairs that generate amplicons of different lengths makes it possible to distinguish the sequences produced by each primer pair. This information was used to determine the taxonomic resolution obtained with each primer pair. For all the taxa obtained in this study, the primary resolution of both arthropod primer pairs (ZBJ-ArtF1c/ZBJ-ArtR2c and mlCOIintF/HC0219) was on the species level (84.31% and 95.96%, respectively) (Fig. S4). The primary resolution of the plant primer pairs (ITS-S2F/ITS4R and cA49325/trnL110R) was on the species level (81.82%) and the family level (81.91%), respectively (Fig. S4). These finding align with those metabarcoding and barcoding studies using the same pairs of primers (da Silva *et al.*, 2019; Suchan *et al.*, 2019; Zhu *et al.*, 2019). The high level of resolution obtained with both arthropod primers and with ITS for plants increases the certainty of the results of the present study (Biffi *et al.*, 2017; McInnes *et al.*,

2017; Deagle *et al.*, 2018). Taxonomic resolution should be considered in the selection of the primer pairs, particularly for plant primers, which have more varied taxonomic resolution.

Trophic interactions

In this study, we assumed that plant DNA obtained from the whole bodies of cleaned insects came from gut contents and corresponded to the specimens' diets. The plant DNA retrieved from the washing solutions was assumed to represent visited plants, either from pollen deposited on the insects' bodies during foraging, or from walking on leaves with pollen deposited by anemophilous plants in the surrounding vegetation. We only detected plant DNA in the washing solutions of *Rhagonycha fulva*, which are larger and hairier than *Anthocoris nemoralis.* The fact that we did not detect plant DNA from the *Anthocoris nemoralis* washing solutions indicates that it may not be necessary to wash such small, glabrous insects. Although it is well known that anthocorids such as *Orius* spp feed on plant resources in laboratory conditions (Naranjo & Gibson, 1996), no plant taxa were detected in the whole specimens, either Some factors may impact the detection of ingested DNA (Kamenova *et al.*, 2018), such as a long period of starvation or a long period of preservation prior to DNA extraction. In this case, the specimens' most recent feeding episode might also has been exclusively on arthropod prey. Their small size and sucking mouthparts may also explain why no plant food was detected in this anthocorid species, especially compared to *Rhagonycha fulva*. In another study, plant DNA was identified in only 30% of the analyzed individuals of another predatory bug present on tomato plants (Pumariño *et al.*, 2011).

All the plant taxa ingested by *R. fulva* were assigned to the Phylum Streptophyta or to a family (Convolvulaceae, Solanaceae, Fabaceae or Poaceae) (Table S3; Fig. 1). Poaceae and Solanacea were the most common taxa (Fig. S5A). However, in the analysis of the washing solutions, more OTUs were assigned to a genus or a species. This difference may be due to

the different quantity and quality of the DNA isolated from digested and fresh (non-digested) samples. These plant taxa indicate that *R. fulva* forages on a wide range of plants, including *Olea europaea, Dactylis glomerata, Poa annua, Beta vulgaris, Pinus* sp., *Sonchus* sp., the family Asteraceae, and the order Caryophyllales (Table S3; Fig. 1). This diet is much more diverse than the single plant species cited by Rodwell *et al.* (2018), *Heracleum sphondylium* L (Apiaceae). These plant taxa can be present in the ground cover of peach crops, in field margins, or in alfalfa crops in the studied region (Juarez-Escario *et al.*, 2010; Juarez-Escario *et al.*, 2018; Solé-Senan *et al.*, 2018). Some of the taxa, such as *Dactilys glomerata, Poa annua,* and *Medicago sativa,* belong to families that were also detected in ingested DNA (Poaceae and Fabaceae), which may indicate that the insects' bodies contacted pollen from tassels or flowers while they were consuming on the plant.

