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Molecular methods to study the movement of predatory arthropods between elements of the landscape: from topical marking to metagenomics

Iván Batuecas Huertas

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RECERCA I TECNOLOGIA AGROALIMENTÀRIES

Doctoral thesis

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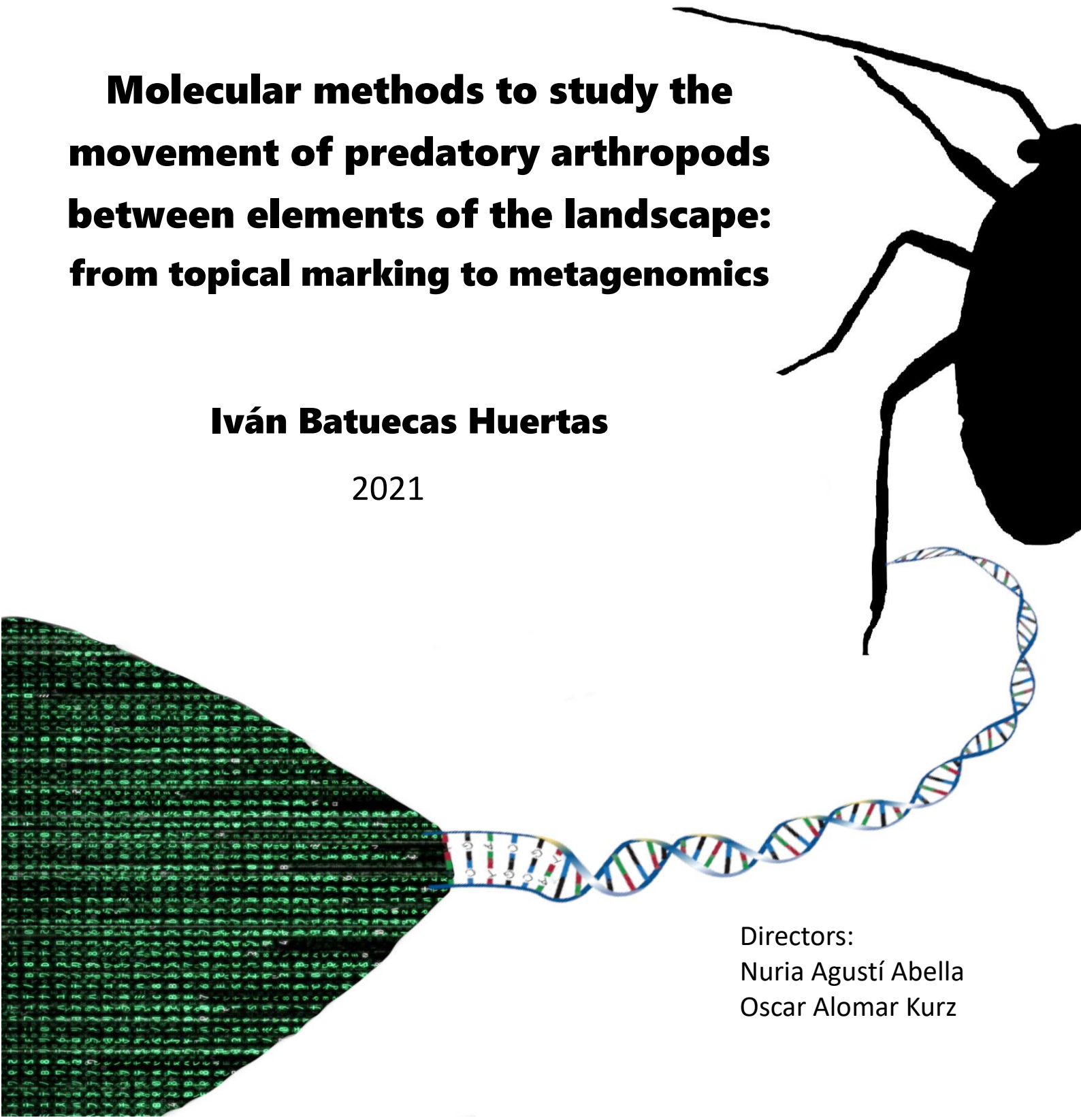

Iván Batuecas Huertas

2021

Directors:
Nuria Agustí Abella
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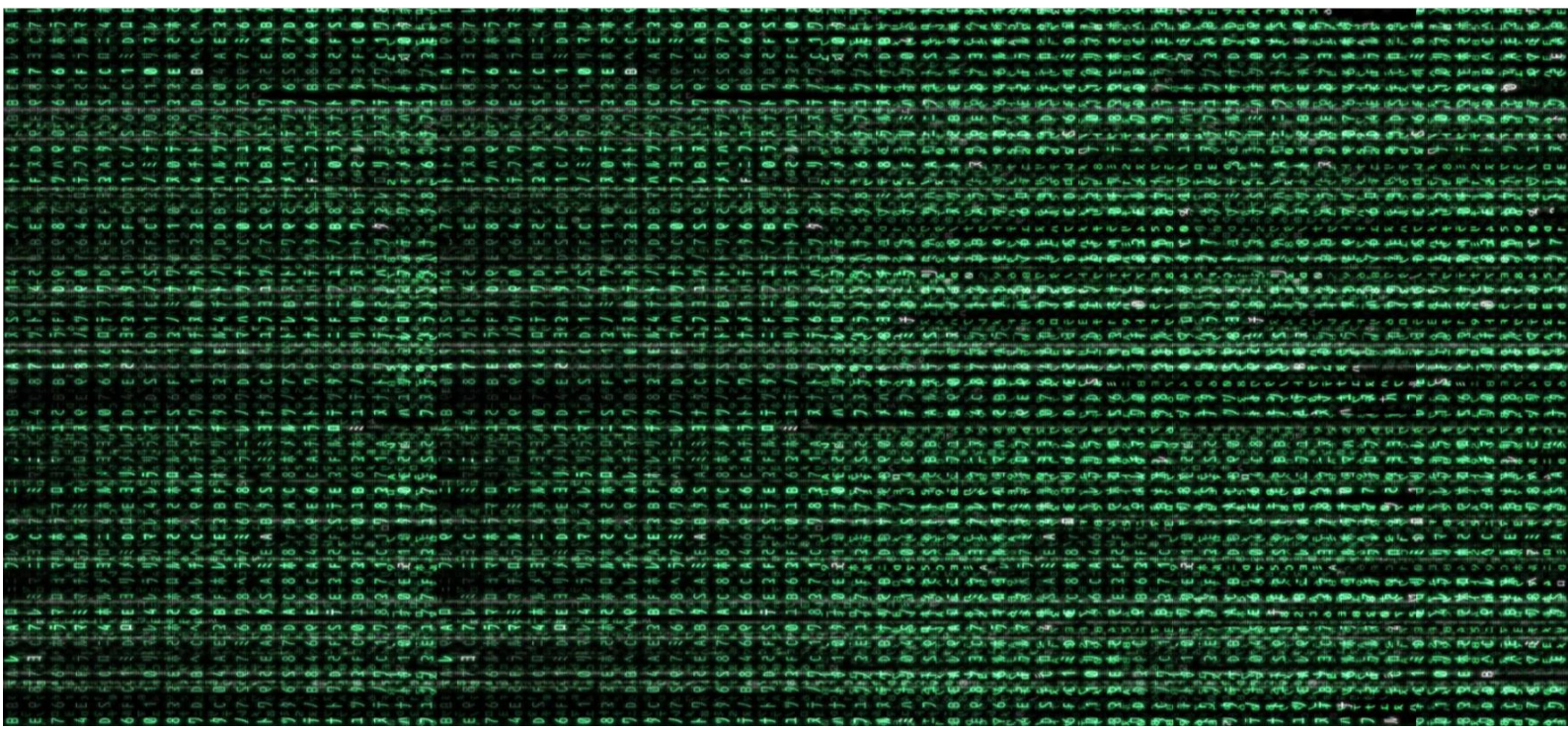

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DOCTORAL THESIS

Molecular methods to study the movement of predatory arthropods between elements of the landscape: from topical marking to metagenomics

Dissertation to obtain the degree of Doctor with international mention by the University of Lleida

Memòria presentada per optar al grau de Doctor amb menció internacional per la Universitat de Lleida

Iván Batuecas Huertas

Programa de Doctorat Ciència i Tecnologia Agrària i Alimentàri

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Carne de yugo, ha nacido
más humillado que bello,
con el cuello perseguido
por el yugo para el cuello.

…

Me duele este niño hambriento
como una grandiosa espina,
y su vivir ceniciento
revuelve mi alma de encina.

…

Me da su arado en el pecho,
y su vida en la garganta,
y sufro viendo el barbecho
tan grande bajo su planta.

¿Quién salvará a este chiquillo
menor que un grano de avena?
¿De dónde saldrá el martillo
verdugo de esta cadena?

Que salga del corazón
de los hombres jornaleros,
que antes de ser hombres son
y han sido niños yunteros.

El niño yuntero

Miguel Hernández

A la memoria de mis Abuelos, que nos dejaron durante el transcurso de esta tesis …

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Summary

Faced with global agricultural production losses due to pests' action, further increased by global warming, conservation biological control (CBC) is a sustainable alternative to mitigate these losses, mainly through natural populations of biological control agents (BCAs). The study of the role that the different habitats that compound the landscape play in the maintenance of the BCAs in crops is necessary, and for this reason, to know the movement of BCAs between crops and other habitats in the landscape is essential. The general objective of this Doctoral Thesis is to study the movement of polyphagous predatory arthropods among landscape elements (crops and non-cultivated areas) through molecular methods, for a future improvement of CBC programs in alfalfa and peach crops in the Ebro basin. To do this, firstly were performed the optimization of a topical marking technique with a DNA non present in the study agroecosystem (the crustacean Artemia spp.) and its amplification by conventional PCR. Results demonstrated the movement from alfalfa to peach by predatory arthropods of interest for the biological control (BC) of peach pests, such as some coccinellids, anthocorids of the genus *Orius*, mirids and chrysopids (Chapter 1). Subsequently, the development of a metagenomic analysis method was carried out through a multi-primer HTS approach (Chapter 2) to detect the main arthropod and plant consumed resources by the predatory arthropods collected in two contiguous plots of peach and alfalfa in the study area, in order to demonstrate its movement between elements of the landscape. To achieve this objective, the optimal parameters for using this massive sequencing methodology were determined by grouping insects by pools. The need to wash larger insects with greater hairiness before metagenomic analysis was also demonstrated, due to their remarkable ability to retain pollen on the cuticle, and thus be able to detect ingested pollen exclusively. In total, 13 predatory taxa belonging to the Cantharidae, Aanthocoridae, Coccinellidae, Miyridae and Lygaeidae families were analysed, detecting 69 and 65 arthropod-arthropod and arthropod-plant trophic interactions, respectively (Chapters 2, 3 and 4). These were trophic relationships already known on important pests of alfalfa and peach, such as *Aphis craccivora*, *Therioaphis trifolii*, *Liriomyza* sp., *Hypera* sp. and *Frankliniella occidentalis*, which confirmed the predatory role of species such as *Orius laevigatus*, *Orius niger*, *Nabis* and *Hippodamia variegata*; or other trophic interactions unknown until now on important pests in peaches, such as *Grapholita molesta*, *Myzus persicae* and *Thrips fuscipennis* consumed by *Anthocoris nemoralis*. The plants detected, mainly Asteraceae, Poaceae and Solanaceae, were consumed by most of the analysed predators, thus demonstrating their importance to conserve those predators near the crops. The movement of predators from the margins to the crops, and between crops, could be demonstrated, either through the detection of ingested prey, such as *Diaphorina lycii*, an oligophagous insect of the *Lycium europaeum* shrub, which was consumed by *Anthocoris nemoralis* and *Adelphocoris lineolatus*; or through the direct ingestion of plant tissues, such as Prunus persica consumed by Orius niger captured in alfalfa. Intra-guild predation was also detected among the complex of analysed predators and other natural enemies present in the agroecosystem, such as among species of the genus *Orius*; or predator-parasitoid relationships such as *Dinocampus coccinellae* parasitizing *Coccinella septempunctata*, or *Leucostoma* sp. parasitizing *Nabis* sp. This methodology has also made possible to demonstrate the omnivory of the majority of the predatory species analysed, even in those taxa with a known phytophagous character, such as *Adelphocoris lineolatus*, *Nysius* and *Lygus*. Even also allowed the detection of the ingestion by Orius niger of insect taxa vectors of diseases that affect peaches, such as the Cicadellidae family; or even diseases that affect animals, such as the family Ceratopogonidae or *Aedes caspius*, suggesting a potential effect of this predator to minimize the transmission of these diseases, to be considered in the future. Finally, this methodology has also made possible to detect species of difficult morphological identification such as *Nysius cymoides* and *Nysius graminícola*. *Nysius graminícola*, a peach pest, was the prey consumed in a higher number of predatory species. All these results have demonstrated the great potential of the HTS methodology used in this Doctoral Thesis to study trophic interactions and movement in agroecosystems with insects of diverse morphology, information necessary to fine-tune future CB programs in peach and in alfalfa.

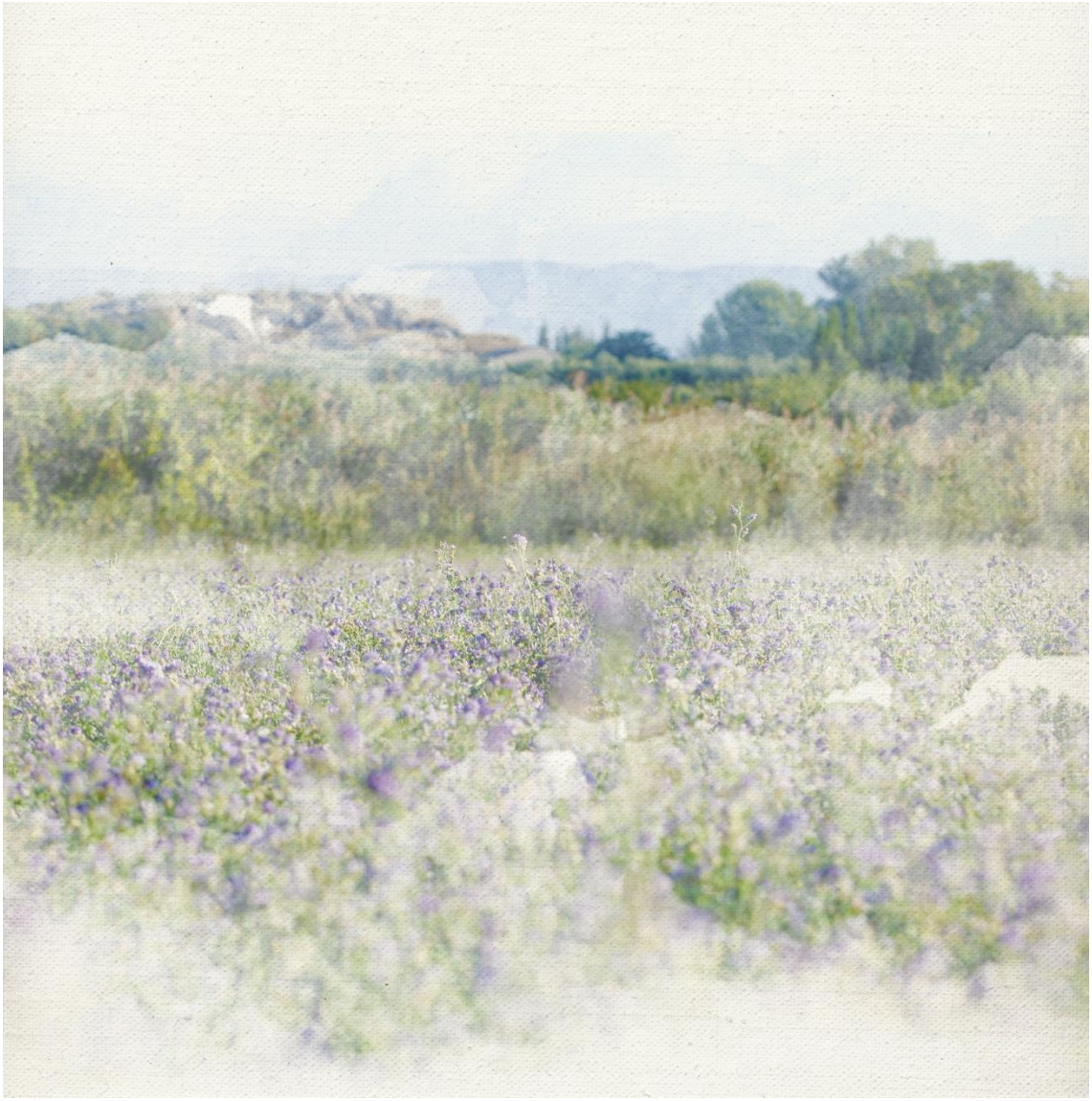
Resumen

Ante las pérdidas de la producción agrícola global debido a la acción de las plagas, incrementadas aún más por el calentamiento global, el control biológico por conservación (CBC) es una alternativa sostenible para mitigar estas pérdidas, en particular mediante poblaciones naturales de agentes de control biológico (ACBs). El estudio del papel que desempeñan los diferentes hábitats que componen el paisaje en el mantenimiento de estos ACB en los cultivos es necesario, y para ello también conocer el movimiento de los ACBs entre cultivos y otros hábitats del paisaje. El objetivo general de esta Tesis Doctoral es el estudio del movimiento de artrópodos depredadores polífagos entre elementos del paisaje (cultivos y áreas no cultivadas) a través de métodos moleculares, para una futura mejora de los programas de CBC en los cultivos de alfalfa y melocotón de la cuenca del Ebro. Para ello, en primer lugar, se llevó a cabo la optimización de una técnica de marcaje tópico con un ADN no presente en el agroecosistema de estudio (el crustáceo *Artemia* spp.) y su amplificación por PCR convencional. Los resultados demostraron el movimiento desde la alfalfa al melocotón por parte de artrópodos depredadores de interés para el control biológico (CB) de las plagas del melocotón, como algunos coccinélidos, antocóridos del género Orius, míridos y crisópidos (Capítulo 1). Posteriormente, se llevó a cabo la puesta a punto de un método de análisis metagenómico mediante un análisis multi-primer HTS (Capítulo 2), para detectar los artrópodos y las plantas consumidas por los principales artrópodos depredadores recolectados en dos parcelas contiguas de melocotón y de alfalfa en el área de estudio, con la finalidad de estudiar su movimiento entre elementos del paisaje. Para ello, se determinaron los parámetros óptimos para la utilización de esta metodología de secuenciación masiva mediante la agrupación de los insectos en pools. También se demostró la necesidad de lavar los insectos de mayor tamaño y con mayor pilosidad de manera previa al análisis metagenómico, debido a su mayor capacidad de retener el polen sobre la cutícula, y poder así detectar exclusivamente el polen ingerido. En total, se analizaron 13 taxones depredadores pertenecientes a las familias Cantharidae, Anthocoridae, Coccinellidae, Miridae y Lygaeidae, llegando a detectar 69 y 65 interacciones tróficas artrópodo-artrópodo y artrópodo-planta, respectivamente (Capítulos 2, 3 y 4). Estas fueron desde relaciones tróficas ya conocidas sobre importantes plagas de alfalfa y melocotón, como *Aphis craccivora*, *Therioaphis trifolii*, *Liriomyza* sp., *Hypera* sp. y *Frankliniella occidentalis*, que confirmaron el rol depredador de especies como *Orius laevigatus*, *Orius niger*, *Nabis* e *Hippodamia variegata*; hasta interacciones tróficas desconocidas hasta el momento y de importancia para el control de plagas en melocotón, como *Grapholita molesta*, *Myzus persicae* y *Thrips fuscipennis*, por parte de *Anthocoris nemoralis*. Las plantas detectadas, mayoritariamente asteráceas, poáceas y solanáceas, fueron consumidas por parte de la mayoría de los depredadores analizados, poniendo así de manifestando su importancia para conservar los depredadores analizados cerca de los cultivos. Se pudo demostrar el movimiento de depredadores desde los márgenes a los cultivos y entre cultivos, ya fuera a través de la detección de presas, como *Diaphorina lycii*, insecto oligófago del arbusto *Lycium europaeum*, que fue consumido por *Anthocoris nemoralis* y por *Adelphocoris lineolatus*; o a través de la ingestión directa de tejidos vegetales, como Prunus persica consumido por Orius niger capturados en alfalfa. También se detectó depredación intragremial entre el complejo de depredadores analizados, así como con otros enemigos naturales presentes en el agroecosistema, como entre especies del género *Orius*; o relaciones depredador-parasitoide como *Dinocampus coccinellae* parasitando *Coccinella septempunctata*, o *Leucostoma* sp. parasitando *Nabis* sp. Esta metodología ha permitido también demostrar la omnivoría de la mayoría especies depredadoras analizadas, incluso en aquellos taxones con un conocido carácter fitófago, como *Adelphocoris lineolatus*, *Nysius* y *Lygus*. Incluso también detectar la ingestión por parte de *Orius niger* de taxones de insectos vectores de enfermedades que afectan al melocotón, como la familia Cicadellidae; o incluso de enfermedades que afectan a animales, como la familia Ceratopogonidae o *Aedes caspius*, sugiriendo un potencial efecto de este depredador para minimizar la transmisión de estas enfermedades, a tener en cuenta en un futuro. Finalmente, esta metodología también ha permitido detectar la presencia de especies de difícil identificación morfológica como *Nysius cymoides* y *Nysius graminícola*. *Nysius graminícola*, plaga de melocotón, fue la presa consumida en un mayor número especies depredadoras. Todos estos resultados han demostrado el gran potencial de la metodología HTS utilizada en esta Tesis Doctoral para el estudio de las interacciones tróficas, así como del movimiento en agroecosistemas con insectos de diversa morfología, información necesaria para poner a punto futuros programas de CB en melocotón y en alfalfa.

Resum

Davant les pèrdues de la producció agrícola global a causa de l'acció de les plagues, incrementades encara més per l'escalfament global, el control biològic per conservació (CBC) és una alternativa sostenible per mitigar aquestes pèrdues, en particular mitjançant poblacions naturals d'agents de control biològic (ACBs). L'estudi del paper que juguen els diferents hàbitats que componen el paisatge en el manteniment d'aquests ACBs en els cultius és necessari, i per a això també conèixer el moviment dels ACBs entre conreus i altres hàbitats del paisatge. L'objectiu general d'aquesta Tesi Doctoral és l'estudi del moviment d'artròpodes depredadors polífags entre elements del paisatge (cultius i àrees no conreades) a través de mètodes moleculars, per a una futura millora dels programes de CBC en els cultius d'alfals i préssec de la conca de l'Ebre. Per això, en primer lloc, es va dur a terme l'optimització d'una tècnica de marcatge tòpic amb un ADN no present en l'agroecosistema d'estudi (el crustaci *Artemia* spp.) i la seva amplificació per PCR convencional. Els resultats van demostrar el moviment des de l'alfals al préssec per part d'artròpodes depredadors d'interès pel control biològic (CB) de les plagues del préssec, com alguns coccinèl·lids, antocòrids del gènere *Orius*, mírids i crisopes (Capítol 1). Posteriorment, es va dur a terme la posta a punt d'un mètode d'anàlisi metagenòmic mitjançant un anàlisi multi-*primer* HTS (Capítol 2), per detectar els artròpodes i les plantes consumides pels principals artròpodes depredadors recol·lectats en dues parcel·les contigües de préssec i d'alfals a l'àrea d'estudi, amb la finalitat d'estudiar el seu moviment entre elements del paisatge. Per a això, es van determinar els paràmetres òptims per a la utilització d'aquesta metodologia de seqüenciació massiva mitjançant l'agrupació dels insectes en *pools*. També es va demostrar la necessitat de rentar els insectes més grans i amb major pilositat de manera prèvia a l'anàlisi metagenòmica, per la seva major capacitat de retenir el pol·len sobre la cutícula, i poder així detectar exclusivament el pol·len ingerit. En total, es van analitzar 13 taxons depredadors pertanyents a les famílies Cantharidae, Anthocoridae, Coccinellidae, MIridae i Lygaeidae, arribant a detectar 69 i 65 interaccions tròfiques artròpode-artròpode i artròpode-planta, respectivament (Capítols 2, 3 i 4). Aquestes van ser, des de relacions tròfiques ja conegudes sobre importants plagues d'alfals i préssec, com *Aphis craccivora*, *Therioaphis trifolii*, *Liriomyza* sp., *Hypera* sp. i *Frankliniella occidentalis*, que van confirmar el paper depredador d'espècies com *Orius laevigatus*, *Orius niger*, *Nabis* i *Hippodamia variegata*; fins interaccions tròfiques desconegudes fins al moment i d'importància per al control de plagues en préssec, com *Grapholita molest*a, *Myzus persicae* i *Thrips fuscipennis*, per part de *Anthocoris nemoralis*. Les plantes detectades, majoritàriament asteràcies, poàcies i solanàcies, van ser consumides per la majoria dels depredadors analitzats, posant així de manifestant la seva importància per a conservar els depredadors analitzats a prop dels cultius. Es va poder demostrar el moviment de depredadors des dels marges als cultius i entre cultius, ja fos a través de la detecció de preses, com *Diaphorina lycii*, insecte oligòfag de l'arbust *Lycium europaeum*, que va ser consumit per *Anthocoris nemoralis* i per *Adelphocoris lineolatus*; o mitjançant la ingestió directa de teixits vegetals, com *Prunus persica* consumit per *Orius niger* capturats en alfals. També es va detectar depredació intragremial entre el complexe de depredadors analitzats, així com amb altres enemics naturals presents en l'agroecosistema, com entre espècies del gènere *Orius*; o relacions depredador-parasitoide com *Dinocampus coccinellae* parasitant *Coccinella septempunctata*, o *Leucostoma* sp. parasitant *Nabis* sp. Aquesta metodologia ha permès també demostrar la omnivoria de la majoria espècies depredadores analitzades, fins i tot en aquells tàxons amb un conegut caràcter fitòfag, com *Adelphocoris lineolatus*, *Nysius* i *Lygus*. Fins i tot també detectar la ingestió per part d'*Orius niger* de tàxons d'insectes vectors de malalties que afecten el préssec, com la família Cicadellidae; o fins i tot de malalties que afecten animals, com la família Ceratopogonidae o *Aedes caspius*, suggerint un potencial efecte d'aquest depredador per minimitzar la transmissió d'aquestes malalties, a tenir en compte en un futur. Finalment, aquesta metodologia també ha permès detectar la presència d'espècies de difícil identificació morfològica com *Nysius cymoides* i *Nysius graminicola*. *Nysius graminicola*, plaga del préssec, va ser la presa consumida en un major nombre espècies depredadores. Tots aquests resultats han demostrat el gran potencial de la metodologia HTS utilitzada en aquesta Tesi Doctoral per a l'estudi de les interaccions tròfiques, així com del moviment en agroecosistemes d'insectes de diversa morfologia, informació necessària per posar a punt futurs programes de CB en préssec i en alfals.

General introduction



1-Control biológico.

Según la FAO, actualmente se pierde entre el 20 y el 40 por ciento de producción global de alimento anual debido a las plagas (FAO, 2020). Esta tendencia sigue al alza y se prevé que aumente aún más debido a la influencia del calentamiento global, que provocará un incremento mayor de la incidencia de las plagas sobre los cultivos (Deutsch et al., 2018). El control biológico (CB) es de gran importancia en la producción agrícola hoy en día, ya que ayuda a controlar las plagas de una manera sostenible, y se define como aquel servicio ecosistémico que a través del uso de un organismo permite reducir la densidad de población de otro organismo. Estos organismos que contribuyen a reducir las poblaciones de otros organismos plaga se conocen como enemigos naturales (EN) o agentes de CB (van Lenteren, 2009).

Según Eilenberg et al. (2001) existen cuatro estrategias principales de CB: el clásico, basado en la introducción de EN alóctonos; el CB inoculativo, centrado en la introducción de un EN que permita el control de la plaga diana; el CB inundativo, basado en la suelta masiva de agentes de CB para controlar la plaga; y el CB por conservación (CBC), cuyo objetivo es proteger y mejorar las poblaciones de EN presentes en los cultivos mediante prácticas de manejo del medio para contribuir a la reducción y el control de las plagas en los agroecosistemas (Landis, 2000; Eilenberg et al., 2001; Begg et al., 2017).

Existen una serie de factores que pueden contribuir a la mejora del CBC y que por lo tanto merecen ser estudiados, como la influencia del paisaje, el movimiento de artrópodos entre los elementos que componen el paisaje, y las interacciones tróficas que se dan entre los artrópodos componentes del agroecosistema.

2- Influencia del paisaje agrícola en el control biológico

Todos los artrópodos presentes en un agroecosistema, incluyendo tanto las plagas como los EN, utilizan recursos de las zonas cultivadas y no cultivadas que se encuentran a distancias próximas de los cultivos durante su ciclo de vida, tanto para alimentarse, como para reproducirse. La abundancia y la diversidad de EN depende de la presencia y de la utilización de los recursos tróficos locales (néctar, polen, melaza, hospedantes y presas) (Landis et al., 2000). Por tanto, para contribuir al aumento de las poblaciones de EN de una manera efectiva, es necesario conocer los recursos que consumen, tanto en referencia a las plantas, como a otros animales. Estos recursos se encuentran en las diferentes estructuras que componen el paisaje, como los propios cultivos, los hábitats naturales, como los bosques, o los hábitats seminaturales herbáceos y márgenes creados por el hombre (Jervis et al. 2004). La presencia de estos recursos en las infraestructuras está directamente influenciada por las prácticas culturales, y por tanto, el manejo de los diferentes hábitats debe estar enfocado a favorecer el aumento de las poblaciones de EN para la supresión de las plagas, especialmente cuando la densidad de huéspedes y presas es baja en el agroecosistema (Denys and Tscharntke 2002).

En las últimas décadas hay una tendencia hacia la intensificación de la agricultura tanto a escala global como local. Este incremento de los monocultivos y la fragmentación de los hábitats naturales y seminaturales ha producido grandes cambios en el paisaje agrícola (Tscharntke et al. 2005; Baessler and Klotz, 2006). La reducción de los hábitats naturales genera un declive de la biodiversidad, que en última instancia afecta al CB de las plagas (Bianchi et al., 2006; Kleijn et al., 2009). En la cuenca del Ebro (Noreste de la Península Ibérica), el paisaje agrícola ha estado dominado tradicionalmente por cultivos extensivos manejados por la rotación de invierno y verano de cereal y alfalfa,

acompañado de pequeñas parcelas de frutales y de fracciones de hábitats seminaturales (Clemente-Orta et al., 2020). En los últimos años, debido a las demandas del mercado se ha producido una modificación en la composición del paisaje agrícola en esta región, basada principalmente en un incremento notable de la proporción de frutales de hueso (National Bureau of Statistics of Spain, 2017). Este incremento parece tener un impacto negativo sobre el CB en otros cultivos como la alfalfa o el maíz (Clemente-Orta et al., 2020).

Dentro de esta complejidad paisajística, se ha demostrado el importante papel que juegan los márgenes existentes entre los cultivos, así como otras infraestructuras ecológicas en la cuenca del Ebro (Albajes et al., 2011; Ardanuy et al., 2018). Sin embargo, también hay otros autores que defienden que la diversidad paisajística no afecta significativamente al CB (Martin et al., 2016; Rusch et al., 2016; Tscharntke et al., 2016; Landis, 2017; Karp et al., 2018). Por tanto, es necesario un conocimiento detallado del papel que juegan las infraestructuras ecológicas, tanto a escala local a nivel de parcela, como a escala global a nivel del paisaje (Avilla et al., 2009). Este conocimiento puede permitir un manejo del paisaje a largo plazo enfocado a diseñar infraestructuras apropiadas como la plantación de setos y plantas, o la conservación de hábitats seminaturales (Batáry et al. 2011; Kleijn et al. 2011), que favorezcan la presencia y la acción de los EN.

3- El cultivo de la alfalfa y del melocotón en la cuenca del Ebro

La alfalfa es una leguminosa perenne originaria de Asia central y Oriente próximo, que ha sido destinada desde tiempos ancestrales a la alimentación ganadera (Bolton et al. 1972). Actualmente se cultiva a nivel mundial, y es el cultivo forrajero más importante de España en cuanto a extensión, con 300.000 ha (Pons et al., 2011).

Según Nuñez (2002), en la cuenca del Ebro la alfalfa es un cultivo tradicional de regadío que se ha cultivado en rotación con el maíz y el trigo en parcelas de tamaño moderado, aunque ha ido perdiendo importancia con los años en detrimento de otros cultivos. Se recolecta a través del cortado de la planta, entre tres y seis veces al año, desde la primavera hasta el verano. Desde el punto de vista del control de plagas, el número de tratamientos insecticidas oscila entre tres anuales y un máximo de uno por corte (Nuñez, 2002).

Hay una gran variedad de insectos que habitan en la alfalfa y que pueden estar presentes en la cuenca del Ebro. Entre los que pueden ocasionar daños económicos considerables en la producción están *Smithurus viridis* L. (Collembola: Sminthuridae), que se alimenta de las hojas provocando manchas amarillas en ellas; y los pulgones *Acyrthosiphum pisum* Harris, *Aphis craccivora* Koch, *Therioaphis trifolii* Monell (Hemiptera: Aphididae), que causan daños directos que dificultan la cosecha, e indirectos por la trasmisión de diversos virus a la alfalfa (Pons et al., 2009). Otras especies, como *Hypera postica* Gyllenhal o *Sitona lineatus* L. (Coleoptera: Curculionidae), *Apion pisi* F. (Coleoptera: Brentidae), y *Colaspiderma atrum* Oliv. (Coleoptera: Chrysomelidae) causan daños en brotes y en hojas Nuñez (2002), así como el género *Liriomyza* (Diptera: Agromyzidae), que se considera una plaga menor, y que raramente causa daños en las hojas (Parrella and Keil, 1978; Pons and Nuñez, 2020).

El cultivo de la alfalfa, a diferencia de otros, está considerado como un reservorio de EN que actúa como dador de estos insectos, facilitando el establecimiento y desarrollo de determinados depredadores que pueden ayudar en el control de las plagas de los

cultivos adyacentes de manera natural (Nuñez 2002; Pons et al., 2005; Samaranayake and Costamagna, 2019; Sisterson et al., 2020). Hay estudios que muestran la presencia de un amplio rango de EN en la alfalfa en la cuenca del Ebro, entre los que destacan varios heterópteros de las familias Anthocoridae, Nabidae, Miridae y Lygaeidae; coleópteros de las familias Sthaphylinidae, Carabidae y Coccinellidae; dípteros de la familia Syrphidae; y neurópteros de las familias Chrysopidae y Hemerobiidae; además de algunas especies de parasitoides (Nuñez, 2002; Pons et al., 2005, 2009, 2011, 2013).

El melocotonero es un árbol de hoja caduca que pertenece a la familia Rosaceae, nativo de China. Según la Food and Agriculture Organization (FAO) (FAOSTAT, 2018), España fue el principal productor de melocotón en Europa en el año 2018, donde el 30% de la producción de España se concentraba en Cataluña, con una superficie de 21.000 hectáreas (MAPA, 2019).

Entre los insectos plaga que habitan en los cultivos de melocotón de la cuenca del Ebro destacan *Anarsia lineatella* Zeller (Lepidoptera: Gelechiidae) y *Grapholita molesta* Busck (Lepidoptera: Tortricidae), cuyas larvas provocan daños en brotes y frutos (Rothschild and Vickers, 1991; Avilla et al., 2009; Torá et al., 2010), así como *Ceratitis capitata Wiedemann* (Diptera: Tephritidae), la mosca mediterránea de la fruta, cuyas larvas se alimentan de la pulpa de la fruta y favorece la entrada de patógenos secundarios que desembocan en la pudrición de la fruta (Bergsten et al., 1999; Avilla et al., 2009). También se encuentran habitualmente varias especies de trips en esa zona, entre las que destacan *Frankliniella occidentalis* Pergande y *Thrips fuscipennis* Haliday (Thysanoptera: Thripidae) (Alford, 1984; Avilla et al., 2009; Torá et al., 2010), provocando daños principalmente en la flor, que posteriormente darán lugar a daños de tipo estético en la fruta. Aunque quizás los pulgones representan la plaga más importante en el cultivo del melocotón, tanto a nivel mundial como en la cuenca del Ebro. Las dos especies principales son *Myzus persicae* Sulzer y *Hyalopterus* spp. (Hemiptera: Aphididae) (Avilla et al., 2009; Dedryver et al., 2010; Torá et al., 2010; Barbagallo et al., 2017; Aparicio, 2019), que provocan daños en las hojas y decoloración de los frutos, además de trasmitir enfermedades como la Sharka (Isac et al., 1998; Katis et al., 2007; Barbagallo et al., 2017). *Asymetrasca decedens* Paoli (Hemiptera: Cicadellidae) también puede estar presente en el cultivo del melocotón en la zona, produciendo debilitamiento del árbol, aunque es considerada plaga secundaria (Alvarado et al., 1994; Torres et al., 2000, Torá et al., 2010).

A pesar del carácter permanente que representa una plantación de frutales, como la de melocotón, ésta proporciona unos recursos mucho más favorables para las plagas que para los EN, haciendo que las plagas estén asociadas al cultivo de una manera más permanente. Este hecho, junto con una estrategia de control de plagas basada casi en su totalidad en un control químico intensivo, ha llevado a la fauna útil a niveles poblacionales muy bajos (Avilla et al., 2009). Solo algunos estudios muestran la presencia de antocóridos, sírfidos y parasitoides como agentes de CB en la cuenca del Ebro (Avilla et al., 2009; Aparicio et al., 2019). A pesar del aumento de la superficie de cultivo del melocotón y de la importancia económica del cultivo en la zona (Clemente-Orta et al., 2020), aún faltan muchos aspectos importantes por clarificar en el CBC de este cultivo, como el rol de los EN y el papel de las infraestructuras ecológicas que forman parte del paisaje (Avilla et al., 2009).

