



Disentangling arthropod and plant resources consumed by *Orius* spp. in peach and alfalfa crops by metagenomic analysis

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Abstract

Agricultural intensification is affecting the biological control of insect pests, an important component for sustainable crop production. To understand the changing patterns of insect abundance within an agroecosystem, it is necessary to disentangle the trophic interactions between species, and metabarcoding is an excellent alternative to show them. In the Ebro Basin (NE Iberian Peninsula), agricultural landscapes are composed of a mosaic of crops scattered with natural and semi-natural habitats, where the presence of *Orius* spp., used as biocontrol agents, is well known. To shed light on their predatory role in this area, a previously developed metabarcoding multi-primer method has been used to study the arthropod and plant most frequently resources consumed by some field-collected *Orius*, sampled at different dates in a peach and an alfalfa adjacent crop. Their high-throughput sequencing (HTS) analysis showed the consumption of 15 arthropod and 12 plant taxa. Eight of them were consumed by *O. laevigatus*, six by *O. majusculus* and 23 by *O. niger*. The obtained results showed that HTS is a powerful tool in studies of trophic interactions in agroecosystems. Among the detected arthropods, other natural enemies were present, showing a certain degree of intraguild predation, which has been demonstrated by developing a new added strategy in the bioinformatic analysis. Detected plant consumption indicates that *Orius* forages on several plant species, showing their movement from them to the sampled crops. These plants could have a potential role in attracting or maintaining these predators in both crops in biological control strategies.

Keywords High-throughput sequencing · Intraguild predation · Molecular diet analysis · Multi-primer approach · *Orius* spp. · Trophic interactions

Key message

- Metabarcoding approaches helped to reveal complex trophic interactions in a Mediterranean agroecosystem.
- *Orius* species used animal and plant diseases insect vectors as food resources.

- *Orius* species frequently engaged in intraguild predation interactions.
- Plant DNA detection in *Orius* gut content evidenced their movement between crop and non-crop habitats.

Introduction

Agricultural intensification, which is causing the loss of biodiversity and landscape simplification (Gámez-Virués et al. 2015), and the global environmental changes caused by climate change are reducing essential ecosystem services vital for human societies (McMeans et al. 2015). Among them, the biological control of pests using natural enemies has become an important component of sustainable crop production in agroecosystems (Bale et al. 2008). The appropriate habitat manipulation to enhance the presence of these natural enemies increases the effectiveness of

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conservation biological control (Landis et al. 2008), which is the only cost-effective biological method in arable crops in the Mediterranean region nowadays (Pons and Starý 2003; Lumbierres et al. 2007; Pons and Eizaguirre 2009; Pons et al. 2011; Meseguer et al. 2021; Levi-Mourao et al. 2022). To understand and predict the changing patterns of insect abundance in the agroecosystems, it is necessary to consider some factors, such as the trophic interactions between species, the landscape structure (i.e. composition and configuration), the management of the crop fields (i.e. tillage, irrigation, pesticide inputs, harvesting/cutting or rotation) or the constant changes in agricultural policy (Clemente-Orta et al. 2020).

In the Ebro Basin (NE Iberian Peninsula), agricultural landscapes are composed of a mosaic of arable crops, including cereals and alfalfa, together with fruit orchards, such as peach, apple and pear scattered with natural and semi-natural habitats that can condition relationships between predators and pests (Pons et al. 2005; Ardanuy et al. 2018; Clemente-Orta et al. 2020). Numerous studies have been performed in this area to relate insect predator abundance with the plant variability of the landscape. Some of them highlighted the role of some predatory species of *Orius* (Hemiptera: Anthocoridae) on the biological control of thrips and aphids, such as *Orius laevigatus* Fieber, *Orius majusculus* Reuter and *Orius niger* Wolff in peach, apple, maize and alfalfa crops (Avilla et al. 2008; Sarasúa et al. 2000; Pons et al. 2005; Aparicio et al. 2021).

To better understand the potential role of each *Orius* species as a biocontrol agent, it is important to know their trophic interactions in the studied agroecosystem. Studying trophic interactions is inherently complicated because predation is an ephemeral process often difficult to visualise, particularly in the field. Omnivorous predators, such as *Orius*, are well known to consume pollen or plant juices, which is also very difficult to evaluate in the field. For this reason, molecular tools have been used since a few decades ago to disentangle trophic relationships in agroecosystems (Agustí et al. 2003; Sheppard and Harwood 2005; Pumariño et al. 2011; Romeu-Dalmau et al. 2012; González-Chang et al. 2016). Currently, metabarcoding is starting to be used to assess biodiversity and to understand the food web structure in ecosystems (Brown et al. 2015; Taberlet et al. 2018) and, more recently, in agroecosystems (Gomez-Polo et al. 2015, 2016; Sow et al. 2020). For example, a metabarcoding multi-primer approach was recently developed to simultaneously identify the most frequent arthropod and plant resources ingested by omnivorous arthropod predators collected in peach crops (Batuecas et al. 2022).

The main aim of this study was to use this metabarcoding multi-primer approach to disentangle the most frequent trophic interactions of small populations of three *Orius* species present in two adjacent fields of peach and alfalfa. The

gathered information wants to shed light on the role of *Orius* as predator of major pests in these crops, as well as on alternative prey species (including other natural enemies). The bioinformatic analysis has included a new step to identify the intraguild predation (IGP) among *Orius* species. Detecting non-crop vegetation ingestion also sheds light on the role of some plants in attracting these predators to both crops, important information to further improve biological control programmes in those crops.