In the *Analysis of field-collected predators* trial, we demonstrated the efficacy of this multi-primer approach for detecting and identifying arthropods ingested by two predator species (Table S3; Fig. 1). Previous literature cites *Rhagonycha fulva* as a predator of some insects, such as aphids (Pons & Eizaguirre, 2000; Rodwell *et al.*, 2018). Our results indicate that this predator also consumes *Nysius graminicola*, which was detected in 25% of the analyzed *Rhagonycha fulva* sample-pools (Fig. S5B). *Nysius graminicola* is an important pest of several summer crops in Italy, including peaches (Blando & Mineo, 2005). In Spain, another species of the same genus, *Nysius ericae,* has been identified as a secondary pest in peaches (Del Rivero & García-Marí, 1983), thus suggesting the potential of *Rhagonycha fulva* as a biocontrol agent. Our results also show that *Rhagonycha fulva*'s intraguild predation (IGP) on Coccinellidae and *Cantharis livida* is a very common trophic interaction (Fig. S5B). It is well known that IGP is widespread in agroecosystems (Lucas & Rosenheim, 2011), and HTS has been successfully used to demonstrate IGP in, for example, field-collected predators in lettuce (Gomez-Polo *et al.*, 2015, 2016). The IGP observed here should

be further studied in order to determine whether it could negatively impact the biological control of peach pests, because some coccinellids, such as *Coccinella septempunctata* or *Stethorus punctillum* (Weise), are efficient biocontrol agents in peach orchards (Trandafirescu *et al.*, 2004; Biddinger *et al.*, 2009).

Anthocoris nemoralis is a well-known biocontrol agent in fruit orchards, particularly for the pear psylla (Solomon *et al.*, 2000; Agustí *et al.*, 2003). Our findings indicate that this species is in fact a polyphagous predator, since its most common prey in our study were two very important peach pests, the green peach aphid *Myzus persicae*, and the peach moth *Grapholita molesta* (Fig. S5; Table S3; Fig. 1). This was not known before the present study. This predator also fed on *Diaphorina lycii,* a hemipteran species that is oligophagous on *Lycium* plants (Solanaceae). Since *Lycium europaeum* L. is planted in hedges to separate agricultural plots in the study area, it can be assumed that *Anthocoris nemoralis* must have moved from peach to those hedges to feed on this particular prey species and then returned to the crop where it was collected. This finding demonstrates how HTS analysis could also be used to understand predator movement, in this case from the peach crop to the surrounding vegetation and back. Finally, we also detected IGP in *A. nemoralis* (Fig. 1), which fed on several coccinellid species and on the genus *Orius*. These included *Oenopia conglobata,* a very common species in urban landscapes (Lumbierres *et al.*, 2018), and *Orius laevigatus*, a known biocontrol agent in vegetables (Gomez-Polo *et al.*, 2015, 2016). The latter trophic interaction should be also considered in further biological control studies.

Four arthropod taxa were also detected in the diet of the *Anthocoris nemoralis* analyzed in the *Taxonomic coverage* trial (Table S3; Fig. 1), reinforcing its role as a generalist predator. One of these was *Thrips fuscipennis,* which damages peaches during ripening (Tavella *et al.*, 2006). The subfamily Cecidomyiinae was also detected; this subfamily includes some predator species and some gall-producing pests in forestry and horticulture (Kolesik, 2014).

The other two prey taxa were in the genus *Orius* and in the family Coccinellidae, which are known predators in both alfalfa and peach crops in the area of study (Trandafirescu *et al.*, 2004; Pons *et al.*, 2009; Aparicio *et al.*, 2020). Our results reinforce the role of *Anthocoris nemoralis* as a potential biological control agent; this finding should also be considered in further studies of peach orchards and alfalfa crops. This is especially important in the study area, where these crops coexist and insects are very likely to move between them.

In this study, we used a multi-primer approach to HTS analysis to detect arthropod and plant resources ingested by two insect predators collected from a peach crop. We have demonstrated that, with this method of analysis, pooling predators into groups of 10 or 23 individuals has no significant influence on the analysis of their diet. We also showed that the use of two primer pairs improves the detection of ingested taxa, increasing the number of arthropod and plant taxa detected. Finally, we have shown that washing predators prior to HTS analysis is particularly important for large insects with hairy structures but may not be necessary for small, glabrous ones. The multi-primer approach developed in this study reduces the time and cost of the HTS analysis and demonstrates both expected and unexpected trophic relationships. The HTS techniques used in the present study revealed that trophic interactions between natural enemies and other organisms present in agroecosystems are highly complex involving all trophic levels. The detection of a broader than expected range of ingested arthropod and plant items highlights the importance of maintaining a diverse landscape composition to enhance the conservation of biological control agents in crops and contribute to more sustainable agriculture.