4- El movimiento de insectos entre elementos del paisaje

En el contexto paisajístico de la cuenca del Ebro donde existe una coincidencia temporal de los cultivos extensivos, es conocida la capacidad del movimiento de algunos EN, como algunos coccinélidos, antocóridos y carábidos que se mueven de manera bidireccional entre los cultivos de maíz y de alfalfa (di Lascio et al., 2016; Madeira et al., 2014, 2019), o entre estos cultivos y los márgenes que se encuentras alrededor de ellos (Madeira et al., 2015). Sin embargo, dentro del mosaico de cultivos presentes en esta zona, el movimiento desde estos cultivos extensivos a los frutales o viceversa se desconoce completamente, así como desde o hacia los otros elementos del paisaje, como márgenes u otras infraestructuras ecológicas.

El movimiento de insectos en los agroecosistemas ha sido ampliamente estudiado, tanto desde hábitats naturales a cultivos (Rand et al. 2006), como entre cultivos adyacentes (Prasifka et al., 1999, 2004; Forbes and Gratton 2011). Para ello se han desarrollado una gran variedad de técnicas, desde métodos que utilizan diferentes tipos de tintas, los isotopos estables, los métodos serológicos de detección proteica, como el ELISA, hasta los métodos moleculares de detección de ADN (Reynolds et al., 1997, Lavandero et al., 2004, Agustí et al., 2020). Dependiendo del propósito del estudio, los insectos se pueden marcar de diversas maneras, comprendiendo desde el marcaje del propio insecto por contacto directo con elementos propios del medio, a elementos añadidos al medio que permitan la detección del marcaje (Reynolds et al., 1997). El marcaje también puede ser externo, es decir aplicado tópicamente sobre el insecto y detectado comúnmente de manera visual; o interno, para el cual se requiere de métodos de detección más sofisticados (Lavandero et al. 2004). Algunos de estos métodos de marcaje de los insectos nos permiten entender no solo su movimiento entre elementos del paisaje, sino también indirectamente conocer los recursos (animales o vegetales) de los que se alimentan. Por tanto, estas técnicas nos permiten una mejor compresión de las dinámicas ecológicas que se producen en los agroecosistemas.

Los métodos moleculares basados en la detección de ADN pueden llegar a jugar un papel fundamental en ese aspecto. Aunque todavía no existen muchos ejemplos de estudios de movimiento de insectos mediante análisis de ADN, recientemente se ha puesto a punto un nuevo método de marcaje de plantas con una solución de cistos de un pequeño crustáceo acuático (*Artemia* spp. (Anostraca: Artemiidae)) que vive exclusivamente en ecosistemas acuáticos salinos, y por tanto nunca se encuentra presente en ecosistemas agrícolas terrestres. Este marcaje tópico, seguido de un análisis mediante PCR convencional con cebadores específicos de este crustáceo, permite la detección de la presencia del ADN de *Artemia* sobre los insectos marcados, permitiendo así conocer su movimiento desde las plantas previamente marcadas (Agustí et al., 2020). Este método se ha utilizado en esta Tesis Doctoral para estudiar el movimiento de los insectos desde un cultivo de alfalfa a uno de melocotón (Capitulo 1).

En esta Tesis Doctoral se plantea además una nueva posibilidad de estudiar el movimiento a nivel de detección de ADN, que se basa en el estudio molecular de las interacciones tróficas. Esta metodología se aborda en los Capítulos 2, 3 y 4. La base de esos estudios ha sido la de considerar como marcador del movimiento la detección de los artrópodos y/o las plantas en el contenido estomacal de los depredadores recolectados en los cultivos diana (melocotón y alfalfa). La hipótesis planteada es que si las especies (artrópodos o plantas) ingeridas (y detectadas) no están presentes de manera habitual en estos cultivos, significa que el depredador analizado (recolectado en

el cultivo diana) debe provenir de una planta diferente a la del cultivo, indicando por tanto el movimiento del depredador analizado de esa planta al cultivo, pudiendo además saber de qué planta proviene sin necesidad de ningún tipo de marcaje. Además de la información que aporta esta metodología en referencia al movimiento del insecto, también aporta indirectamente una gran cantidad de información con relación a las interacciones tróficas que se dan en el agroecosistema estudiado, información de gran utilidad para la puesta a punto o la mejora de los programas de CB en estos dos cultivos.

5- Interacciones tróficas entre componentes del agroecosistema

La ecología trófica se basa en el estudio de los organismos y sus interacciones alimentarias en un ecosistema. Bajo una perspectiva de ecología funcional, se centra en los roles o funciones que desempeñan las especies en el ecosistema. Por tanto, la ecología trófica está estrechamente relacionada con las interacciones tróficas, y en última instancia con los servicios ecosistémicos. Estos se definen como la variedad de condiciones y procesos a través de la cual los ecosistemas y su biodiversidad confieren beneficios a la humanidad (Constanza et al., 1997; Daily, 1997).

Las interacciones tróficas se producen tanto a nivel de individuo como a nivel del ecosistema, incluyendo las interacciones depredador-presa, las redes tróficas y la comunidad ecológica (Siegenthaler et al., 2019). En el ámbito del CB, se considera una red trófica aquella que describe la relación entre los artrópodos plaga y los EN, así como otras relaciones tróficas, como las que se dan entre depredadores y presas alternativas que no son plaga, como las previamente observadas por Agustí et al. (2003), o la depredación intragremial ((IGP) *Intraguild Predation*)), como las observadas previamente por Gómez-Polo et al. (2015, 2016), que se dan entre EN en un ecosistema (depredador-depredador o depredador-parasitoide). Por otra parte, también se encuentran las relaciones tróficas depredador-planta, en el caso de que los depredadores sean omnívoros en mayor o menor grado. El estudio de las interacciones tróficas aporta información que puede contribuir a la selección de plantas que proveen de recursos a los EN, además de información acerca de la efectividad de los EN en el control de determinadas plagas.

La dificultad inherente a la observación directa, sumado a la complejidad de reproducir las interacciones tróficas que tienen lugar en el medio natural en condiciones de laboratorio, hace que su estudio sea especialmente complejo (Symondson, 2002). En el pasado, el estudio de las interacciones tróficas se llevó a cabo a través de observaciones directas de la depredación en campo en un principio. Posteriormente se realizó a través de la disección del abdomen del depredador y la caracterización morfológica de los restos semidigeridos presentes (Bjugstad et al., 1970; Holechek et al., 1982; Moreby, 1988). Debido a la complejidad de estos ensayos, también se han realizado multitud de ensayos de depredación en laboratorio, aunque en realidad no permiten evaluar la depredación real en situaciones naturales.

La utilización de métodos postmortem, basados en el análisis del contenido estomacal del depredador para detectar e identificar de los recursos consumidos, facilita enormemente este tipo de estudios. Diferentes técnicas moleculares se utilizan habitualmente hoy en día para estudiar las interacciones tróficas en agroecosistemas, como las basadas en la detección del ADN de la presa, la identificación de macromoléculas de ácidos grasos, o el análisis de isotopos estables (Agustí, 1998; Sydmonson, 2002; González-Chang et al., 2016; Nielsen et al., 2018). La utilización de la

PCR (*Polimerase Chain Reaction*) convencional en este tipo de estudios ha sido muy utilizada, y se basa en la amplificación mediante cebadores específicos para cada especie presa que se pretenda detectar (Agusti et al. 1998; Symondson, 2002). Es importante puntualizar que los estudios de detección de la presa ingerida mediante PCR convencional ofrecen una información cualitativa de la depredación, es decir indican presencia o ausencia de la especie diana que se pretende detectar en el digestivo del depredador, y no permite la cuantificación de esta ingestión. Aunque se han llevado a cabo estudios que utilizan la PCR a tiempo real (Gómez-Polo 2015; 2016), esta técnica no permite tampoco cuantificar, ya que no es posible discernir entre la cantidad de ADN ingerida y el tiempo transcurrido después de la ingestión, con lo que no se ha profundizado en este aspecto en este tipo de estudios.

La mayoría de estos estudios con PCR convencional abordan la detección de una especie plaga diana en concreto. En algunos estudios la presa objeto de estudio no era una plaga, sino una presa alternativa, y su detección en el depredador sirve para determinar su rol en el mantenimiento de estos depredadores en el cultivo (Agustí et al., 2003). La utilización de la PCR convencional para estudios en los que pueda existir un número elevado de presas diana en el ecosistema presenta una limitación importante debido a la gran cantidad de cebadores específicos que se deberían diseñar, y la gran cantidad de análisis independientes que se debería hacer. Es necesario realizar una PCR independiente para cada especie de presa, o bien diseñar una PCR multiplex que permita la detección de varias presas de manera simultánea. Aunque algunos estudios han abordado este tema (Traugott et al., 2006; Sint et al., 2014), éstos suponen una gran complejidad y una gran cantidad de PCRs multiplex a realizar cuando el número de presas presentes en un ecosistema es elevado. Aun así, nunca sería posible detectar todas las interacciones tróficas presentes en un ecosistema.

Para solventar estos problemas, en los últimos años se han puesto a punto estudios de HTS (*High-Throughput Sequencing*) o NGS (*Next Generation Sequencig*). Las plataformas HTS de secuenciación masiva o en paralelo son capaces de secuenciar simultáneamente cientos de miles de fragmentos de ADN, generando millones de secuencias con una extensión de decenas o cientos de gigabytes en un corto espacio de tiempo y con un coste cada vez más reducido (Kulski, 2016). Mediante la utilización de cebadores pretendidamente universales de artrópodos es posible detectar muchas de las especies de artrópodos ingeridas por un depredador polífago mediante un análisis metagenómico (Tablerlet et al., 2018). Estos análisis parten de muestras de origen ambiental (eDNA) para llegar a identificar los taxones presentes mediante metabarcoding.

Existen diversas plataformas HTS que funcionan con diferentes procedimientos de secuenciación, como Ion Torrent o Illumina (de segunda generación), o incluso otras posteriores, de tercera y cuarta generación, como PacBio y Nanopore, que permiten la secuenciación en tiempo real (Krishna et al., 2019). Estas plataformas y métodos de secuenciación difieren sustancialmente en los procedimientos de preparación de las muestras y en el tamaño de la generación de los datos. Las plataformas HTS han sido utilizadas para diversos fines, desde la secuenciación de genomas de seres vivos (bacterianos, humano, etc.) o el diagnóstico médico, a estudios ecológicos en diferentes tipos de ecosistemas (Soon et al., 2014; Goodwin et al., 2016; Heather et al., 2016; Levy and Myers, 2016; Kchouk et al., 2017; Taberlet et al., 2018; McCombie et al., 2019). El hecho que ha facilitado el uso generalizado de esta tecnología por parte de la comunidad científica ha sido la progresiva reducción del coste de los análisis. En los últimos años la

plataforma Illumina se ha consolidado como la más utilizada para estudios de ecología trófica, con estudios tanto en ecosistemas de agua dulce, marinos y terrestres; de microbiota del suelo o de huéspedes, así como en estudios del contenido estomacal o de heces (Taberlet et al., 2018). Recientemente se ha publicado algunos trabajos que estudian las interacciones tróficas en agroecosistemas (Gómez-Polo 2015, 2016; Aizpurúa et al., 2018; Sow et al., 2018, 2019, 2020). Estas técnicas pueden aumentar considerablemente el conocimiento de las relaciones tróficas presentes en un agroecosistema, e incluso mostrar relaciones tróficas desconocidas hasta el momento, por tanto, permiten un conocimiento detallado de las redes tróficas, y de la importancia de determinadas especies en el manejo de los agroecosistemas (Bohan et al., 2013), pudiendo ayudar a optimizar los programas de CB de plagas.

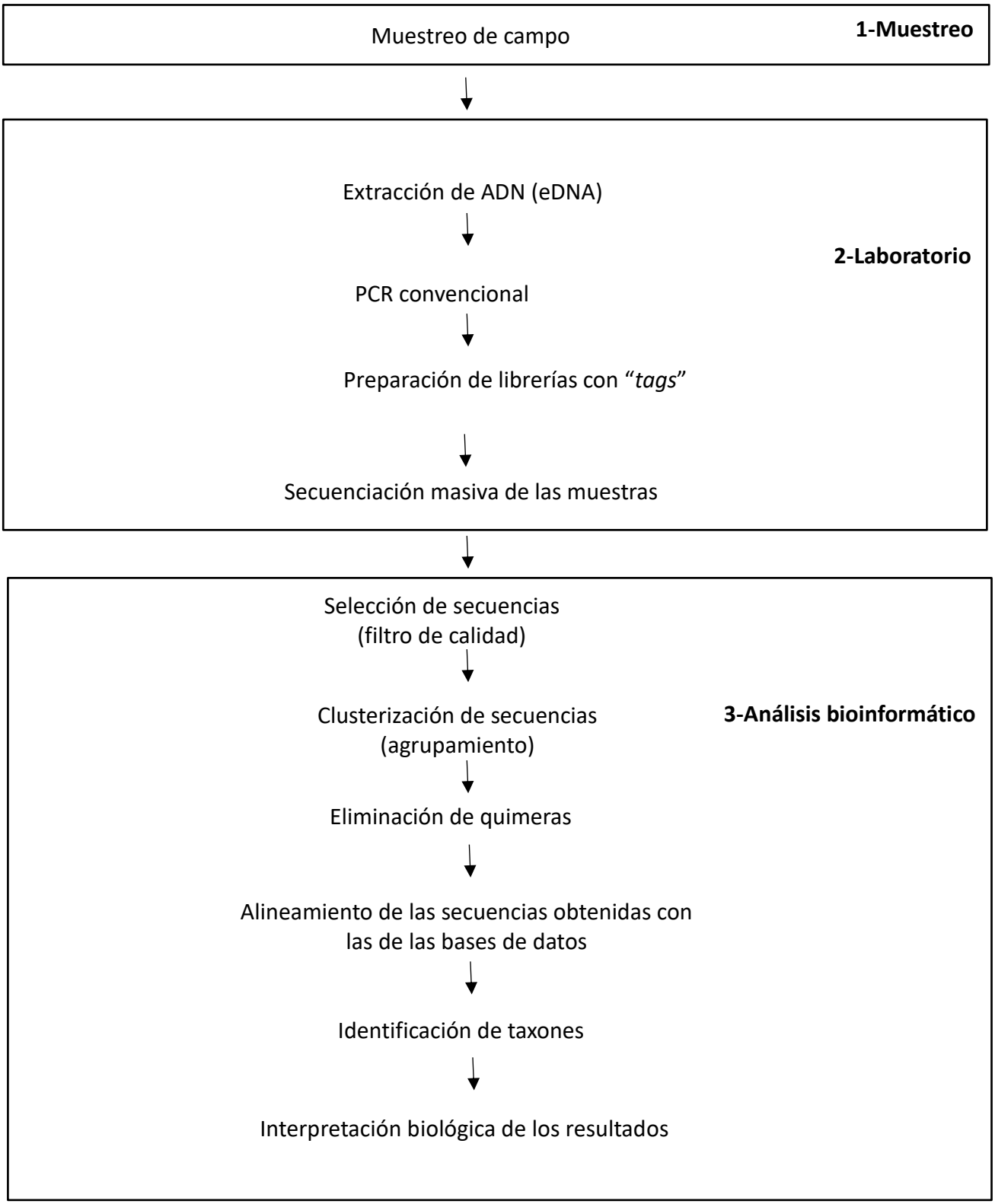
A pesar del gran potencial de estas técnicas HTS, éstas no están exentas de limitaciones. Una de ellas radica en la falta de disponibilidad de secuencias de todas las especies presentes en el ecosistema a estudiar (Galán et al., 2018). Aunque el número de especies representadas en las bases de datos, como NBCI, va aumentando cada día, es muy posible que no estén todas las especies secuenciadas, y si la secuencia no está disponible en la base de datos, o las disponibles contienen errores, no será posible identificar las especies mediante este método. Otro problema a tener en cuenta de estas técnicas es que, debido a su alta sensibilidad, pueden llegar a detectar taxones ingeridos por la presa ingerida, fenómeno conocido como depredación secundaria (Deagle et al., 2018; Da Silva et al., 2019).

La naturaleza de la información obtenida en los análisis HTS, tal como también ocurre con la PCR convencional es cualitativa. Tal como apunta Piñol et al. (2019), los diversos sesgos que se generan en el flujo de trabajo del análisis bioinformático, hacen que no sea posible una cuantificación del ADN a través de estas técnicas de secuenciación masiva. A pesar de ello, debido a que en este tipo de análisis se suele utilizar un número de muestras considerable, se han descrito varias métricas como el porcentaje RRA (*Relative Read Abundance*), o el porcentaje FOO (*Frecuency of Occurrence*) que nos sirven para dar una estimación de la importancia de cada taxon detectado (Deagle et al., 2018).

La utilización de las técnicas HTS conlleva un extenso flujo de trabajo (Fig. 1) que requiere de conocimientos en varias disciplinas, como la biología molecular, necesaria para desarrollar el análisis de las muestras en las plataformas HTS, y la informática, para el análisis de los miles de secuencias obtenidas en el proceso de secuenciación. El procedimiento de preparación de las muestras previo a la secuenciación masiva se realiza mediante la amplificación por PCR convencional del ADN diana mediante cebadores universales. Posteriormente tiene lugar la preparación de las librerías, en la que los fragmentos de ADN obtenidos a partir de cada muestra se marcan con cebadores de pequeño tamaño o "*tags*" para indicar la muestra de la que proceden. Posteriormente tiene lugar la secuenciación masiva de las muestras y su análisis bioinformático. Éste se realiza utilizando diferentes softwares y algoritmos ejecutados a través de lenguajes de programación como R, Python o Bash. Todo ello conforma la*pipeline* (conjunto de instrucciones informáticas para la ejecución de instrucciones de los softwares), que es la herramienta bioinformática con la que se lleva cabo el análisis de las secuencias en cada paso del flujo de trabajo. Esta *pipeline* comienza con el filtrado de las secuencias obtenidas en función de la calidad de las bases que conforman las secuencias; la clusterización o agrupamiento de las secuencias, que consiste en agruparlas en función

de su porcentaje de similitud; la eliminación de quimeras (artefactos creados a lo largo del proceso HTS que pueden provocar sesgos en la identificación de los taxones); el alineamiento de las secuencias obtenidas con las secuencias presentes en las bases de datos; la asignación taxonómica de esas secuencias; y por último la interpretación de los resultados, que requiere de conocimientos biológicos del área de estudio para la correcta asignación de los taxones (Tablerlet et al., 2018).

Figura 1. Esquema del flujo de trabajo en la utilización de técnicas HTS en estudios de metabarcoding.



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Objectives

Objetivo general

El objetivo general de esta Tesis Doctoral es el estudio del movimiento de artrópodos depredadores polífagos entre elementos del paisaje, ya sea entre cultivos, como desde áreas no cultivadas a los cultivos, para una futura mejora de los programas de CBC en cultivos de alfalfa y melocotón de la cuenca del Ebro.

Este objetivo se divide en dos objetivos específicos:

- **1**. Optimización de una técnica de marcaje tópico con un ADN externo al agroecosistema estudiado para el análisis por PCR convencional del movimiento de artrópodos depredadores entre dos parcelas contiguas de melocotón y de alfalfa (**Capítulo 1**).

- **2.** Puesta a punto y utilización de un método multi-primer HTS para estudiar el movimiento de artrópodos depredadores a través del estudio de sus interacciones tróficas (depredador-artrópodo y depredador-planta) presentes en dos parcelas contiguas de melocotón y de alfalfa.

  Este objetivo específico se divide en tres subobjetivos:

  - **I.** Puesta a punto de un método multi-primer HTS para el estudio de las interacciones tróficas más comunes de dos artrópodos depredadores de diferente morfología (*Anthocoris nemoralis* y *Ragonycha fulva*) con respecto a los artrópodos y a las plantas ingeridas (**Capítulo 2**).

  - **II.** Optimización del análisis bioinformático del método multi-primer HTS, para que la detección de las interacciones tróficas presentes en el agroecosistema estudiado permita incluir las relaciones intragremiales (IGP) entre depredadores del género *Orius* (**Capítulo 3**).

  - **III.** Estudio del grado de omnivoría de ocho especies de insectos depredadores (heterópteros y coccinélidos) presentes en el agroecosistema estudiado para detectar e identificar los recursos ingeridos **(Capítulo 4**).

Chapter 1

Molecular tracking of insect dispersal to verify arthropod predator movement from an alfalfa field to a peach orchard.

Abstract
Implementation of landscape approaches to conservation biological control programs requires the confirmation of putative sources that contribute to predator colonization of crops. This study aims to confirm predator dispersal from an alfalfa field to a neighboring peach orchard with a DNA mark-capture procedure based on a topical application of a solution of grinded brine shrimp cysts, *Artemia* spp. (Anostraca: Artemiidae), followed by a conventional PCR.
To optimize the marking procedure, a well-known predator present in orchards as well as in arable crops, *Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae), was used as a model in this study. In greenhouse trials, the acquisition and the retention time of the *Artemia* markings were determined, either directly by spraying them with the *Artemia* solution or indirectly via residual contact on caged plants after the spray. The topical mark remained detectable on *O. laevigatus* after 6 days, and 50% of the tested predators were positive 3 days after walking on the sprayed plants.
After that, a 25m^2 strip of an alfalfa crop neighboring to a peach orchard was sprayed with the *Artemia* solution just after the alfalfa cuts, and several common predator species were collected using sticky traps placed between both crops. After PCR analysis with the *Artemia* specific primers, 32% of the analysed predators (coccinellids, anthocorids, chrysopids, and mirids) showed the mark. The results of this study confirm the usefulness of this marking method to monitor dispersal of biological control agents between neighboring crops, in this case alfalfa and peach.

Key words: alfalfa crop, peach orchard, *Artemia* spp. cysts, mark-capture, PCR analysis, predator movemen

1. Introduction

Conservation Biological Control (CBC) represents a sustainable way to enhance naturally occurring Biological Control Agents (BCAs) to control crop pests (Eilenberg et al., 2001). This control strategy is based on the provision of food and shelter to BCAs, and field margins and flower strips are increasingly being used in order to enhance them (Landis et al., 2000; Aguilar-Fenollosa et al., 2011; Amaral et al., 2013; Pollier et al., 2018; Gontijo, 2019). Semi-natural habitats and crops are also important sources of BCAs, and their movement from crop to crop occur especially in agricultural areas with spatial and temporal heterogeneity. An increasing amount of research links landscape composition and configuration with pest and prey abundances in focal crops. Such results help to identify crop and non-crop habitats contributing to higher populations of target insects, particularly of key predators (Haan et al., 2020). However, there is no simple and consistent response of pest or natural enemy abundances to a landscape composition (Karp et al., 2018, Chaplin-Kramer et al., 2019). Samaranayake and Costamagna (2019) indicate the need to study the role that landscape habitats (i.e. crop fields surrounding the target field) play in contributing with BCAs, with studies that evaluate the movement of natural enemies between crops and other habitats. More specifically, landscape approaches to the development of CBC of arthropod pests requires the confirmation of the movement of predators between neighboring crops. Such information is important to implement IPM strategies.

In the Ebro Basin (NE Iberian Peninsula) cropping landscapes that were traditionally dominated by rotation of arable crops (alfalfa, maize and other cereals) have experimented a great increase of orchard production, specially peaches, resulting in a mixed mosaic of arable crops and orchards together with semi-natural habitats (Madeira et al., 2014, Clemente-Orta et al., 2020). According to the Food and Agriculture Organization (FAOSTAT, 2018), Spain was the main peach producer in Europe, with a 30% of the Spanish production concentrated in Catalonia (MAPA, 2019). The coexistence of annual and perennial crops could be advantageous if they share mutual natural enemies which disperse from one to another along the season, searching for refuges and prey. Alfalfa is known to act as a reservoir and source of many insect natural enemies in agricultural landscapes (Samaranayake and Costamagna, 2019; Sisterson et al., 2020). Several important predatory groups have been recorded in alfalfa in the area (*Orius* spp, mirids, nabids and coccinellids), that are shared with other arable crops (Pons et al., 2005, 2009). There are some shared pests with peach too, as the western flower thrips, *Frankliniella occidentalis* (Pergande), that could migrate to the orchards when cutting the alfalfa. Besides, peaches have other pests that cause the application of several pesticides for their control. Among them, the Mediterranean fruit fly, *Ceratitis capitata* Wiedemann; lepidoptera as *Grapholita molesta* Busck and *Anarsia lineatella* Zeller; aphids as *Myzus persicae* (Sulzer) and *Brachycaudus schwartzi* (Börner); and scales as *Diaspidiotus perniciosus* (Comstock) (Avilla et al., 2009).

A wide range of marking techniques have been developed to evaluate the dispersal patterns of arthropods (Lavandero et al., 2004; di Lascio et al., 2016; Madeira and Pons, 2016; Jiao et al., 2019; Kenne et al., 2019; Tavares et al., 2019; Hagler and Machtley, 2020). El Sheikha (2019) reviews the advantages and disadvantages of several tracking techniques. Among them, DNA gut content analyses, that are increasingly being used to identify prey or plant consumption by arthropods of agricultural importance (González-Chang et al., 2016), can also address the movement or dispersal of insects. Examples are

the gut content PCR analyses using specific primers of a particular insectary plant (Pumariño et al., 2011; Wang et al., 2017; Hayashi et al., 2020), using universal plant primers and sequencing (Wang et al., 2019; Avanesyan and Lamp, 2020) or the DNA analysis of microbial communities associated with insects (El Sheikha and Menozzi 2019).

Recently, a new marking method based on spraying plants with an aqueous solution of a grinded aquatic invertebrate (*Artemia* spp. (Anostraca: Artemiidae) that exclusively lives in saline waters, followed by a conventional PCR with specific primers for its DNA detection has been developed (Agustí et al., 2020). In that study, the movement of the mirid bug *Macrolophus pygmaeus* (Rambur) from a banker plant (*Calendula officinalis* L.) to the tomato crop was confirmed under greenhouse conditions. The aim of the present study was to further optimize that procedure and to apply it in open field commercial crops in order to track predator's movement between neighboring crops. This marking method was improved with added laboratory and semi-field experiments using *Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae) as a model. *Orius* spp. are known to be common in several crops in the growing area of Lleida, like alfalfa and maize. *Orius laevigatus* has been found in peach, apple, and pear (Sarasúa et al., 2000; Pons et al., 2005; Albajes et al., 2011). The improved marking method was then applied to confirm predator dispersal from an alfalfa crop to a neighboring peach orchard, and confirms the utility of this technique to identify the sources of beneficial insects that colonize crops.

2. Materials and Methods

The marking solution was prepared by grinding dry *Artemia* spp. cysts (Inve Aquaculture, Inc.), in order to make the *Artemia* DNA more accessible, and mixing them with water at a concentration of 0.1 gr/ml as explained in Agustí et al. (2020), except that Tween-20 (0.02%) was added to the solution as surfactant. The obtained *Artemia* solution was always used in the following 24h.

2.1 Efficiency of the marking on O. laevigatus.

The marking was topically applied to *O. laevigatus* adults in order to know whether it was also effective for marking a smaller predator than *M. pygmaeus* used in Agustí et al. (2020) (*O. laevigatus* 1.4-2.4mm and *M. pygmaeus* 3-6mm). Predators were purchased in Agrobío S.L. (Almería, Spain). To improve PCR detection, the addition of Tween-20 (0, 1 µl and 2.5 µl) to the marking solution was compared. PCR analysis were also tested just after spraying *O. laevigatus* dead adults with the *Artemia* solution, and after 24h of the spray (n=4-10 and n=14 adults, respectively) (Table 1.1). In addition, two different concentrations of the *Artemia* solution (0.1 gr/ml and 0.01 gr/ml) were tested (n=34 and n=20 adults, respectively). All individuals were analysed by conventional PCR, using the specific pair of primers of *Artemia* spp., as described in Agustí et al. (2020). Each specimen was DNA extracted using the Speedtools Tissue DNA Extraction Kit (Biotools, CA, USA) following the manufacturer protocol and using the whole body of the insect. The obtained DNA was eluted in 100 µl of elution buffer provided by the manufacturer and stored at -20 ºC. A negative extraction control was added to each set of DNA extractions.

2.2. Semi-field trials to study the extent of the marking

Acquisition and retention time of the DNA marker was tested after spraying the *Artemia* solution on alfalfa plants containing stationary (dead) or freely roaming (alive) individuals.

A first trial aimed to verify the effectiveness and persistence of the *Artemia* mark when spraying stationary *O. laevigatus* placed at two heights within the alfalfa plant canopy. The trial was arranged in a randomised complete block design consisting of three glasshouse compartments (4x6 m), each one with 10 closely placed pots (5L capacity) containing 4-5 alfalfa plants each. All plants were ca. 50 cm height. *O. laevigatus* cadavers (killed by freezing) were glued with their dorsum facing up on the upper side and on the lower side of a yellow sticky label (9 cm long and 2 cm wide). Nine individuals were glued on each side, in three sections of three individuals, one section for each sample date. Two labels were attached horizontally at two heights (at 25cm and 40cm from the top of the plant) on a wooden stick that was placed in the middle of each pot (Fig. 1.1). Overall, 1080 predators were exposed. Twenty-seven pots (9 per compartment) were sprayed with the *Artemia* solution (0.1 gr / ml plus the surfactant Tween-20 at 0.02% until run-off) with a commercial backpack sprayer (Matabi Super Green 16L, Goizper Spraying, Spain). Three other pots (one from each compartment) were sprayed only with water in another compartment and afterwards each one placed in each of the three compartments, as controls. The effectiveness of the sprays was assessed with water sensitive spray cards placed below the labels. Twelve hours after spraying, the outer section of all labels was cut and predators were individualised in 1.5 ml centrifuge tubes, labelled and frozen at -20ºC until PCR analysis. Similarly, the middle and the inner sections of each label were cut 3 and 6 days later, respectively. For the analysis, one adult was randomly chosen from the three corresponding to each sample date section.

A second trial aimed to verify the acquisition of the mark by alive *O. laevigatus* adults when walking on dry residues of the *Artemia* solution after spraying the alfalfa canopy. For this, eight pots of alfalfa were placed in a greenhouse compartment. Two of these pots were sprayed with water outside the compartment (control pots) and the other six were sprayed with the *Artemia* solution, as done in the previous test. When plants were dry, each pot was then covered with a fine mesh (eight threads/mm) sleeve cage, and 10 live adult *O. laevigatus* were released on each cage. The insects could roam freely on the alfalfa for 12 hours or 3 days. After each of those times, all insects of three sprayed pots and one control pot were collected. Each collected insect was individually placed in a clean 1.5 ml centrifuge tube and frozen at -20 ºC for further PCR analysis.

The results from the first trial were analysed with a generalised linear model (GLM) assuming a binomial distribution and logit function. The initial model included the proportion of marked individuals as dependent variable, and the factors height (middle, bottom), side (upper, lower), and time (12h, 3d, 6d) as well as all their interactions as predictors. Akaike's information criterion (AIC) by multi-model inference using the 'MuMIn' package (Bartoń, 2018), and analysis of deviance (with Chi-squared test) were used to compare fitted models and test the significance of predictor terms (Burnham and Anderson, 2002; Hastie and Pregibon, 1992). To ensure there was no violation of the normality and homoscedasticity assumptions, model residuals were graphically inspected with Q-Q plot, and a residual versus fitted values plot (Zuur et al., 2010; Crawley, 2013). For the second trial, the proportion of marked individuals obtained at two different times (12h and 3d) were analysed with a test of equal or given proportions

using the prop.test function (Newcombe, 1998). All data were analysed with R version 3.5.1) (R Development Core Team, 2018).

2.3. Field effectiveness of the marking method and PCR detection

The effectiveness of the DNA mark was finally tested under open-field conditions in a commercial alfalfa field (1.3 ha) adjacent to an organic peach orchard (2 ha) located in Vilanova de Segrià, Lleida, Spain (41º43'3"N, 0º37'7"E). Alfalfa plants were about 50 cm high at the time of study, which is when the crop was ready to be cut. As in other studies (Madeira and Pons, 2016), a strip of the alfalfa field (2.5 m width x 10 m long, and 2-4 m from the peach orchard margin) was sprayed with 8L of the *Artemia* solution 2h before being cut. The spray was done with a knapsack sprayer (Matabi Super Green 16L, Goizper Spraying, Spain). Effectiveness of the spray was assessed with water sensitive spray cards. After the spray, 10 (1st cut), and 20 (2nd and 3rd cut) unfolded Pherocon® Unbaited AM Yellow Sticky Traps (Trécé Inc., OK, USA) were placed between the alfalfa and the peach orchard. Traps, separated ca. 2 m between them, were placed at 60 and 80cm from the ground, in order to catch insects flying at different heights. Sticky traps were collected 24h (1st and 2nd cuts) or 3h (3rd cut) after being placed, and they were stored at 4ºC in a portable cooler. Once in the laboratory, predators collected on the sticky traps were picked up carefully, individualised in order to avoid cross-contamination, and stored at -20ºC. Finally, they were all analysed by PCR for the topical presence of *Artemia* DNA. The experiment was repeated three times, at the time of the cuttings of the alfalfa field (6th of July, 6th of August, and 6th of September).

Both crops were sampled during the experiments in order to determine key predators present in them. The alfalfa field was sampled before the spray with a sweep net. The branches of the peach trees that were facing the sprayed alfalfa strip were vigorously shaken in order to remove most of the predators present, both before the alfalfa cutting and after traps were removed.

Only adults from major aerial predator groups (Heteroptera, Coccinellidae and Neuroptera) were finally collected. Those predators were identified to family and species level when possible using taxonomic keys, except the *Orius,* which were identified using a molecular method previously developed (Gomez-Polo et al., 2013).

3. Results

3.1. Efficiency of the marking on O. laevigatus.

When testing the effect of adding Tween-20 as a booster in the PCR reactions of sprayed *O. laevigatus*, all tested individuals sprayed with the highest *Artemia* concentration (0.1 gr/ml) at t=0 were amplified, regardless whether Tween-20 was added or not (Table 1.1). However, with the lowest *Artemia* concentration (0.01 gr/ml), higher amplification percentages were obtained with the highest amount of Tween-20. Based on that, we tested the highest *Artemia* concentration (0.1 gr/ml) together with the highest Tween-20 amount (2.5 µl) after 24h of spraying, obtaining a 100% amplification (Table 1.1). From these results, this methodology was used in the following semi-field and field trials. No phytotoxic effects were observed on the alfalfa plants after being sprayed with the *Artemia* solution in any case.

3.2. Semi-field trials to study the extent of the marking

The first trial, conducted to verify the effectiveness and persistence of the *Artemia* mark on *O. laevigatus* placed at two different heights within the alfalfa plant canopy, indicated significant differences only regarding the sides of the labels, with a higher number of marked individuals (77-96 %) on the upper side (Fig. 2.1, Fig. 3.1, Table 1.1). Therefore, the efficiency of the spray in marking those predators was not affected by their location in the plant canopy (either 25cm or 40cm from the top of the plant), nor by the time lapse after spraying (12 h, 3 days or 6 days). Water sensitive spray cards also indicated that the sprays done with the knapsack sprayer had an effective coverage of the plant. The lowest percentages of marked insects were obtained on the lower sides of the labels, 3 and 6 days after the spray (ca. 15%).

The second trial conducted to verify the acquisition of the mark by adults of *O. laevigatus* when freely walking on dry residues of the *Artemia* solution after spraying the alfalfa canopy showed that they were able to self-mark in that way. From the 30 released adults, 80% of them were marked 12h after the spray. After 3 days 56.6 % were still marked. Although there was a major reduction in the efficiency of the mark, differences were not significative (Chi= 2.7728, df = 1, P-value = 0.09588). None of the control insects showed PCR amplification.

3.3. Field effectiveness of the marking method and PCR detection

When the effectiveness of the mark was tested in open-field, several predator species were captured on the yellow sticky traps. In total, 102 adult predators were collected in the sticky traps: 35 in the first cut (34 %), 47 in the second cut (45 %) and 21 in the third cut (21%), which belonged to the families Coccinellidae (61%), Anthocoridae (21%), Chrysopidae (12%), and Miridae (6%). Overall, 33 of them (32%) scored positive by PCR for *Artemia* DNA (Table 3.1), indicating that they had dispersed from the sprayed alfalfa strip to the peach orchard after the alfalfa cuttings.

From the three samplings conducted in the alfalfa field before the sprays, 372 predators were collected, comprising Coccinellidae (*Coccinella septempunctata* L., *Hippodamia variegata* (Goeze), *Propylea* sp., *Scymnus* sp., *Hyperaspis campestris* Herbst, *H. reppensis* (Herbst)), Cantharidae, Anthocoridae (*O. majusculus* (Reuter)*, O. laevigatus, O. minutus* L*., O. niger* (Wolff)*, Anthocoris nemoralis* (Fabricius)), Lygaeidae (*Nysius* sp.), Miridae and Aeolothripidae. From the intensive sampling of peach trees facing the sprayed alfalfa, 110 predators were collected. Seventy-five of them were collected before the spray: Coccinellidae (*Propylea* sp., *Oenopia conglobata* L., *H. variegata*, *Stethorus* sp., *Scymnus* sp.), Anthocoridae (*O. albidipennis* (Reuter)*, O. minutus, A. nemoralis*), Lygaeidae (*Nysius* sp.), Dermaptera, Chrysopidae and Syrphidae; and 35 after the spray, thus indicating that they may have moved from the nearby alfalfa: Coccinellidae (*O. conglobata*, *H. variegata*, *Stethorus* sp., *Scymnus* sp.), Anthocoridae (*O. majusculus, O. laevigatus, O. minutus, A. nemoralis*), Lygaeidae (*Nysius* sp.), Miridae and Dermaptera.

4. Discussion

Predator movement into crops is crucial to ensure pest control. The present study successfully validates a mark-capture method for dispersal studies, based on spraying a putative source habitat with a solution of the shrimp *Artemia* spp. and detecting its DNA by conventional PCR with specific primers, as previously proposed by Agustí et al.,

(2020). Our findings demonstrate that spraying such a DNA solution from a species that is not naturally present in the agroecosystem, is able to effectively mark several predator species within a range of very different insect families in an open-field environment.