Materials and methods

Sample collection and DNA extraction

Orius spp. adult specimens ($n=97$) were collected in two adjacent plots of peach and alfalfa located in Vilanova de Segrià (Lleida), Spain (UTM 10×10 : 31TCG01), in June and August 2016 and in July and September 2017. Peach trees were sampled by beating their branches and alfalfa with a vacuum sampler (McCulloch MAC320BV). Each collected specimen was individualised in a DNA-free tube and placed in a portable freezer to avoid DNA degradation. Once in the laboratory, they were stored at $-20\text{ }^{\circ}\text{C}$ until the DNA extraction. A previous study (Batuecas et al. 2022) showed that no plant DNA could be identified from the washing solution of another anthocorid (*Anthocoris nemoralis* (Fabricius)). Therefore, the collected *Orius* specimens, which are also glabrous and smaller (1–3 mm vs. 3–5 mm for *A. nemoralis*), were not washed before the pooling because the risk of *Orius* retaining pollen grains on their surface was highly unlikely.

The DNA of each insect or plant sample (1 cm²-diameter leaf of peach (*Prunus persica* (L.) Batsch) or alfalfa (*Medicago sativa* L.) was extracted using the Speedtools Tissue DNA Extraction Kit (Biotools, Germany; protocol for animal tissues). Total DNA was eluted in 100 μL of AE buffer provided by the manufacturer and stored at $-20\text{ }^{\circ}\text{C}$. A negative control without DNA (just DNA-free water) was added to each DNA extraction set.

Orius molecular identification and pooling

The collected *Orius* were molecularly identified by following the molecular protocol and the F2/R2 primers described in Gomez-Polo et al. (2013), with some modifications. PCR volumes (20 μL) contained 2 μL of resuspended DNA, 10 μL of master mix (Biotools, Madrid, Spain) and 1 μL of each primer [10 μM]. Amplifications were conducted in a 2720 thermal cycler (Applied Biosystems, CA, USA). Target DNA from some morphologically identified adult *Orius* and water were always included as positive and negative controls, respectively.

PCR products were separated by electrophoresis using 2.4% agarose gels stained with SYBR[®]Safe (Invitrogen, Karlsruhe, Germany) and visualised under UV light. Each *Orius* specimen was identified by comparing the molecular weight of the obtained PCR product with those of the positive controls, as done in Gomez-Polo et al. (2013).

After molecular identification, the concentration of each DNA extraction was measured using a Qubit[®] 2.0 fluorometer with the dsDNA HS assay kit (Invitrogen, Carlsbad, CA, USA). Equimolar amounts of each *Orius* individual DNA extraction (5 ng/ μ L) were finally pooled by species, crop and date in seven sample pools (Table 1; sample pools 1–7).

To save time and cost, predators were pooled (up to 25 in the same sample pool), and both pairs of arthropod primers were used together in the same library, as well as both pairs of plant primers, as done in Batuecas et al. (2022). Both universal arthropod pairs of primers (ZBJ-ArtF1c/ZBJ-ArtR2c, 157 bp, and mlCOIintF/HC02198, 313 bp) amplify different amplicon sizes of the mitochondrial cytochrome oxidase I (COI) region (Table S1). They were selected like that to avoid competition for the same primer binding sites. Similarly, both pairs of universal plant primers used (ITS-S2F/ITS4R, 350 bp, and cA49325/trnL110R, 80 bp) were from very different regions (Table S1), the first from the nuclear internal transcribed spacer 2 (ITS2) and the second from the chloroplast *trnL* intron. Two plant sample pools, namely *P. persica* and *M. sativa*, were used as positive

controls (Table 1; sample pools 8 and 9), as recommended by Jusino et al. (2019).

PCR amplification, library preparation and sequencing

All sample pools were amplified using the multi-primer approach described in Batuecas et al. (2022), with the two previously mentioned pairs of universal arthropod primers (Table S1). Each PCR volume (50 μ L) contained 15 μ L of DNA of each equimolar pool, 25 μ L of multiplex master mix (Qiagen, Hilden, Germany) and 1 μ L of each primer [10 μ M]. PCR conditions for both arthropod primer pairs 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, and a final extension at 72 °C for 10 min. PCR conditions for both pairs of plant primers were as follows: 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. Amplifications were conducted in a 2720 thermal cycler (Applied Biosystems, CA, USA). Target DNA and DNA-free water were included as positive and negative controls, respectively. The resulting PCR products were cleaned with the QIAquick PCR purification kit (Qiagen), and 5 μ L of each clean PCR product was used as a template to prepare the libraries to be sequenced. Libraries were built by mixing the PCR products either both pairs of arthropod primers or both pairs of plant primers. DNA-free water from PCR amplification for sequencing was included as PCR blank (sample pool 10, Table 1). All libraries were processed

Table 1 Sample pools analysed by HTS, indicating the number of individuals included in each sample pool and the number of the library with the primers used