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Disclosure

The authors declare that they have no conflict of interest.

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Supporting Information

Table S1. Arthropod (ZBJ-ArtF1c/ZBJ-ArtR2c and mlCOIintF/HC02198) and plant (ITS-S2F/ITS4R and cA49325/trnL110R) primer pairs used in this study, indicating the sequence of each forward (F) and reverse (R) primer and the length of the amplified fragment.

Table S2. Relative read abundance (RRA) obtained from each arthropod and plant primer pairs in each library (L) in the three trials included in the study: (1) *Taxonomic coverage*; (2) *Plant primer resolution*; and (3) *Analysis of field-collected predators*. 3A corresponds to arthropods, 3B to plants, and 3C to washing solutions. Those percentages eliminated from the analysis for not reaching the 1% threshold are shown in bold. Art1 = ZBJ-ArtF1c/ZBJ-ArtR2c; Art2 = mlCOIintF/HC02198; Pl1 = ITS-S2F/ITS4R; Pl2 = cA49325/trnL110R; NA = Not amplified.

Table S3. Taxa obtained and primer pairs used in each trial and from each library. NT= not tested; NAmp = not amplified

Table S4. Comparison of the number of arthropod taxa obtained in each library in the *Taxonomic coverage* trial with*:* (A) either 10 or 23 *Anthocoris nemoralis* specimens; (B) either one or two pairs of primers.

Table S5. Number of template-mismatches of each arthropod pair of primers with each taxon amplified. NS = no sequence present in the databases at the moment of the analysis.

Fig. S1. Location of the COI primer pairs tested in the present study.

Fig. S2. Percentage of taxa obtained for each pair of arthropod (A) and plant (B) primers amplified in all libraries, in each HTS batch.

Fig. S3. Representation by Venn's diagrams of the artropod and plant taxa obtained by HTS (including all trials) with each primer pair. Numbers in each circle indicate the number of taxa amplified by each primer and how many are shared by both pairs of primers (overlaping area). (A) artropod primers; (B) plant primers. n = Number of taxa obtained with each primer pair.

Fig. S4. Accuracy of the taxonomic assignation according to the primer pair used. Data is presented as the percentage OTUs assigned to each taxonomic level.

Fig. S5. Percentage of frequency of occurrence (FOO%) of the obtained taxa items: (A) plant consumed by *R. fulva* and detected in *R. fulva* washing solutions; and (B) arthropod consumed by *A. nemoralis* and *R. fulva*.

Table 1 Arthropod and plant sample-pools analyzed using HTS in the three trials conducted in the present study. The table also shows the number of the HTS batch where each library was analyzed, the number of individuals (or amount of plant material) in each sample-pool, and the primer pairs used in each library. The arthropod universal primers ZBJ-ArtF1c/ZBJ-ArtR2c and mlCOIintF/HC02198 were used; the plant universal primersITS-S2F/ITS4R and CA49325/trnL110R were used.

HTS batch number	Trial	Species	Sample size (number of specimens or amount of plat leaf)	Sample-pool number	Primer pair	Library number
1	Taxonomic coverage	*Anthocoris nemoralis*	10	1	ZBJ-ArtF1c/ZBJ-ArtR2c	L1
					mlCOIintF/HC02198	L2
					ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L3
		Anthocoris nemoralis	23	2	ZBJ-ArtF1c/ZBJ-ArtR2c	L4
					mlCOIintF/HC02198	L5
					ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L6
	Plant primer resolution	*Medicago sativa* *Prunus persica* *Convulvulus arvensis* *Picris echiodies* *Setaria pumila*	1 cm^2	3	ITS-S2F/ITS4R; CA49325/trnL110R	L7
		Prunus persica	1 cm^2	4	ITS-S2F/ITS4R; CA49325/trnL110R	L8
2		*Medicago sativa*	1 cm^2	5	ITS-S2F/ITS4R; CA49325/trnL110R	L9
		Medicago sativa	1 cm^2	6	ITS-S2F/ITS4R; CA49325/trnL110R	L10

				ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L11
		26	7	ITS-S2F/ITS4R; CA49325/trnL110R	L12
				ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L13
		24	8	ITS-S2F/ITS4R; CA49325/trnL110R	L14
	Rhagonycha fulva			ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L15
		23	9	ITS-S2F/ITS4R; CA49325/trnL110R	L16
				ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L17
		5	10	ITS-S2F/ITS4R; CA49325/trnL110R	L18
Analysis of field-collected predators				ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L19
		26	11	ITS-S2F/ITS4R; CA49325/trnL110R	L20
	Anthocoris nemoralis			ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L21
		16	12	ITS-S2F/ITS4R; CA49325/trnL110R	L22
		26	13	ITS-S2F/ITS4R; CA49325/trnL110R	L23
	Rhagonycha fulva	24	14	ITS-S2F/ITS4R; CA49325/trnL110R	L24
	washing solutions	23	15	ITS-S2F/ITS4R; CA49325/trnL110R	L25
		5	16	ITS-S2F/ITS4R; CA49325/trnL110R	L26
	Anthocoris nemoralis washing	42	17	ITS-S2F/ITS4R; CA49325/trnL110R	L27

		solutions				
1	Blanks	PCR blank of batch 1	-	18	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L28
					ITS-S2F/ITS4R;	L29
					CA49325/trnL110R	
2		PCR blank of batch 2	-	19	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L30
					ITS-S2F/ITS4R;	L31
					CA49325/trnL110R	
		DNA Extraction blank	-	20	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L32
					ITS-S2F/ITS4R;	L33
					CA49325/trnL110R	

			Arthropod universal primers				Plant universal primers			
			ZBJ-ArtF1c/ZBJ-ArtR2c		mlCOIintF/HC02198		ITS-S2F/ITS4R		CA49325/trnL110R	
Step	Action	Total reads	# reads	# OTUs	# reads	# OTUs	# reads	# OTUs	# reads	#OTUs
0	Raw reads	9 047 294	NA	NA	NA	NA	NA	NA	NA	NA
1	Merged reads	4 297 098	NA	NA	NA	NA	NA	NA	NA	NA
2	Quality filtering	4 256 516	NA	NA	NA	NA	NA	NA	NA	NA
3	Length splitting	4 203 223	655 153	NA	515 905	NA	727 948	NA	2 304 227	NA
4	Clustering	4 203 223	655 153	4 096	515 905	5 527	727 948	894	2 304 227	2 322
5	Chimera	4	653	4	515 012	5 323	727	846	2 304 225	2 051

	removing	200 610	605	050			875			
6	Taxonomy assignment	4 153 413	648 171	278	501 486	1 270	726 404	174	2 277 352	482
7	OTUs contaminants filtering	4 145 004	647 497	59	499 650	250	725 486	33	2 272 371	180
8	OTUs secondary predation filtering	4 142 718	646 432	51	499 630	248	725 486	33	2 271 169	173

Table 2 Number of reads and OTUs obtained in each step of the bioinformatic analysis. The total data for each arthropod and plant primer pair for each step of the bioinformatic analysis are presented. NA = not applicable.

Table 3 Summary of all arthropod (*n* =14) and plant (*n* = 20) taxa obtained after bioinformatic analysis of the HTS data (33 libraries of 20 different sample-pools: see Table 1). The lowest taxonomic rank reached is indicated in bold.

Kingdom	Phylum	Order	Family/Subfamily	Genus	Species
Animalia	Arthropoda	Hemiptera	Anthocoridae		***Anthocoris nemoralis* Fabricius**
				Orius	
					***Orius laevigatus* Fieber**
			Aphididae		
					***Myzus persicae* Sulzer**
			Lygaeidae		***Nysius graminicola* Kolenati**
			Liviidae		***Diaphorina lycii* Loginova**
		Coleoptera	**Coccinellidae**		
					***Oenopia conglobata* L.**
			Cantharidae		***Cantharis livida* L.**
					***Rhagonycha fulva* Scopoli**
		Diptera	**Cecidomyiinae**		
		Lepidoptera	Tortricidae		***Grapholita molesta* Busck**
		Thysanoptera	Thripidae		***Thrips fuscipennis* Haliday**
Plantae	**Streptophyta**				
		Asterales	**Asteraceae**		
				Sonchus	
					***Picris echioides* L.**
		Solanales	**Convolvulaceae**		
					***Convolvulus arvensis* L.**
		Solanales	**Solanaceae**		
		Fabales	**Fabaceae**		
					***Medicago sativa* L.**
				Trifolium	
		Lamiales	Oleaceae		***Olea europaea* L.**
		Pinales	Pinaceae	***Pinus***	
		Poales	**Poaceae**		
				Setaria	
					***Dactylis glomerata* L.**
					***Poa annua* L.**
		Caryophyllales			
			Amaranthaceae		***Beta vulgaris* L.**
		Rosales	**Rosaceae**		
					***Prunus persica* (L.) Batsch**