When spraying stationary insects, more than 77% of those placed on the upper side of the labels were still marked after 6 days in a greenhouse, even when they were located at the bottom of the alfalfa plant canopy, indicating that the backpack sprayer provided a uniform coverage of the plant. The water sensitive spray cards confirmed this. Less individuals were marked on the lower side of the labels and the mark was also lost quicker. For this reason, it is of a great importance to try to spray both surfaces of the leaves when conducting this kind of marking experiments, in order to ensure a correct spray coverage and to reduce untreated parts of the leaves. In addition, at least 50% of those predators that could roam freely on previously sprayed plants were able to self-mark up to 3 days after the spray due to the contact with the residues, indicating that, under field conditions, it is more likely that more insects than those directly sprayed would be able to acquire the mark. On the other hand, it is also possible that some predators are self-marked by feeding on unbroken hydrated cysts of *Artemia*, even if it is expected to show a weak detection by this way, as already stated by Agustí et al. (2020). It is well known that *Artemia* cysts are accepted as prey by some predators, since they are used as supplemental food to sustain populations of several species when establishing in greenhouse crops (Castañé et al., 2006; Labbé et al., 2018; Seko et al., 2019). The fact that in our study the mark could last up to 6 days indicates that this technique is suitable to track local short-term dispersal into fields.

Exposure to direct sunlight in some parts of the plant canopy has been argued to be a cause for degradation of protein markers (Hagler et al., 2014) and it could also be the case with DNA. Nevertheless, in the greenhouse trial the mark persisted for 6 days on those insects located on the upper side of the labels with a high detection percentage (around 80% in both cases: top and middle height in the plant canopy), which was in principle more exposed to the UV light than the lower side of the label. Agustí et al. (2020) reported a similar persistence (6 days) of the *Artemia* solution when sprayed on the whitefly predator *M. pygmaeus* in tomato greenhouses during spring. In the present study, marked insects were also recovered from sticky traps placed in the ecotone between alfalfa and peach. In this case, sprays were conducted during summer months. The study area is a continental interior that features warm to hot dry summers, classified as a cold semi-arid climate (type BSk, Kottek et al., 2006). During the field trial days, mean temperatures and irradiation levels were high (33.6ºC; 22-30 MJ/m^2). Nevertheless, DNA persistence under those conditions was enough to ensure the marking of the insects.

This DNA mark–capture technique proved useful for uniquely tagging the predators inhabiting the alfalfa crop. Overall, 32% (n = 33 out of 102) of all the focal predators captured on the sticky traps showed the DNA mark. As expected, most of the trapped species (except *Stethorus punctillum* (Weise) and *O. albidipennis*) were also captured when sampling the alfalfa crop. In addition, most of those species (*P. quatordecimpunctata*, *H. variegata*, *Stethorus* sp., *Scymnus* sp., *O. albidipennis* and *O. minutus*) were also captured when sampling the peach orchard before the alfalfa cut, indicating that they are also part of the predator complex present in peach. After the alfalfa cuts, some predator species (*H. variegata*, *Scymnus*, *O. majusculus*, *O. laevigatus* and *O. minutus*) were collected again in the peach orchard, indicating that the trap

captures confirmed the immigration of common predators into the orchard. Those traps captured five coccinellid species and all of them were represented in the marked individuals. Most of them are aphidophagous, except *S. punctillum* that prey on mites. They are cosmopolitan and commonly found in arable crops (alfalfa and maize) as well as in orchards (de la Poza et al., 2005; Miñarro et al., 2005; Pons et al., 2005, 2009; Dib et al., 2010; Michaud, 2012; Markó et al., 2013; Zhou et al., 2014) which form the crop mosaic in the study area. They all are present in the Iberian Peninsula (Benhadi-Marin et al., 2011). Even if few surveys have been done in peach in the study area, *C. septempunctata* has been cited to be present (Celada, 2000), as well as in apple, where *Stethorus* and *P. quatuordecimpunctata* are common (Happe et al., 2019). *Coccinella septempunctata* and *P. quatuordecimpunctata* are important BCAs of many important aphid pests, but they can also survive feeding on other alternative prey (e.g. scales, psyllids, lepidopteran eggs or mites) and even on plant materials (e.g. pollen and fruits), moving between trees and herbaceous plants along the season (Hodek and Michaud, 2008; Omkar, 2011; Papachristos et al., 2015). The small *Scymnus* species are still poorly known, but recent papers address their importance also as aphid biocontrol agents (Sebastião et al., 2015). *Stethorus* species have been cited as predators of spider mites (Rott and Ponsonby, 2000; Ragkou et al., 2004), and *S. punctillum* has been also identified as an important predator in peach orchards (Ivancich, 1974).

Four species of *Orius* were also captured in the sticky traps and all of them had some marked individuals. *Orius* spp. are well known predators of thrips, while they can also feed on other pests including aphids, mites, whiteflies and lepidopteran eggs (Riudavets, 1995; Arnó et al., 2008; Atakan, 2010; Bán et al., 2010; Gomez-Polo et al., 2016), some of which can be important pests in peach orchards. All four species are common in several weeds and crops, including orchards (Brown and Schmitt, 2001; Bosco and Tavella, 2013; Pehlivan and Atakan, 2020). More specifically, *O. laevigatus* and *O. niger* have been recorded in peach orchards in the area of study (Avilla et al., 2009; Aparicio, 2019) and *O. minutus* also in peach orchards in France (Remaudière and Leclant, 1971). *Orius niger*, *O. minutus*, *O. majusculus* can be abundant in alfalfa (Pons et al., 2009; Bán et al., 2010) and *O. niger* plays a major role in controlling hemipteran pests in maize (Albajes et al., 2011). *Orius albidipennis* is an efficient predator of the thrips *F. occidentalis* (Blaeser et al., 2004), an important pest of peaches and nectarines. Other predators found in alfalfa in the area of study, as *A. nemoralis*, *O. majusculus* and *Nysius* sp. (Heteroptera: Lygaeidae) (Pons et al., 2005; Scaccini and Furlan, 2019), were not captured on the sticky traps, but they were collected in the sampling conducted on peach before and after the alfalfa cutting.

The fact that the same predators were collected both in the alfalfa field and the peach orchard indicate that both crops share a similar predator complex and highlights the importance of neighboring crops as a source of predators as BCAs. Our results confirm the contribution of the alfalfa field as a source of predators in the peach orchard, and that repetitive cuts of the alfalfa provided an influx of predators that should contribute to control peach pests. However, not only the alfalfa cuts trigger the dispersal of predators from alfalfa to the adjacent crops. In the area of study, a bidirectional movement of coccinellids (*C. septempunctata, P. quatuordecimpunctata, H. variegata*) and anthocorids (*O. majusculus, O. niger*) has been documented between arable crops (di Lascio et al., 2016; Madeira et al., 2014, 2019), and the same can also be expected between orchards and arable crops. Predator abundance in apple orchards (*Orius* spp.)

seems to depend on the proportion of extensive arable crops over the landscape (Whalon and Croft, 1986), which is also true for other orchards (Markó et al., 2017). Conversely, intensive pesticide applications in orchards have been related with a reduction of *C. septempunctata* abundance in neighboring maize fields (Clemente-Orta et al., 2020). Our results indicate that conserving beneficial fauna in alfalfa favors key predators in fruit orchards. The development of sustainable pest control practices together with a reduction in intensive pesticide applications in fruit orchards should therefore enhance the biological control functions in surrounding arable crops in such mixed landscapes (Markó et al., 2017; Clemente-Orta et al., 2020).

5. Conclusion.

This study proofs the efficacy of a novel DNA topical marking method to identify putative sources of predators colonizing crops and to study dispersal of arthropod species of agronomic interest under natural conditions. Spraying different habitats with different DNA solutions could provide unique tags (El-Sheikha and Menozzi 2019), which should make possible to trace captured insects to their sources and produce more accurate food webs of key predators. Such method offers prospects to be integrated with other molecular approaches in order to improve pest management strategies. For example, the DNA extraction of each predator can be further used to identify the ingested prey, thus determining the contribution of each predator species to the biological control of selected target crop pests (Moreno-Ripoll et al., 2012), or confirm the consumption of plant resources by omnivorous predators (Pumariño et al., 2011; Wang et al., 2017).

Table 1.1. Percentage (%) of PCR amplification of the *O. laevigatus* adults sprayed with two different concentrations (0.1 and 0.01 gr/ml) of the *Artemia* solution, regarding the time elapsed after the spray (h) and the amount of Tween-20 added in the PCR reactions (µl). The number of *O. laevigatus* tested in each case is also indicated (n).

Time (h)	Tween-20 (µl)	n	0,1gr/ml (%)	0.01gr/ml (%)
0	0	4	100	0
0	1	6	100	66.7
0	2.5	10	100	100
24	2.5	14	100	-

Table 2.1. Statistical parameters of the percentage of marked *O. laevigatus* adults by the *Artemia* solution after different times after spraying (12h, 3 days, 6 days) on two yellow sticky labels placed at different hights (middle= 25cm, bottom=40cm) from the top of the plant and in both sides of the label (upper, lower).

Factors	Degrees of freedom	Deviance	Residual Degrees of freedom	Residual Deviance	Pr(>Chi)
Time (12h, 3d, 6d)	2	5.041	9	129.135	0.08041
Height (middle, bottom)	1	2.937	8	126.198	0.08659
Side (upper, lower)	1	119.695	7	6.503	< 2e-16 ***

Table 3.1. Number of field-collected predators scoring positive by conventional PCR for the topical presence of *Artemia* DNA from the total number tested (N).

Family	Species	N	Nº Positives
Coccinellidae		**45**	**20**
	Propylea quatuordecimpunctata L.	21	8
	Coccinella septempunctata L.	1	0
	Hippodamia variegata Goeze	4	0
	Stethorus punctillum (Weise)	11	8
	Scymnus sp.	2	1
	Unidentified	6	3
Anthocoridae		**34**	**7**
	Orius niger Wolff	22	4
	Orius laevigatus (Fieber)	3	1
	Orius albidipennis Say	6	1
	Orius minutus L.	3	1
Miridae		**11**	**4**
Chrysopidae		**12**	**2**
TOTAL		**102**	**33**

Figure 1.1 Alfalfa pot with the two labels attached to a stick at two different heights in the plant canopy, and detail of a label. Dead *Orius laevigatus* were glued on both sides and on three sections of the label, each section to be cut after 12h, 3 days and 6 days after spraying.

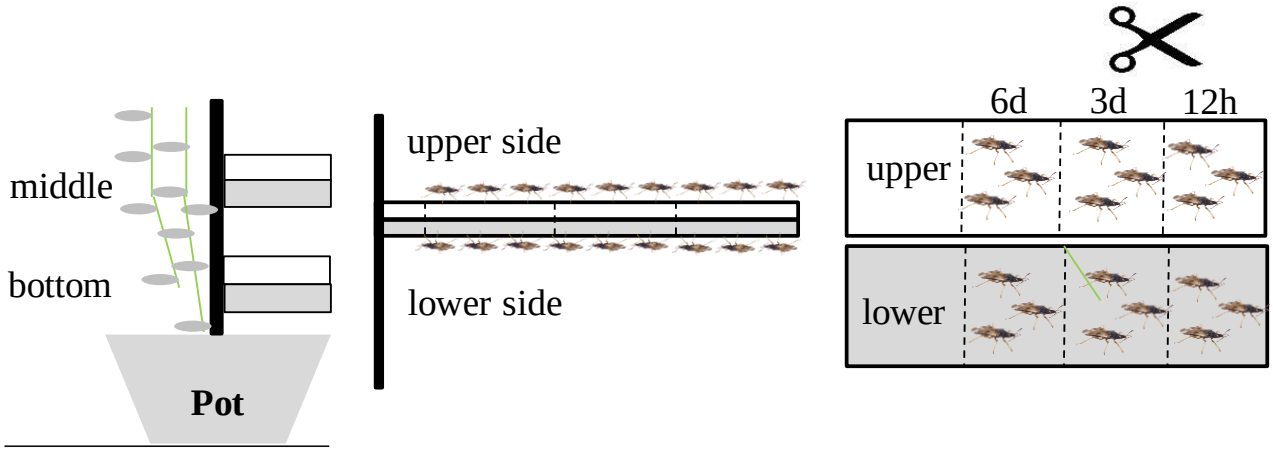


Figure 2.1. Percentage of *Orius laevigatus* individuals scoring positive by PCR for the presence of *Artemia* DNA. Dead adult insects were glued on both sides (upper/lower) of labels placed at two heights (25 cm (middle) and 40 cm (bottom) from the top) within the canopy of alfalfa. Plants were sprayed with the *Artemia* DNA solution, and predators were collected after 12h, 3 days and 6 days.

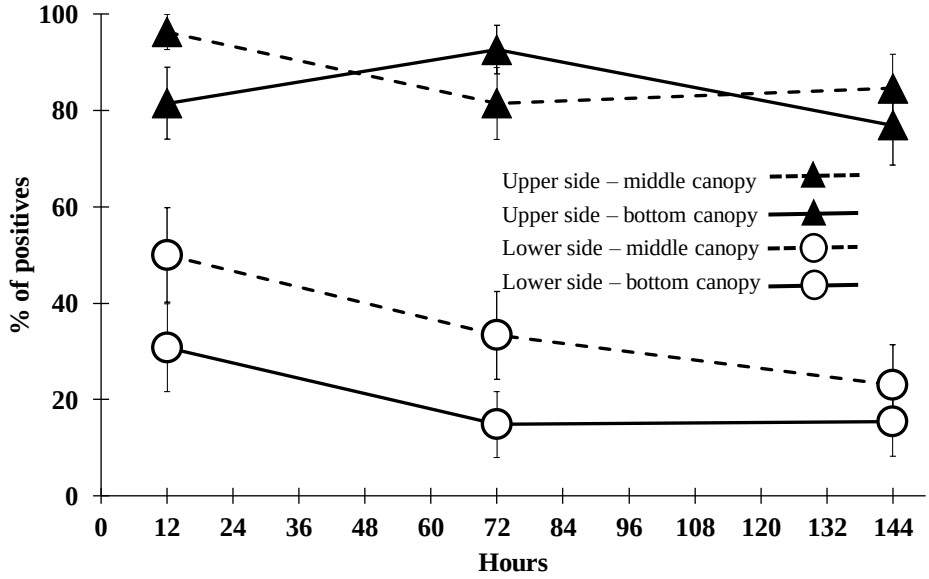
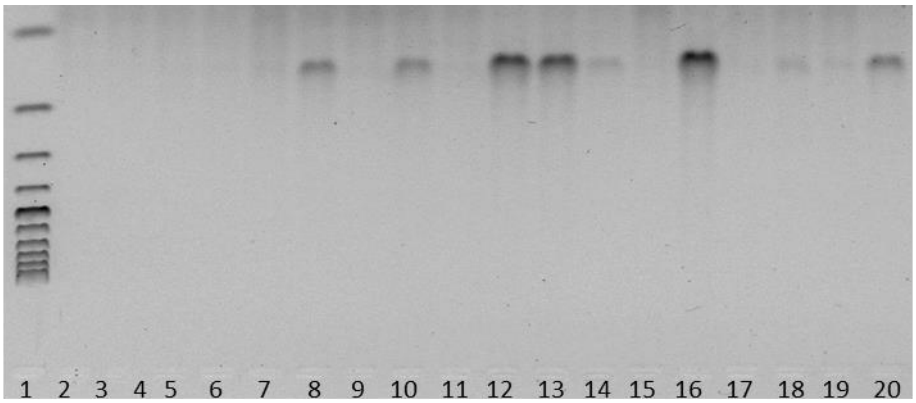


Figure 3.1. Agarose gel electrophoresis of amplified DNA from *Orius laeviatus* specimens tested in the semi-field trial by PCR using the *Artemia*-specific primers ARTF2 and ARTR3 (146 bp). Lane 1: 100bp molecular-size marker; lane 2: PCR negative control; lanes 3 to 11, *O. laevigatus* placed on the lower side of the labels; lanes 9 to 20, *O. laevigatus* placed on the upper side of the labels.



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Chapter 2

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

Abstract

Knowing which arthropod and plant resources are used by generalist predators in agroecosystems is important to understand trophic interactions and the precise ecological role of each predatory species. To achieve this objective, molecular approaches, such as the use of high-throughput sequencing (HTS) platforms are key. This study develops a multi-primer metabarcoding approach and explores its suitability for the screening of the most common trophic interactions of two predatory species of arthropod with contrasted morphology, *Rhagonycha fulva* (Coleoptera: Cantharidae) and *Anthocoris nemoralis* (Hemiptera: Anthocoridae) collected in an organic peach crop. To save time and cost in this metabarcoding approach, we first evaluated the effect of two different predator-pool sizes (10 and 23 individuals of the same species), as well as the performance of using one or 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 optimizes the HTS analysis. With these best-performing conditions, we analysed whole bodies of field-collected predators as well as the washing solutions used to clean the insect bodies. Results showed that we were able to identify both, gut content (i.e. diet) as well as external pollen load (i.e. on the insects' body), respectively. This study also demonstrates the need of washing predatory insects prior to HTS analysis when the target species have a considerable size and hairy structures. This metabarcoding approach has a high potential for the study of trophic links in agriculture, revealing both expected and unexpected trophic relationships.

Keywords: high-throughput sequencing, metabarcoding, molecular diet analysis, multi-primer approach, predatory arthropods, trophic interactions.

Introduction

The management of ecosystem services in agroecosystems is key for food production. One of these ecosystem services is pest control, carried out by natural enemies, such as insect generalist predators. Commonly, these beneficial insects do not only require prey as food, but they also need plant resources as food and/or as habitat supply (Demestihas et al. 2017). A more thorough understanding of how generalist insect predators use these resources in an agroecosystem is important to further utilize these predators in pest control programs.

Studying trophic interactions within an ecosystem is inherently difficult, because predation is an ephemeral process often difficult to visualize. Different methods have been used to measure insect predation, from their direct observation in the field, to the molecular analyses of their gut contents (Agustí et al. 2003; Pumariño et al. 2011; Nielsen et al. 2018). Molecular approaches to study predation increases the precision of the diet description (Nielsen et al. 2018), particularly with the use of high-throughput sequencing (HTS) platforms, which allow the detection of more realistic trophic interactions conducted in the field. Within these HTS (also called next generation sequencing or NGS) approaches, DNA metabarcoding, understood as the identification of organisms from a sample containing DNA from more than one organism, has been used to describe interactions in both terrestrial and aquatic ecosystems (Kennedy et al. 2020). Metabarcoding can be very helpful in agroecosystems, particularly for an initial screening of the gut content analysis of generalist predators (Pompanon et al. 2012), as already shown in few other cases (Piñol et al. 2014; Gomez-Polo et al. 2015, 2016; González-Chang et al. 2016).

DNA metabarcoding studies usually follow a well-established workflow that includes the DNA extraction often from the whole specimens, a PCR amplification with barcoded primers, high-throughput DNA sequencing, and a tailored bioinformatic analysis to obtain the desired taxonomic classification (Deagle et al. 2018). Nevertheless, recent literature highlights that several factors can affect the final result, indicating that certain technical aspects need to be improved (Lamb et al. 2019). These factors include the need for an external washing of the predator specimens to remove foreign external contamination (e.g. pollen grains) from their exoskeleton (Jones, 2012); the need for pooling samples, particularly when ingested DNA template is low; the use of biological replicates to obtain robust estimates of diet diversity and composition (Mata et al. 2019); the number of primer pairs used (Gibson et al. 2014); the availability of comprehensive reference databases with regards to the taxonomic groups of interest (Bohmann et al. 2011); or the use of different pipelines and data cleaning procedures during the bioinformatic analysis (Plummer et al. 2017). The use of more than one primer set has been previously recommended in order to minimize the effect of set biases and to recover a higher taxonomic coverage of the diet (Piñol et al. 2015; Krehenwinkel et al. 2017; Hajibabaei et al. 2019). With that in mind, we developed a new metabarcoding approach using two arthropod and two plant universal primer pairs per library to describe the main consumed taxa of predator diets by HTS, and we have tested it on two generalist insect predator species.

The main aim of this study was to explore the suitability of a multi-primer metabarcoding approach to provide a screening of the most common trophic interactions in the agroecosystem with pooled samples, whilst considering the reduction on time and cost

when field-collected predatory arthropod specimens have to be analysed. 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 Lleida region (NE Spain), as well as in other fruit and arable crops in the same area of study, like maize or alfalfa (Pons and Eizaguirre, 2000; Jauset et al. 2007). *Anthocoris nemoralis* is known as one of the most important biocontrol agents of the pear psyllids *Cacopsylla pyricola* (Foerster) and *Cacopsylla pyri* L. (Hemiptera: Psyllidae) (Agustí et al. 2003). However, this predatory species has also been described to feed on pollen (Naranjo and Gibson 1996). *Rhagonycha fulva* is mainly present in wooded agricultural landscapes and arable lands (Meek et al. 2002; Rodwell et al. 2018). Even if this species is mainly known to feed on pollen and nectar from umbellifers (Apiaceae) (Meek et al. 2002), it has also been cited as predator of some insect species (Pons and Eizaguirre, 2000; Rodwell et al. 2018). Nevertheless, its role as biocontrol agent is not well-known, as it is also the case for *A. nemoralis* in other fruit crops than pears, like peaches. The selected predator species are morphologically different regarding their potential to retain pollen grains on their body. *Rhagonycha fulva* is large (10-15 mm) and pubescent, particularly on its head and ventral side, while *A. nemoralis* is much smaller (3 mm) and glabrous. These different morphological characteristics make them good candidates to study pollen retention on their bodies, and therefore the need of washing them before HTS analysis.

In this study, we have investigated the effect of a variable sample-pool size on the range of prey taxa detected (taxonomic coverage); as well as the effect of using one or two primer pairs in the same library. We then validated the developed methodology by analysing the arthropod and plant diet of two small populations of *A. nemoralis* and *R. fulva*, two omnivorous insects with contrasted morphology. Plant and other arthropod DNA content in their washing solutions was also analysed as a mean to identify the pollen present on their body while foraging on diverse plants in the landscape.

Materials and Methods

Predator collection, cleaning and DNA extraction

Anthocoris nemoralis (n=42) and *R. fulva* (n=78) were collected by beating branches in a peach orchard in Vilanova de Segrià (Lleida), Spain (UTM 10x10: 31TCGO1) in June and July 2016, and May 2017, respectively. Each specimen was individualised in a DNA-free tube and placed in a portable freezer to avoid DNA degradation. Once in the lab, specimens were morphologically identified and stored at -20ºC until metabarcoding analysis.

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

The DNA of each insect specimen or washing solution was extracted using the Speedtools Tissue DNA Extraction Kit (Biotools, Germany; protocol for animal tissues). DNA from washing solutions was extracted with an additional disruption step using 0.15 g of 500–750 µm diameter glass beads (ACROS Organics™), and vortexed for 15 min at 50 Hz in a Gene2 vortex (MoBio Laboratories), for a suitable breakage of the potentially present pollen grains. Plastic pestles were used for whole insects instead. After the DNA extraction process, total DNA was eluted in 100 µl of AE buffer provided by the manufacturer and stored at −20°C. A negative control without 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 1.2.

PCR amplification, library preparation and sequencing

Two pairs of universal arthropod primers which partially amplify the mitochondrial Cytochrome Oxidase subunit I (COI) region were used to amplify 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 S1.2; Fig. S1.2), thereby avoiding competition for the same primer binding sites. Similarly, we used two pairs of universal plant primers also amplifying different amplicon sizes (ITS-S2F/ITS4R, 350 bp; and cA49325/trnL110R, 80 bp) (Table S1.2). In this case, primer pairs were chosen to amplify fragments in different regions, the first in the nuclear Internal Transcribed Spacer 2 (ITS2) and the second in the chloroplast *trnL* intron.

Sample-pools shown in Table 1.2 were amplified using a universal multi-primer approach with these four pairs of universal primers for arthropods and plants, performing one PCR with both pairs of arthropod primers, and another one 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 µM], 8 µL of free-DNA water and 15 µL of DNA of each sample-pool. PCR conditions used with the arthropod primers were: 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 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. Amplifications were conducted in a 2720 thermocycler (Applied Biosystems, CA, USA). Target DNA and DNA-free water were included in each PCR run as positive and negative controls, respectively. Resulting PCR products were purified with QIAquick PCR Purification kit (Qiagen), and 5 µl of each PCR product was used afterwards as template to prepare the libraries to be sequenced. HTS analysis was conducted in two batches (Table 1.2), 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 Nextera XT Index kit. Amplicons were purified with magnetic beads and 5 µl of each library were pooled and sequenced with a paired-end approach (2 X 225 bp).

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

Two different sample-pools of *A. nemoralis* were build: sample-pool 1, with 10 individuals; and sample-pool 2, with 23 individuals (Table 1.2, *Taxonomic coverage*). Only 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 in different libraries (L1, L2, L4 and L5). The effects of the sample-pool size (sample-pool 1 vs 2) and the use of one or both primer pairs together in the same library on the number of taxa obtained (taxonomic coverage) after HTS was compared using the non-parametric Kruskal-Wallis rank sum test. The statistical analyses were performed with 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 level of taxonomic resolution, we built a plant sample-pool with five plant species that are common in orchard ground covers and field margins of the study area (Table 1.2, sample-pool 3) (Ibáñez-Gastón, 2018; Juarez-Escario et al. 2010). In addition, to validate the accurate parameterization of the bioinformatic pipeline (Jusino et al. 2019), we included three positive controls containing only the crop plants (sample-pools 4-6). Unlike arthropods, plant samples (1 cm^2 leaf disc) were not washed prior to DNA extraction, which was conducted in the same way as for arthropod samples.

Analysis of field-collected predators

Field-collected predators were analysed with the multi-primer approach described above and using the most appropriate sample-pool size and number of primer pairs, according to the results of the previous *Taxonomic coverage* and *Plant primer resolution* trials. Four sample-pools were tested for *R. fulva* (Table 1.2, sample-pools 7-10) and two for *A. nemoralis* (sample-pools 11 and 12). In addition, five sample-pools were analysed in order to identify pollen load on the insects' body: four sample-pools from *R. fulva* washing solutions (sample-pools 13-16), and one from *A. nemoralis* washing solutions (sample-pool 17). In order to determine whether both predators only foraged on plants or also consumed plant resources, we compared the obtained plant taxa from their washing solutions with those obtained from their gut contents. Finally, in order to increase the amount of taxa detetected with the aim to show the highest diet diversity, we have considered each sample-pool of the same predator species as a different biological replicate, which provides greater variability than technical replicate for the taxa detected (Mata et al. 2019).

Bioinformatics

Raw Illumina reads were merged using VSEARCH 2.0 algorithm (Rognes et al. 2016), and then analysed using a restrictive strategy to reduce biases. The assembled reads were quality filtered using the FASTX-Toolkit tool (Gordon and 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 with 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 custom-made databases using BLAST 2.2.31+ (BLASTN, E-value 1e-10, minimum coverage of the query sequence: 97%, numbers of alignments: 9) (Camacho et al. 2009). The custom-made databases contained all arthropod and plant sequences present in the study area and available in the NCBI database (http://www.ncbi.nlm.nih.gov) at the moment of the analysis (October 2019). For this, we used 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 ≥97% identity by Last Common Ancestor algorithm (LCA) with BASTA (Kahlke and Ralph 2019). To remove possible contaminants from the OTUs assigned to different taxa for each group of primer pairs (arthropods or plants), we considered in the analysis only those OTUs that strictly had more than five reads and that had been detected in at least two sample-pools of the same species (Boyer et al. 2013). When the OTUs were obtained only in one sample-pool, they were used 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 plant or arthropod in each case. Obtained OTUs were then categorised as predator or prey based on their taxonomy.

To reduce other biases, such as secondary predation (an important limitation of HTS when studying food webs (da Silva et al. 2019)), and also with the aim of showing the most important taxa ingested, 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 considering 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, a filter to eliminate resources <1% of the amplified taxa was applied, as recommended by Deagle et al. (2018). This was applied for each primer pair in each library. With the taxa obtained, the second metric was calculated, which was the percentage of Frequency of Occurrence (FOO%), being the percentage of the total number of pools of each species analysed that contain a resource items obtained, indicating the most common resources consumed.

Results

The analysis of 33 libraries (120 predators and 20 sample-pools) conducted in two HTS batches (Table 1.2) generated 9,047,294 raw paired-end reads, 95% of which were successfully merged (Table 2.2, step 1). After that, 40,582 (step 2) and 53,286 reads (step 3) that did not match our quality and/or length requirements were discarded, as well as 2,512 chimera reads (step 5). After the taxonomic assignment (step 6), 1,548 arthropod and 649 plant OTUs were filtered (step 7 and Step 8). From the initial raw paired-end reads, only 8,930 (0.098%) came from the DNA extraction blank (sample-pool 20) and both PCR blanks (sample-pools 18 (batch 1) and sample-pool 19 (batch 2)) (Table 1.2). Those reads were eliminated at the step 7. After calculating RRA% and FOO% percentages and eliminating taxa with a number of reads lower than 1% (Table 2.2, step 8; Table S2.2), we finally obtained 299 arthropod and 206 plant OTUs (Table 2.2), which corresponded to 14 arthropod and 20 plant taxa (Table 3.2).

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

The six libraries analysed in this trial (Table 1.2, L1-L6), yielded 10 arthropod taxa (Table S3.2; Table 3.2). Besides the predator itself (*A. nemoralis*), we detected other anthocorids (*Orius* and *O. laevigatus* Fieber), other potential predator (Cecidomyiinae), as well as some pest (Aphididae, *Grapholita molesta* Busck*, Myzus persicae* Sulzer (Aphididae)*, Thrips fuscipennis* Haliday) and non-pest prey (*Diaphorina lycii* Loginova).

The number of arthropod taxa obtained was not significantly different between libraries mode of 23 or 10 *A. nemoralis* individuals (Kruskal-Wallis chi-squared = 0.78, df = 1, p-value = 0.37) (Table S4.2). Similarly, when comparing the number of arthropod taxa obtained using only one or two pairs of primers together in the same library, no significant differences were observed (Kruskal-Wallis chi-squared = 0.16, df = 1, p-value = 0.68) (Table S4.2). Therefore, in order to save 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 analysed in this trial (Table 1.2, L7-L10), yielded 11 plant taxa (Table S3.2; Table 3.2). Most of these taxa were expected because they were present in the composition of the sample-pools 3-6 (Table 1.2) (*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, like Streptophyta, which corresponds to a clade that show just plant DNA amplificated without additional taxonomic level information, and the families Fabaceae, Rosaceae, Convolvulaceae and Asteraceae, which were the families of the plant species of the sample-pools 3-6 (Table S3.2; Table 3.2). The genus *Trifolium* (Fabaceae) in the library L7 was also detected (Table S3.2). Nevertheless, it represented only 0.026% of the total reads obtained, and for this reason, it was not considered in further analysis.

Analysis of field-collected predators

The 17 libraries analysed in this trial (Table 1.2, L11-L27), yielded 28 taxa (14 of arthropods and 14 of plants (Table S3.2; Fig. 1)). Regarding the diet of *R. fulva* (Cantharidae), besides the predator itself, we detected three other arthropod taxa: *Nysius graminicola* Kolenati (Lygaeidae), *Cantharis livida* L. (Cantharidae) and Coccinellidae; and five plant taxa: Streptophyta, Convolvulaceae, Solanaceae*,* Fabaceae and Poaceae (Table S3.2; Fig. 1; Table 3.2). Regarding the diet of *A. nemoralis* (Anthocoridae), besides the predator itself, we detected 9 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 S3.2; Fig. 1.2).

We obtained amplification in two of the four libraries from the washing solutions of *R. fulva* analysed (Table 1.2, L23-L26) (Table S3.2). 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 S3.2; Fig. 1.2). No plant taxa were detected from *A. nemoralis* washing solutions (Table 1.2, L27; Table S3.2).

Discussion

Methodological issues

The present study addresses the challenge of developing a multi-primer approach for DNA metabarcoding analysis to disentangle the most common plants and arthropods resources ingested by field-collected omnivorous predators. The digestion process reduces the likelihood of detecting ingested DNA from gut or whole specimens. One way to improve PCR success in insect diet analyses is to increase the amount of DNA template by pooling individual specimens of the same species. Such pooling has been performed 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), and leads to the detection of most commonly ingested taxa (Mata et al. 2019). This strategy reduces cost and time, like other strategies, as the nested tagging, that have been also used in insect predation studies (Kitson et al. 2019). However, nested tagging can be highly sensitive to cross-contamination between the analysed samples and the control, introducing other biases avoidable with our approach.

Our first objective aimed to determine the effect of a variable sample-pool size (10 or 23 *A. nemoralis*) on the taxonomic coverage. As the number of taxa detected was not significantly different between both sample-pool sizes (Table S3.2, *Taxonomic coverage* trial), we conducted the *Analysis of field-collected predators* trial by pooling up to 26 individuals together in the same library, in order to save time and cost of the HTS run. Our second objective aimed to compare the performance of using either one or two pairs of primers in the same library. The use of one pair of primers per library is common practice in HTS studies for arthropod (Burgar et al. 2014) and plant-focused studies (Richardson et al. 2015). Here we showed the benefit of using two pairs of arthropod primers together in the same library. On one hand, no significant differences were observed in the number of taxa obtained in both cases in the *Taxonomic coverage* trial. On the other hand, the use of two primer pairs in the same library reduced the number of libraries by half, which consequently decreased the cost, as well as the time needed for the preparation of the libraries. For this reason, both arthropod and both plant pairs of primers were used in one library in the *Analysis of field-collected predator trial*.

When studying the diet of omnivorous species, a multi-primer approach is needed to characterize the full diet, and the choice of the primer pairs is key, because the richness of the taxa obtained depends on it (Hajibabaei et al. 2019). However, some aspects like the taxonomic coverage or the taxonomic resolution of each primer pair used, or their complementarity are not well known, despite their potential impact on the final results (Deagle et al. 2018; Corse et al. 2019).

Considering the three trials of the present study, we observed that the arthropod primer pair mlCOIintF/HC02198 amplified a slightly higher percentage of taxa, around 20% more than ZBJ-ArtF1c/ZBJ-ArtR2c in both batch (Fig. S2.2 (A)). Both arthropod primer pairs amplify a short fragment within the multicopy COI region, improving the detection of degraded DNA by the digestion process (Agustí et al. 2003). But, even if they amplify

fragments in the same region, both primer pairs have different primer binding sites (Fig. S1.2), which increases the chances to amplify different taxa (Table S3.2). As suggested by Piñol et al. (2015), this is probably due to the different number of template-mismatches of each arthropod primer pair for each taxon, because a high number of template-mismatches has a negative impact on the amplification efficiency, and reduces the number of amplified taxa. Seven arthropod taxa were detected when using ZBJ-ArtF1c/ZBJ-ArtR2c and 11 with mlCOIintF/HC02198 (Fig. S3.2 (A)). However, when both pairs of primers were used together, we were able to increase the detection rate up to 14 different arthropod taxa (only four of them were amplified by both primer pairs), showing a higher taxonomic coverage when using both primers instead of only one.

Both plant primer pairs were also selected to have different primer binding sites. The primer pair ITS-S2F/ITS4R amplifies a fragment of the nuclear ITS region, and cA49325/trnL110R of the chloroplast *trnL* region. The first was chosen because it is the most common region to identify mixed pollen loads from insects (Suchan et al. 2019), and the second one because it was recommended for the analysis of degraded DNA (Taberlet et al. 2007). Our results confirmed this statement, as a higher percentage of taxa was amplified with cA49325/trnL110R compared to ITS-S2F/ITS4R in both batch (Fig. S2.2 (B)), especially in the second batch where DNA was mainly ingested (Table 1.2). The number of plant taxa detected when considering both trials was 11 for each primer pair (Fig. S3.2 (B)). However, when using both primer pairs together, we detected 20 different plant taxa, showing a higher taxonomic coverage, as only three taxa were shared by both pairs of primers.

The use of two arthropod primer pairs that generate amplicons of different lengths allow discriminating between those sequences produced by each primer pair. This information was very useful to determine the taxonomic resolution obtained with each primer pairs. Considering all taxa obtained in this study, resolution of both arthropod primer pairs (ZBJ-ArtF1c/ZBJ-ArtR2c and mlCOIintF/HC0219) was mainly to species level (84.31% and 95.96%, respectively) (Fig. S4.2). On the other hand, resolution of both plant primer pairs (ITS-S2F/ITS4R and cA49325/trnL110R) was mainly to species level (81.82%) and to family level (81.91%), respectively (Fig. S4.2). These results corroborate those obtained in other studies using the same pairs of primers for metabarcoding and barcoding studies (da Silva et al. 2019; Suchan et al. 2019; Zhu et al. 2019). Such high-level resolution obtained with both arthropod primers and with ITS for plants increases the certainty of the obtained results (Biffi et al. 2017; McInnes et al. 2017; Deagle et al. 2018). Taxonomic should be a factor to consider in the selection of the primer pairs, particularly for plant primers, where the taxonomic resolution is more variable.

Trophic interactions

In this study, we assumed that plant DNA obtained from whole body extraction of cleaned insects came from their gut contents and corresponded to their diet. On the contrary, plant DNA retrieved from washing solutions is taken to represent visited plants, either from the pollen deposited on their bodies when foraging on them, or from walking on leaves with deposited pollen from anemophilous plants of the surrounding vegetation. We only detected plant DNA from the washed bodies of *R. fulva*, which are larger and hairier than *A. nemoralis.* The fact that we did not detect plant DNA from *A.*

nemoralis washing solutions indicates that it may not be necessary to wash such small and glabrous insects. Even if it is well known that anthocorids like *Orius* spp feed on plant resouces in laboratory conditions (Naranjo and Gibson 1996), no plant taxa were detected using the whole specimens either. If their most recent feeding episode was on arthropod prey, that may explain this result. Their small size and their sucking mouthparts, may also explain why no plant food was detected in this species, especially in comparison with *R. fulva*. Plant DNA was identified in only 30% of the analysed individuals of another predatory bug which were present on tomato plants in a greenhouse (Pumariño et al. 2011).