Species/sample	Crop	Date	Sample- pool #	# of individuals	Primer pair	Library number
<i>Orius laevigatus</i>	Peach	June 2016	1	7	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L1
					ITS-S2F/ITS4R; CA49325/trnL110R	L2
<i>Orius majusculus</i>	Peach	June 2016	2	21	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L3
					ITS-S2F/ITS4R; CA49325/trnL110R	L4
<i>Orius niger</i>	Alfalfa	June 2016	3	9	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L5
					ITS-S2F/ITS4R; CA49325/trnL110R	L6
					ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L7
	June 2016	4	25	ITS-S2F/ITS4R; CA49325/trnL110R	L8	
				ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L9	
				ITS-S2F/ITS4R; CA49325/trnL110R	L10	
September 2017	6	22	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L11		
			ITS-S2F/ITS4R; CA49325/trnL110R	L12		
			ITS-S2F/ITS4R; CA49325/trnL110R	L13		
<i>Prunus persica</i>	Peach	–	7	1 cm ²	ITS-S2F/ITS4R; CA49325/trnL110R	L13
<i>Medicago sativa</i>	Alfalfa	–	8	1 cm ²	ITS-S2F/ITS4R; CA49325/trnL110R	L14
PCR blank	–	–	9	–	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L15
					ITS-S2F/ITS4R; CA49325/trnL110R	L16

in a unique high-throughput sequencing (HTS) batch, done 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. Amplicons were purified with magnetic beads, and 5 µL of each library was grouped and sequenced with a paired-end approach (2 × 225 bp).

Bioinformatics

Raw Illumina reads were merged using VSEARCH 2.0 algorithm (Rognes et al. 2016). The assembled reads were quality filtered using the FASTX-Toolkit tool (Gordon and Hannon 2010) with a minimum of 75% of bases \geq Q30. The resulting reads were then split by the length of the expected amplicon from each primer pair with custom Python scripts. Primer sequences were removed from sequencing reads using Cutadapt 1.11 (Martin 2017). The obtained reads were clustered into OTUs with a similarity threshold of 97% using VSEARCH 2.0. Chimaeras were removed using the UCHIME algorithm (Edgar et al. 2011). The remaining OTUs were queried against custom-made databases using BLAST 2.2.31 + (BLASTN, *E*-value 1e-10, the minimum coverage of the query sequence: 97%, the number of alignments: 9) (Camacho et al. 2009). The custom-made databases contained all arthropod and plant sequences present in the study area available in the NCBI database (<http://www.ncbi.nlm.nih.gov/>) at the moment of the analysis (October 2019). For this, we used two European and regional biodiversity databases: GBIF.org (<http://www.gbif.org/>) and *Banc de dades de biodiversitat de Catalunya* (<http://biodiver.bio.ub.es/biocat/>). Taxonomy was assigned at \geq 97% identity by the Last Common Ancestor algorithm with BASTA (Kahlke and Ralph 2019). To remove possible contaminants from the OTUs obtained from each group of primer pairs (arthropods or plants), we only considered those OTUs that had more than five reads and were detected in at least two sample pools of the same species (Boyer et al. 2013). When the OTUs were obtained in only one sample pool, they were considered if there were more than five reads with both primer pairs or if they exceeded the 0.01% of the total reads from OTUs filtered for plant or arthropod in each case as recommended by Alberdi et al. (2018). The obtained OTUs were categorised as predator or prey based on their taxonomy. To reduce other biases, like the secondary predation, and show the most important taxa ingested, two dietary metrics were calculated, as done by Deagle et al. (2018) and Batuecas et al. (2022), the percentage of the relative read abundance (RRA%) and the percentage of frequency of occurrence (FOO%). The first was the total number of reads of each consumed resource (arthropod or plant) amplified with each primer pair and for each library, divided by the

number of total reads of all resources obtained with each primer pair for each library. After that, those resources $<$ 1% of RRA were eliminated. The second metric was calculated from the taxa obtained, which was the percentage of the resource items obtained per species, thus indicating the most frequent resources consumed.

Because low divergence is expected between congeneric species (Jung et al. 2011), some additional steps in the bioinformatic analysis were developed to detect the potential IGP between *Orius* species. To validate whether the adopted similarity threshold (\geq 97%) was the most suitable to obtain a proper taxonomic assignment between the ingested *Orius* species and the predator species of *Orius* itself, the *Orius* sequences present in GenBank and Bold databases belonging to the regions amplified by each pair of arthropod primers used were aligned and taken as reference (Table S2). The interspecific percentage of similarity in both binding sites within the COI region for the *Orius* species was calculated from the sequences obtained in the HTS process and compared with those interspecific percentages of similarity from the sequences found in the databases. We only considered those OTUs assigned to the species level, whose sequences came from the amplification with each pair of primers within each analysed pool. These OTUs were then aligned, and the interspecific percentages of similarity were calculated using R v3.4.3 in RStudio v1.1.419 by the function pairwiseAlignment (parameters of the alignment: Match: 1, Mismatch: 0, gapOpening/Extension:0) of the R package Biostring (Pagès et al. 2017a).

Results

Orius molecular identification

All *Orius* specimens collected in both peach and alfalfa plots showed a specific band pattern that allowed their identification at the species level as done in Gomez-Polo (2013). The predominant species varied according to the crop sampled, with *O. niger* as the only species found in alfalfa on all sampled dates (34 in June 2016, 13 in July 2017 and 22 in September 2017) and *O. majusculus* and *O. laevigatus* found only in peach in June 2016 (21 and 7, respectively). All of them were used to build the sample pools for the following HTS analysis (Table 1).