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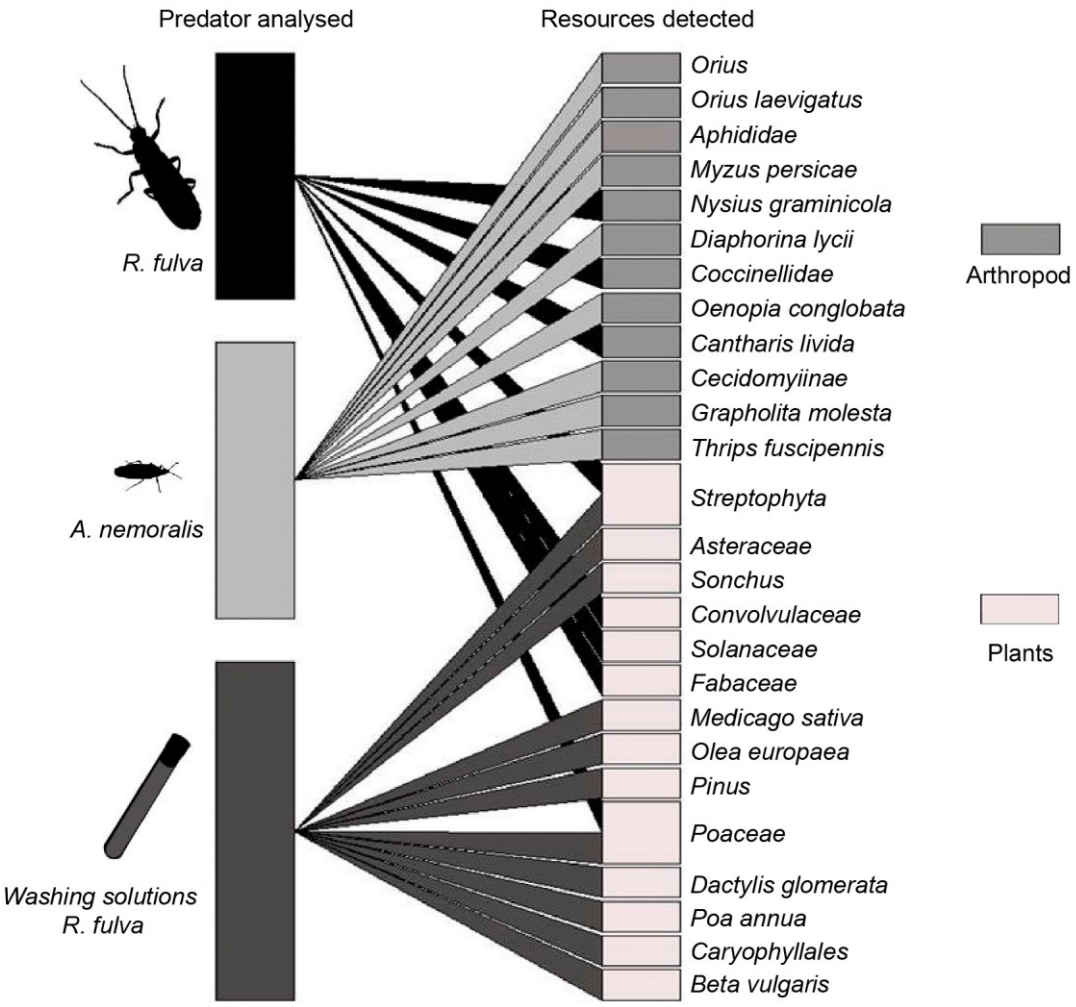
Fig. 1 Trophic web of the predators *Ragonycha fulva* and *Anthocoris nemoralis* from arthropod and plant taxa detected from their whole body DNA extractions using HTS analysis, and plant taxa detected in the washing solutions of *R. fulva* bodies.



Graphical Abstract text

Interaction network of the arthropod and plant taxa detected