When analyzing the plant taxa ingested by *R. fulva*, we observed that they were all assigned to the Phylum Streptophyta or to a family (Convolvulaceae, Solanaceae, Fabaceae or Poaceae) (Table S3.2; Fig. 1.2), being Poaceae and Solanacea the most common detected taxa (Fig. S5.2 (A)). However, when analyzing their washing solutions, more OTUs were assigned to genera or to species level, possibly because plant DNA from pollen grains attached to the insects' body is not as degraded as the ingested one. These plant taxa indicate that *R. fulva* forages on a wide range of plants, like *O. europaea, D. glomerata, P. annua, B. vulgaris, Pinus* sp., *Sonchus* sp., one of their family (Asteraceae) and one of their order (Caryophyllales) (Table S3.2; Fig. 1.2). This diet is much more diverse than the single plant species cited by Rodwell et al. (2018), *Heracleum sphondylium* L (Apiaceae). The detected plant taxa can be present in ground covers of peach crops, field margins or alfalfa crops in the area of study (Juarez-Escario et al. 2010; Juarez-Escario et al.,2018; Solé-Senan et al. 2018), and some of them, like *D. glomerata, P. annua* and *M. sativa* belong to families that were also detected by ingestion (Poaceae and Fabaceae), which may indicate that their body was in contact with pollen from tassels or flowers while consuming it.

In the *Analysis of field-collected predators* trial, we have also demonstrated the efficacy of this multi-primer approach to detect and identify arthropods ingested by both predator species (Table S3.2; Fig. 1.2). Even if previous literature cites *R. fulva* as predator of some insects, such as aphids (Rodwell et al. 2018; Pons and Eizaguirre, 2000), our results indicate that this predator also consumed *N. graminicola*, because it was detected in 25% of the analysed *R. fulva* sample-pools (Fig. S5.2 (B)). *Nisius graminicola* is cited as an important pest of several summer crops in Italy, including peaches (Blando and Mineo, 2005). In Spain, another species of the same genus, *N. ericae,* has been described as secondary pest in peaches (Del Rivero and García-Marí, 1983), thus suggesting the potential of *R. fulva* as biocontrol agent. Our results also show that intraguild predation (IGP) by *R. fulva* on Coccinellidae and *C. livida,* is a very common trophic interaction (Fig. S5.2 (B)). It is well known that IGP is widespread in agroecosystems (Lucas and Rosenheim, 2011), and HTS has been successful at demonstrating IGP for example in field-collected predators in lettuce (Gomez-Polo et al. 2015, 2016). The IGP observed here should be further studied in order to know whether it could have a negative effect on the biological control of peach pests, because some coccinellids such as *C. 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 of the pear psylla (Solomon et al. 2000; Agustí et al. 2003). Our results 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 *M. persicae*, and the peach moth *G. molesta* (Fig. S5.2; Table S3.2; Fig. 1.2), information unknown until now. This predator also fed on *D. lycii,* an hemipteran species which 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 *A. nemoralis* must have moved from peach to those hedges to feed on this particular prey species and then back to the crop was it was collected. This result demonstrates how HTS analysis could also be used as a tool to understand predator movement, in this case from the peach crop to the surrounding vegetation and backwards. Finally, we also detected IGP in *A. nemoralis* (Fig. 1.2), which fed on several species coccinellid in the genus Orius. These included *O. conglobata,* a very common species in urban landscapes (Lumbierres et al., 2018), and *O. laevigatus*, a known biocontrol agent in vegetables (Gomez-Polo et al. 2015, 2016). The latter trophic interaction should be also taken in consideration in further biological control studies.
Four arthropod taxa were also detected in the diet of *A. nemoralis* analysed in the *Taxonomic coverage* trial (Table S3.2; Fig. 1.2), reinforcing its role as generalist predator. One of them was *T. fuscipennis,* which damages peaches during ripening (Tavella et al. 2006). Also detected, the subfamily *Cecidomyiinae* 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 predators known to be present in both crops in the area of study (Trandafirescu et al. 2004; Pons et al. 2009; Aparicio et al. 2020). Our results reinforce the role of *A. nemoralis* as potential biological control agent, which should be considered in further studies in peach orchards and alfalfa crops. This is especially important in the study area where both crops coexist and the movement of insects between them is very likely.

In this study, we have detected arthropod and plant resources ingested by two insect predators present in a peach crop by HTS analysis using a multi-primer approach. We have demonstrated that pooling predators in groups of 10 or 23 individuals has no significant influence on the analysis of their diet when analysed this way. We also showed that the use of two primer pairs improves the detection of ingested taxa, with an increased number of arthropod and plant taxa. Finally, we have shown that washing predators prior to HTS analysis is particularly needed for large insects with hairy structures, but may not be useful for small and glabrous ones. The developed multi-primer approach reduces time and cost of the HTS analysis and shows both expected and unexpected trophic relationships. The description of the most common trophic interactions by HTS with multi-primer approach could lead to an improvement of the biological control of pest species in agroecosystems, contributing to a more sustainable agriculture. The detection of a wider than expected range of ingested arthropod and plant items highlights the importance of keeping a diverse landscape composition in order to enhance the conservation of biological control agents in crops.

Table 1.2 Arthropod and plant sample-pools analysed by HTS in the three trials conducted in the present study. Also indicated the number of the HTS batch where each library was analysed, the number of individuals (or amount of plant material) in each sample-pool, and the primer pairs used in each library. ZBJ-ArtF1c/ZBJ-ArtR2c and mlCOIintF/HC02198 are the arthropod universal primers used, and ITS-S2F/ITS4R and CA49325/trnL110R are the plant universal primers 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
		Medicago sativa	1 cm^2	5	ITS-S2F/ITS4R; CA49325/trnL110R	L9
2		*Medicago sativa*	1 cm^2	6	ITS-S2F/ITS4R; CA49325/trnL110R	L10
	Analysis of field-collected predators	*Rhagonycha fulva*	26	7	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L11
					ITS-S2F/ITS4R; CA49325/trnL110R	L12
			24	8	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L13
					ITS-S2F/ITS4R; CA49325/trnL110R	L14
			23	9	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L15
					ITS-S2F/ITS4R; CA49325/trnL110R	L16
			5	10	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L17
					ITS-S2F/ITS4R; CA49325/trnL110R	L18
		Anthocoris nemoralis	26	11	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L19
					ITS-S2F/ITS4R; CA49325/trnL110R	L20
			16	12	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L21
					ITS-S2F/ITS4R; CA49325/trnL110R	L22
		Rhagonycha fulva washing solutions	26	13	ITS-S2F/ITS4R; CA49325/trnL110R	L23

			24	14	ITS-S2F/ITS4R; CA49325/trnL110R	L24
			23	15	ITS-S2F/ITS4R; CA49325/trnL110R	L25
			5	16	ITS-S2F/ITS4R; CA49325/trnL110R	L26
		Anthocoris nemoralis washing solutions	42	17	ITS-S2F/ITS4R; CA49325/trnL110R	L27
1	Blanks	PCR blank of batch 1	-	18	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L28
					ITS-S2F/ITS4R; CA49325/trnL110R	L29
2		PCR blank of batch 2	-	19	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L30
					ITS-S2F/ITS4R; CA49325/trnL110R	L31
		DNA Extraction blank	-	20	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L32
					ITS-S2F/ITS4R; CA49325/trnL110R	L33

Table 2.2 Number of reads and OTUs obtained at each step of the bioinformatic analysis. Data is presented in total and according to each arthropod and plant primer pair in each step of the bioinformatic analysis. NA = not applicable.

			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 removing	4,200,610	653,605	4,050	515,012	5,323	727,875	846	2,304,225	2,051
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 3.2 Summary table of all arthropod (n=14) and plant (n=20) taxa obtained after bioinformatic analysis of 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**

Figure 1.2 Interaction network of the arthropod and plant taxa detected from whole body extractions of *Ragonycha fulva* and *Anthocoris nemoralis*, as well as from the washing solutions of *R. fulva*.

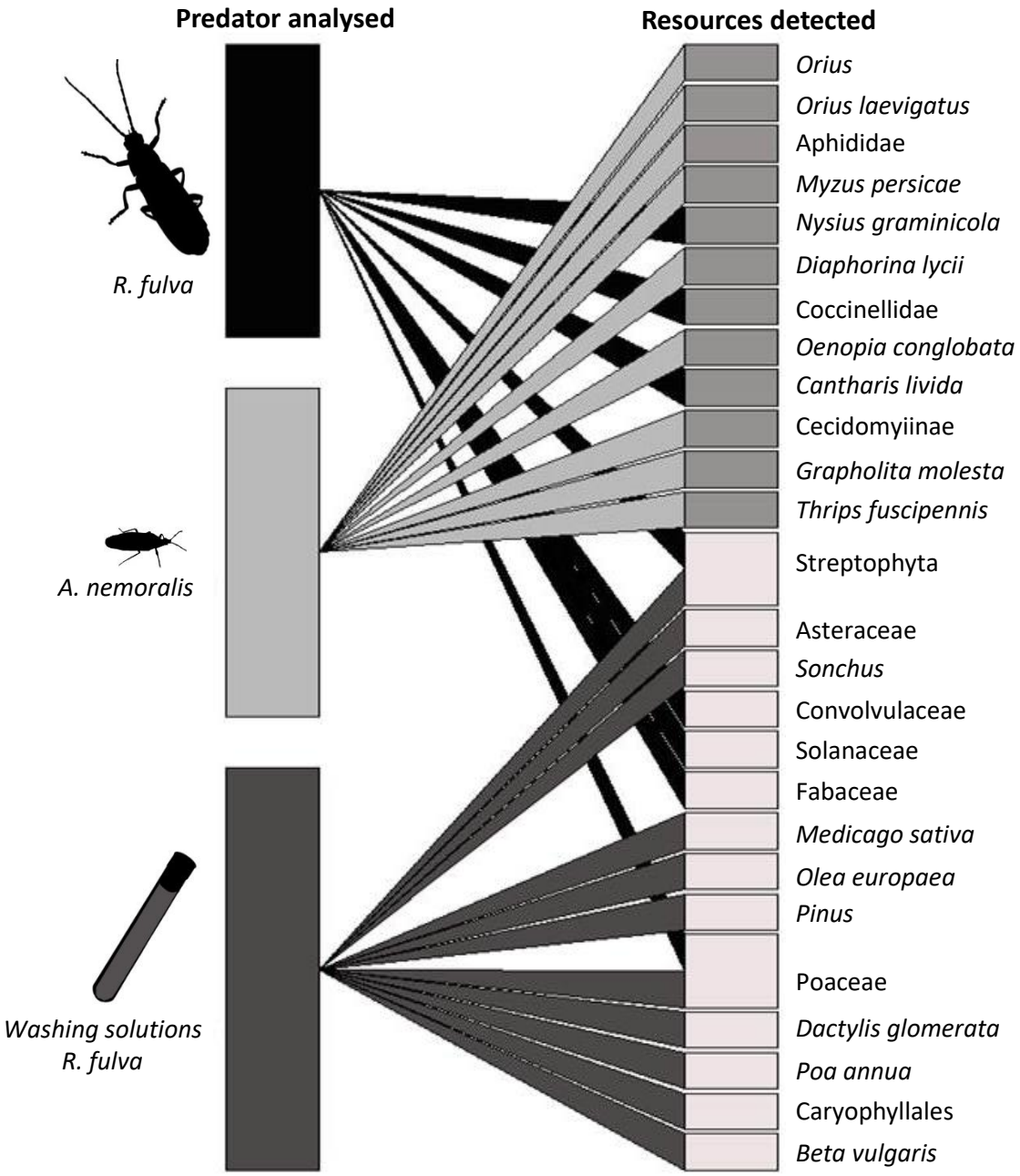


Table S1.2. Arthropod (ZBJ-ArtF1c/ZBJ-ArtR2c and mlCOIintF/HC02198) and plant (ITS-S2F/ITS4R and cA49325/trnL110R) primer pairs used in this study, indicating the sequence of each forward (F) and reverse (R) primer and the length of the amplified fragment.

	Sequence 5´- 3´(F)	Sequence 5´- 3´(R)	Reference	Fragment (bp)
ZBJ-ArtF1c/ZBJ-ArtR2c	AGATATTGGAACWTTATATTTTATTTTTGG	WACTAATCAATTWCCAAATCCTCC	Zeale et al. 2011	157
mlCOIintF/HC02198	GGWACWGGWTGAACWGTWTAYCCYCC	TAAACTTCAGGGTGACCAAAAAATCA	Leray et al. 2013/Folmer et al. 1994	313
ITS-S2F/ITS4R	ATGCGATACTTGGTGTGAAT	TCCTCCGCTTATTGATATGC	Chen et al. 2010/White et al. 1990	350
cA49325/trnL110R	CGAAATCGGTAGACGCTACG	GATTTGGCTCAGGATTGCCC	Taberlet et al. 2007/Borsch et al. 2003	80

Table S2.2 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.

(1)

	L1	**L2**	**L3**		**L4**	**L5**	**L6**	
	Art1	**Art2**	**Art1**	**Art2**	**Art1**	**Art2**	**Art1**	**Art2**
Orius	-	-	-	-	-	20,44	**-**	18,15
Orius laevigatus	-	-	-	-	22,72	**0,03**	22,19	**-**
Aphididae	-	-	18,17	**-**	28,69	**-**	28,74	**-**
Myzus persicae	-	21,92	-	27,16	-	31,71	**-**	35,67
Diaphorina lycii	-	1,57	-	1,33	-	1,36	-	1,09
Coccinellidae	**0,02**	-	-	-	**0,35**	**-**	**0,35**	**-**
Cecidomyiinae	2,18	**-**	1,93	**-**	3,91	**-**	3,67	**-**
Grapholita molesta	97,80	76,51	79,90	71,52	44,06	45,15	44,77	43,93
Thrips fuscipennis	-	-	-	-	**-**	1,32	**-**	1,16
Sitona discoideus	-	-	-	-	**0,27**	**-**	**0,28**	**-**

(2)

	L7		**L8**		**L9**		**L10**	
	Pl1	**Pl2**	**Pl1**	**Pl2**	**Pl1**	**Pl2**	**Pl1**	**Pl2**
Streptophyta	**-**	**0,17**	**-**	**-**	**-**	**-**	-	-
Asteraceae	**-**	39,94	**-**	**-**	**-**	**-**	-	-
Picris echioides	16,13	**-**	**-**	**-**	**-**	**-**	-	-
Convolvulaceae	**-**	**0,64**	**-**	**-**	**-**	**-**	-	-
Convolvulus arvensis	15,36	**-**	**-**	**-**	**-**	**-**	-	-
Fabaceae	**-**	2,29	**-**	**-**	**-**	99,97	-	99,94
Medicago_sativa	23,75	**-**	**-**	**-**	100	**-**	100	**0,05**
Trifolium	**-**	2,79	**-**	**-**	**-**	**-**	-	-
Setaria	5,11	34,19	**-**	**-**	**-**	**-**	-	-
Rosaceae	**-**	20	**-**	100	**-**	**0,03**	-	**0,004**
Prunus persica	39,65	-	100	-	**-**	-	-	

(3A)

	L11		L13		L15		L17		L19		L21	
	Art1	**Art2**	**Art1**	**Art2**	**Art1**	**Art2**	**Art1**	**Art2**	**Art1**	**Art2**	**Art1**	**Art2**
Orius	-	-	-	-	-	-	NA	-	-	-	-	18,32
Orius laevigatus	-	-	-	-	-	-	NA	-	-	-	21,09	-
Aphididae	-	-	-	-	-	-	NA	-	100	-	22,54	-
Myzus persicae	-	-	-	-	-	-	NA	-	-	24,39	-	24,29
Nysius graminicola	**0,06**	-	50	42,11	-	-	NA	-	-	-	-	-
Diaphorina lycii	-	-	-	-	-	-	NA	-	-	37,80	-	1,90
Coccinelidae	-	-	-	57,89	-	**0,31**	NA	100	-	-	**0,70**	-
Oenopia conglobata	-	-	-	-	-	-	NA	-	-	37,80	-	5,70
Cantharis_livida	99,94	100	50	**-**	100	99,69	NA	-	-	-	-	-
Cecidomyiinae	-	-	-	-	-	-	NA	-	-	-	5,00	-
Grapholita molesta	**-**	-	-	**-**	-	-	NA	-	-	-	50,68	39,76
Thrips fuscipennis	**-**	-	-	**-**	-	-	NA	-	-	-		10,04

(3B)

	L12		L14		L16		L18		L20		L22	
	Pl1	**Pl2**	**Pl1**	**Pl2**	**Pl1**	**Pl2**	**Pl1**	**Pl2**	**Pl1**	**Pl2**	**Pl1**	**Pl2**
Streptophyta	NA	-	-	13,04	NA	-	NA	22,29	NA	NA	NA	NA
Convolvulaceae	NA	-	-	56,52	NA	-	NA	33,76	NA	NA	NA	NA
Solanaceae	NA	37,65	-	6,52	NA	-	NA	35,03	NA	NA	NA	NA
Fabaceae	NA	62,35	-	**-**	NA	47,37	NA	-	NA	NA	NA	NA
Poaceae	NA	-	100	23,91	NA	52,63	NA	8,92	NA	NA	NA	NA

(3C)

Libraries	**L23**		**L24**		**L25**		**L26**		**L27**	
Primers pairs	**Pl1**	**Pl2**	**Pl1**	**Pl2**	**Pl1**	**Pl2**	**Pl1**	**Pl2**	**Pl1**	**Pl2**
Streptophyta	-	63,95	-	6,11	-	86,48	NA	NA	NA	NA
Asteraceae	-	7,08	-	81,22	-	4,44	NA	NA	NA	NA
Sonchus	12,75	-	-	-	28,41	-	NA	NA	NA	NA
Medicago sativa	1,24	-	-	-	2,21	-	NA	NA	NA	NA
Olea europaea	5,17	-	-	-	6,09	1,25	NA	NA	NA	NA
Pinus	-	23,01	-	-	-	-	NA	NA	NA	NA
Poaceae	28,33	4,43	-	9,61	41,70	7	NA	NA	NA	NA
Dactylis glomerata	4,30	-	-	-	4,06	-	NA	NA	NA	NA
Poa annua	19,88	-	-	-	3,69	-	NA	NA	NA	NA
Caryophyllales	-	1,53	-	3,06	-	**0,83**	NA	NA	NA	NA
Beta vulgaris	28,33	-	-	-	13,84	-	NA	NA	NA	NA

Table S3.2. Taxa obtained and primer pairs used in each trial and from each library. NT= not tested; NAmp = not amplified

Trial	Species/sample	# of individuals/ Sample size	Library	Assigned taxa with each arthropod primer pair		Assigned taxa with each plant primer pair	
				ZBJ-ArtF1c/ZBJ-ArtR2c	mlCOIintF/HC02198	ITS-S2F/ITS4R	CA49325/trnL110 R
Taxonomic coverage	*Anthocoris nemoralis*	10	L1	Aphididae Cecidomyiinae *Grapholita molesta*	NT	NT	NT
			L2	NT	*Anthocoris nemoralis* *Myzus persicae* *Diaphorina lycii* *Grapholita molesta*	NT	NT
			L3	Aphididae Cecidomyiinae *Grapholita molesta*	*Anthocoris nemoralis* *Myzus persicae* *Diaphorina lycii* *Grapholita molesta*	NT	NT
		23	L4	*Orius laevigatus* Aphididae Cecidomyiinae *Grapholita molesta*	NT	NT	NT
			L5	NT	*Anthocoris nemoralis* *Orius* *Myzus persicae* *Diaphorina lycii* *Grapholita molesta* *Thrips fuscipennis*	NT	NT
			L6	*Orius laevigatus* Aphididae Cecidomyiinae *Grapholita molesta*	*Anthocoris nemoralis* *Orius* *Myzus persicae* *Diaphorina lycii* *Grapholita molesta* *Thrips fuscipennis*	NT	NT

Plant primer resolution	*Medicago sativa* *Prunus persica* *Convulvulus arvensis* *Picris echiodies* *Setaria pumila*	1 cm^2	L7	NT	NT	*Picris echioides* *Convolvulus arvensis* *Medicago sativa* *Setaria* *Prunus persica*	Streptophyta Asteraceae Convolvulaceae Fabaceae Trifolium *Setaria* Rosaceae
	Prunus persica	1 cm^2	L8	NT	NT	*Prunus persica*	*Rosaceae*
	Medicago sativa	1 cm^2	L9	NT	NT	*Medicago sativa*	Fabaceae *Rosaceae*
	Medicago sativa	1 cm^2	L10	NT	NT	*Medicago sativa*	Fabaceae Rosaceae
Analysis of field-collected predators	*Rhagonycha fulva*	26	L11	*Rhagonycha fulva* *Cantharis lívida*	*Rhagonycha fulva* *Cantharis lívida*	NT	NT
			L12	NT	NT	NAmp	Fabaceae Solanaceae
		24	L13	*Rhagonycha fulva* *Nysius graminicola* *Cantharis lívida*	*Rhagonycha fulva* *Nysius graminicola* Coccinellidae	NT	NT
			L14	NT	NT	Poaceae	Streptophyta Convolvulaceae Solanaceae Poaceae

		23	L15	*Rhagonycha fulva* *Cantharis lívida*	*Rhagonycha fulva* *Cantharis lívida*	NT	NT
			L16	NT	NT	NAmp	Fabaceae Poaceae
		5	L17	*Rhagonycha fulva*	*Rhagonycha fulva* Coccinellidae	NT	NT
			L18	NT	NT	NAmp	Streptophyta Convolvulaceae Fabaceae Poaceae
	Anthocoris nemoralis	26	L19	Aphididae	*Diaphorina lycii* *Myzus persicae* *Oenopia conglobata* *Anthocoris nemoralis*	NT	NT
			L20	NT	NT	NAmp	NAmp
		16	L21	Aphididae *Grapholita molesta* *Orius laevigatus* Cecidomyiinae	*Anthocoris nemoralis* *Diaphorina lycii* *Grapholita molesta* *Myzus persicae* *Oenopia conglobata* *Orius* *Thrips fuscipennis*	NT	NT

			L22	NT	NT	NAmp	NAmp
	Rhagonycha fulva *washing solution*	26	L23	NT	NT	*Sonchus* *Medicago sativa* *Olea europaea* *Dactylis glomerata* Poaceae Poa annua Beta vulgaris	Streptophyta *Asteraceae* *Pinus* Poaceae Caryophyllales
		24	L24	NT	NT	NAmp	NAmp
		23	L25	NT	NT	*Sonchus* *Medicago_sativa* *Olea_europaea* *Dactylis_glomerata* Poaceae *Poa annua* *Beta vulgaris*	Streptophyta Asteraceae Poaceae Caryophyllales
		5	L26	NT	NT	NAmp	NAmp
	Anthocoris nemoralis *washing solution*	42	L27	NT	NT	NAmp	NAmp

Table S4.2. 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.

(A)

Library	# of individuals per library	# of arthropod taxa
L1	10	2
L2	10	4
L3	10	7
L4	23	4
L5	23	6
L6	23	10

(B)

Library	# of primers per library	# of arthropod taxa
L3	2	7
L6	2	10
L1, L2	1	6
L4, L5	1	10

Table S5.2. 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.

	mlCOIintT/HC02198		ZBJ-ArtF1c/ ZBJ-ArtR2c	
Taxon	**Forward primer (mlCOIintT)**	**Reverse primer (HC02198)**	**Forward primer (ZBJ-ArtF1c)**	**Reverse primer (ZBJ-ArtR2c)**
Anthocoris nemoralis	0	0	4	5
Aphididae	1	NS	3	2
Cantharis livida	0	NS	1	2
Cecidomyiinae	0	NS	1	1
Coccinellidae	1	3	3	3
Diaphorina lycii	1	3	10	9
Grapholita molesta	1	3	0	0
Myzus persicae	2	2	3	3
Nysius graminicola	1	2	0	0
Oenopia conglobata	0	NS	12	2
Orius	2	4	3	3
Orius laevigatus	0	NS	2	0
Rhagonycha fulva	1	NS	2	2
Thrips fuscipennis	1	NS	8	12

Figure S1.2. Location of the COI primer pairs tested in the present study.

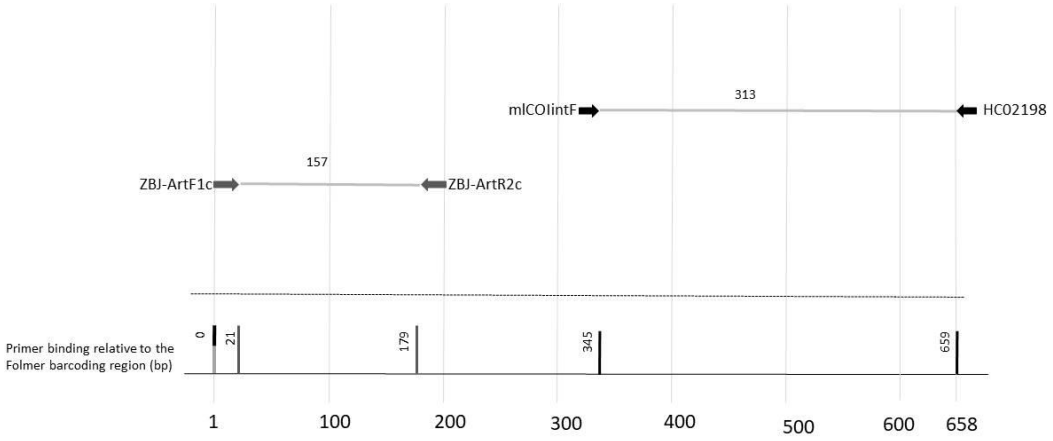


Figure S2.2. Percentage of taxa obtained for each pair of arthropod (A) and plant (B) primers amplified in all libraries, in each HTS batch.

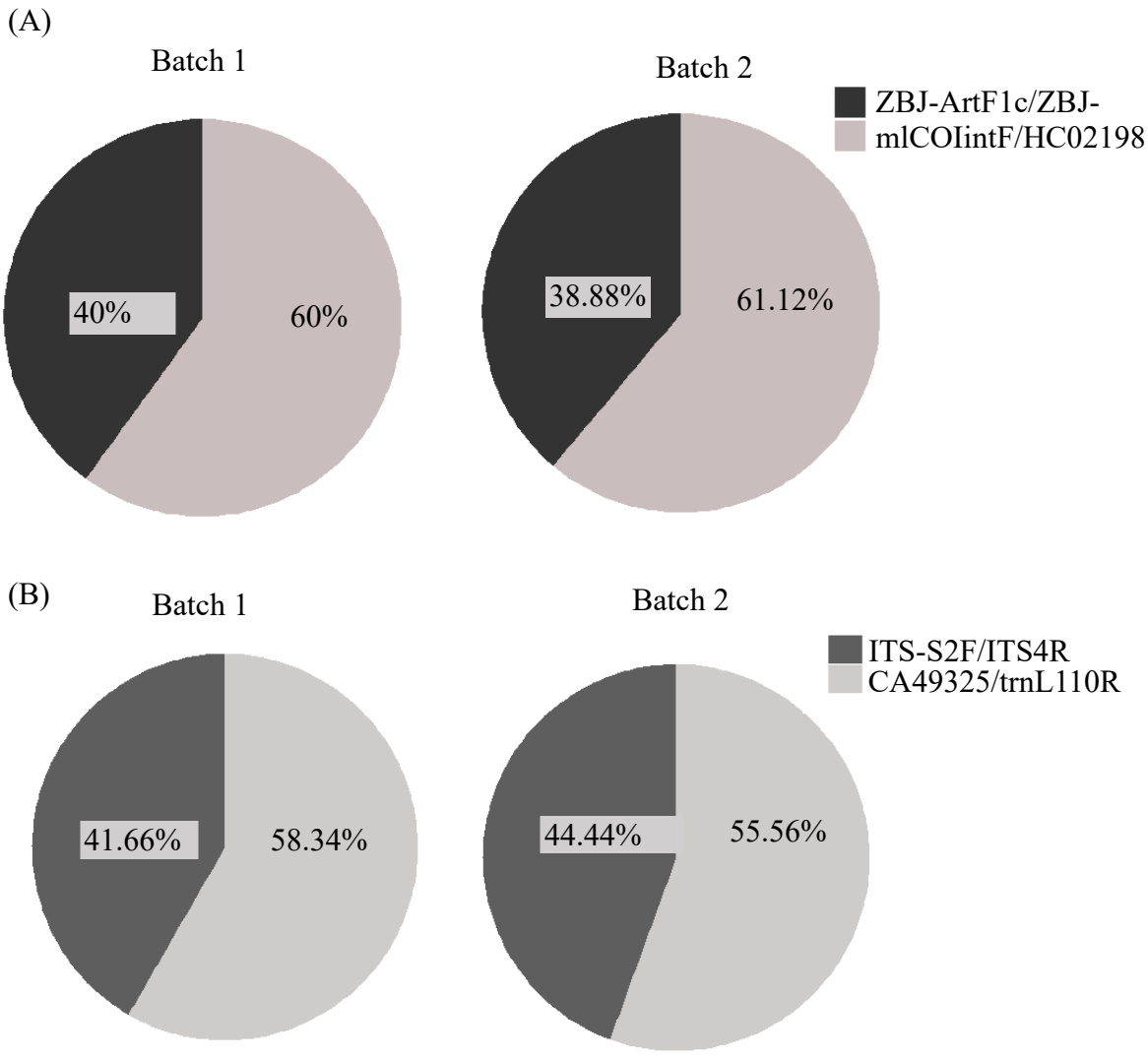
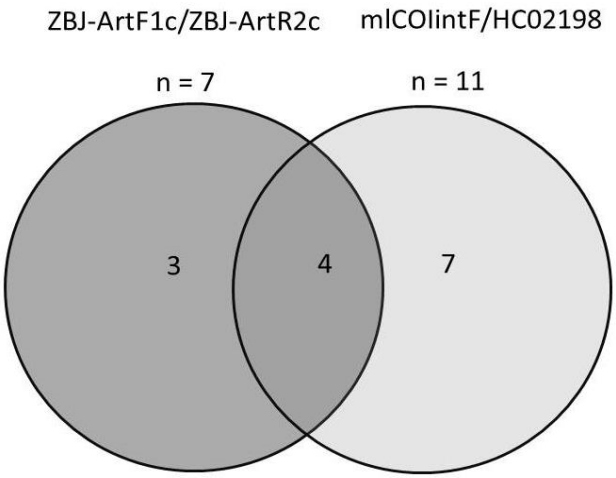


Figure S3.2. Representation by Venn´s diagrams of the artropod and plant taxa obtained by HTS (including all trials) with each primer pair. Numbers in each circle indicate the number of taxa amplified by each primer and how many are shared by both pairs of primers (overlaping area). (A) artropod primers; (B) plant primers. n = Number of taxa obtained with each primer pair.

(A)



(B)

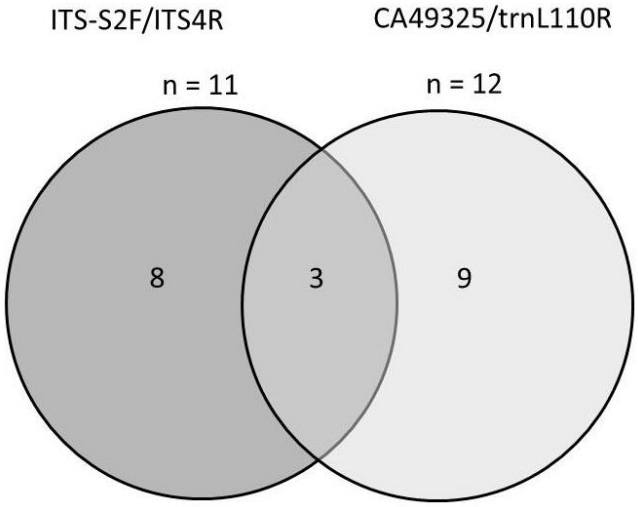


Figure S4.2. Accuracy of the taxonomic assignation according to the primer pair used. Data is presented as the percentage OTUs assigned to each taxonomic level.

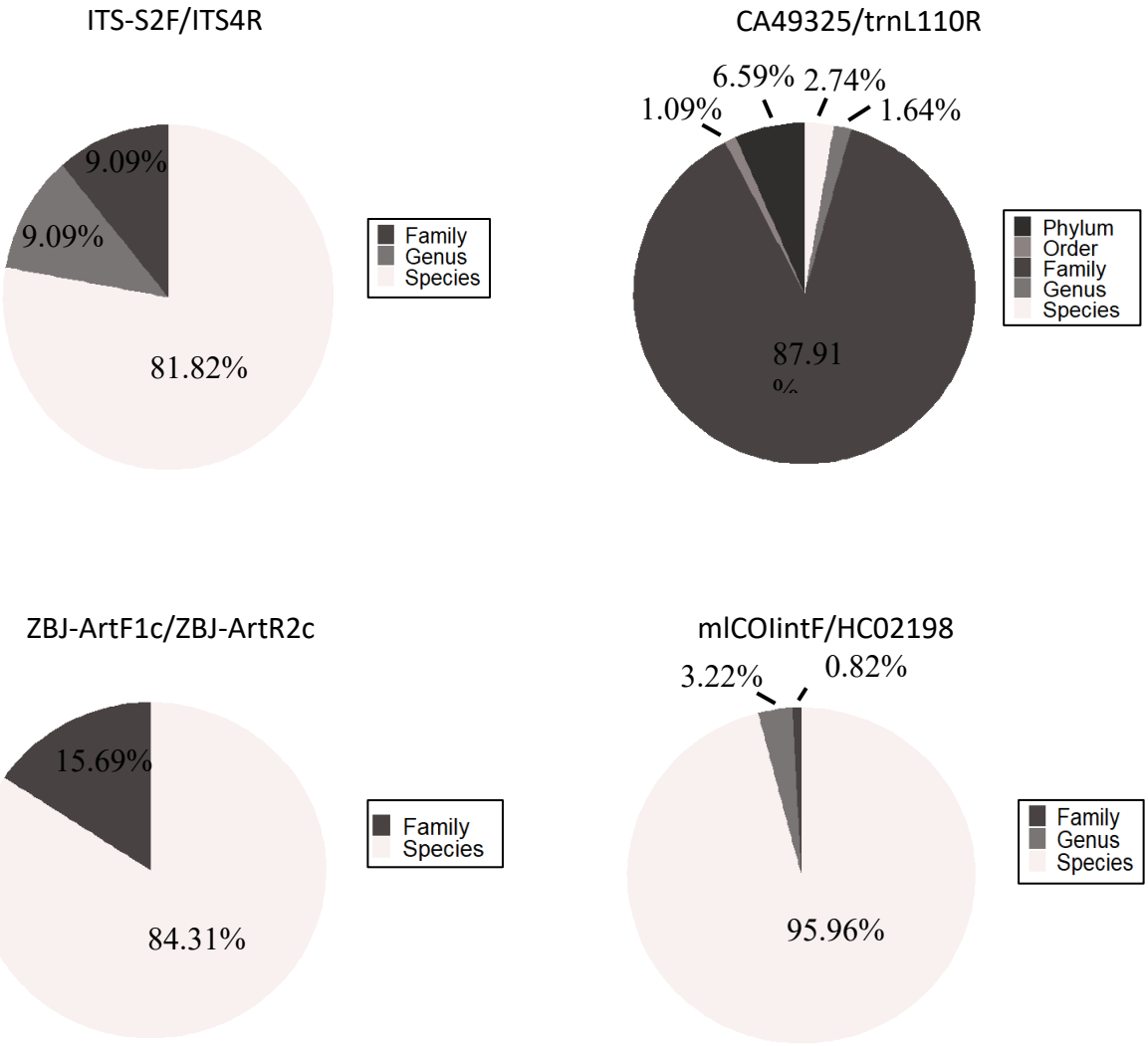
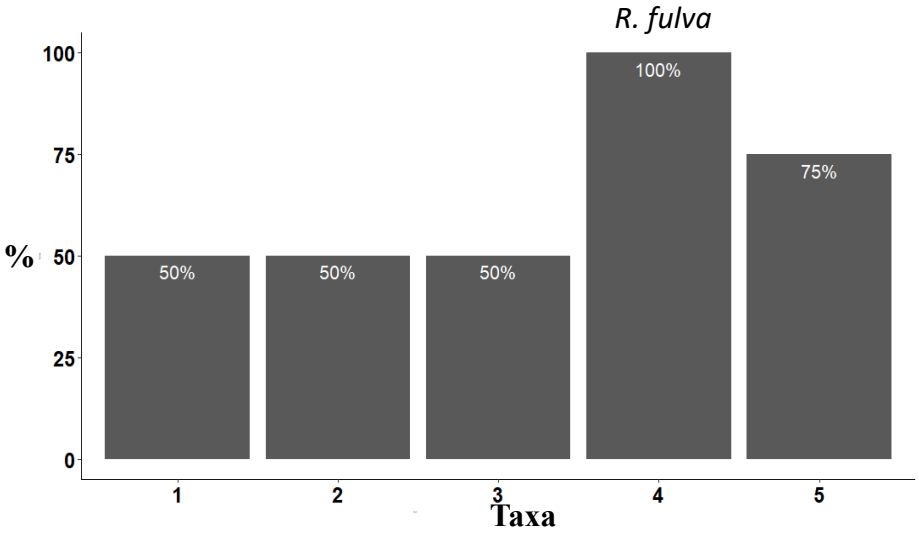
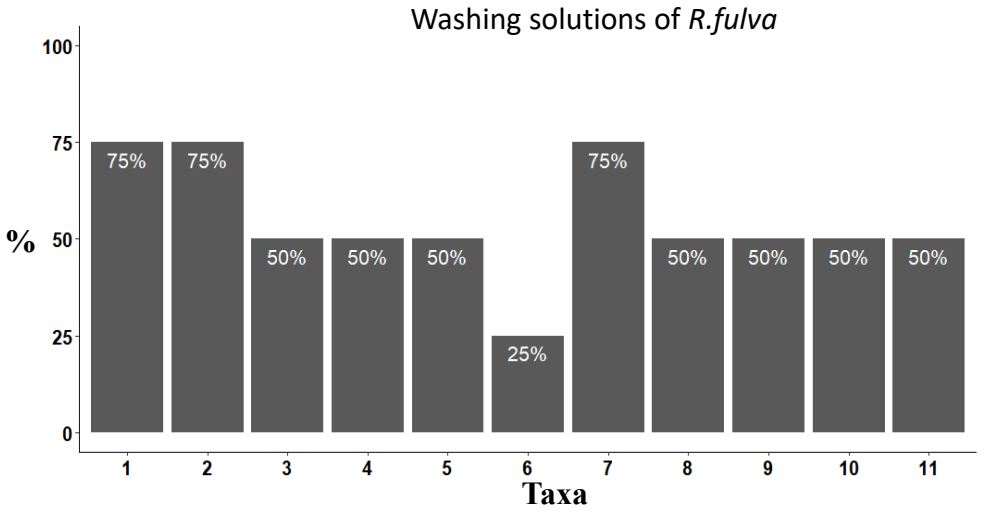


Figure S5.2. Percentage of frequency of occurrence (FOO%) of the obtained taxa items: (A) plant consumed by *R. fulva* and detected in *R. fulva* washing solutions; and (B) arthropod consumed by *A. nemoralis* and *R. fulva*.