HTS analysis of field-collected *Orius*

The HTS analysis of the 16 libraries (Table 1) generated 1,104,574 raw paired end reads. Of these, 94.8% were successfully merged, quality filtered and assigned to one of the four primer pairs (85.4% to arthropod primers and 14.6% to plant primers (step 3, Table 2)). After clustering, chimaera

Table 2 Total number of reads and OTUs obtained with each universal arthropod and plant primer pair in each step of the bioinformatic analysis

Step	Action	Total reads	Arthropod primers				Plant primers			
			ZBJ-ArtF1c/ZBJ-ArtR2c		mlCOIintF/HC02198		ITS-S2F/ITS4R		CA49325/trnL110R	
			#Reads	#OTUs	#Reads	#OTUs	#Reads	#OTUs	#Reads	#OTUs
0	Raw reads	1,104,574	NA	NA	NA	NA	NA	NA	NA	NA
1	Merged reads	530,729	NA	NA	NA	NA	NA	NA	NA	NA
2	Quality filtering	528,720	NA	NA	NA	NA	NA	NA	NA	NA
3	Length splitting by	523,726	354,641	NA	92,690	NA	6836	NA	69,559	NA
4	Clustering	523,726	354,641	542	92,690	745	6836	139	69,559	112
5	Chimera removing	523,293	354,394	524	92,505	707	6835	138	69,559	112
6	Taxonomy assignment	511,090	347,177	175	87,754	246	6647	60	69,512	76
7	OTUs filtering	510,254	346,845	64	87,393	66	6562	14	69,402	27
8	OTUs secondary predation filtering	510,156	346,799	60	87,393	66	6562	14	69,402	27

NA Not applicable

discarding and taxonomy assignment, we obtained 421 arthropod and 136 plant OTUs (step 6, Table 2). After the OTUs filtering to eliminate contaminants (step 7, Table 2), the taxa with a number of reads lower than 1% were also eliminated (step 8, Table 2). From the *Orius* sample pools analysed (sample pools 1–6; Table 1), we obtained 126 arthropod and 41 plant OTUs, which were finally assigned to 15 arthropod taxa (eight to species level) and 12 plant taxa (three to species level) (Table 3; Table S3).

The HTS analysis of *O. laevigatus* (sample pool 1, Table 1) showed arthropod and plant amplification. A part of the predator itself, we detected *Orius* as predatory taxon, and two pest taxa: the family Aphididae and the species *Frankliniella occidentalis* Pergande (Thysanoptera: Thripidae) (Fig. 1). Plant taxa detected corresponded to one of the sampled crops (*P. persica*), its family (Rosaceae), the alfalfa family (Fabaceae), the family Solanaceae and the genus *Pinus* (Fig. 1; Table 3).

Regarding *O. majusculus* (sample pool 2, Table 1), a part of the predator itself, there was amplification of two other predatory taxa (*Orius* and *O. laevigatus*) and four plant taxa corresponding to three families (Asteraceae, Rosaceae and Fabaceae) and the genus *Pinus* (Fig. 1; Table 3).

The four sample pools of *O. niger* (sample pools 3–6, Table 1) showed amplification of four pest taxa: the genus *Hypera* and *Liriomyza*, the species *Therioaphis trifolii* Monell (Aphididae) and *F. occidentalis*; three predator taxa corresponding to one genus (*Orius*) and two species (*O. laevigatus* and *Aeolothrips Intermedius* Bagnall (Thripidae)); five non-pest taxa belonging to two families (Ceratopogonidae and Cicadellidae), one subfamily (Orthoclaadiinae), and two dipteran species (*Aedes caspius* Pallas (Culicidae) and *Tanytarsus volgensis* Miseiko (Chironomidae)) (Fig. 1; Table 3; Table S3). Regarding plant taxa, the

two sampled crops (*P. persica* and *M. sativa*) were detected, as well as nine other plant taxa: Streptophyta, two orders (Asparagales and Caryophyllales), four families (Asteraceae, Fabaceae, Poaceae and Rosaceae), one genus (*Pinus*), and one species (*P. annua*) (Fig. 1; Table S3; Table 3).

Regarding the positive controls of peach and alfalfa, we obtained the expected amplification of both plant sample pools (sample pools 7 and 8, respectively; Table 1), amplifying *P. persica* and *M. sativa* with the plant primers ITS-S2F/ITS4R, as well as their corresponding families (Rosaceae and Fabaceae) with the plant primers cA49325/trnL110R (Table S3).

Regarding the validation of the IGP between *Orius* species, we calculated the percentages of similarity between the three *Orius* species sequences (*O. majusculus*, *O. laevigatus* and *O. niger*) found in the databases. They ranged from 92 to 94% for ZBJ-ArtF1c/ZBJ-ArtR2c and from 87 to 91% for mlCOIintF/HC02198 (Table S4). We also calculated the percentages of similarity between the obtained number of OTUs for each predator (*O. majusculus* or *O. niger*) and for each prey (other *Orius* species) using the ZBJ-ArtF1c/ZBJ-ArtR2c pair of primers (Table S5), being in all cases below the cluster similarity threshold of 97% used. On the other hand, to show the high taxonomic resolution obtained, we want to indicate that the 423,697 obtained (95 OTUs) were assigned to only three *Orius* species (Table S6). Almost all these reads were obtained with the primer pair ZBJ-ArtF1c/ZBJ-ArtR2c (81.54%). The rest (18.46%) were obtained with the primer pair mlCOIintF/HC02198.

Table 3 Summary table of detected arthropod ($n = 15$) and plant ($n = 12$) taxa (in bold) after the bioinformatic analysis of HTS data (16 libraries of 9 sample pools (Table 1))

Kingdom	Phylum/Clade	Order	Family/Subfamily	Genus	Species	
Animalia	Arthropoda	Coleoptera	Curculionidae	Hypera		
		Diptera	Agromyzidae	Liriomyza		
			Ceratopogonidae			<i>Tanytarsus volgensis</i> Miseiko
			Chironomidae			<i>Aedes caspius</i> Pallas
			Culicidae			
			Orthocladinae			
			Anthocoridae	Orius		<i>Orius laevigatus</i> Fieber
						<i>Orius majusculus</i> Reuter
						<i>Orius niger</i> Wolff
					Aphididae	
Plantae	Streptophyta	Thysanoptera	Cicadellidae		<i>Aeolothrips intermedius</i> Bagnall	
			Aeolothripidae		<i>Frankliniella occidentalis</i> Pergande	
			Thripidae			
			Asparagales			
			Asterales	Asteraceae		<i>Medicago sativa</i> L.
			Caryophyllales			
			Fabales	Fabaceae		
			Pinales		Pinus	
			Poales			<i>Poa annua</i> L.
			Rosales	Rosaceae		<i>Prunus persica</i> (L.) Batsch
				Solanaceae		

Table 4 Percentages of relative read abundance (RRA%) of the reads amplified from resources consumed by each arthropod and plant primer pairs in each library (L) in the trials included in the study: (1) arthropod taxa detected in the analysed *Orius*; (2) plant taxa detected in the analysed *Orius*

Primers used	L1		L3		L5		L7		L9		L11	
	Art1	Art2	Art1	Art2	Art1	Art2	Art1	Art2	Art1	Art2	Art1	Art2
(1)												
<i>Orius</i>	-	-	-	NA	-	-	-	-	-	-	-	-
<i>Orius laevigatus</i>	-	-	100	NA	8,88	-	-	-	-	-	87,23	-
<i>Orius majusculus</i>	-	-	-	NA	-	-	-	-	-	-	-	-
<i>Orius niger</i>	-	-	-	NA	-	-	-	-	-	-	-	-
<i>Aeolothrips intermedius</i>	-	-	-	NA	-	-	73,78	16,72	-	-	0,21	-
<i>Frankliniella occidentalis</i>	-	100	-	NA	-	100	-	74,62	-	-	-	73,11
Aphididae	100	-	-	NA	-	-	-	-	-	-	-	-
<i>Therioaphis trifolii</i>	-	-	-	NA	-	-	-	-	-	-	0,29	10,27
<i>Hypera</i>	-	-	-	NA	5,33	-	2,32	-	-	-	-	-
<i>Liriomyza</i>	-	-	-	NA	3,55	-	21,58	8,66	-	-	-	-
<i>Aedes caspius</i>	-	-	-	NA	-	-	-	-	-	-	0,38	8,07
Ceratopogonidae	-	-	-	NA	-	-	-	-	-	-	0,21	4,89
<i>Tanytarsus volgensis</i>	-	-	-	NA	68,64	-	-	-	-	-	-	-
<i>Orthocladinae</i>	-	-	-	NA	13,61	-	2,32	-	-	-	-	-
<i>Cicadellidae</i>	-	-	-	NA	-	-	-	-	100	-	11,68	-
Primers used	L2		L4		L6		L8		L10		L12	
Taxa detected	PI1	PI2	PI1	PI2	PI1	PI2	PI1	PI2	PI1	PI2	PI1	PI2
(2)												
Streptophyta	-	-	NA	-	-	-	NA	-	-	-	100	31,69
Asparagales	-	-	NA	-	-	7,07	NA	-	-	-	-	-
Caryophyllales	-	-	NA	-	-	-	NA	-	-	-	-	8,69
Asteraceae	-	-	NA	12,95	-	2,18	NA	-	-	-	-	42,04
Poaceae	-	-	NA	-	-	1,25	NA	-	-	-	-	-
Solanaceae	-	2,67	NA	-	-	-	NA	-	-	-	-	-
<i>Poa annua</i>	-	-	NA	-	-	-	NA	-	-	-	-	1,78

Table 4 (continued)

Primers used	L2		L4		L6		L8		L10		L12	
	PI1	PI2	PI1	PI2	PI1	PI2	PI1	PI2	PI1	PI2	PI1	PI2
<i>Pinus</i>	-	9,45	NA	15,83	-	2,18	NA	-	-	-	-	1,36
Fabaceae	-	40,86	NA	41,73	-	87,32	NA	100	-	42,93	-	58,26
Rosaceae	-	47,02	NA	29,50	-	-	NA	-	-	11,08	-	-
<i>Medicago sativa</i>	-	-	NA	-	100	-	NA	-	59,65	-	-	-
<i>Prunus persica</i>	100	-	NA	-	-	-	NA	-	40,35	-	-	-

Those percentages eliminated from the analysis for exceeding the 1% threshold are shown in bold