(A)

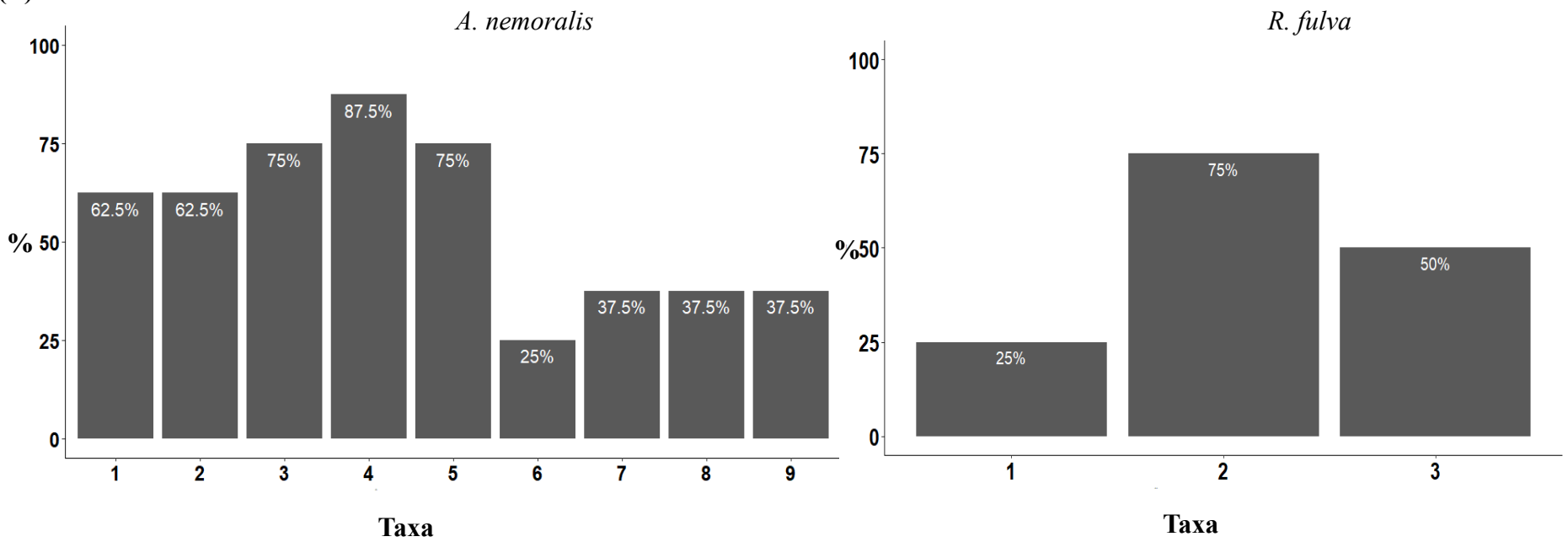


1-Strepthopyta
2-Convolculaceae
3-*Fabaceae*
4-Poaceae
5-Solanaceae



1-Strepthopyta
2-Asteraceae
3-*Sonchus*
4-Medicago sativa
5-Olea europaea
6-*Pinus*
7-*Poaceae*
8-*Dactylys glomerata*
9-*Poa annua*
10-Caryophyllales
11-*Beta vulgaris*

(B)



1-Aphididae
2-Cecidomyiinae
3-*Diaphorina lycii*
4-*Grapholita molesta*
5-*Myzus persicae*
6-*Oenopia conglobata*
7-*Orius*
8-Orius laevigatus
9-*Thrips fuscipennis*

1-*Nysius graminicola*
2-*Cantharis livida*
3-Coccinellidae

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Chapter 3

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

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 by a mosaic of crops scattered with natural and semi-natural habitats, where the presence of *Orius* spp. as biocontrol agent is well known. In order to shed light about their predator role in this area, a metabarcoding multi-primer approach has been used to study the arthropod and plant most common resources consumed by a small population of field-collected *Orius laevigatus* Fieber, *Orius majusculus* Reuter and *Orius niger* Wolff, sampled at different dates in a peach and an alfalfa adjacent crops. Their high throughput sequencing 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*. Among the detected arthropods, other natural enemies were present, showing a certain degree of intraguild predation, as well as some arthropod vectors that cause zoonotic and plant diseases. Intraguild predation among *Orius* species has been demonstrated by developing a new added strategy in the bioinformatic analysis. Plant consumption indicates *Orius* foraging on some non-crop plant species, indicating the potential role of certain plants in attracting or maintaining these predators in both crops.

Keywords: high-throughput sequencing, intra-guild predation, molecular diet analysis, multi-primer approach, *Orius* spp, trophic interactions.

Introduction

Agricultural intensification, which is causing the loss of biodiversity and the landscape simplification (Gámez-Virués et al. 2015), together with the global environmental changes caused by climate change, is reducing essential ecosystem services vital for human societies (McMeans et al., 2015). Among them, the biological control (BC) of pests by natural enemies has become an important component for sustainable crop production in agroecosystems (Bale et al., 2008). The appropriate environmental manipulation to enhance the presence of these natural enemies increases the effectiveness of conservation biological control (CBC) (Landis et al., 2008), which is the only cost-effective biological method in arable crops in the Mediterranean region nowadays (Pons and Eizaguirre, 2009; Pons et al., 2011). Nevertheless, in order to understand and predict the changing patterns of insect abundance in the agroecosystems, it is necessary to take into account some factors, like 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 by a mosaic of arable crops, including cereals and alfalfa, together with fruit orchards, like 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 in order to relate insect predator abundance with the plant variability of the landscape. Some of them highlighted the role of some predatory *Orius* on thrips and aphids, like *Orius laevigatus* Fieber, *Orius majusculus* Reuter and *Orius niger* Wolff in peach, apple, maize and alfalfa crops (Sarasúa et al., 2000; Avilla et al., 2008; Pons et al., 2005; Aparicio et al., 2020).

To better understand the potential role of each *Orius* species as biocontrol agent (BCA), 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 visualize, particularly in commercial fields. Omnivorous predators, 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; Romeu-Dalmau et al. 2012, Pumariño et al., 2011; González-Chang et al., 2016). Nowadays, metabarcoding is starting to be used to assess biodiversity and to understand the food web structure in an ecosystem (Brown et al., 2015, Taberlet et al., 2018), as it has been recently done in agricultural ecosystems (Gomez-Polo et al. 2015, 2016; Sow et al., 2020). More recently, a metabarcoding multi-primer approach has been developed to simultaneously identify the most common arthropod and plant resources ingested by arthropod omnivorous predators (Chapter 2). The main aim of this study is to apply this metabarcoding multi-primer approach to disentangle the most common trophic interactions of a small populations of three *Orius* species present in two adjacent fields of peach and alfalfa. The gathered information wants to shed light about the role of *Orius* as predator of major pests in these crops, as well as on alternative prey species (including other natural enemies). A new step in the

bioinformatic analysis has been developed in order to show the intraguild predation among *Orius* species, that is to say when predator and prey belongs to the same genus. Ingestion on non-crop vegetation will also shed light about the role of some plants in attracting or maintaining these predators within both crops important information to further improve BC 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 10x10: 31TCGO1) 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 lab, they were stored at -20ºC until the DNA extraction. A previous study (Chapter 2) showed that no plant DNA could be identified from the washing solution of another anthocorid (*Anthocoris nemoralis* (Fabricius)). Therefore, these *Orius* specimens, which are also glabrous, and smaller (1 to 3 mm vs. 3 to 5 mm for *A. nemoralis*) were not washed before the pooling, as the risk of *Orius* retaining pollen grains when walking on the surface of leaves seemed very unlikely.

The DNA of each insect or plant sample (1 cm^2 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°C. A negative control without DNA (just DNA-free water) was added to each DNA extraction set.

Orius molecular identification and pooling

Collected *Orius* were molecularly identified by following the amplification protocol of Gomez-Polo et al. (2013), with some modifications. PCR reaction 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] F2/R2 (Table S1). 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) that were 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 and the dsDNA HS Assay kit (Invitrogen, Carlsbad, CA, USA). Equimolar amounts of each *Orius* individual DNA extraction (5 ng/µl) were finally pooled by species, crop and date in seven sample-pools (Table 1.3; sample-pools 1 to 7).

In order to save time and cost, they were pooled up to 25 predators in the same sample-pool when possible, and both pairs of arthropod primers were used together in the same library, as well as both pairs of plant primers, as done in Chapter 2. The two plant sample-

pools (*P. persica* and *M. sativa*) were used as positive controls (Table 1.3; sample-pools 8 and 9), as recommended by Jusino et al. (2019).

PCR amplification, library preparation and sequencing

The obtained sample-pools were amplified using the multi-primer approach described in Chapter 2, with two pairs of universal arthropod primers (Table S1.3). Each PCR reaction (50 µL) contained 15 µL of DNA of each equimolar pool, 25 µL of Multiplex Master Mix (Qiagen, Hilden, Germany) and 1 µL of each primer [10 µM]. PCR conditions for both arthropod primer pairs were: 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: 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 thermocycler (Applied Biosystems, CA, USA). Target DNA and DNA-free water were included as positive and negative controls, respectively. Resulting PCR products were cleaned with QIAquick PCR Purification kit (Qiagen), and 5 µl of each clean PCR product was used as 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 1.3). All libraries were processed in a unique batch of *High Throughput Sequencing* (HTS), 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 were grouped and sequenced with a paired-end approach (2 X 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 ≥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. Chimeras 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, minimum coverage of the query sequence: 97%, number of alignments: 9) (Camacho et al. 2009). The custom-made databases contained all arthropod and plant sequences present in the study area and 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 ≥97% identity by 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 considerer 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 like recommended by Alberdi et al. (2018). Obtained OTUs were categorised as predator or prey based on their taxonomy. In order to reduce other biases, like the secondary predation, and show the most important taxa ingested, two dietary metrics were calculated, as done in Deagle et al. (2018) and Chapter 2, 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 common 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. First, we aligned the following *Orius* sequences from GenBank NCBI and Bold databases (Table S2.3), all belonging to the regions amplified by each pair of arthropod primers used. From them, the interspecific percentage of similarity in both regions between the *Orius* species were calculated as follows. In order to validate whether the similarity threshold adopted (≥97%) was the most suitable to obtain a proper taxonomic assignation between the ingested *Orius* species and the species of *Orius* predator itself, we only considered those OTUs assigned to species level. These OTUs, coming from the amplification of one particular pair of primers within one library were aligned and the interspecific percentages of similarity were calculated by 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). Customed scripts will be available in GitHub (https://github.com/Ivanbh214/Validation_predation).

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 in all sampled dates (34 in June and 9 in August 2016, and 13 in July 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 1.3).

HTS analysis of field-collected Orius

The HTS analysis of the 16 libraries (Table 1.3) 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 2.3)). After clustering, chimaera discarding, and taxonomy assignment we obtained 421 arthropod and 136 plant OTUs (step 6, Table 2.3). After the OTUs filtering (step 7)

for eliminate contaminats, were eliminated the taxa with a number of reads lower than 1% (Step 8, Table S3), from the *Orius* sample-pools analysed (sample-pools 1-7; Table 1.3) we obtained 126 arthropod and 41 plants OTUs which were finally assigned to 15 arthropod taxa (eight to species level) and 12 plant taxa (three to species level) (Table 3.3; Table S4.3).

The HTS analysis of *O. laevigatus* showed arthropod and plant amplification in the only sample-pool of this species (sample-pool 1, Table 1.3). Besides the predator itself, we detected another predator taxon (*Orius*) and two pest taxa belonging to one family (Aphididae) and one species (*Frankliniella occidentalis* Pergande (Thripidae)); five plant taxa corresponding to the sampled crops (*P. persica*) and three families (Solanaceae, Fabaceae, Rosaceae) and one genus (*Pinus*) (Fig. 1.3; Table S4.3).

Regarding *O. majusculus* (sample-pool 2, Table 1.3), besides the predator itself, there was amplification of two other predator taxa (*Orius* and *O. laevigatus*), four other plant taxa corresponding to three families (Asteraceae, Fabaceae, Rosaceae) and one genus (*Pinus*) (Fig. 1.3; Table S4.3).

The five sample-pools of *O. niger* (sample-pools 3-7, Table 1.3) showed amplification of four pest taxa belonging to two genus (*Hypera, Liriomyza*) and two species (*Therioaphis trifolii Monell* (Thripidae) and *F. occidentalis*), three predator taxa corresponding to one (*Orius*) and two species (*O. laevigatus* and *A. intermedius*), five non-pest taxa belonging to two families (Ceratopogonidae, Cicadellidae) one subfamily (Orthocladiinae) and two species (*Aedes caspius* Palla s(Culicidae) and *Tanytarsus volgensis* Miseiko (Chiromonidae)), two taxa corresponding to the sampled crops (*P. persica* and *M. sativa*,) and nine other plant taxa (Streptophyta), two orders (Asparagales and Caryophyllales), four families (Asteraceae, Poaceae, Fabaceae, Rosaceae) one genus (*Pinus*) and one species (*P. annua*) (Fig. 1.3; Table S4.3).

Regarding the positive controls of peach and alfalfa, we obtained the expected amplification of both plant sample-pools (sample-pools 8 and 9, respectively; Table 1.3), 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 S1.3).

To validate the IGP between *Orius* species, we calculated the percentages of similarity between the three amplified *Orius* species (*O. majusculus, O. laevigatus* and *O. niger*), which ranged from 92-94% for ZBJ-ArtF1c/ZBJ-ArtR2c and from 87-91% for mlCOIintF/HC02198 (Tables S5.3 and S6.3), being in all cases below the cluster similarity threshold of 97% used. By other hand, to show the high taxonomic resolution that allow detected the *Orius* taxa to species level we obtained, 423,697 of them (95 OTUs) were assigned to three species of *Orius* (Table S7.3). 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.

Discussion

This study was addressed to identify the arthropod and plant resources ingested by the *Orius* species complex present in peach and alfalfa adjacent fields by an HTS multi-primer approach. Results showed how, with a discrete number of analysed *Orius*, this method is a reliable tool to identify a broader range of the most common ingested resources than the described in previous field studies based on the observation of field predatory

episodes (Pericart, 1972; Riudavets, 1995; Riudavets and Castañé, 1998; Lattin, 1999; Pons et al., 2005). On the other hand, to our knowledge this is the first time that plant consumption is detected in field-collected *Orius* by molecular methods. Detected plant taxa were all present outside the sampled crop, confirming that *Orius* individuals use the neighboring habitats to forage, which has also been observed in Chapter 1 using a new marking method using an aqueous solution of an aquatic invertebrate (*Artemia* spp.) followed by a conventional PCR with *Artemia* specific primers.

Methodological issues

Plant positive controls used in the HTS analysis (sample-pools 8 and 9) allowed a confident taxonomic identification of plant species. Both plant primer pairs gave a correct identification of each analysed piece of leaf, either to species (using ITS-S2F/ITS4R) or to family level (using CA49325/trnL110R) (Table S4.3), as observed in Chapter 2. 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 correct identification of each *Orius* sample-pool to species level (Table S4.3).

Field-collected *Orius* specimens were pooled by species and, in the case of *O. niger,* also by sampling dates (Table 1.3), which allowed to have biological replicates, as recommended by Mata et al. (2018). On the other hand, the calculation of the two dietary metrics RRA% and FOO% provided a more reliable evidence of their consumption, demonstrating that consumption on a particular taxon is not spurious or is not indirectly ingested (secondary predation).

In this study, a suitable taxonomic resolution was obtained, where a certain number of arthropod species were obtained (8 species from 15 taxa) (Table 3.3). The analysed *Orius* showed a high detection of the predator taxa (Fig. S1.3), representing the 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 S8.3). Despite this fact, we still detected some prey taxa (Table 3.3), because according to Agustí et al. (2003), those primer pairs amplifying shorter amplicons within the COI region improve the detection of the degraded DNA by digestion. Our results confirm this statement because the primer pair that amplified the shortest fragment gave a higher deep sequencing in the used Illumina platform (Table S1.3; Table 2.3).

Trophic interactions

Some crop pests were detected in 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 the present study in predators collected in both crops. *Orius* is well-known genus of predators present in several crops (Riudavets, 1995; Riudavets and Castañé, 1998). In the present study, *F. occidentalis* has been 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 common arthropod taxa consumed by the analysed *Orius* specimens (Fig. 1.3; Fig. S2.3 A). 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 either on peach or on alfalfa, highlighting *Hypera* and *Liriomyza* as the most common trophic interaction after *F. occidentalis* (Fig. S2.3 A and B). In peach, we detected consumption of Aphididae, a family that includes important pests of peach orchards and important vectors of the disease plum pox virus or Sharka (Aparicio et al., 2019). These trophic interactions were previously described by Barbagallo et al., (2017) in peach, particularly by *O. laevigatus*. In *O. niger* collected in alfalfa, we detected the aphid *T. trifolii* (Fig. 1.3; Table S4.3), 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 economically loss (Parrella and Keil, 1978), and has been cited in alfalfa crops in the same area of study (Pons and Nuñez, 2020).

Other prey taxa were detected in both crops, like *A. intermedius* and *O. laevigatus*, both known predators of thrips (Riudavets, 1995), showing a certain degree of IGP. *A. 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. 1.3, Table S4.3). Intraguild predation using metabarcoding analysis of field-collected predators in agricultural systems had been already shown in other previous HTS study (Gomez-Polo 2015, 2016; Chapter 2). In the present study, IGP is present between species of the same genus (*Orius*), which makes their detection more difficult to demonstrate because of their close taxonomic similarity. For this reason, a particular bioinformatic process was conducted, which allowed an accurate *Orius* detection. The obtained percentages of similarity between the sequences of *Orius* species from the database (NCBI) in the region amplified by both arthropod primer pairs (Table S5.3), together with the percentages of similarity between the OTUs of the predator itself and each *Orius* species as prey (Table S7.3), showed an accurate way in the clustering process to allow the differentiation between the detected *Orius* species (Table S6.3), because these percentages were always under 97% (Table S7.3). These results demonstrate predation between congeneric *Orius* species, showing trophic interactions where the taxonomical distance between species was low. This intraguild IGP predation should be considered in further studies, as a potential negative effect on the biological control of key pests, like *F. occidentalis*.

Some non-pest taxa were also detected in lower percentages in those *O. niger* collected in alfalfa, including Ceratopogonidae, *T. volgensis*, *A. caspius*, Orthocladiinae and Cicadellidae (Fig. 1.3, Table S2.3.B). The fact that more arthropod species have been detected within those predators collected on alfalfa than those collected on peach (Fig. 1.3) 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 pests on this crop (Nuñez, 2002; Pons et al., 2005).

Orius predation on some dipteran taxa has been previously reported, like *O. majusculus* feeding on Syrphidae in lettuce, which was also detected by HTS (Gomez- Polo et al., 2016). Regarding Ceratopogonidae and *A. caspius,* they have both been cited to cause

zoonotic diseases with an important socio-economic 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 a floodwater mosquito species widely distributed in the Western Palearctic. As an anthropophilic species, its role as arbovirus vector is key for understanding the transmission cycle of certain diseases in Europe, like with the Rift Valley fever virus (Moutailler et al.,2008) and the West Nile virus, which has been recently reported in Lleida (Catalonia (NE Spain)), the same region that the area of study (Busquets et al., 2018). *T. volgensis* and Orthocladinae belong to the family Chironomidae (Table 3.3), which is the most abundant insect group in all types of freshwater and even saltwater (Armitage et al., 1995). The presence of these dipterans in alfalfa, is probably due to that the alfalfa plot was irrigated by flood, causing the proliferation of these taxa. Areas with high levels of water have a high probability of colonization by mosquitoes, provoking the presence of zoonotic diseases (Bett et al., 2017).

Orius niger predated Cicadellidae in alfalfa (Fig 1.3; Table S4.3). The family Cicadellidae is a vector 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) also in alfalfa in the same area of study. One of the key pests in alfalfa in Spain, *E. fabae* 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., 2020). The identified plant taxa within the three *Orius* species further indicate that they do forage a wide range of plants under field conditions, highlighting Fabaceae (Fig. 1.3; Table S2.3 C), the family of *M. sativa*. This result indicates the use of alfalfa crops as resources by *Orius* (Nuñez, 2002; Pons et al., 2005). The rest of the detected plant taxa (Table S2.3 C) have been cited either in ground covers, in in field margins of peach crops, or in alfalfa crops in the same area of study (Ibáñez-Gastón, 2018; Clemente-Ortega et al., 2020), shows that they used these plant resources, and then moved to peach and alfalfa crops, as previously suggested by Ardanuy et al., (2018). 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, which may lead the *Orius* to disperse in the landscape, as previously indicated in the movement between crops by Madeira et al. (2019). The detection of *P. persica* within *O. niger* sampled in alfalfa in September (Table S4.3; Table S2.3 D), is particularly interesting because 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* 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 decay after 14 days (Schield et al., 2015) particularly with the high summer temperatures in the area of study. The *Orius* movement from alfalfa to peach had been previously demonstrated in Chapter 1 using a DNA mark-capture method, particularly of *O. laevigatus* and *O. majusculus*. Results obtained in the present study indicate that *Orius*

movement could be from alfalfa to peach that along with the results obtained in Chapter 1 show the bidirectional movement between crops, showing this multi-primer approach as a useful tool to track predator movement between crops.

Some *Orius* were also fed on *Pinus* (Fig. 1). Several anthocorids have been described on pinetrees (Pericart, 1972), and some *Orius* species have also been occasionally recorded on pines, like, *O. niger* and *Orius albidipennis* Reuter (Heidari et al., 2015) and *Orius tristicolor* White (Lattin and Stanton, 1992). The area of study has 88% of the soil occupied by crops, and the presence of *Pinus* species is low (www.creaf.uab.cat/iefc/pub/Regions/Comarques/CobertesSegria.htm). *Pinus* is a wind-pollinated genus that produces abundant pollen dispersed over long distance, and the species present in the area of study are *Pinus nigra* Arnold and *Pinus halepensis* Mill, both flowering in spring (www.creaf.uab.cat/iefc/pub/Regions/EstratArbustiuRF8.htm). Most of the pine pollen of these species is shed between April and July in the area of study, and even some pollen has been recovered from aerial palynology studies in summer and autumn (https://www.polenes.com/home). Therefore, it is plausible that *Orius* had fed on pollen deposited on leaves or had previously foraged on pine trees before entering the alfalfa crop.The use of windspread pollen by predatory mites, have been described to feed on other wind spread pollen in Spain (González-Fernández et al., 2009).

In this study, we have detected the most common arthropod and plant resources ingested by *Orius* predators present in a peach and an alfalfa crop by HTS analysis, confirming their role as predators and suggesting the influence of the landscape on their presence in peach and alfalfa crops. We also showed unknown trophic relationships, like the predation by *O. niger* on a cicadellid vector of a peach disease, and some dipteran vectors of animal and even human diseases, suggesting that this *Orius* species could have a potential role in minimizing the risk of plant and zoonotic diseases in the area of study. We also showed the presence of IGP between *Orius* species and on *A. intermedius*, which should be further considered in future BC programs in peach and alfalfa crops. Finally, we also showed the contrasted omnivory of these *Orius* species, feeding on some plant resources present in the different elements that compounds the landscape of the area of study, and suggesting the importance of plant biodiversity in the landscape, and the need of preserving this diversity for a more sustainable agriculture.

Table 1.3. 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
Orius laevigatus	Peach	June 2016	1	7	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L1
					ITS-S2F/ITS4R; CA49325/trnL110R	L2
Orius majusculus	Peach	June 2016	2	21	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L3
					ITS-S2F/ITS4R; CA49325/trnL110R	L4
Orius niger	Alfalfa	June 2016	3	9	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L5
					ITS-S2F/ITS4R; CA49325/trnL110R	L6
		June 2016	4	25	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L7
					ITS-S2F/ITS4R; CA49325/trnL110R	L8
		July 2017	5	13	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
Prunus persica	Peach	-	7	1 cm^2	ITS-S2F/ITS4R; CA49325/trnL110R	L13
Medicago sativa	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

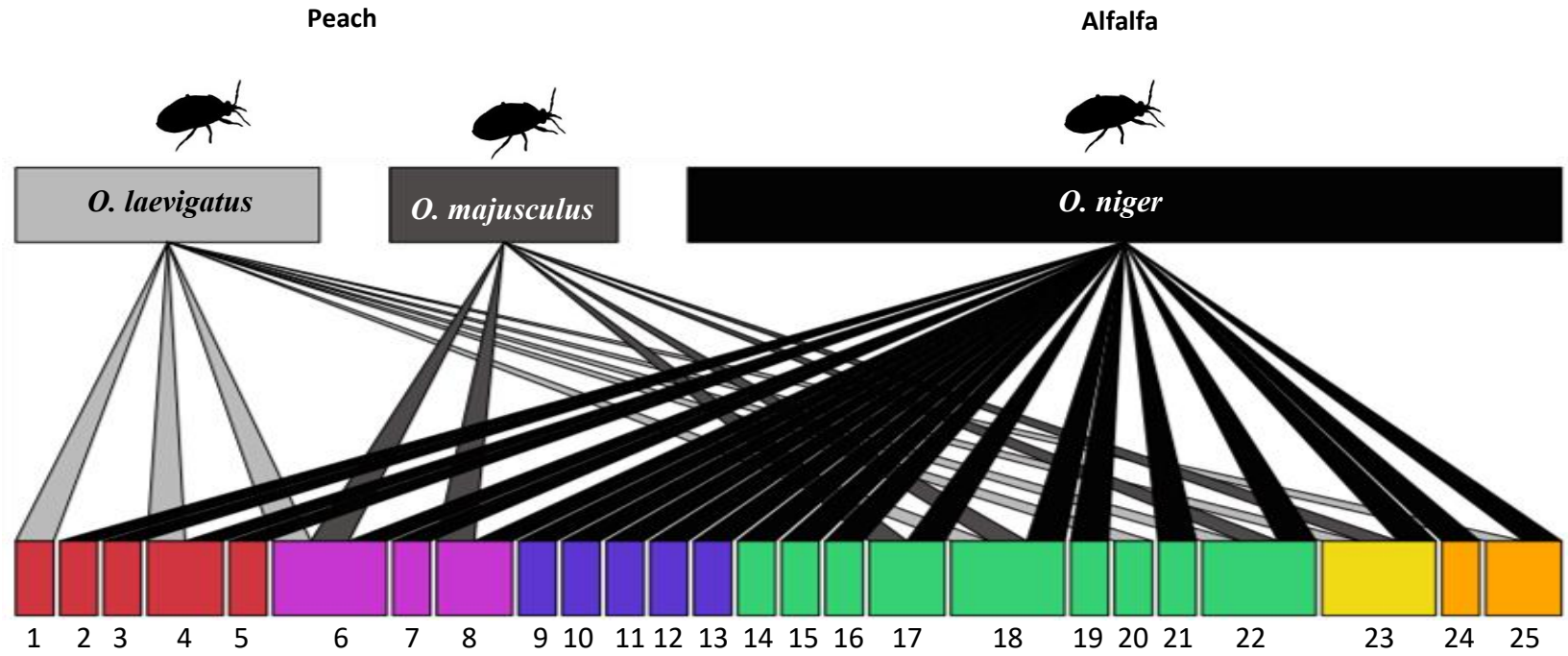
Table 2.3. Total number of reads and OTUs obtained with each universal arthropod and plant primer pair in each step of the bioinformatic analysis. NA = not applicable.

			Arthropod primers				Plant 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	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	6,836	NA	69,559	NA
4	Clustering	523,726	354,641	542	92,690	745	6,836	139	69,559	112
5	Chimera removing	523,293	354,394	524	92,505	707	6,835	138	69,559	112
6	Taxonomy assignment	511,090	347,177	175	87,754	246	6,647	60	69,512	76
7	OTUs filtering	510,254	346,845	64	87,393	66	6,562	14	69,402	27
8	OTUs secondary predation filtering	510,156	346,799	60	87,393	66	6,562	14	69,402	27

Table 3.3. Summary table of all 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		
			Chironomidae		***Tanytarsus volgensis*** **Miseiko**
			Culicidae		***Aedes caspius*** **Pallas**
			Orthocladiinae		
		Hemiptera	Anthocoridae	***Orius***	
					Orius laevigatus **Fieber**
					Orius majusculus **Reuter**
					Orius niger **Wolff**
			Aphididae		
					Therioaphis trifolii **Monell**
			Cicadellidae		
		Thysanoptera	Aeolothripidae		***Aeolothrips intermedius*** **Bagnall**
			Thripidae		***Frankliniella occidentalis*** **Pergande**
Plantae	**Streptophyta**				
		Asparagales			
		Asterales	**Asteraceae**		
		Caryophyllales			
		Fabales	**Fabaceae**		
					Medicago sativa **L.**
		Pinales	Pinaceae	***Pinus***	
		Poales	**Poaceae**		
					Poa annua **L.**
		Rosales	**Rosaceae**		
					Prunus persica **(L.) Batsch**
			Solanaceae		

Figure 1.3. 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.



Pest
1- Aphididae**, 2**- *Hypera*, **3**- *Liriomyza,* **4**- *F. occidentalis,* **5**- *T. trifolii*

Predator
6- Orius, **7**- *A. intermedius*, **8**- *O. laevigatus*

Non-pest
9- Ceratopogonidae, **10**- Cicadellidae, **11-** Orthocladiinae
12- *A. caspius*, **13-** *T. volgensis*

Weeds
14- Streptohphyta, **15**- Asparagales, **16**- Caryophyllales, **17**- Asteraceae, **18-** Fabaceae, **19-** Poaceae, **20-** Solanaceae, **21-** Rosaceae, **22-** *P.annua*

Non-weeds
23- *Pinus*

Crops
24- *M. sativa,* **25**- *P. persica*

Table S1.3. Arthropod (ZBJ-ArtF1c/ZBJ-ArtR2c and mlCOIintF/HC02198) and plant (ITS-S2F/ITS4R and cA49325/trnL110R) primer pairs used in this study. Also indicated the sequence of each forward (F) and reverse (R) primer and the length of the amplified fragment.

	Sequence 5´- 3´(F)	**Sequence 5´- 3´(R)**	**Reference**	**Fragment (bp)**
F2/R2	GTCGCTACTACCGATTGAATGG	GTGTCCTGCAGTTCACATGG	Hinomoto et al., 2004	200-400
ZBJ-ArtF1c/ZBJ-ArtR2c	AGATATTGGAACWTTATATTTTATTTTTGG	WACTAATCAATTWCCAAATCCTCC	Zeale et al. 2011	157
mlCOIintF/HC02198	GGWACWGGWTGAACWGTWTAYCCYCC	TAAACTTCAGGGTGACCAAAAAATCA	Leray et al. 2013/Folmer et al. 1994	313
ITS-S2F/ITS4R	ATGCGATACTTGGTGTGAAT	TCCTCCGCTTATTGATATGC	Chen et al. 2010/White et al. 1990	350
cA49325/trnL110R	CGAAATCGGTAGACGCTACG	GATTTGGCTCAGGATTGCCC	Taberlet et al. 2007/Borsch et al. 2003	80

Table S2.3. List of the accession numbers (NCBI or BOLD) of the sequences used as reference to obtain the percentages of similarity by pairs from region amplified by each arthropod primer pairs used.

Accession number	**species**	**Databases used**
MG007856.1	*Orius laevigatus*	NCBI
MG007857.1		
KM021482.1	*Orius niger*	
KM021745.1		
KM021890.1		
KM022197.1		
KM022760.1		
KM022882.1		
KM022999.1		
SONE138-11	*Orius majusculus*	Bold
SONE141-11		

Table S3.3. 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 three trials included in the study: (1) Taxonomic coverage; (2) Plant primer resolution; and (3) Analysis of field-collected predators. Those percentages eliminated from the analysis for exceeding the 1% threshold are shown in bold and grey. Art1= ZBJ-ArtF1c/ZBJ-ArtR2c; Art2= mlCOIintF/HC02198; Pl1= ITS-S2F/ITS4R; Pl2= cA49325/trnL110R; NA= Non amplified.

(1)

Primers used	**L1**		**L3**		**L5**		**L7**		**L9**		**L11**	
Taxa detected	**Art1**	**Art2**	**Art1**	**Art2**	**Art1**	**Art2**	**Art1**	**Art2**	**Art1**	**Art2**	**Art1**	**Art2**
Orius	-	-	-	100	-	-	-	-	-	NA	-	-
Orius laevigatus	-	-	100	-	8,88	-	-	-	-	NA	87,23	-
Orius majusculus	-	-	-	-	-	-	-	-	-	NA	-	-
Orius_niger	-	-	-	-	-	-	-	-	-	NA	-	-
Aeolothrips intermedius	-	-	-	-	-	-	73,78	16,72	-	NA	**0,21**	-
Frankliniella occidentalis	-	100	-	-	-	100	-	74,62	-	NA	-	73,11
Aphididae	100	-	-	-	-	-	-	-	-	NA	-	-
Therioaphis trifolii	-	-	-	-	-	-	-	-	-	NA	**0,29**	10,27
Hypera	-	-	-	-	5,33		2,32	-	-	NA	-	-
Liriomyza	-	-	-	-	3,55		21,58	8,66	-	NA	-	-
Aedes caspius	-	-	-	-	-	-	-	-	-	NA	**0,38**	8,07
Ceratopogonidae	-	-	-	-	-	-	-	-	-	NA	**0,21**	4,89
Tanytarsus volgensis	-	-	-	-	68,64	-	-	-	-	NAm	-	-
Orthocladiinae	-	-	-	-	13,61	-	2,32	-	-	NAm	-	-
Cicadellidae	-	-	-	-	-	-	-	-	100	NAm	11,68	-

(2)

Primers used	**L2**		**L4**		**L6**		**L8**		**L10**		**L12**	
Taxa detected	**Pl1**	**Pl2**	**Pl1**	**Pl2**	**Pl1**	**Pl2**	**Pl1**	**Pl2**	**Pl1**	**Pl2**	**Pl1**	**Pl2**
Streptophyta	-	-	NA	-	-	-	NA	-	-	2,17	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	-	-	-	-	-
Poa annua	-	-	NA	-	-	-	NA	-	-	1,78	-	-
Pinus	-	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	-	-
Medicago sativa	-	-	NA	-	100	-	NA	-	59,65	-	-	-
Prunus persica	100	-	NA	-	-	-	NA	-	40,35	-	-	-

Table S4.3. Taxa obtained regarding each primer pairs from each library. NT= not tested.

species/sample	Crop	Date	# of individuals/ Sample size	Library #	Arthropod primer pairs		Plant primer pairs	
					ZBJ-ArtF1c/ZBJ-ArtR2c	mlCOIintF/HC02198	ITS-S2F/ITS4R	CA49325/trnL110R
Orius laevigatus	Peach	June-2016	7	L1	*Orius laevigatus* Aphididae	*Orius* *Orius laevigatus* *Frankliniella occidentalis*	NT	NT
				L2	NT	NT	*Prunus persica*	Solanaceae *Pinus* Rosaceae Fabaceae
Orius majusculus			21	L3	*Orius laevigatus* *Orius majusculus*	*Orius* *Orius majusculus*	NT	NT
				L4	NT	NT	NAmp	Asteraceae *Pinus* Fabaceae Rosaceae
Orius niger	Alfalfa	June-2016	9	L5	*Hypera* *Tanytarsus volgensis* Orthocladiinae *Liriomyza* *Orius laevigatus* *Orius niger*	*Orius niger* *Frankliniella occidentalis*	NT	NT
				L6	NT	NT	Streptophyta	Streptophyta Caryophyllales Fabaceae *Pinus*
		June-2016	25	L7	*Hypera* Orthocladiinae *Liriomyza* *Orius niger* *Aeolothrips intermedius*	*Liriomyza* *Orius niger* *Frankliniella occidentalis* *Aeolothrips intermedius*	NT	NT
				L8	NT	NT	*Medicago sativa*	Asparagales Asteraceae Poaceae *Pinus*

								Fabaceae
		July-2017	13	L9	*Orius* *Orius niger* Cicadellidae	*Orius* *Orius niger*	NT	NT
				L10	NT	NT	NAmp	*Fabaceae*
		September-2017	22	L11	*Orius* *Orius laevigatus* *Orius niger* Cicadellidae	*Aedes caspius* Ceratopogonidae *Orius* *Orius niger* *Therioaphis trifolii* Cicadellidae *Frankliniella occidentalis*	NT	NT
				L12	NT	NT	*Medicago sativa* *Prunus persica*	Streptophyta Asteraceae Fabaceae Rosaceae *Poa annua*
Prunus persica	Peach		1 cm^2	L13	NT	NT	*Prunus persica*	*Rosaceae*
Medicago sativa	Alfalfa		1 cm^2	L14	NT	NT	*Medicago sativa*	Fabaceae

Table S5.3. Percentages of similarity means by pairs from the region amplified by each arthropod primer pair used calculated from the sequences obtained in the databases of three amplified *Orius* species.