Art1 ZBJ-ArtF1c/ZBJ-ArtR2c; *Art2* mICOLintF/HC02198; *PI1* ITS-S2F/ITS4R; *PI2* cA49325/trnL110R; NA Not amplified

Discussion

This study identified the arthropod and plant resources consumed by the *Orius* species complex in two peach and alfalfa adjacent fields by an HTS multi-primer approach. The results showed how, with a discrete number of analysed *Orius* specimens, this method allowed studying trophic interactions in agroecosystems since a broad range of the most frequently ingested resources than the described in previous field studies based on the observation of field predatory episodes were revealed (Pericart 1972; Riudavets 1995; Riudavets and Castañé 1998; Latin 1999; Pons et al. 2005). However, we must consider that the results obtained do not show an overview of the complete diet of each of these *Orius* species, but rather what the population present in the sampled plot fed on. On the other hand, to our knowledge, this is the first time that plant consumption has been detected in field-collected *Orius* by molecular methods. Some of the detected plant taxa were from outside the sampled crop, confirming that these natural enemies are highly mobile between crops and non-crop habitats and use the neighbouring habitats to forage. This was also observed in Batuecas et al. (2021) using a new marking method with an aqueous solution of an aquatic invertebrate (*Artemia* spp.) followed by a conventional PCR test using *Artemia*-specific primers.

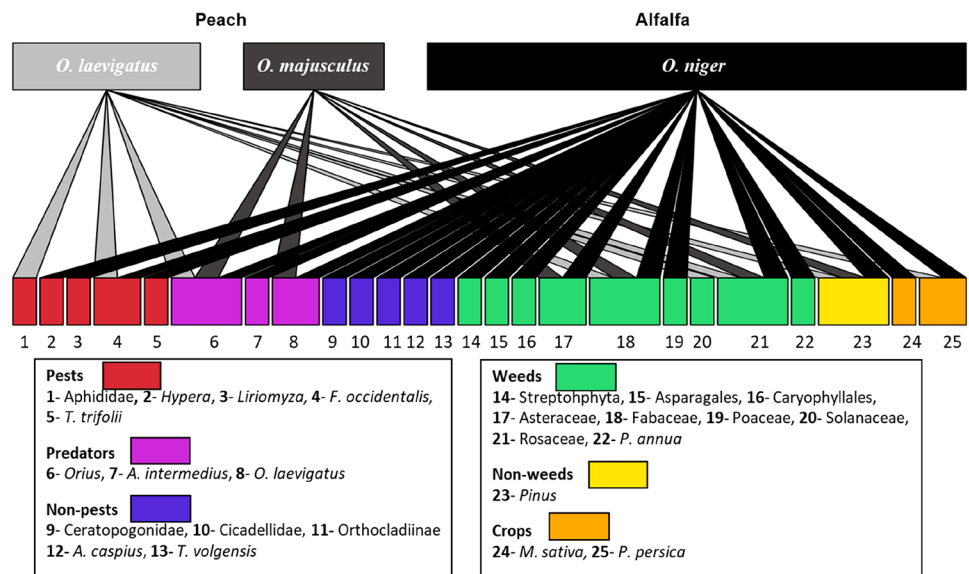
Methodological issues

Plant positive controls used in the HTS analysis (sample pools 7 and 8) allowed a confident taxonomic identification of plant species. Both plant primer pairs gave a suitable identification of each analysed piece of leaf, either to species (using ITS-S2F/ITS4R) or to family level (using CA49325/trnL110R) (Table S3), as also observed in Batuecas et al. (2022). Also acting as positive controls, the analysed *Orius* specimens, which were previously identified by conventional PCR, allowed a confident taxonomic identification of themselves by showing a suitable identification of each *Orius* sample pool to species level (Table 3).

Field-collected *Orius* specimens were pooled by species and, in the case of *O. niger*, also by sampling dates (Table 1), which allowed having biological replicates in this case, as recommended by Mata et al. (2018). On the other hand, the dietary metrics RRA% and FOO% showed more reliable evidence of their consumption giving an estimation from the reads obtained in each sample and the frequency that the taxa are detected in the analysed samples, demonstrating that consumption on a particular taxon is not spurious or is not indirectly ingested (secondary predation).

This study obtained a suitable taxonomic resolution, where a certain number of arthropod species were obtained

Fig. 1 Interaction network of the arthropod and plant taxa detected from the analysed predators (*O. laevigatus*, *O. majusculus* and *O. niger*) collected in alfalfa and peach



(eight species from 15 taxa) (Table 3). The analysed *Orius* showed an expected high detection of the predator taxa (Fig. S1), representing 97.58% of the total reads obtained with the arthropod primer pairs. This is due to the low number of primers mismatches between the detected *Orius* species (Table S7). Despite this, we still detected some prey taxa (Table 3). According to Agustí et al. (2003), the primer pairs used, which amplify short amplicons within the COI region, improve the detection of degraded DNA due to the digestion process. Our results confirm this statement because the primer pair that amplified the shortest fragment showed a higher deep sequencing in the HTS process (Table S1; Table 2). Using two different primer pairs should also be considered in further studies since it increases the chance of detecting a broader range of resources consumed. This recommendation is based on the results obtained in this study for *O. niger*, where 23 different resources were detected (Fig. 1), showing that the use of only one pair of primers would reduce the obtained results by half (Table S5: L5, L7, L9, L11).