Species comparison	ZBJ-ArtF1c/ZBJ-ArtR2c (%)	mlCOIintF/HC02198 (%)
O. majusculus - O. laevigatus	92,04	87,29
O. majusculus - O. niger	92,42	90,85
O. laevigatus - O. niger	94,34	91,53

Table S6.3. Mean of the calculated percentages of similarity between the obtained number of OTUs for the predator (*O. majusculus* or *O. niger*) and for the prey (other *Orius* species) using the ZBJ-ArtF1c/ZBJ-ArtR2c pair of primers.

Predator species	Prey species	Library	# Predator OTUs	# Prey OTUs	Mean % similarity between OTUs
O. majusculus	*O. laevigatus*	L3	5	5	91,90
O. niger	*O. laevigatus*	L5	6	1	92,73
O. niger	*O. laevigatus*	L11	2	1	93,94

Table S7.3. Number of reads and OTUs obtained per library regarding the amplified *Orius* species and the taxon finally assigned with each pair of arthropod primer used. The species with the highest number of reads and OTUs within a library and pair of primer used is indicated in grey.

Predator	Library	Primer pair	*Orius* species		
			# Reads	**# OTUs**	**Taxon assigned**
Orius laevigatus	L1	ZBJ-ArtF1c/ZBJ-ArtR2c	56868	23	***O. laevigatus***
		mlCOIintF/HC02198	2015	3	***O. laevigatus***
Orius majusculus	L3	ZBJ-ArtF1c/ZBJ-ArtR2c	24906	5	***O. majusculus***
			6668	5	*O. laevigatus*
		mlCOIintF/HC02198	1635	2	***O. majusculus***
Orius niger	L5	ZBJ-ArtF1c/ZBJ-ArtR2c	109935	6	***O. niger***
			15	1	*O. laevigatus*
		mlCOIintF/HC02198	10679	2	***O. niger***
	L7	ZBJ-ArtF1c/ZBJ-ArtR2c	79012	3	***O. niger***
		mlCOIintF/HC02198	21255	2	***O. niger***
	L9	ZBJ-ArtF1c/ZBJ-ArtR2c	25677	1	***O. niger***
		mlCOIintF/HC02198	19202	1	***O. niger***
	L11	ZBJ-ArtF1c/ZBJ-ArtR2c	38716	2	***O. niger***
			3668	1	*O. laevigatus*
		mlCOIintF/HC02198	23446	38	***O. niger***
		Total	423697	95	

Table S8.3. Number of template-mismatches of each arthropod pair of primers with each amplified taxon. NS = no sequence present in the databases.

	mlCOIintT/HC02198		**ZBJ-ArtF1c/ ZBJ-ArtR2c**	
Taxon	**Forward (mlCOIintT)**	**Reverse (HC02198)**	**Forward (ZBJ-ArtF1c)**	**Reverse (ZBJ-ArtR2c)**
Hypera	1	NS	0	2
Liriomyza	1	3	1	1
Tanytarsus volgensis	1	NS	3	8
Orthocladiinae	0	NS	0	6
Ceratopogonidae	1	NS	3	4
Aedes caspius	1	1	1	5
Orius	2	4	3	3
Orius laevigatus	0	NS	1	0
Orius majusculus	1	NS	1	0
Orius niger	0	NS	0	2
Aphididae	1	NS	3	2
Therioaphis trifolii	2	NS	3	8
Cicadellidae	1	NS	4	2
Aeolothrips intermedius	0	NS	3	6
Frankliniella occidentalis	1	NS	4	5

Figure S1.3. Percentage of predator and prey taxa amplified by each arthropod primer pairs from each library: **(A)** Taxa amplified by ZBJ-ArtF1c/ZBJ-ArtR2c primer pairs; **(B)** Taxa amplified by mlCOIintF/HC02198 primer pairs. Predator (green); Prey (orange)

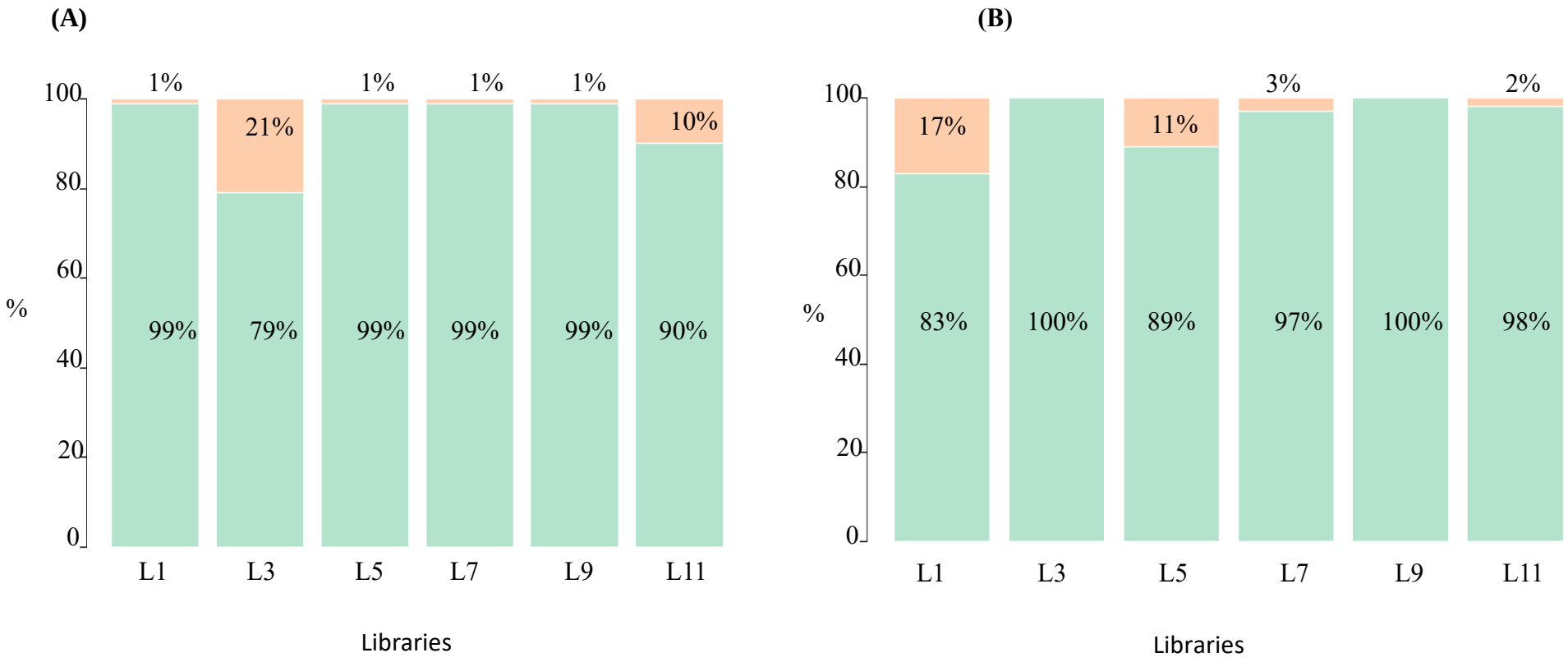
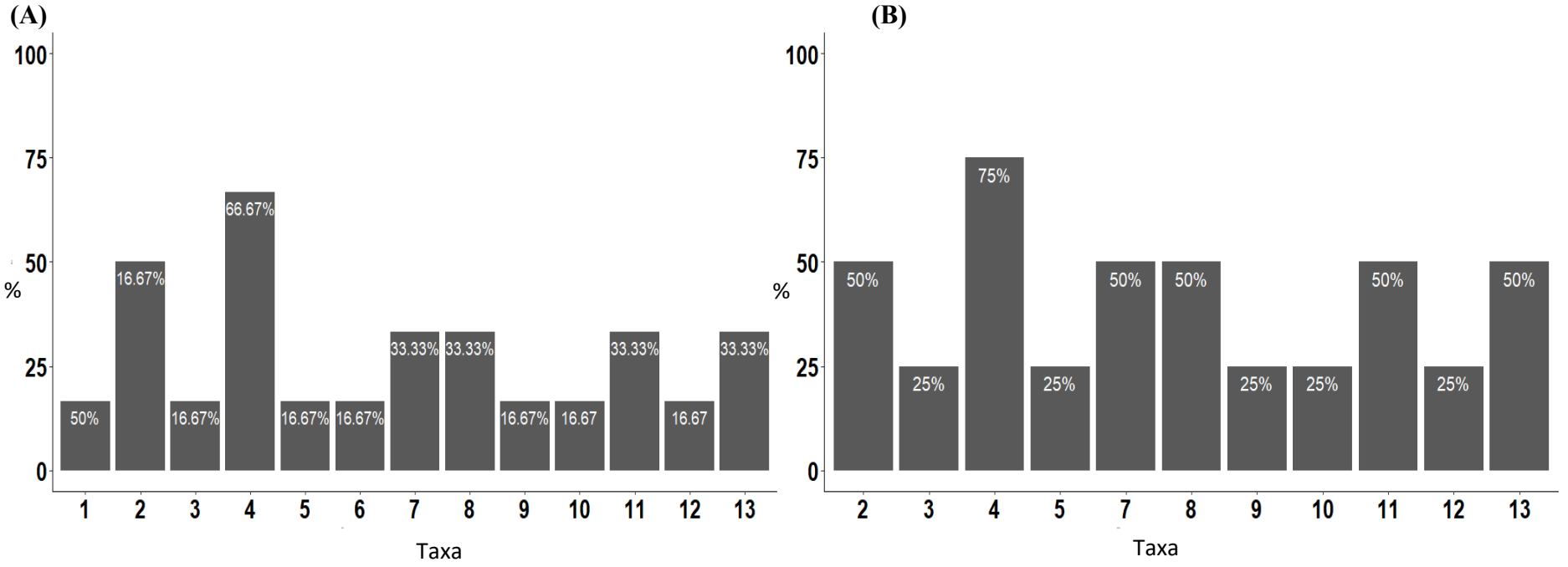
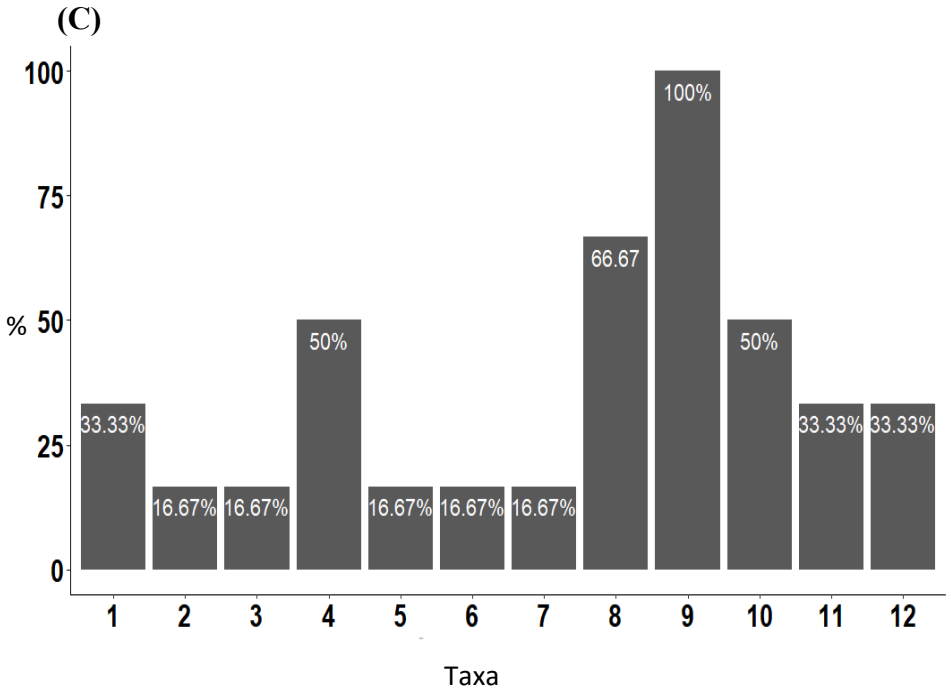
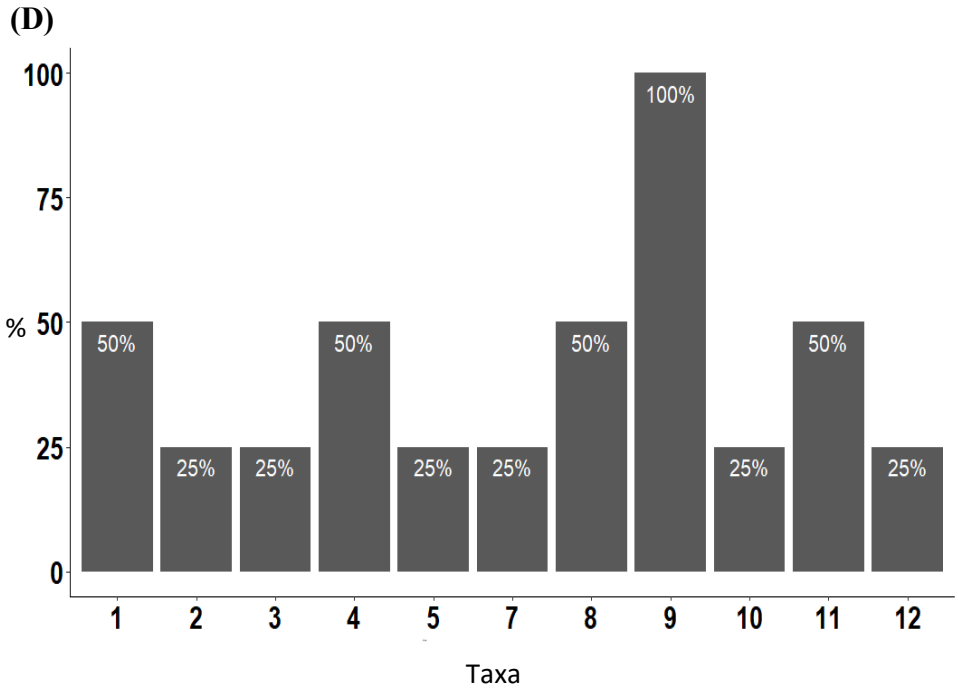


Figure S2.3. Percentage of frequency of occurrence (FOO%) of the (A) arthropod taxa resources items consumed by all *Orius*; (B) arthropod taxa resources items consumed by *Orius niger;* (C) plant taxa resources items consumed by all *Orius*; (D) of the plant taxa resources items consumed by *Orius niger***.**



1-*Orius*	5-Aphididae	9- *Aedes caspius*	13-Cicadellidae
2-*Orius laevigatus*	6- *Therioaphis trifolii*	10- Tanytarsus volgensis	
3-*Aeolothrips intermedius*	7-*Hypera*	11- Orthocladiinae	
4-Frankliniella occidentalis	*8-Liriomyza*	*12- Ceratopogonidae*	





1-Streptophyta
2-Asparagales
3-Caryophyllales
4-Asteraceae
5-Poaceae
6-Solanaceae
7-*Poa annua*
8-*Pinus*
9-Fabaceae
10-Rosaceae
11-*Medicago sativa*
12-*Prunus persica*

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Chapter 4

Disentangling the omnivory of heteropteran and coccinellid predators present in peach and alfalfa crops by metabarcoding analysis

Abstract

Ecosystem management is essential to develop the biological control of pests in agriculture. For this, it is necessary to know which arthropod and plant resources are the most used by the generalist predators present in the studied agroecosystem. Molecular approaches, like high-throughput sequencing (HTS) are nowadays a key tool to disentangle the resources consumed by each predator species. In this study we use a multi-primer metabarcoding approach with pooled samples to screen the most common trophic interactions of four heteropteran and four coccinellid species. They were collected in a peach and in an adjacent alfalfa crops at different dates in two consecutive years. The HTS analysis of those 433 predators showed that they ingested 27 arthropod and 14 plant taxa, confirming their omnivory. Among the detected arthropods, we found a potential pest of peach non cited until now. Predation on non-pest taxa indicated predator movement from the field margins to both crops. Detection of ingested plant DNA showed that those predator species foraged on non-crop plants, which play a role in attracting or maintaining these arthropods close or in the crops. This metabarcoding approach showed the omnivory of those heteropteran and coccinellid species, important information to improve biological control programs.

Keywords: high-throughput sequencing, molecular diet analysis, multi-primer approach, trophic interactions, omnivory

Introduction

Nowadays, the studies related with ecosystem services in agroecosystems are focused on understanding the functional features provided by insects, like biological control (Perović et al., 2018; Demestihas et al., 2017). It is estimated that 20 to 40% of the global crop production is annually lost by the action of insect pests (FAO, 2020), increasing in the last years due to the climate warming that alters relevant biological insect features (Deutsch et al., 2018). It is known that the efficiency of biological control depends on the food web of the agroecosystem, in this context the role of each species could become fundamental to manage species or functional groups (Bohan et al., 2013). Despite the numerous studies in insect food webs, some factors that influence them are still not well known, like predator diversity and its importance in the ecosystem management, or how the role of omnivorous insects affects other organisms in an agroecosystem (Schoenly et al., 1991; Lancaster et al., 2005; Krimmel, 2011). Insect omnivory is common and widespread across agricultural ecosystems (Eubanks et al., 2003), and to know which plant and prey resources are consumed allows developing efficient pest control programs (Krimmel, 2011). Even if the study of food webs is complicated and often requires years of ecological observations and considerable taxonomic expertise (Clare et al., 2019), nowadays some approaches like the analysis of fatty acids, the use of stable isotopes, or DNA molecular approaches are of great help (Nielsen et al., 2018). High throughput sequencing (HTS) has emerged as a suitable option for dietary studies of environmental samples (González-Chang et al., 2016; Roslin and Majaneva, 2016; Nielsen et al., 2018; Chapter 2 and Chapter 3 of this Doctoral Thesis), showing some advantages with respect to other methods, like a higher taxonomic resolution, the detection of unexpected trophic interactions or the correct identification of species that are not easy to identify (Galan et al., 2018; Taberlet et al., 2018, Nichols et al., 2018). The use of plant and arthropod primers to study omnivory and the analysis of a higher number of samples in sample-pools, saving time and cost, have been reported when using this method (Chapter 2 and 3).

In the study of a food web within an agroecosystem, both the landscape configuration and composition must be considered, because they influence the abundance of insects (pests and predators) and the insect community structure (Marino and Landis., 1996; Bianchi et al., 2006; Rusch et al., 2010; Tscharntke et al., 2012), particularly considering the local variables of the area of study (Clemente-Orta et al., 2020) and the insect's movement between crops (Madeira et al., 2014, 2019). The influence of the landscape is especially relevant in the Ebro Basin (NE Iberian Peninsula), composed by a mosaic of crops dominated by arable crops and alfalfa, scattered with fruit orchards and natural or semi-natural habitats (Clemente-Orta et al., 2020). In this area, alfalfa fields host major insect predators, like coccinellids and heteropterans, which also play an essential role in the conservation biological control (CBC) of adjacent crops, like maize (Pons, 2005, 2009; di Lascio et al., 2016; Madeira et al., 2014, 2019). Even if peach orchards in the same area of study have a growing economic importance, their role in CBC has been much less studied than alfalfa crops. Some studies show the presence of anthocorids, syrphids and hymenopteran parasitoids as biocontrol agents (Avilla et al., 2009; Aparicio et al., 2019, 2020, 2021), but their role is still not well known.

Heteropterans are the most abundant arthropod predators in the Ebro Basin (Pons et al., 2005). It is known that the species of this Suborder belong to two main lineages with

different feeding habits according to their adaptative evolutions, either omnivorous or strict herbivorous (Eubanks et al., 2017). In this area, some heteropterans, like nabids and mirids have been traditionally considered as predators of aphids, as well as other prey species, but information about their potential plant consumption is unknown (Pons et al., 2009). On the other hand, mirids and lygaeids have been considered as phytophagous in other regions, even as pests of alfalfa or peach (Del Rivero and García-Marí, 1983; Schaber and Entz, 1994; Blando and Mineo, 2005). Coccinellids have been traditionally considered as true predators because most of the species of this family have been shown to feed on aphids, although they could also diversify their diet with additional food including other invertebrates, and even pollen, nectar and spores (Giorgi et al., 2009; Escalona et al., 2017). Nevertheless, in the Ebro Basin, they have been considered as aphids predators exclusively (Pons et al., 2009).
In the present study, we use an HTS multi-primer metabarcoding approach to analyse four heteropteran and four coccinellid taxa to detect their most common trophic interactions regarding arthropod, as well as plant resources. They were collected in an alfalfa and in a peach crop in the Ebro basin, both adjacent and surrounded by other natural and semi-natural habitats. The obtained information sheds light about the omnivory of these coccinellid and heteropteran taxa analysed, and therefore about their role as potential biological control agents. Ingestion of non-crop vegetation indicates the role that some plants could play in attracting or maintaining these predators within both crops, important information to further improve biological control programmes.

MATERIALS AND METHODS

Sample collection and DNA extraction

Adult heteropteran and coccinellid specimens were collected in two adjacent plots of peach, *Prunus persica* (L.) Batsch and alfalfa, *Medicago sativa* L., located in Vilanova de Segrià (Lleida), Spain (UTM 10x10: 31TCGO1) from June to September 2016, and from May to September of 2017. Peach trees were sampled by beating their branches and alfalfa by using a vacuum sampler (Mc Culloch MAC320BV, Spain). Each collected specimen was individualised in a DNA-free tube and placed in a portable freezer to avoid DNA degradation. Once in the lab, they were stored at -20ºC until the DNA extraction.

Before DNA extraction, all collected specimens were individually washed to remove contaminants from their cuticle, as described in Chapter 2. Each insect and three plant samples of 1 cm^2 diameter leaf of both *P. persica* and *M. sativa*, that were not washed were DNA 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°C. A negative control without DNA (just DNA-free water) was added to each DNA extraction set.

The concentration of each DNA extraction was measured using a Qubit® 2.0 fluorometer and the dsDNA HS Assay kit (Invitrogen, Carlsbad, CA, USA). Equimolar amounts of each insect individual DNA extraction (5 ng/µl) were finally pooled by species, crop and date in 33 sample-pools (Table 1.4; sample-pools 1-33). In order to validate the accurately parameterised bioinformatic pipeline, three plant sample-pools built with *P. persica* and *M. sativa* DNAs, were used as positive controls (Table 1.4; sample-pools 34-36), as recommended by Jusino et al. (2019) t

PCR amplification, library preparation and sequencing

The obtained sample-pools were amplified using the multi-primer approach described in Chapter 2, with two pairs of universal arthropod primers (ZBJ-ArtF1c/ZBJ-ArtR2c and mlCOIintF/HC02198) and two pairs of universal plant primers (ITS-S2F/ITS4R and cA49325/trnL110R). Target DNA and DNA-free water were included as positive and negative controls, respectively. Resulting PCR products were cleaned with QIAquick PCR Purification kit (Qiagen), and 5 µl of each clean PCR product was used as template to prepare the libraries to be sequenced. HTS analysis was conducted in two batches (Table 1.4) and libraries were built by mixing the PCR products either of both pairs of arthropod primers, or those of both pairs of plant primers. In each batch, PCR and extraction negative controls were included (Table 1.4, sample- pools 37, 38 and 39). Both 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 Nextera XT Index kit. Amplicons were purified with magnetic beads and 5 µl of each library were grouped and sequenced with a paired-end approach (2 X 225 bp).

Bioinformatics

We performed the same steps of the bioinformatic analysis as those described in Chapter 2. Raw Illumina reads were merged, and assembled reads were filtered for quality with a minimum of 75% of bases ≥Q30. Resulting reads were split by length and clustered into OTUs with a similarity threshold of 97%. Chimeras were removed and the remaining OTUs were queried against custom-made databases with restrictive parameter for BLAST (BLASTN, E-value 1e-10, minimum coverage of the query sequence: 97%, numbers of alignments: 9). Taxonomy was assigned at ≥97% identity by Last Common Ancestor algorithm (LCA) with BASTA (Kahlke and Ralph, 2019). To remove possible contaminants, we only considered those OTUs that strictly had more than five reads and were detected in at least two sample-pools (Boyer et al., 2013). In those cases where the OTUs were obtained in only one sample-pool, they were considered for the analysis if they had an abundance of more than five reads with both primer pairs or if they exceeded the 0.03 % threshold of the total reads from OTUs filtered for plant or arthropod in each case.

To reduce biases not eliminated before, or spurious results as secondary predation, and with the aim of showing the most important taxa ingested, diet data were represented 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 from the total number of reads of each consumed resource group (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, a new filter was applied, which eliminated those resources with a value of RRA < 2.5 %, which was even higher than the 1% threshold recommended by Deagle et al. (2018). With the taxa obtained, a second metric was calculated, which was the percentage of Frequency of Occurrence (FOO%), being the percentage of the number of pools of each analysed specimens analysed that contain a particular resource.

Results

Predator identification

A total of 433 heteropteran (*Adelphocoris lineolatus* Goeze*, Nysius* sp.*, Lygus* sp. and *Nabis* sp.) and coccinellid (*Coccinella septempunctata* L.*, Hippodamia variegata* Goeze*, Oenopia conglobata* L. and *Stethorus punctillum* Weise) specimens were collected in both peach and alfalfa crops. They were morphologically identified to species level, except *Nysius*, *Lygus* and *Nabis*, that were identified to genus level because of the morphological detail required that was incompatible with DNA analysis. The predominant taxa varied according with the crop and the sampled date. *Adelphocoris lineolatus, Nysius* and *C. septempunctata* were found in both crops; *Lygus* and *Nabis* were found only in alfalfa; and *O. conglobata* and *S. punctillum* were only found in peach. All specimens were used to build the sample-pools for the following HTS analysis (Table 1.4).

HTS analysis of field-collected predators

The HTS analysis of the 75 libraries (Table 1) generated 17,022,304 raw paired-end reads. The number of reads and OTUs obtained in each step of the bioinformatic process is reported in Table S1. From the raw paired-end reads obtained in step 0, only 8,930 (0.039%) came from the DNA extraction blank (sample-pool 39) and both PCR blanks (sample-pools 37 (batch 1) and 38 (batch 2)) (Table 1). After step 1, 97. 55% of the initial reads were successfully merged, discarding 207,704 raw reads. After that, 207,704 (step 2) and 161,548 (step 3) reads were discarded, respectively. After clustering the reads (step 4), 62,619 chimera reads were discarded (step 5). After the taxonomy assignment (step 6), 2,270 arthropod and 828 plant OTUs were filtered (step 7). After the OTUs filtering (step 7) to eliminate contaminants (the reads obtained from both PCR blanks and the DNA extraction blank), the taxa with RRA% lower than 2.5% were also eliminated (Table S1 step 8; Table S2). From the 39 sample-pools analysed (Table 1), we obtained 795 arthropod and 210 plant OTUs, which were finally assigned to 27 arthropod taxa (17 to species level) and 14 plant taxa (four to species level) (Table 2). The predator itself was detected in all the analysed sample-pools, either to genus or to species level (Table S3). Regarding the analysed Heteroptera, the mirid *A. lineolatus* sampled in alfalfa and peach (sample-pools 1, 2, 3 and 22, Table 1.4), showed arthropod and plant amplification in all sample-pools (Table S3.4). From those *A. lineolatus* collected in alfalfa, we detected one pest species (*Nysius graminicola* Kolenati & F.A. (Lygaeidae)); five predator taxa belonging to two families (Coccinellidae and Miridae), one genus (*Adelphocoris)* and two species (*O. conglobata* and *Rhagonycha fulva* Scopoli (Cantharidae)); one non-pest species (*Diaphorina lycii* Loginova (Liviidae)); and six plant taxa corresponding to *Medicago sativa* L. (the crop where they were collected)), some unidentified plants of the Clade Streptophyta, three families (Asteraceae, Fabaceae and Poaceae), and one species (*Beta vulgaris* L. (Amaranthaceae)) (Table 2). From those *A. lineolatus* collected in peach, we detected one arthropod non-pest taxon *(D. lycii)* and three plant taxa (one corresponding to alfalfa (*M. sativa*), and two plant families (Asteraceae and Fabaceae) (Table S3.4; Fig. 1.4; Fig. 2.4).

The other mirid bug, *Lygus*, which was only collected in alfalfa (sample-pools 23 to 25, Table 1.4), showed arthropod and plant amplification in all of the analysed sample-pools (Table S3.4). *Lygus* did prey on the same pest species than *A. lineolatus* (*N. graminicola)*,

as well as on Streptophyta and three particular families (Asteraceae, Fabaceae and Solanaceae) (Table S3.4; Fig. 1.4 and Fig. 2.4). The species of *Lygus* could not be identified (see discussion).

From the lygaeid *Nysius* sampled in alfalfa and peach (sample-pools 4 to 10, Table 1.4), we also obtained arthropod and plant amplification in all of the tested sample-pools (Table S3.4). In the case of *Nysius* sampled in alfalfa, we identified two *Nysius* species in those pools, *N. graminicola* and *Nysius cymoides* Spinola. They consumed three arthropod taxa, corresponding to two families (Coccinellidae and Miridae) and one species (*R. fulva*); and three plant families (Asteraceae, Fabaceae and Solanaceae). From *Nysius* collected in peach, we identified only one *Nysius* species (*N. graminicola*), and we detected two other arthropod taxa, corresponding to one family (Coccinellidae) and one species (*R. fulva);* and three plant taxa, one species (*M. sativa*) and two another families (Asteraceae and Fabaceae) (Table S3.4; Fig. 1.4 and Fig. 2.4).

Regarding the diet of the analysed *Nabis,* which were collected exclusively in alfalfa (sample-pools 26 to 29, Table 1.4), showed arthropod and plant amplification in all of the tested sample-pools (Table S3). We detected five pest taxa, corresponding to two genus (*Aphis* and *Hypera* (Curculionidae)) and three species (*Aphis craccivora* Koch & C.L., *Therioaphis trifolii* Monell (Aphididae), and *N. graminicola*); six predator taxa (two families (Cecidomyiidae and Miridae), one genus (*Sphaerophoria* (Syrphidae)), and three species *(Aeolothrips intermedius* Bagnall (Aeolothripidae), *Deraeocoris serenus* Douglas & Scott (Miridae) and *Orius niger Wolff* (Anthocoridae)). We also identified one parasitoid, the tachinid *Leucostoma* sp., known to parasitize Heteroptera, as well as three plant taxa (*M. sativa* and two families (Asteraceae and Fabaceae) (Table S3.4; Fig. 1.4 and Fig. 2.4; Table 2).

Regarding the analysed coccinellid predators, *C. septempunctata* sampled in alfalfa and peach, showed arthropod and plant amplification in all sample-pools (sample-pools 11, 12, 13 and 30, Table 1.4). Those collected in alfalfa showed ingestion on two pest taxa corresponding to one genus (*Aphis*) and one species (*N. graminicola*); the coccinellid parasitoid *Dinocampus coccinellae* Schrank (Braconidae); two predator taxa corresponding to one family (Coccinellidae) and one species (*R. fulva)*; and five plant taxa (Streptophyta, two families (Asteraceae and Fabaceae), one genus (*Pinus*) and one species (*Poa annua* L. (Poaceae)). From *C. septempunctata* collected in peach, we detected one pest species (*N. graminicola*); one parasitoid (*D. coccinellae*)*;* two predator taxa corresponding to one family (Coccinellidae) and one species *(R. fulva*); and four plant taxa (Streptophyta, two families (Asteraceae and Poaceae), and one genus (*Pinus*) (Table S3.4; Fig. 1.4 and Fig. 2.4).

Hippodamia variegata, which was only found in alfalfa (sample-pools 31 to 33, Table 1.4), showed arthropod and plant amplification in all the analysed sample-pools. We detected two pest taxa (the genus *Aphis* and the species *N. graminicola*)*;* six predator (two families (Coccinellidae and Miridae), one genus (*Nabis*), and three species (*C. livida*, *D. serenus* and *R. fulva*); and ten plant taxa (Streptophyta, one order (Caryophyllales), five families (Asteraceae, Brassicaceae, Fabaceae, Poaceae and Solanaceae, and *M. sativa*), and two genera (*Malva* and *Pinus*) (Table S3.4; Fig. 1.4 and Fig. 2.4).

Oenopia conglobata, which was only found in peach (sample-pools 14 to 16, Table 1.4), just amplified the predator itself. No other arthropods or plants were detected (Table S3.4; Fig. 1.4 and Fig. 2.4).

Stethorus. punctillum, which was also only found in peach (sample-pools 17 to 21, Table 1.4), showed arthropod and plant amplification in all of the sample-pools. We detected two arthropod taxa corresponding to one family (Coccinellidae) and one pest species (N. *graminicola*), as well as three plant families (Asteraceae, Fabaceae and Rosaceae) (Table S3.4; Fig. 1.4 and Fig. 2.4).

Regarding the plant positive controls built with a piece of leaf of peach (sample-pool 34; Table 1.4) and alfalfa (sample-pools 35 and 36; Table 1.4), we amplified *P. persica* and *M. sativa*, respectively, as well as their corresponding families (Rosaceae and Fabaceae) (Table S3.4). Rosaceae and Streptophyta were also detected in sample-pools 34 and 35 (Table S3.4). Nevertheless, they represented only 0.0056% of the total plant reads obtained after applying all filters. For this reason, they were not considered in further analysis.

Discussion

This study was performed to assess the most common trophic interactions of some heteropterans and coccinellids in a peach and an alfalfa adjacent crop by an HTS multi-primer metabarcoding approach. Results showed this method as a reliable tool to understand the trophic interactions of these two groups of omnivorous insects present in this agroecosystem, and to elucidate their role as omnivorous predators. This methodology was also able to detect potential pests of both crops, to detect intraguild predation among predators (including those analysed), to show the importance of some plant species as predator resources, and to demonstrate insect movement between elements of the landscape, particularly from the field margins to the crops.

Methodological issues

HTS techniques allow amplifying a high diversity of ingested taxa (Forin-Wiart et al., 2018). In the present study, we have performed the HTS analysis by using sample-pools in order to increase the robustness of the results. Those predator species collected in the same crop and date were grouped in sample-pools, which were used as biological replicates (Table 1). It has been described that pooling samples reduces the detection of the less frequent trophic interactions, allowing the detection of the most ingested species and avoiding the detection of spurious trophic interactions with less importance (Mata et al., 2019). We prioritised the use of biological replicates instead technical replicates of the PCR reactions because biological replicates in HTS led to obtain a more significant variation in the prey species composition detected than those obtained by different PCR replicates per sample (Mata et al., 2019). The analysis of a single PCR replicate per sample, like in the present study, allows the identification of the most abundant consumed resources present in a sample-pool (Leray and Knowlton, 2017). Therefore, the use of biological replicates shows that if a taxon is detected in all sample-pools, it must be a common resource (Chapter 2 and Chapter 3).

We built our own customed database with sequences downloaded from the NCBI public database. It is well known that some errors could be present in those sequences (Shen

et al., 2013), and that the absence of sequences for some species potentially present in the studied ecosystems is frequent (Corse et al., 2019). The completeness and reliability of the databases are the most critical limitations of metabarcoding studies (Galan et al., 2018). The absence of sequences in those databases generate false negative results, not detecting the occurrence of those species not included in the databases (Sow et al., 2019).

The restrictive sequence analysis conducted in the present study shows that 85% and 73% of the OTUs obtained of arthropods and plants, respectively, were not identified after the assignment using our own customed database (Step 6, Table S1). This high percentage of unassigned OTUs is probably due to the scarcity of representative sequences of arthropods and plants from the Ebro basin in the NBCI database. After this assignment and the use of both filters (Steps 7 and 8), the obtained trophic interactions came from only 25% of the obtained OTUs, but this still gives a good overview of the main trophic interactions with a high level of confidence due to the restrictive conducted bioinformatic analysis. Particular caution should be taken to discriminate between primary and secondary predation (hyperpredation) in metabarcoding studies, that is to say, when one predator feeds on another one that has recently eaten another prey, or the predator itself is parasitised. This has been reported as one of the most important limitations of the HTS technique (Galan et al., 2018; Da Silva et al., 2019). In order to decrease its impact, we have calculated the RRA% (Relative Read Abundance) of the detected taxa, as done in Chapter 2 and Chapter 3. In the present study we detected secondary predation of *L. gracilis* in two *Nabis* sample-pools (25 and 27, Table 1) with a RRA of 2.29% and 1.09%, respectively (Table S2.4 (2)). *Lipolexis gracilis* is a primary parasitoid of Aphididae, which included *A. craccivora* in alfalfa (Pons et al., 2011), being evident in this case that *Nabis* indirectly ingested it. This is why, to avoid the detection of secondary predation we decided to applied a threshold of 2.5 % in the bioinformatic analysis. The detection of this parasitoid in *Nabis* could be because this parasitoid is one of the most prevalent in alfalfa in the area of study (Pons et al., 2011). The high FOO% (Frequency of Occurrence) of *Aphis* and *A. craccivora* (75% *Aphis*, 100% *A. craccivora*; Fig. S1.4 A) in the *Nabis* samples indicated that this predator has a preference for aphids, and that they were very common in the agroecosystem, which could also favor the presence of the parasitoid. *Lipolexis* spp. have also been reported from the same peach plot parasitizing *Myzus persicae* (Sulzer) (Aparicio et al., 2019; Kocić et al., 2020).

Our results showed that the methodology used allows distinguishing between species of difficult identification, like *N. graminicola* and *N. cymoides* in the *Nysius* sample-pools (Table S3). However, in other cases, we did not identify different species of one genus, as in the *Lygus* or *Nabis* sample-pools. The explanation could be the lack of sequences in the public databases, as previously stated. The genus *Lygus* is represented in the study area mainly by *Lygus rugulipennis* Poppius (López-Marin et al., 2017), but also by *Lygus pratensis* L., *Lygus punctatus* Remane and *Lygus wagneri* Remane. The genus *Nabis* is represented by *Nabis pseudoferus* Remane, *Nabis provencalis* Remane and *Nabis punctatus* A. Costa in the same area of study (Nuñez, 2002; Pons and Eizaguirre, 2009; López-Marín et al., 2017). When we built the customed database, the available sequences in NCBI database for *Lygus* were: *L. rugulipennis*, *L. pratensis*, *L. punctatus*, *L. lineolaris* and *L. gemellatus*. However, the analysed specimens could also belong to *Lygus italicus* Wagner o *Lygus maritimus* Wagner, as they had been also cited in Catalonia

(Goula et al., 2020). Regarding Nabis, the sequences available in the database when analysis were conducted belonged to *N. pseudoferus* and *N. punctatus*. It is possible that the anaylsed specimens belonged to *N. provencalis*, the most abundant in the area of study in alfalfa (Nuñez, 2002), but its sequence is not available until now.