Trophic interactions

Orius is a well-known genus of predators present in several crops (Riudavets 1995; Riudavets and Castañé 1998). Some crop pests were detected within the field-collected *Orius*. One of them was the thrips *F. occidentalis*, a well-known key pest of several crops, including alfalfa and peach (Lacasa et al. 2008), which was detected in predators collected in both crops. In the present study, *F. occidentalis* was consumed by *O. laevigatus* in peach in June 2016 and by *O. niger* in alfalfa also in June 2016 and in September 2017, being the most frequent arthropod taxa detected in the analysed *Orius* specimens (Fig. 1; Fig. S2).

Considering that thrips are attracted by flowers (Frey et al. 1994) and that they feed on pollen to increase their fecundity (Zhi et al. 2005), it makes sense that this pest was detected in peach at the end of spring when high numbers of thrips were still present after the orchard flowering.

The rest of the pest taxa were detected in those *Orius* collected in both crops sampled, highlighting *Hypera* and *Liriomyza* as the most frequent trophic interactions after *F. occidentalis* (Fig. S2). In peach, we detected consumption of Aphididae, a family that includes important pests of peach orchards and important vectors of the plum pox virus or Sharka disease (Aparicio et al. 2019). These trophic interactions were previously described in peach by Barbagallo et al. (2017), particularly by *O. laevigatus*. In *O. niger* collected in alfalfa, we detected the aphid *T. trifolii* (Fig. 1; Table 4), a pest that causes important economic damages in this crop (Pons 2002) as well as the curculionid genus *Hypera*, another important pest of this crop (Pons and Eizaguirre 2009). Trophic interactions between *Orius* spp. and *T. trifolii* or *Hypera* have been previously described by Pons et al. (2005) in the same area of study. The genus *Liriomyza* has previously been classified as a minor pest that rarely produces economic loss (Parrella and Keil 1984) and has been cited in alfalfa crops in the same study area (Pons and Nuñez 2020).

Other prey taxa were detected in both crops, namely *A. intermedius* and *O. laevigatus*, which are known predators of thrips (Riudavets 1995), showing a certain degree of IGP. *Aeolothrips intermedius* was consumed by *O. niger* (collected in alfalfa), whereas *O. laevigatus* was detected within *O. majusculus* (collected in peach) and *O. niger* (collected in alfalfa) (Fig. 1, Table S3). Intraguild predation using metabarcoding analysis of field-collected predators in agricultural systems has already been shown

in other HTS studies (Gomez-Polo et al. 2015, 2016; Batuecas et al. 2022). In the present study, IGP was also present between species of the same genus (*Orius*), which makes its detection more difficult to demonstrate because of their close taxonomic similarity. For this reason, a new bioinformatic process was added, allowing differentiation between the amplified *Orius* species. These results demonstrate predation between congeneric *Orius* species, showing trophic interactions where the taxonomical distance between species was low. This IGP should be considered in further studies, as a potential negative effect on the biological control of key pests, such as *F. occidentalis*.

Some non-pest taxa were also detected in lower percentages in *O. niger* (collected in alfalfa), including Ceratopogonidae, *T. volgensis*, *A. caspius*, Orthoclaadiinae and Cicadellidae (Fig. 1, Table S3). The fact that more arthropod species have been detected within those predators collected on alfalfa than in those collected on peach (Fig. 1) could be due to the higher number of specimens analysed in alfalfa (69 from 97 analysed). Nevertheless, alfalfa has been recognised as an important reservoir of natural enemies due to the presence of a high number of different phytophagous arthropod species in this crop (Nuñez 2002; Pons et al. 2005).

Orius predation on some dipteran taxa has also been reported before, like *O. majusculus* feeding on Syrphidae in lettuce, also detected by HTS (Gomez-Polo et al. 2016). Both Ceratopogonidae and *A. caspius* have been cited to cause zoonotic diseases with a significant socioeconomic impact (Aranda et al. 1998; Pagès et al. 2017b). Ceratopogonidae is the vector of the bluetongue epizootics, which affects ungulates, sheep, cattle and goats (Nolan et al. 2008), and *A. caspius* is described as a floodwater mosquito species widely distributed in the Western Palearctic. As an anthropophilic species, its role as an arbovirus vector is key to understanding the transmission cycle of certain diseases in Europe, like as the Rift Valley fever virus (Moutailler et al. 2008) and the West Nile virus, which has been recently reported in Lleida (Busquets et al. 2018), which is in the same region of the area of study. *Tanytarsus volgensis* and Orthoclaadiinae belong to the family Chironomidae (Table 3), which is the most abundant insect group in all types of freshwaters and even in saltwater (Armitage et al. 1995).

Orius niger also predated Cicadellidae in alfalfa (Fig. 1; Table 3). This family includes vectors of some plant diseases (McClure 1980), like *Asymmetrasca decedens* (Paoli) present in Spain and Italy (Alvarado et al. 1994; Torres et al. 2000), which transmit peach diseases as the almond witches-broom (Abou-Jawdah et al. 2014). Other species of this family are known to be the vector of Pierce's disease caused by *Xylella fastidiosa* in *Prunus* spp. (Bragard et al. 2019), which is a serious problem also in peaches. Trophic interactions between *Orius* and Cicadellidae had been previously suggested by Pons et al. (2005) in alfalfa

in the same area of study. One of the key pests in alfalfa in Spain, *Empoasca fabae* Harris, also belongs to this family (Pons and Nuñez 2020). Albajes et al. (2011) and Ardanuy et al. (2018) also indicated *Orius* predation on the cicadellid *Zyginidia scutellaris* (Herrich-Schaffer) in maize plots in the same area of study.

It is well known that *Orius* benefit from feeding on pollen and plant juices on several plant species (Lundgren 2009; Pumariño and Alomar 2012; Mendoza et al. 2021). The identified plant taxa within the three *Orius* species further indicate that they forage a wide range of plants under field conditions, highlighting Fabaceae (Fig. 1; Table 3), the family of alfalfa. This result indicates the use of alfalfa as resource used by *Orius*, as it was described by Nuñez (2002) and Pons et al. (2005). The rest of the detected plant taxa (Table 3) have been cited either in ground covers, in field margins of peach crops or in alfalfa crops in the same area of study (Ibáñez-Gastón 2018; Clemente-Ortega et al. 2020). This detection shows that they used these plant resources and then moved to peach and alfalfa crops, as Ardanuy et al. (2018) suggested. In the case of *O. niger* (collected in alfalfa), with a high number of plant taxa detected, a potential trigger effect of the alfalfa cuts was present, leading *Orius* individuals to disperse in the landscape, as previously indicated by Madeira et al. (2019). Detection of *P. persica* within *O. niger* sampled in alfalfa in September (Table 3) is particularly interesting because it indicates that these *Orius* have visited the peach crop and then moved to alfalfa. Peach trees bloom in spring, which makes unlikely that those *Orius* were fed on pollen deposited on alfalfa leaves, and even if that was the case, it could not be easily amplified because pollen DNA detection by conventional PCR strongly decays after 14 days (Schield et al. 2015), particularly with the high summer temperatures present in the area of study. The *Orius* movement from alfalfa to peach had been previously demonstrated by Batuecas et al. (2021) using a PCR-based detection method, particularly for *O. laevigatus* and *O. majusculus*. The results obtained in the present study indicate that *Orius* movement is possible from alfalfa to peach and agree with the results obtained in Batuecas et al. (2021), showing the bidirectional movement between both crops. This shows this multi-primer approach as a valuable tool to track predator movement.

Some *Orius* were also fed on *Pinus* (Fig. 1). Several anthocorids have been described on pine trees (Pericart 1972), and some *Orius* species have also been occasionally recorded on pines, such as *O. niger* and *Orius albidipennis* Reuter (Heidari et al. 2015), and *Orius tristicolor* White (Lattin and Stanton 1992). Nevertheless, the area of study has 88% of the soil occupied by crops, and the presence of *Pinus* species is relatively low (www.creaf.uab.cat/iefc/pub/Regions/Comarques/CobertesSegria.htm). *Pinus* is a wind-pollinated genus that produces abundant pollen dispersed

over long distances. The species present in the study area are *Pinus nigra* Arnold and *Pinus halepensis* Mill, both flowering in spring (www.creaf.uab.cat/iefc/pub/Regions/EstraArbustiRF8.htm). In the area of study, most of the pine pollen of these species is shed between April and July, and some pollen has been even recovered in aerial palynology studies in summer and autumn (<https://www.polenes.com/home>). Therefore, it is plausible that *Orius* fed on pollen deposited on leaves or had previously foraged on pine trees before entering the alfalfa crop.

In this study, we have exposed the main advantages that this HTS method could offer to trophic studies in agroecosystems, like detecting the most frequently ingested arthropod and plant resources by *Orius* specimens present in a peach and an alfalfa crop. HTS analysis confirmed their role as predators and suggested the influence of the landscape on their presence in peach and alfalfa crops. HTS also contributed to showing unknown trophic interactions, like predation on Cicadellidae and some dipteran vectors of animal and human diseases by *O. niger*. This methodology also showed the presence of IGP between *Orius* species and between *Orius* and *A. intermedius*, which could be further considered in the future biological control studies in peach and alfalfa crops. Finally, even if we analysed just a few specimens, we showed the omnivory of these three *Orius* species that fed on some plant resources present in the different elements of the landscape in the area of study, which suggest the importance of plant biodiversity in the landscape and the need of preserving it for a more sustainable agriculture. Despite these advantages, HTS also has some limitations. The main one is the impossibility of quantifying prey DNA consumed by the predator because there is no correlation between the number of reads obtained and the quantity of DNA consumed (Piñol et al. 2015). Therefore, the results from HTS must be considered from a qualitative perspective. Only a certain estimation of the frequency of ingested resources can be given by the metrics RRA % and FOO %. Also, HTS shows which resources are being consumed but reduces the chances of detecting resources consumed by scavenging. Nevertheless, HTS techniques are an indispensable tool for studying the trophic food web in an agroecosystem, and they will have a significant implication in the biological control pest discipline.

Author contributions

IB and NA conceived and designed the HTS analysis and wrote the manuscript. IB, LG and NA conducted the HTS analysis. IB, CC, OA, NA and LG conducted the field samplings. IB and JP analysed the data. All authors revised and approved the manuscript.

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

Code availability (software application or custom code) Bioinformatic pipeline to analyse the data: <https://github.com/Ivanbh214/MMAP>. Bioinformatic analysis to detect the potential IGP between *Orius* species: https://github.com/Ivanbh214/Validation_predation.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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