In the used HTS analysis, most of the detected taxa (70%) were identified to genus/species level, which is a suitable taxonomic resolution, according to a previous HTS study of omnivory conducted with mammals, that reached a 60 % (De Barba et al., 2014). The obtained taxonomic resolution agrees with the one obtained in chapter 2 and 3 and in other previous studies, detecting mainly species when using the COI region (Elbrecht et al., 2016), or the ITS2, and mainly family level when using *trnL* plant region (De Barba et al., 2014), as happens in the present study.

Trophic interactions

The used HTS multi-primer approach showed the omnivory of all the heteropteran and coccinellid analysed species, except for *O. conglobata,* from which no arthropod or plant resources were detected. Even if this species is a well-known predator in urban green and herbaceous plants (Hodek et al., 1966, Mehrnejad et al., 2004, Lumbierres et al., 2018), we did not detect any feeding episode of this species. The taxa amplified by both arthropod and plant primer pairs showed a complex food web structure with species feeding on different trophic levels (phytophagues, other predators and parasitoids, and plants) (Fig. 1.4 and Fig.2.4). This food web is represented by 18 trophic interactions, five pest taxa, ten polyphagous predators and two parasitoids (*Leucostoma* and *D. coccinellae*) (Table S2.4). Other previous HTS studies conducted in agroecosystems have also detected parasitism (Lefort et al., 2017; Sow et al., 2019). The parasitism of *C. septempunctata* by *D. coccinellae* detected agree with those field observations conducted by Pons et al. (2015). Regarding to *Leucostoma*, several species are present in southern Europe, included the Ebro basin, like some heteropteran parasitoids, as well as *Nabis* parasitoids in alfalfa (Lattin, 1989; Tschorsnig and Herting, 1992).

From the four analysed heteropteran species, three of them (*A. lineolatus, Nysius* and *Lygus*) have been traditionally considered exclusively phytophagous, being either pests of alfalfa or peach (Del Rivero and García-Marí, 1983; Schaber and Entz, 1994; Blando and Mineo 2005). Our results demonstrated their predation on pests (*N. graminicola*), other predators (*Nabis, O. conglobata* and *R. fulva*), and non-pest prey (*D. lycii*) for the first time (Fig. 1.4). Some of these predation episodes had been suggested by Nuñez (2002), based on field observations of *A. lineolatus* and *Lygus,* but no information is available in the literature regarding predatory episodes of *Nysius.* This genus is mainly composed by secondary pests of different crops, like olive, grapevine or tomato, being found in a wide range of other host plant species (Scacinni and Fuland, 2019).

In the present study, *A. lineolatus* fed on the non-pest species *D. lycii*, being the most common trophic interaction of this predator (Fig. S1A). *Diaphorina lycii* is oligophagous on *Lycium* plants (Solanaceae) (Burckhardt et al. 1984), and *Lycium europaeum* L. is commonly planted in hedges to separate agricultural plots in the study area (Bolòs and Vigo, 1996). Therefore, it can be assumed that *A. lineolatus* might have moved from those plants located in the margins to the peach crop, and from the same margins to the alfalfa crop. Probably they find more plant resources there, like in the crops themselves or in the plants that are present in the ground covers. Other movements were also observed among other elements of the landscape in the same area of study, like

Anthocoris nemoralis Fabricius moving from the field margins or from other more distant plants to the peach crop (Chapter 2), or *Orius* spp. moving from peach to alfalfa (Chapter 3).

The fourth analysed heteropteran taxon was *Nabis*, which showed the highest number of trophic interactions (Fig. 1.4). These results confirmed its predatory nature. It is a known biological control agent in alfalfa (Pons et al., 2009). In the present study they were detected to prey on alfalfa pests like some Aphididae (*Aphis, A. craccivora* and *T. trifolii*) and *Hypera*, trophic interactions already cited as well by Nuñez (2002) and Pons et al. (2009). On the other hand, *Nabis* was also detected to prey on *N. graminicola*, as well as on other predatory taxa (Fig. 1.4).

Regarding the four analysed coccinellids, in three of them some crop pests, like aphids or *N. graminicola* were detected (Fig. 1.4). *Coccinella septempunctata* and *H. variegata* collected in alfalfa, showed their well-known aphidophagous nature, already cited by Pons et al., (2009) in the same area of study. Nevertheless, the most important trophic interaction was on *N. graminicola,* which was detected in seven of the eight analysed predator species (Fig. 1.4). This species has been cited to be an important pest of several summer crops in Italy, including peaches (Blando and Mineo, 2005). *Nysius graminicola* has been also described in citrus in Catalonia, but it has not been considered a crop pest (Ribes et al., 2004). On the other hand, *N. cymoides* was also detected in two sample-pools (Table S3.4). This species causes damages in some crops in the Middle East and Europe, including alfalfa (Scaccini and Fuland, 2019). Its presence in the area of study had not been documented until now.

However, the most detected trophic interactions were related to intraguild predation (IGP), shown in 13 of the 18 detected arthropod taxa. In this IGP is included *R. fulva* and *O. niger* analysed predators in Chapter 2 and 3; *C. livida and A. intermedius* taxa obtained, that were detected as well in Chapter 2 and 3 and *D. serenus*, that were not sampled but these results show their presence in the agroecosystem. As well *Nabis* and *O. conglobata* analysed in present study and all of them present in the area of study.

Plant DNA was detected in all analysed heteropterans and coccinellids (except *O. conglobata*), showing their omnivorous trend in both sampled years. The most consumed plant taxa were Asteraceae, Poaceae, Solanaceae, Fabaceae and *M. sativa* (Fig. S1.4 C and D; Fig. 1.4 B). These predators were collected in summer, when green plants are scarce in the field margin and ground covers. In the area of study, all plants are dry at this time of the year because of the extremely high temperature, and the only green plant at that time is alfalfa, together with the ground cover of irrigated peaches (Clemente-Orta et al., 2021). Identical plant taxa were obtained when *Ragonycha fulva* and *Orius* spp. collected in the same area of study and in the same time of the year were analysed by the same HTS analysis (Chapter 2 and Chapter 3), emphasizing the importance of these plant taxa for several predator species in the studied agroecosystem.

Other plant taxa had a high FOO% (Fig. S1.4 C and D), showing that they could also be important plants in the diet of the analysed predators, like Caryophyllales, Brassicae, *Malva* and *Pinus* for *Nabis; B. vulgaris* for *A. lineolatus*; and Caryophyllales, Brassicae, *Malva* and *Pinus* for coccinellids. These results seem to indicate that different groups of predators may use different plants, demonstrating the need, of conserving those plant taxa to maintain the complex of predators close to these crops. These plants should be

considered when establishing flower margins for predator conservation or attraction to the crop.

Pinus was detected within *C. septempunctata* from both alfalfa and peach, and within *H. variegata* from alfalfa, as well as within *Orius* collected in the same alfalfa and peach field crops (Chapter 3). This detection can be only explained by the presence of windspread *Pinus* pollen deposited on both crops, because pine trees were not close to the crops. *Pinus* pollen could have a significant effect on these biological control agents as a secondary resource, particularly in this challenging period of the year when plant resources are so limited. The use of windspread maize pollen by predatory mites has been described (González-Fernández et al., 2009).

This study showed the most common trophic interactions of seven insect predators present in an agroecosystem composed by a peach and an alfalfa plots in the Ebro Basin, using a multi-primer HTS approach, and elucidates the movement of insects between neighboring habitats. This information allows to demonstrate the omnivorous role of the four heteropteran (*A. lineolatus, Nysius, Lygus* and *Nabis*) and three coccinellid (*C. septempunctata*, *H. variegata* and *S. punctillum*) analysed taxa. The detection of predator movement between crops, as well as from other plants located in the margins of those crops demonstrate the importance of the landscape biodiversity in the area of study. This information could be useful to improve pest management in agroecosystems enhancing biological control programs in these crops. The detection of *N. cymoides,* a potential pest in peach crops in the area of study, should be further investigated, showing the potential of this tool also for biomonitoring the presence of new threats.

Table 1.4. 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.

HTS batch number	Species/sample	Crop	Date	Sample-pool number	number of individuals	Primer pairs	Library number
1	*Adelphocoris lineolatus*	Alfalfa	September 2017	1	22	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L1
						ITS-S2F/ITS4R; CA49325/trnL110R	L2
				2	10	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L3
						ITS-S2F/ITS4R; CA49325/trnL110R	L4
		Peach		3	6	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L5
						ITS-S2F/ITS4R; CA49325/trnL110R	L6
	Nysius	Alfalfa	June 2017	4	25	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L7
						ITS-S2F/ITS4R; CA49325/trnL110R	L8
				5	22	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L9
						ITS-S2F/ITS4R; CA49325/trnL110R	L10
			July 2017	6	11	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L11
						ITS-S2F/ITS4R; CA49325/trnL110R	L12
			September 2017	7	4	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L13
						ITS-S2F/ITS4R; CA49325/trnL110R	L14
		Peach	June 2017	8	7	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L15
						ITS-S2F/ITS4R; CA49325/trnL110R	L16
			July 2017	9	3	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L17
						ITS-S2F/ITS4R; CA49325/trnL110R	L18
			September 2017	10	3	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L19
						ITS-S2F/ITS4R; CA49325/trnL110R	L20
	Coccinella septempunctata	Alfalfa	June 2016	11	6	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L21
						ITS-S2F/ITS4R; CA49325/trnL110R	L22
			June 2017	12	7	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L23
						ITS-S2F/ITS4R; CA49325/trnL110R	L24
		Peach	June 2016	13	17	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L25
						ITS-S2F/ITS4R; CA49325/trnL110R	L26
	Oenopia conglobata	Peach	June 2016	14	5	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L27
						ITS-S2F/ITS4R; CA49325/trnL110R	L28
			July 2016	15	15	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L29
						ITS-S2F/ITS4R; CA49325/trnL110R	L30

			September 2017	16	7	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L31
						ITS-S2F/ITS4R; CA49325/trnL110R	L32
	Stethorus punctillum	Peach	June 2016	17	10	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L33
						ITS-S2F/ITS4R; CA49325/trnL110R	L34
			September 2017	18	7	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L35
						ITS-S2F/ITS4R; CA49325/trnL110R	L36
				19	25	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L37
						ITS-S2F/ITS4R; CA49325/trnL110R	L38
				20	25	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L39
						ITS-S2F/ITS4R; CA49325/trnL110R	L40
				21	25	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L41
						ITS-S2F/ITS4R; CA49325/trnL110R	L42
2	*Adelphocoris lineolatus*	Alfalfa	September 2016	22	10	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L43
						ITS-S2F/ITS4R; CA49325/trnL110R	L44
	Lygus	Alfalfa	July 2016	23	11	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L45
						ITS-S2F/ITS4R; CA49325/trnL110R	L46
			August 2016	24	8	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L47
						ITS-S2F/ITS4R; CA49325/trnL110R	L48
			September 2016	25	9	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L49
						ITS-S2F/ITS4R; CA49325/trnL110R	L50
	Nabis	Alfalfa	July 2016	26	23	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L51
						ITS-S2F/ITS4R; CA49325/trnL110R	L52
			August 2016	27	14	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L53
						ITS-S2F/ITS4R; CA49325/trnL110R	L54
			June 2017	28	16	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L55
						ITS-S2F/ITS4R; CA49325/trnL110R	L56
			July 2017	29	25	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L57
						ITS-S2F/ITS4R; CA49325/trnL110R	L58
	Coccinella septempunctata	Alfalfa	May 2017	30	7	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L59
						ITS-S2F/ITS4R; CA49325/trnL110R	L60
	Hippodamia variegata	Alfalfa	June 2016	31	7	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L61
						ITS-S2F/ITS4R; CA49325/trnL110R	L62
			July 2016	32	16	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L63
						ITS-S2F/ITS4R; CA49325/trnL110R	L64

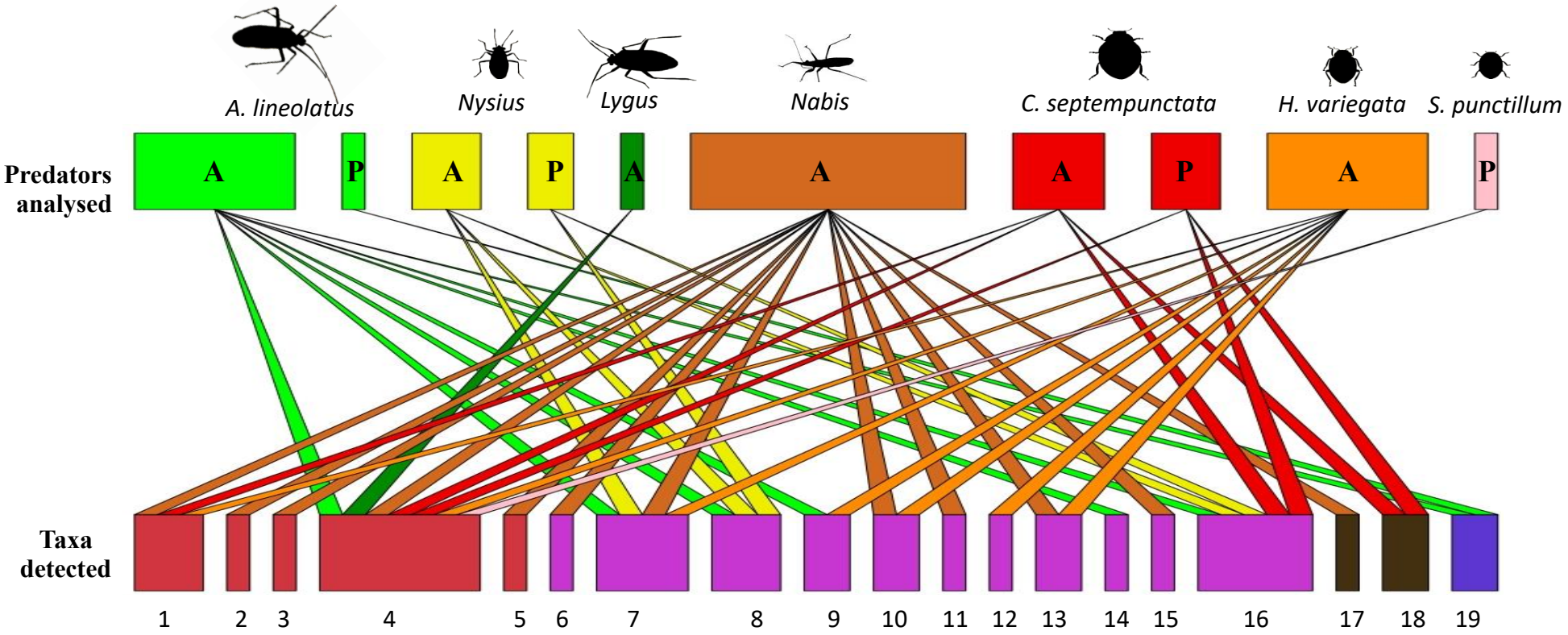
			August 2016	33	25	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L65
						ITS-S2F/ITS4R; CA49325/trnL110R	L66
1	*Prunus persica*	Peach	-	34	1 cm^2	ITS-S2F/ITS4R; CA49325/trnL110R	L67
	Medicago sativa	Alfalfa	-	35	1 cm^2	ITS-S2F/ITS4R; CA49325/trnL110R	L68
2	*Medicago sativa*	Alfalfa	-	36	1 cm^2	ITS-S2F/ITS4R; CA49325/trnL110R	L69
1	PCR blank	-	-	37	-	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L70
						ITS-S2F/ITS4R; CA49325/trnL110R	L71
2	PCR blank	-	-	38	-	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L72
						ITS-S2F/ITS4R; CA49325/trnL110R	L73
	Extraction blank	-	-	39	-	ZBJ-ArtF1c/ZBJ-ArtR2c; mlCOIintT/HC02198	L74
						ITS-S2F/ITS4R; CA49325/trnL110R	L75

Table 2.4. Summary table of all detected arthropod (n=27) and plant (n=14) taxa after the bioinformatic analysis (in bold) of HTS data (75 libraries of 39 different sample-pools (see Table S1)).

Kingdom	Phylum/Clade	Order	Family/Subfamily	Genus	Species
Animalia	Arthropoda	Coleoptera	Cantharidae		***Cantharis livida* L.**
					***Rhagonycha fulva* Scopoli**
			Curculionidae	***Hypera***	
			Coccinellidae		
					***Coccinella septempunctata* Linnaeus**
					***Hippodamia variegata* Goeze**
					Oenopia conglobata L.
					***Stethorus punctillum* Weise**
		Diptera	**Cecidomyiidae**		
			Syrphidae	***Sphaerophoria***	
			Tachinidae	***Leucostoma***	
		Hemiptera			
			Anthocoridae		***Orius niger* Wolff**
			Aphididae	***Aphis***	
					***Aphis craccivora* Koch & C.L.**
					***Therioaphis trifolii* Monell**
			Liviidae		***Diaphorina lycii* Loginova**
			Lygaeidae		***Nysius cymoides* M.Spinola**
					***Nysius graminicola* Kolenati**
			Miridae		
				Adelphocoris	
					***Adelphocoris lineolatus* Goeze**
				Deraeocoris	***Deraeocoris serenus* Douglas & Scott**
				Lygus	
			Nabidae	***Nabis***	
		Thysanoptera	Aeolothripidae		***Aeolothrips intermedius* Bagnall**
		Hymenoptera	Braconidae		***Dinocampus coccinellae* Schrank**
					Lipolexis gracilis Forster

Plantae	**Streptophyta**				
		Asterales	**Asteraceae**		
		Brassicales	**Brassicaceae**		
		Caryophyllales			
			Amaranthaceae		***Beta vulgaris L.***
		Fabales	**Fabaceae**		
					***Medicago sativa* L.**
		Malvales	Malvaceae	***Malva***	
		Pinales	Pinaceae	***Pinus***	
		Poales	**Poaceae**		
					***Poa annua* L.**
		Rosales	**Rosaceae**		
					***Prunus persica* (L.) Batsch**
		Solanales	**Solanaceae**		

Figure 1.4. Interaction network of the arthropod taxa detected within the analysed predators (*A. lineolatus, Nysius, Lygus, Nabis, C. septempunctata, H. variegata* and *S. punctillum*) collected in alfalfa and peach. **A** = alfalfa; **P** = peach.



Pest

1- *Aphis*, **2-** *Hypera*, **3-** *A. craccivora*, **4-** *N. graminicola*, **5-** *T. trifolii*

Parasitoid

17- *Leucostoma*, **18-** *D. coccinellae*

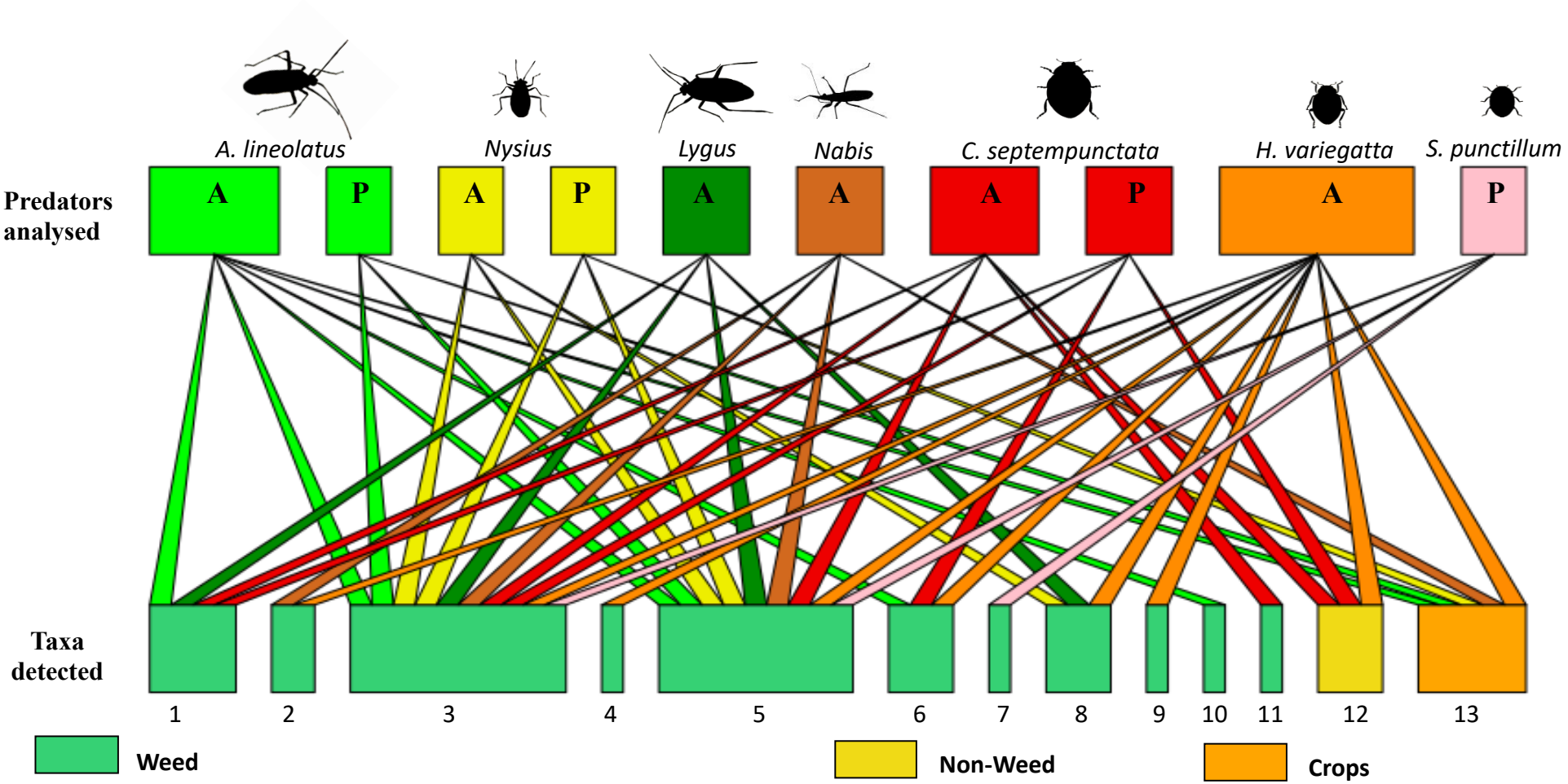
Predator

6- Cecidomyiidae, **7**- Miridae, **8**- Coccinellidae, **9**- *Nabis*, **10**-*Sphaerophoria* **11**- *A. intermedius*, , **12**- *C.livida*, **13**- *D.serenus*, **14**- *O. conglobata*, **15**- *O. niger* , **16**- *R.fulva*,

Non-pest

19- *D. lycii*

Figure 2.4. Interaction network of the plant taxa detected within the analysed predators (*A. lineolatus, Nysius, Lygus, Nabis, C. septempunctata, H. variegata* and *S. punctillum*) collected in alfalfa and peach. **A** = alfalfa; **P** = peach.



1- Streptophyta, **2**- Caryophyllales, **3**- Asteraceae, **4**- Brassicaceae, **5**- Fabaceae, **6**- Poaceae, **7**- Rosaceae, **8**- Solanaceae, **9**- *Malva*, **10**- *B. vulgaris*, **11**- *P. annua*

12- *Pinus*

13- *M. sativa*

Table S1.4. Total number of reads and OTUs obtained with each universal arthropod and plant primer pair in each step of the bioinformatic analysis. NA = not applicable.

			Arthropod primers				Plant primers			
		Total #	ZBJ-ArtF1c/ZBJ-ArtR2c		mlCOIintF/HC02198		ITS-S2F/ITS4R		CA49325/trnL110R	
Step	**Action**	**reads**	**# Reads**	**# OTUs**	**# Reads**	**# OTUs**	**# Reads**	**# OTUs**	**# Reads**	**# OTUs**
0	Raw reads	17,022,304	NA	NA	NA	NA	NA	NA	NA	NA
1	Merged reads	8,303,448	NA	NA	NA	NA	NA	NA	NA	NA
2	Quality filtering	8,227,207	NA	NA	NA	NA	NA	NA	NA	NA
3	Length splitting	8,065,659	1,500,883	NA	3,557,444	NA	706,227	NA	2,301,105	NA
4	Clustering	8,065,659	1,500,883	6,481	3,557,444	10,396	706,227	925	2,301,105	2,510
5	Chimera removing	8,003,040	1,491,896	6,421	3,504,674	8,963	706,155	878	2,300,432	2,236
6	Taxonomy assignment	4,807,367	1,108,733	1,485	702,727	1,285	704,909	202	2,290,998	626
7	OTUs contaminants filtering	4,793,925	1,105,627	618	699,628	194	704,064	24	2,284,606	214
8	OTUs secondary predation filtering	4,792,838	1,105,406	604	699,606	191	704,064	24	2,283,762	186

Table S2.4. Relative read abundance (RRA%) obtained from each arthropod and plant primer pairs in each library (L) included in the study: Arthropod taxa 1, 2, 3 (1) *Adelphocoris lineolatus* and *Nysius;* (2) *Lygus* and *Nabis* to arthropod taxa detected; (3) *Coccinella septempunctata, Hippodamia variegata, Stethorus punctillum*; Plant taxa 4, 5, 6; (4) *Adelphocoris lineolatus* and *Nysius;* (5) *Lygus* and *Nabis;* and (6) *Coccinella septempunctata*, *Hippodamia variegata* and *Stethorus punctillum*. The taxa percentages that were eliminated by the application of the 2.5% threshold are in underlined bold. Art1= ZBJ-ArtF1c/ZBJ-ArtR2c; Art2= mlCOIintF/HC02198; Pl1= ITS-S2F/ITS4R; Pl2= cA49325/trnL110R; NAm= Not amplified.

(1)

Predator species	*Adelphocoris lineolatus*						*Nysius*							
Library	L1		L3	L5	L43		L7	L11		L15		L17	L20	
Primer	Art1	Art2	Art2	Art2	Art1	Art2	Art2	Art1	Art2	Art1	Art2	Art2	Art1	Art2
Nysius graminicola	100	-	-	-	76,69	9,69	-	-	-	-	-	-	-	-
Coccinellidae	-	-	36,67	-	-	75	100	-	-	-	100	-	-	-
Miridae	-	-	-	-	-	8,16	-	70	37,5	-	-	-	-	-
Oenopia conglobata	-	-	63,33	***1,13***	-	-	-	-	-	-	-	-	-	-
Rhagonycha fulva	-	-	-	***1,32***	23,31	-	-	30	62,5	100	-	100	100	100
Diaphorina lycii	-	100	-	97,55	-	8,67	-	-	-	-	-	-	-	-

(2)

Predator species	*Lygus*					*Nabis*							
Library	L45		L47	L49		L51		L53		L55		L57	
Primer	Art1	Art2	Art1	Art1	Art2	Art1	Art2	Art1	Art2	Art1	Art2	Art1	Art2
Aphis	-	-	-	-	-	29,03	-	6,32	-	43,98	-	-	-
Aphis craccivora	-	-	-	-	-		100	-	14,29	-	63,16	-	52,94
Hypera	-	-	-	-	-	51,86	-	-	-	5,54	-	-	-
Nysius graminicola	100	100	100	100	100	-	-	***0,61***	-	18,36	27,19	***1,82***	-
Therioaphis trifolii	-	-	-	-	-	-	-	***0,36***	-	-	-	***1,32***	10,59
Aeolothrips intermedius	-	-	-	-	-	***1,74***	-	-	-	-	-	5,12	-
Cecidomyiidae	-	-	-	-	-	12,16	-		-	***1,34***	-	-	-
Miridae	-	-	-	-	-	5,21	-	***1,03***	-		-	8,91	-
Deraeocoris serenus	-	-	-	-	-	-	-	***1,64***	-	3,25	-	-	-
Orius niger	-	-	-	-	-	-	-	7,96	19,05	-	-	-	-
Sphaerophoria	-	-	-	-	-	-	-	-	-	25,24	9,65	-	-
Leucostoma	-	-	-	-	-	-	-	80,98	66,67	-	-	82,84	36,47
Lipolexis gracilis	-	-	-	-	-	-	-	***1,09***	-	***2,29***	-	-	-

(3)

Predator species	*Coccinella septempunctata*						*Hippodamia variegata*					*Stethorus punctillum*	
Library	L21		L25		L59		L61	L63		L65		L33	
Primer	Art1	Art2	Art1	Art2	Art1	Art2	Art1	Art1	Art2	Art1	Art2	Art1	Art2
Aphis	-	-	-		97,04		25,33	30,64	-	-	-	-	-
Nysius graminicola	-	-	14,58	***1,28***	***1,96***	100	9,33	5,20	-	55,24	54,64	100	100
Cantharis livida	-	-	-	-	-	-	-	6,94	-	***2,13***	-	-	-
Deraeocoris serenus	-	-	-	-	-	-	-	-	-	19,89	8,25	-	-
Dinocampus coccinellae	-	96,09	-	91,88	-	-	-	-	-	-	-	-	-

Miridae	-	-	-	-	-	-	46,67	47,40	30,77	16,52	7,22	-	-
Nabis	-	-	-	-	-	-	-	-	69,23		29,90	-	-
Rhagonycha fulva	100,00	3,91	85,42	6,84	***1,00***	-	18,67	9,83		6,22	0	-	-

(4)

Predator species	*Adelphocoris lineolatus*						*Nysius*							
Library	L4		L6		L44		L8	L10	L12	L14	L16		L18	L20
Primer	Pl1	Pl2	Pl1	Pl2	Pl1	Pl2	Pl2	Pl2	Pl2	Pl2	Pl1	Pl4	Pl4	Pl4
Fabaceae	81,05	100		89,86	-	-	-	***1,14***	5,63	***0,45***	-	10,29	42,99	28,25
Medicago sativa	-	-	100	-	3,65	-	-	-	-	-	100	-	-	-
Rosaceae	-	-	-	-	-	-	***0,23***	-	-	-	-	-	-	-
Asteraceae	18,95	-	-	10,14	-	4,63	99,77	98,86	77,11	97,65	-	87,71	57,01	71,75
Beta vulgaris	-	-	-	-	18,60	-	-	-	-	-	-	-	-	-
Caryophyllales	-	-	-	-	-	***0,78***	-	-	-	***1,90***	-	-	-	-
Poaceae	-	-	-	-	77,74	5,66	-	-	-	-	-	***1,49***	-	-
Solanaceae	-	-	-	-	-	-	-	-	17,25	-	-	***0,23***	-	-
Streptophyta	-	-	-	-	-	88,93	-	-	-	-	-	***0,27***	-	-

(5)

Predator species	*Lygus*			*Nabis*					
Library	L46	L48	L50	L52	L54		L56		L58
Primer	Pl2	Pl2	Pl2	Pl2	Pl1	Pl2	Pl1	Pl2	Pl4
Fabaceae	98,51	75,40	57,95	9,19	-	52,69	-	***0,38***	83,70
Medicago sativa	-	-	-	-	100	-	100	-	-

Asteraceae	-	17,11	30,79	90,65	-	47,31	-	99,62	10,37
Caryophyllales	-	-	-	***0,16***	-	-	-	-	5,93
Solanaceae	***1,49***	-	3,97	-	-	-	-	-	-
Streptophyta	-	7,49	7,28	-	-	-	-	-	-

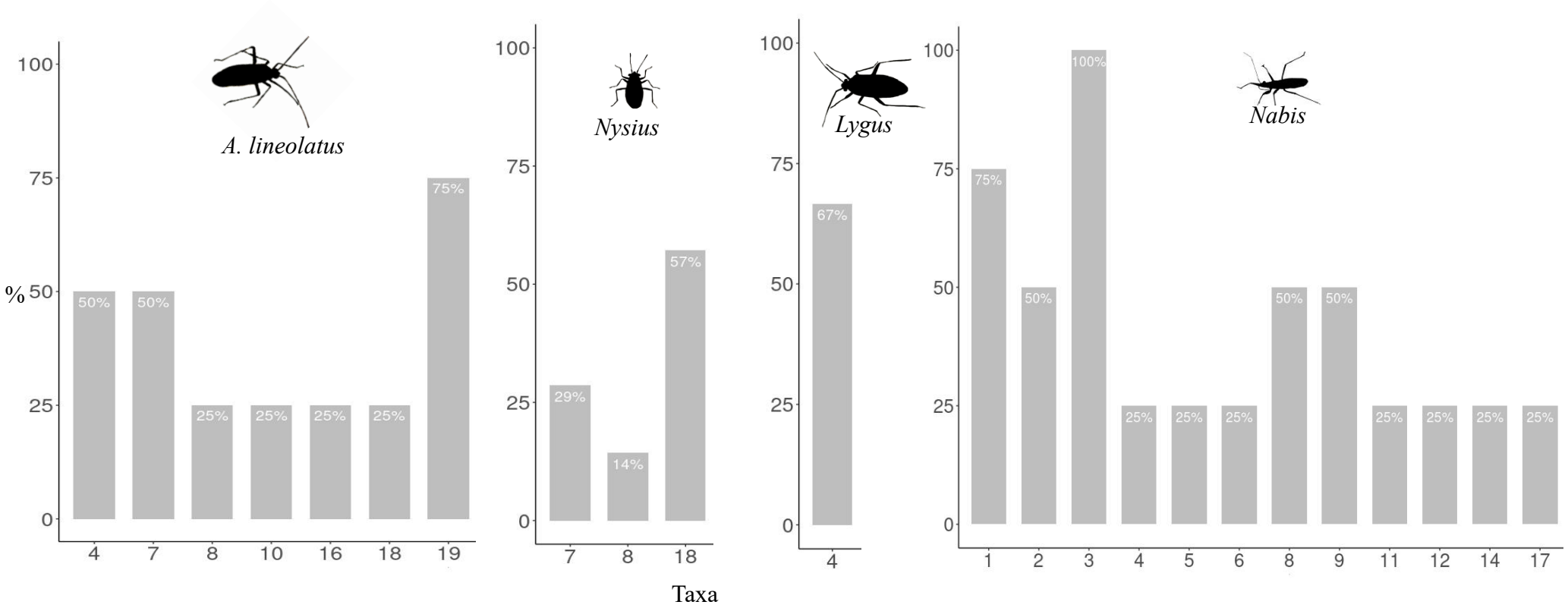
(6)

Predator species	*Coccinella septempunctata*					*Hippodamia variegata*						*Stethorus punctillum*				
Library	L22	L24	L26	L60		L62		L64		L66		L34	L36	L38	L40	L42
Primer	Pl2	Pl2	Pl2	Pl1	Pl2	Pl1	Pl2	Pl1	Pl2	Pl1	Pl2	Pl2	Pl2	Pl2	Pl2	Pl2
Fabaceae	95,98	-	-	-	***1,51***	0	28,56	-	71,89	-	58,11	8,50	-	33,33	***0,14***	-
Medicago sativa	-	-	-	-	-	100	-	100	-	100	-	-	-	-	-	-
Rosaceae	-	-	-	-	-	-	-	-	***1,98***	-	***2,25***	-	-	-	-	-
Asteraceae	-	93,79	13,13	-	90,34	-	-	-	3,09	-	4,17	***1,33***	100	66,67	99,86	-
Brassicaceae	-	-	-	-	-	-	3,31	-	-	-	***1,99***	-	-	-	-	-
Caryophyllales	-	-	-	-	-	-	***0,94***	-	6,32	-	6,09	-	-	-	-	-
Malva	-	-	-	-	-	-	-	-	11,23	-	7,08	-	-	-	-	-
Pinus	4,02	-	22,22	-	-	-	63,13	-	***1,02***	-	-	-	-	-	-	-
Plantago	-	-	-	-	-	-	***0,44***	-	***0,84***	-	-	-	-	-	-	-
Poa annua	-	***2,42***	-	100	-	-	-	-	-	-	-	-	-	-	-	-
Poaceae	-	-	6,06	-	***1,45***	-	-	-	2,52	-	17,54	-	-	-	-	-
Solanaceae	-	-	-	-	-	-	3,06	-	***0,61***	-	2,78	-	-	-	-	-
Rosaceae	-	-	-	-	-	-	-	-	-	-	-	90,17	-	-	-	-
Streptophyta	-	3,79	58,59	-	6,70	-	***0,56***	-	***0,5***	-	-	-	-	-	-	-

Table S3.4. Taxa obtained and primer pairs used from each library. NT= not tested; NAmp = not amplified. (See: https://github.com/Ivanbh214/Table_S3)

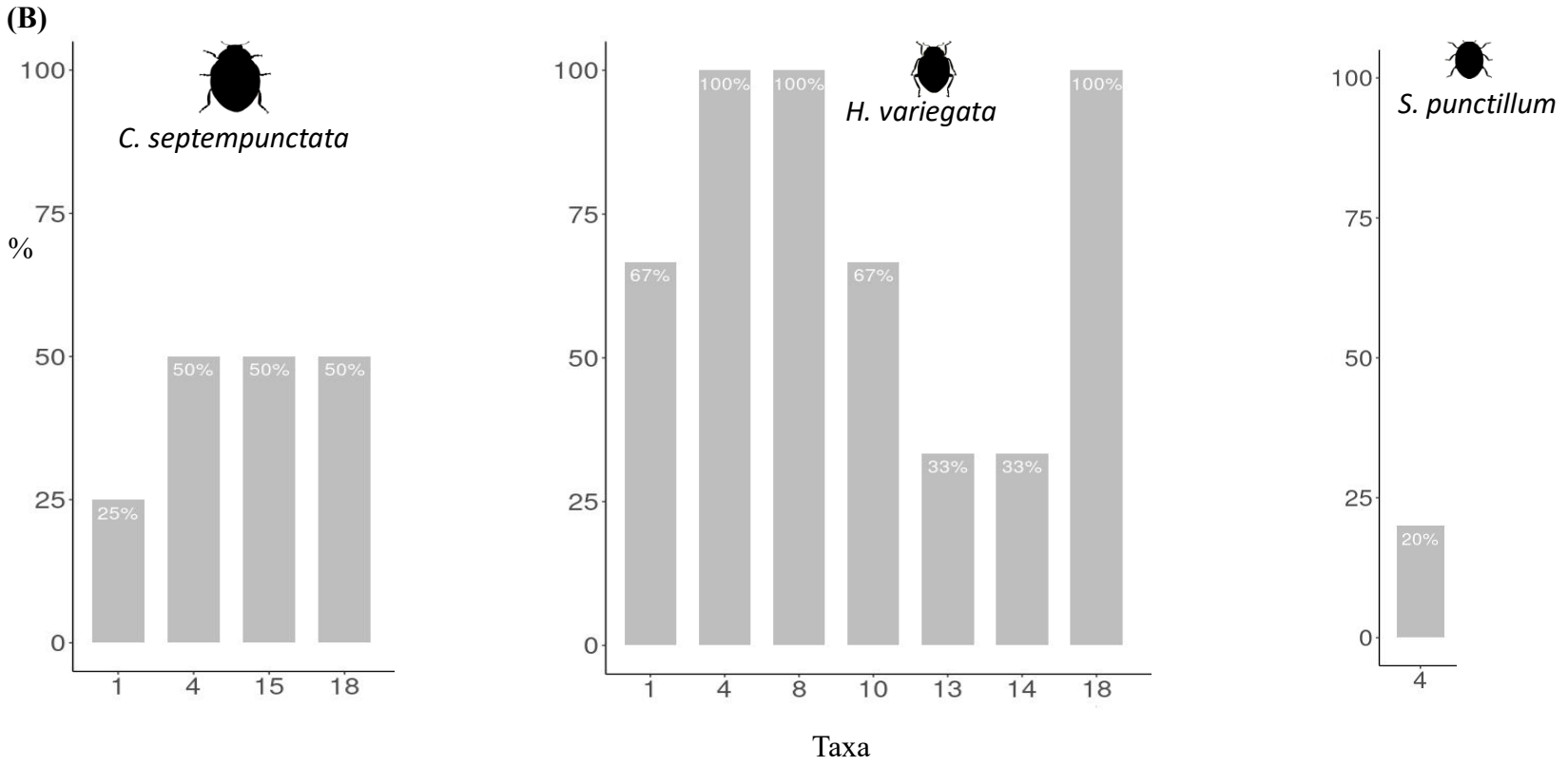
Figure S1.4. Percentage of frequency of occurrence (FOO%) of the total number of pools of (A) arthropod taxa consumed by each heteroptera analysed (B) arthropod taxa resources consumed by each coccinellid analysed (C) heteropteran plant taxa resources consumed by each predator analysed (D) coccinellid plant taxa resources consumed by each predator analysed**.**

(A)



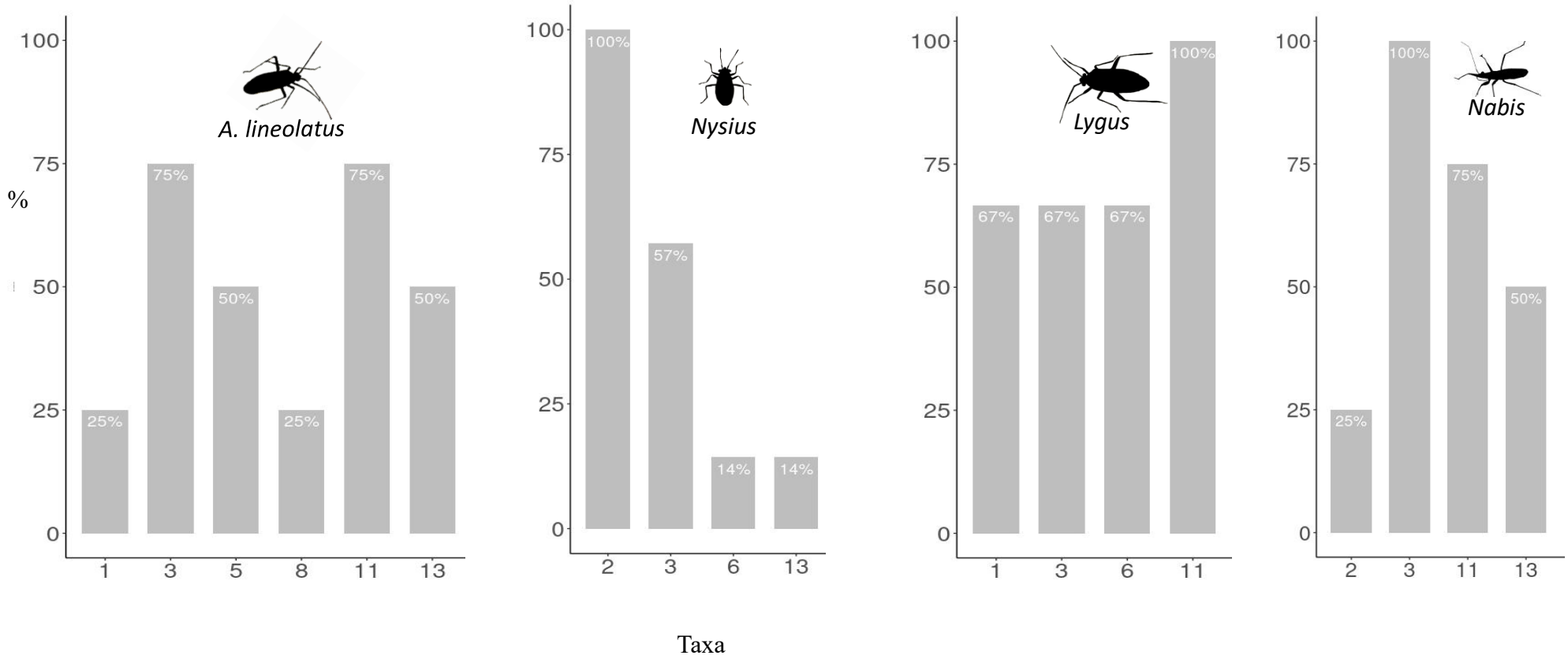
1-*Aphis*, **2**- *Hypera*, **3**- *A. craccivora*, **4**- *N. graminícola*, **5**- *T. trifolii*, **6**- Cecidomyiidae, **7**- Coccinellidae, **8**- Miridae, **9**- *Leucostoma*, **10**- *Nabis*,

11- *Sphaerophoria*, **12**- *A. intermedius*, **13**- *C. livida*, **14**- *D. serenus*, **15**- *D. coccinellae*, **16**- *O.conglobata*, **17**- *O. niger*, **18**- *R. fulva*, **19**- *D. lycii.*

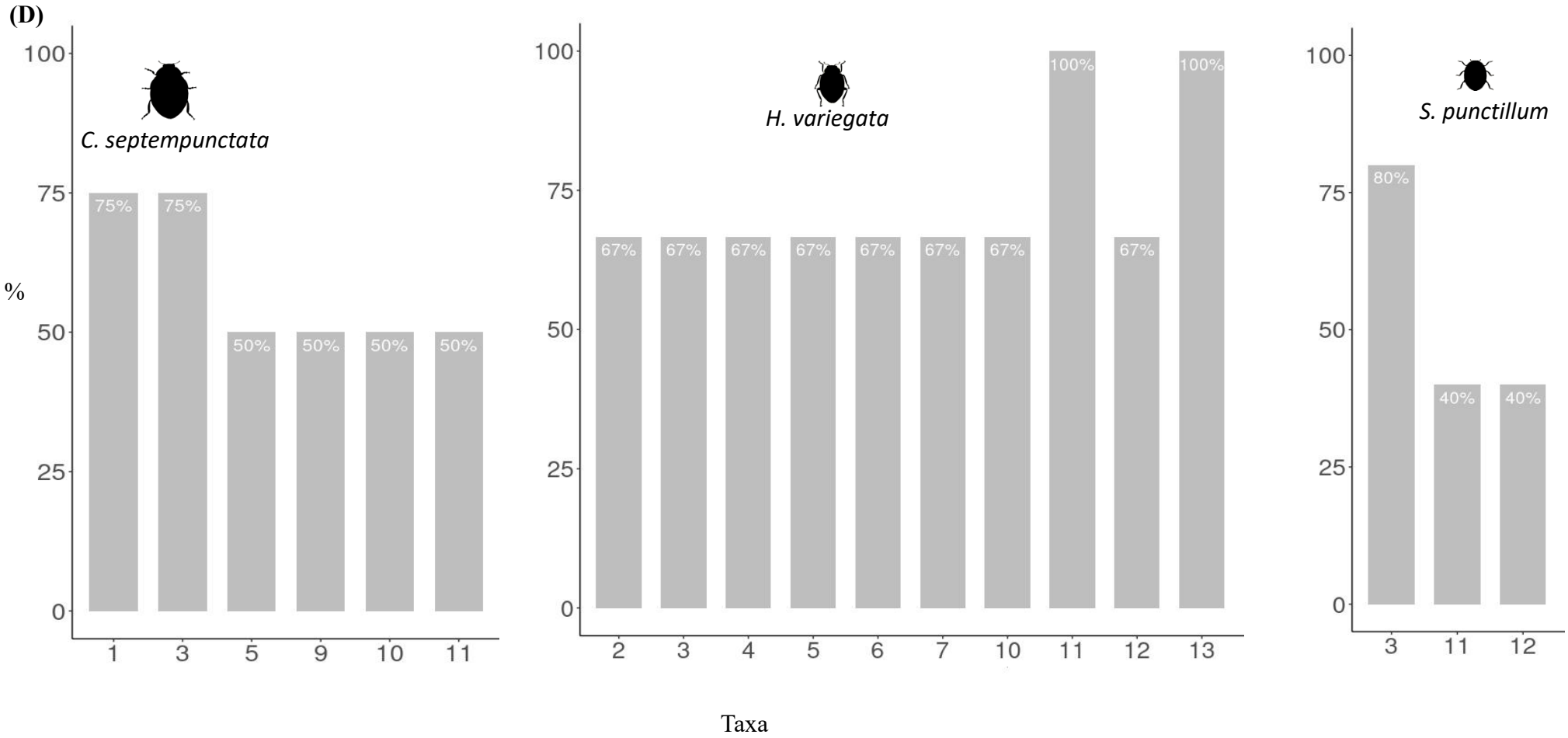


1- *Aphis*, **2**- *Hypera*, **3**- *A. craccivora*, **4**- *N. graminícola*, **5**- *T. trifolii*, **6**- Cecidomyiidae, **7**- Coccinellidae, **8**- Miridae, **9**- *Leucostoma*, **10**- *Nabis*, **11**- *Sphaerophoria*, **12**- *A. intermedius*, **13**- *C. livida*, **14**- *D. serenus*, **15**- *D. coccinellae*, **16**- *O. conglobata*, **17**- *O. niger*, **18**- *R. fulva*, **19**- *D. lycii*

(C)



1- Streptophyta, **2**- Caryophyllales, **3**- Asteraceae, **4**- Brassicaceae, **5**- Poaceae, **6**- Solanaceae, **7**- *Malva*, **8**- *B. vulgaris* ,**9**- *P. annua*, **10**- *Pinus*, **11**- Fabaceae, **12**- Rosaceae, **13**- *M. sativa*



1- Streptophyta, **2**- Caryophyllales, **3**- Asteraceae, **4**- Brassicaceae, **5**- Poaceae, **6**- Solanaceae, **7**- *Malva*, **8**- *B. vulgaris* ,**9**- *P. annua*, **10**- *Pinus*, **11**- Fabaceae, **12**- Rosaceae, **13**- *M. sativa*

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General discussion

El avance hacia una agricultura más sostenible basada en la utilización del control biológico por conservación (CBC), requiere de un conocimiento detallado de la ecología y la biología de los enemigos naturales que habitan en el agroecosistema. Para ello es necesario centrarse en el estudio de la utilización de los recursos, tanto animales como vegetales, por parte de estos enemigos naturales, así como de los movimientos que éstos realizan entre los distintos hábitats presentes en él. Esta Tesis Doctoral se ha centrado en el desarrollo y la utilización de herramientas moleculares para el estudio del movimiento de insectos, así como de sus interacciones tróficas a partir de insectos recolectados en dos parcelas adyacentes de alfalfa y melocotón en la cuenca del Ebro.

El estudio del movimiento de insectos entre ambos cultivos y desde las zonas no cultivadas a éstos ha sido abordado en los cuatro capítulos de esta Tesis. Este estudio se ha llevado a cabo mediante métodos basados en la detección de un ADN tópico, presente sobre la cutícula del insecto (**Capítulo 1**), o bien interno, presente en el contenido estomacal del insecto (**Capítulos 2**, **3** y **4**). Para ello, en los **Capítulos 1** y **2**, se han puesto a punto dos métodos moleculares diferentes que han permitido el estudio del movimiento de los insectos analizados, tanto desde el cultivo de alfalfa al de melocotón (**Capítulo 1**), como desde cualquier cultivo o infraestructura ecológica hacia los cultivos de alfalfa y melocotón (**Capítulos 2**, **3** y **4**).

Consideraciones metodológicas

En el **Capítulo 1**, se ha optimizado un método basado en el marcaje externo con una solución de cistos de *Artemia* spp. y la posterior detección mediante PCR convencional con primers específicos de *Artemia* spp. (Agustí et al. 2020). Se trata de un método de marcaje versátil, que ha sido capaz de detectar el ADN de esta especie de crustáceo, no presente en ecosistemas terrestres, tanto en condiciones de invernadero, como de campo, y en particular en una zona como la cuenca del Ebro con grandes variaciones diarias de temperatura. En esta Tesis se ha optimizado esta herramienta para monitorizar la dispersión de insectos depredadores desde un cultivo de alfalfa a un cultivo de melocotón adyacente. El bajo coste de esta metodología y la inmediatez de los resultados obtenidos mediante este método de marcaje seguido de una detección por PCR convencional, hace que sea un método de fácil utilización. Precisa de un equipamiento habitualmente presente hoy en día en laboratorios de entomología, a diferencia de otros métodos, como la detección de isótopos o los métodos serológicos. Además, los cistos de *Artemia* están fácilmente disponibles debido a su uso amplio en acuariología. Sin embargo, este método de marcaje no está ausente de desventajas, como la pérdida de persistencia en el tiempo, y sobre todo ante determinadas condiciones climáticas como la lluvia, que puede hacer desaparecer el marcaje tópico, o los elevados niveles de radiación ultravioleta que pueden llegar a degradar el ADN presente en él. Además, requiere de un marcaje previo mediante el rociado de aquellas plantas desde las que se desee estudiar el movimiento de los insectos. Estas desventajas no se dan mediante el uso de técnicas de secuenciación masiva del contenido estomacal.

Las técnicas HTS permiten la secuenciación paralela de un gran número de fragmentos de ADN, que en el caso de estudios de metabarcoding como los realizados en esta Tesis Doctoral, da como resultado la detección de un gran número de taxones. Esta

metodología HTS aplicada al análisis del contenido estomacal de los depredadores, ya había sido mostrada como una excelente herramienta para identificar agentes de control biológico potenciales y poder evaluar su rol en los agroecosistemas (Gómez-Polo et al., 2015; 2016; Sow et al., 2020). En esta Tesis, se ha desarrollado una nueva metodología multi-primer HTS para el análisis del contenido estomacal de los insectos que ha permitido determinar los artrópodos y las plantas más consumidos en condiciones reales de campo, pero este análisis HTS no está exento de complicaciones. Debido al elevado nivel técnico de análisis se requieren conocimientos de varias disciplinas, como la biología molecular o la bioinformática. Al basarse en un flujo de trabajo largo, que incluye numerosos pasos, es importante minimizar los sesgos que se puedan generar en el laboratorio y en el proceso de secuenciación, como la contaminación de las muestras, el cruce de *tags* en el proceso de secuenciación (*tag-jumping*), o la creación de quimeras en el mismo proceso de secuenciación, para evitar falsos positivos y evitar conclusiones biológicas erróneas.

La metodología HTS necesaria para la detección e identificación de los principales recursos animales y vegetales ingeridos por los insectos capturados en las dos parcelas adyacentes de melocotón y de alfalfa objeto de estudio, se ha desarrollado en el **Capítulo 2**, en el que se ha se ha puesto a punto un sistema multi-primer, con dos pares de primers universales de artrópodo y dos de planta, para la detección de los principales recursos ingeridos. En el **Capítulo 3,** este sistema se ha optimizado para la correcta detección de la depredación intragremial (IGP) presente en el agroecosistema. Finalmente, en los **Capítulos 2**, **3** y **4** se han analizado los depredadores más abundantes en las parcelas muestreadas y se han podido caracterizar los principales recursos consumidos. Todo ello ha permitido la detección del movimiento entre los cultivos y las infraestructuras ecológicas que componen el paisaje del área de estudio mediante la amplificación de taxones de plantas y de artrópodos presentes en su tracto digestivo.

Quizás la ventaja más importante de las técnicas HTS es la posibilidad de detectar relaciones tróficas desconocidas hasta el momento y con una gran resolución taxonómica (Galan et al., 2018; Taberlet et al., 2018, Nichols et al., 2018), tanto a nivel de plantas como de artrópodos. Así, se ha demostrado la ingestión de determinadas plantas en algunos artrópodos que quizás son más importantes por su rol depredador. Por ejemplo, en el caso de *Orius niger,* la ingestión de determinadas plantas hospedadoras, entre ellas el melocotón que indica su presencia en dicho cultivo y el movimiento por parte de estos depredadores omnívoros hacia el cultivo de la alfalfa (**Capítulo 3**). También se han detectado casos de presas ingeridas que se encuentran únicamente presentes sobre determinadas plantas. Este es el caso de la detección de *Diaphorina lycii* en depredadores como el antocórido *Anthocoris nemoralis* (recolectado sobre melocotón) y mírido *Adelphocoris lineolatus* (recolectado sobre alfalfa y melocotón) (**Capítulos 2** y **4**, respectivamente). Este fitófago de la familia de los psílidos se alimenta únicamente de plantas del género *Lycium* (Burckhardt et al., 1984), entre ellas *Lycium europaeum* presente en los márgenes del área de estudio (Bolòs and Vigo, 1996), sugiriendo que este arbusto es un candidato a considerar en la construcción de los setos colocados alrededor de los cultivos para fomentar la presencia de estos depredadores.

Redes tróficas del agroecosistema de estudio

En las Figuras 1.5 y 2.5 se muestra el conjunto de todas las relaciones tróficas detectadas en esta Tesis, 69 entre artrópodos y 65 artrópodo-planta, mostrando también cuáles han sido los recursos más utilizados por los depredadores analizados, algunos de ellos no descritos previamente. En ellas se observa el movimiento desde la alfalfa al melocotón demostrado en el **Capítulo 1** de esta Tesis coincidiendo con los cortes de la alfalfa. En concreto, se ha demostrado este movimiento en depredadores como los coccinélidos *Propylea quatordecimpunctata*, *Hippodamia variegata*, *Stethorus* sp., *Scymnus* sp.; y los antocóridos *Orius niger*, *Orius laevigatus*, *Orius albidipennis* y *Orius minutus*. La utilización de la metodología HTS (**Capítulos 2**, **3** y **4**) ha permitido demostrar además la presencia de otros depredadores en ambos cultivos, entre los que se encuentran los sírfidos *Sphaerophoria* sp., el trips depredador *Aelothrips intermedius*, el mírido *Deraeocoris serenus* y el cantárido *Cantharis lívida.* Estos resultados confirman el rol de la alfalfa como reservorio de enemigos naturales, como previamente había sido citado por Nuñez (2002) y Pons et al. (2005), así como el rol de la alfalfa como dador de enemigos naturales hacia el melocotón, de la misma manera que fue previamente descrito desde la alfalfa hacia el maíz (Madeira et al., 2014; 2019). Este flujo de depredadores desde la alfalfa al melocotón puede contribuir a la mejora del control biológico de las plagas en el melocotón.

En referencia a la detección de otras plantas consumidas por los insectos analizados (Figura 2.5), aparte de ambos cultivos, se encuentran las plantas presentes en las cubiertas vegetales y márgenes. Esta detección demuestra la omnivoría de la mayoría de los insectos analizados (excepto *Anthocoris nemoralis* y *Oenopia conglobata*), poniendo de manifiesto el importante rol que juegan las plantas como recursos para muchos enemigos naturales. Algunas plantas detectadas en esta Tesis conforman elementos del paisaje, como las cubiertas o los márgenes de *Poa annua* o *Malva sp;* y otras provienen de la vegetación natural más alejada de los cultivos, como *Pinus*. Esta información sugiere la necesidad de avanzar hacia prácticas culturales que fomenten la conservación de entornos diversos con mosaicos de infraestructuras ecológicas habituales en la cuenca del Ebro, o la creación márgenes florales con *Malva*, Asteraceas, Solanaceas y Poaceas, como *Poa annua*.

Acerca del consumo de artrópodos (Figura 1.5), algunas relaciones tróficas ya eran conocidas previamente, como el caso de la depredación de trips por parte de *Orius* spp. (Riudavets, 1995; Riudavets and Castañé, 1998), o de áfidos por parte de coccinélidos y nábidos (Pons et al., 2005), y otras no. La información mostrada en estas redes tróficas es de gran importancia para la puesta a punto y la mejora de programas de control biológico para plagas importantes de estos cultivos, como *G. molesta* y *M. persicae* en melocotón; y *Aphis craccivora* y *Therioaphis trifolii* en alfalfa. En la Figura 1.5 se observa que *N. graminícola* es consumido por la mayoría de los depredadores analizados (excepto *Anthocoris nemoralis* y *Orius* spp.). Este resultado es de importante debido a que varias especies del género *Nysius* son plagas de cultivos. Se sabe que *Nysius cymoides*, dectectado en esta Tesis en pools de *Nysius* recolectados en alfalfa (**Capítulo 4**) es considerada una plaga emergente en Europa (Scaccini and Fuland, 2019), y *Nysius graminicola* detectada en pools de *Nysius* recolectados en alfalfa y en melocotón es una

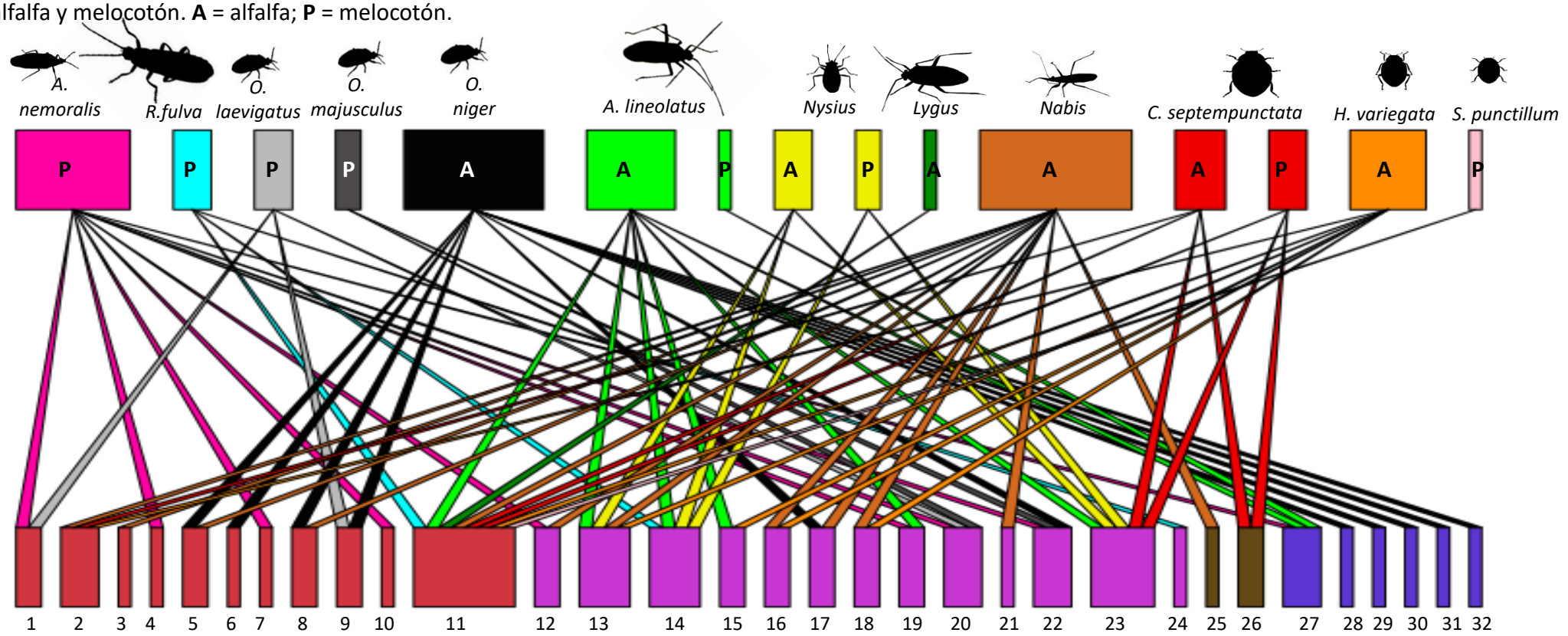
plaga de frutales en Italia (Blando and Mineo, 2005). Hasta el momento, esta especie no constituye un problema como plaga en los frutales de la cuenca del Ebro, quizás debido al control ejercido por los depredadores de ambos cultivos analizados en esta Tesis.

En la Figura 1.5 se muestra también la utilización de otro tipo de presas alternativas (que no son plaga) por parte de algunos depredadores. Este es el caso de *Orius niger*, depredador de algunos taxones de artrópodos que transmiten enfermedades tanto en plantas, como en animales, incluyendo los humanos. Esto sugiere que este depredador podría llegar a ejercer un cierto efecto en minimizar la transmisión de estas enfermedades. Es el caso de algunos cicadélidos que pueden transmitir la enfermedad de Pierce (causada por *Xylella fastidiosa*) (Bragard et al., 2019), o la escoba de brujas (causada por fitoplasmas) (Abou-Jawdah et al., 2014) que afectan al cultivo del melocotón. También es el caso de *Aedes caspius,* que trasmite enfermedades a animales, como el Virus del Nilo que puede afectar a la salud humana; o la familia Ceratopogonidae, que transmite la lengua azul a los ungulados. Estos resultados podrían mostrar la importancia de estos enemigos naturales no solo en el ámbito agrícola si no también en el sanidad vegetal y ambiental.

Perspectivas de futuro

El diagnóstico molecular es una herramienta muy útil y necesaria para el estudio de las redes tróficas que se dan en una comunidad diversa como un agroecosistema, porque muestra las verdaderas relaciones que se dan en condiciones de campo, sin artefactos producidos por la manipulación de los insectos en ensayos de depredación de laboratorio. La aparición de las técnicas HTS ha provocado una cierta revolución y la publicación de un gran número de estudios en ecología (Taberlet et al., 2018). De todas maneras, se deben mejorar una serie de limitaciones, como la falta de secuencias que representen la biodiversidad de los organismos presente en los agroecosistemas (Galán et al., 2018). Por ejemplo, en los **Capítulos 2, 3** y **4** se detecta el clado Streptophyta, lo que muestra la detección de plantas que no han podido ser asignadas a un grado taxonómico más concreto. Por ejemplo, en el **Capítulo 4,** el 85% y 73 % de los OTUs de artrópodos y plantas, respectivamente no se pudieron asignar a ningún taxon en particular. Es por tanto muy importante continuar con la secuenciación de la entomofauna y de las plantas presentes en los cultivos para ir completando las bases de datos y así obtener información aún más precisa de las relaciones tróficas presentes en los agroecosistemas. Estas técnicas HTS aplicadas a los estudios de depredación también nos pueden permitir la detección, o incluso el monitoreo, de la presencia de nuevas plagas en el cultivo, obteniendo una detección precoz de éstas que permita tomar las medidas adecuadas rápidamente. Es posible que en los próximos años las plataformas de 4ª generación, como Nanopore (MinION), vayan ganando terreno en este tipo de estudios. Estas nuevas plataformas, que tienen un coste menor en el proceso de secuenciación, podrían animar a que su uso fuera cada vez más común para la puesta a punto de programas de control biológico de plagas en agroecosistemas.

Figura 1. Red trófica que ilustra los artrópodos detectados en los depredadores analizados (*Rhagonycha fulva, Anthocoris nemoralis, Orius laevigatus, Orius majusculus, Orius niger, Anthocoris lineolatus, Nysius, Lygus, Nabis, Coccinella septempunctata, Hippodamia variegata* y *Stethorus punctillum*) recolectados en alfalfa y melocotón. **A** = alfalfa; **P** = melocotón.



Plagas

1- Aphididae, **2-** *Aphis*, **3-** *A. craccivora*, **4-** *M. persicae*, **5-** *T. trifolii,* ***6-*** *Lyriomyza,* ***7-*** *G. molesta*, ***8-*** *Hypera***, *9-*** *F. occidentalis*, ***10-*** *T. fuscipennis,* ***11-*** *N. graminicola*

Parasitoides

25- *Leucostoma*, **26-** *D. coccinellae*,

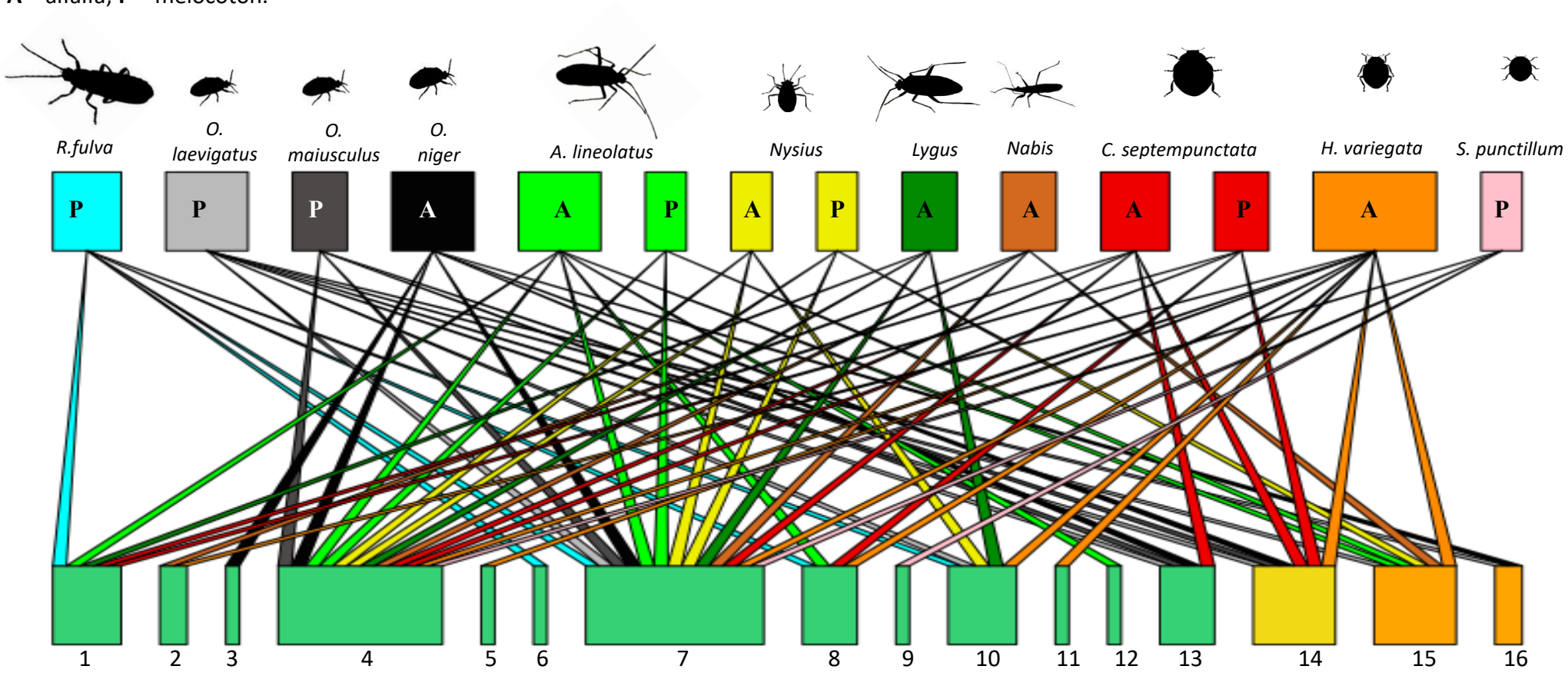
Depredadores

12- Cecidomiyiidae, **13-** Miridae, **14-** *Coccinellidae*, **15-** *Nabis*, **16-** *Sphaerophoria,* **17-***A. intermedius*, **18-** *D.serenus,* **19-** *O. conglobata* **20-** *Orius***, 21-** *O. niger,* **22-** *O. laevigatus,* **23-** *R.fulva,* **24-** *C. livida,*

Presas alternativas

27- *D. lycii*, **28-** Ceratopogonidae, **29-** Cicadellidae, **30-** Orthocladinae, **31-** *A. caspius,* **32-** *T. volgensis*

Figure 2. Red trófica que ilustra las plantas detectadas en los depredadores analizados (*Rhagonycha fulva, Orius laevigatus, Orius majusculus, Orius niger, Adelphocori lineolatus, Nysius, Lygus, Nabis, Coccinella septempunctata, Hippodamia variegata* and *Stethorus punctillum*) recolectados en alfalfa y melocotón. **A** = alfalfa; **P** = melocotón.



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Conclusions

Conclusions

The results obtained in this Doctoral Thesis show the utility of the molecular methods used to study the movement of polyphagous predators among elements of the landscape. These molecular tools provide relevant information regarding the movement of insects and the trophic interactions present in the studied agroecosystem, information that can contribute to the design of new strategies to improve biological control (BC) programs in peach and in alfalfa in the studied area.

The conclusions of this Doctoral Thesis are the following:

1. The topical marking method used with a DNA external to the agroecosystem and its detection by conventional PCR has made possible to detect the movement, from alfalfa to peaches, of predatory arthropods of interest for the BC of peach pests, such as several species of coccinellids, anthocorids of the genus *Orius*, mirids and chrysopids (Chapter 1).

2. The developed multi-primer HTS method has allowed studying the trophic relationships present in the studied agroecosystem, also allowing the detection of arthropods movement, by detecting ingested prey and plants other than peach and alfalfa, which were the target crops (Chapters 2, 3 and 4).

3. The detection of pollen in *Rhagonycha fulva* washing solutions and the non-detection in those of *Anthocoris nemoralis* indicates that some insects must be washed before being analysed by HTS to discriminate between the ingested and the visited plants. Washing solution analyses show that the largest and hairiest insects retain more pollen on the cuticle, than the smallest and glabrous ones (Chapter 2).

4. The HTS methodology used has allowed to detect trophic relationships unknown until now, such as the predation of important pests of the peaches, like *Grapholita molesta*, *Myzus persicae* or *Thrips fuscipennis* by *Anthocoris nemoralis* (Chapter 2). The role of this predator should therefore be considered in future BC programs in this crop.

5. Already known interactions have also been detected on important pests of alfalfa and peach, such as *Therioaphis trifolii*, *Liriomyza* sp., *Hypera* sp. and *Frankliniella occidentalis* fed by *Orius laevigatus* and *Orius niger* (Chapter 3); or *Aphis craccivora*, *Therioaphis trifolii* and *Hypera* sp. fed by *Nabis* and *Hippodamia variegata* (Chapter 4).

6. This multi-primer HTS has also allowed to demonstrate predator movement, both between peach and alfalfa crops, and from ecological infrastructures, such as margins; or even from more remote vegetation towards both crops, showing the importance of the plant biodiversity in this agroecosystem. The high detection of some plant families like Asteraceae, Poaceae or Solanaceae indicates their importance to conserve these predators through habitat management in CBC programs, as long as they do not represent a risk for the crop (Chapters 2, 3 and 4).

7. Intraguild predator-predator interactions have also been detected, such as those between species of the genus *Orius*; or those between *Orius niger* and *Aeolothrips intermedius* (Chapter 3). Also, predator-parasitoid interactions were detected like, *Dinocampus coccinellae* parasitizing *Coccinella septempunctata*; or *Leucostoma* sp. parasitizing *Nabis* sp. (Chapter 4). The knowledge of these trophic interactions is important since intraguild interactions can have a negative effect on the BC of the target pests.

8. This methodology also showed the polyphagia and even the omnivory of the analysed predator taxa, except for *Anthocoris nemoralis* and *Oenopia conglobata*. This information is important to develop BC programs in these crops, particularly because some of those predators were only known for their phytophagous nature, such as *Adelphocoris lineolatus*, *Nysius* and *Lygus,* and now it has been shown that they can exert a predatory role (Chapters 2, 3 and 4).

9. This HTS method has also allowed to detect the ingestion of insects that transmit diseases to plants and animals. This is the case of certain leafhoppers that can be vectors of diseases in peaches; the family Ceratopogonidae, which could be the vector of the bluetongue of livestock; or *Aedes caspius*, the vector of the Nile virus, which can affect human health. All of them were ingested by *Orius niger*, which suggests that this predator could have an effect in order to minimise the transmission of these diseases, which should be further studied (Chapter 3).

10. Using this HTS method, it has been also demonstrated the movement by detecting the ingestion of a phytophagous insect present in a particular plant species. This is the case of the movement of the predator *Adelphocoris lineolatus* towards peach and alfalfa, detected through the ingestion of *Diaphorina lycii*, an oligophagous insect of *Lycium europaeum*, present in the margins between agricultural plots in the study area (Chapter 4). *Anthocoris nemoralis* collected on peaches has also shown this ingestion (Chapter 2).

11. The used HTS methodology has also allowed to identify species that are difficult to identify by morphological methods, like two species of the genus *Nysius*, which were *N. cymoides* and *N. graminicola* (Chapter 4)

12. On the other hand, *Nysius graminicola* has been the prey detected in the largest number of predators (Chapters 2, 3 and 4). These significant trophic interactions should be taken into account in future BC programs of this species in peach.

13. Finally, the HTS methodology used in this Doctoral Thesis for the study of trophic interactions and arthropod movement is potentially usable in other agroecosystems, and in a great variety of arthropods of different morphologies to study trophic webs.