Wild pollination in agroecosystems with a focus on Brachycera and Hymenoptera
an integrative approach

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“The greatest danger to our future is apathy.”

Jane Goodall

Para Thomas, Bernarda y Simone.

To all women in science.

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Preface

This thesis was carried out at the Leibniz-Institute for the Analysis of Biodiversity Change – Museum Koenig and at the Department of Agroecology and Organic Farming in the Institute of Crop Science and Resource Conservation at the University of Bonn, in the framework of the project GBOL II. The project was funded by the German Federal Ministry of Education and Research.

Zusammenfassung

Als Bestäubung versteht man die Übertragung von Pollenkörnern von den Staubbeuteln auf die Narbe von Pflanzen. Diese enge wechselseitige Beziehung zwischen Tieren und Blütenpflanzen, hat die Vielfalt der Angiospermen weltweit geprägt. Insbesondere die Bestäubung durch Insekten zählt zu den wesentlichen Ökosystemleistung für den Menschen, da viele Anbaupflanzen für eine optimale Frucht- und Samenproduktion in hohem Maße von Bestäubung abhängig ist. Die Intensivierung der Landwirtschaft im letzten Jahrhundert hat zu folgenden Widerspruch geführt: Einerseits besteht ein erhöhter Bedarf an erfolgreicher und optimaler Bestäubung zur Sicherung der Produktion; andererseits, trägt die mit intensiven Anbaumethoden verbundene Expansion der Landwirtschaft zu einem weltweiten Rückgang der Bestäuber bei. Eine erfolgreiche Bestäubung kann daher oft nur durch verstärkten Einsatz bewirtschafteter Bestäuber, vor allem der Europäischen Honigbiene *Apis mellifera* (Linnaeus, 1758) und anderen ausgewählten Wildbienenarten, gesichert werden. Die Abhängigkeit von diesen spezifischen Bestäubern führt in Agrarökosystemen zu einer Unterschätzung und verzerrten Wahrnehmung der Vielfalt anderer potenzieller Bestäuber. Zu den relevantesten Bestäubergruppen der Agrarökosysteme gehören Bienen und Wespen (Hymenoptera), sowie Fliegen (Diptera: Brachycera). Diese potenziellen Bestäuber interagieren nicht nur mit den angestrebten Anbaupflanzen, sondern auch mit weiteren Pflanzenarten, die als komplexe Interaktionen zwischen Pflanzen und Insekten in zweiseitigen ökologischen Pflanzen-Bestäuber-Netzwerken analysiert werden. Diese Netzwerke und ihre Struktur geben Aufschluss über die Stabilität von Pflanzen-Insekten-Interaktionen als Ökosystemfunktionen und ihre Widerstandsfähigkeit gegenüber äußeren Einflüssen und Stressfaktoren. Die gängigsten Methoden zur Analyse dieser Pflanzen-Bestäuber-Netzwerke sind Erhebungen der Blütenbesuche, wobei nicht alle Blütenbesucher auch Bestäuber sind, oder die morphologische Bestimmung der Zusammensetzung der Pollenfracht an Insekten. Die morphologische Identifizierung von Pollen ist jedoch zeitaufwändig, erfordert viel Fachwissen und führt in der Regel zu einer geringeren taxonomischen Auflösung. DNA metabarcoding beinhaltet die Analyse einer gemischten DNA-Probe mit *Next Generation Sequencing und* kann einiger dieser Herausforderungen überwinden. In dieser Dissertation verwendete ich einen integrativen Ansatz, bei dem in erster Linie DNA barcoding der *Cytochrom-c-Oxidase-Untereinheit I* (COI) Barcodes zur Identifizierung der Insektenarten und DNA metabarcoding des *internal transcribed spacer 2* (ITS2) Barcodes zur Analyse der Pollenladungen kombiniert wurden, zusätzlich zur teilweisen morphologischen Identifizierung der Insekten und Pollenladungen , um so die

Interaktionen von Pflanzen und Bestäubern zu analysieren. Das erste Ziel der vorliegenden Arbeit war es, in Agrarökosystemen I) die Interaktionen zwischen Pflanzen und Bestäubern aller Hymenoptera und Brachycera, mit Ausnahme von Honigbienen, zu untersuchen, II) die Schlüsselarten von Pflanzen und Bestäubern für die Stabilität der Netzwerke zu identifizieren und III) die potenzielle zeitliche Dynamik der Pflanzen-Bestäuber-Netzwerke zu analysieren. Diese Studien sind die ersten ihrer Art für diese beiden Kulturpflanzenarten.

In **Kapitel 2** wurden die Pflanzen-Bestäuber-Netzwerke von Kümmel (*Carum carvi* L.) mit Schwerpunkt auf wilden Hymenoptera und Brachycera analysiert, die auch komplexe Wechselwirkungen zwischen potenziellen Bestäubern des Kümmels und weiteren Pflanzentaxa, die von denselben Insekten besucht wurden, beinhalten. In den Pflanzen-Bestäuber-Netzwerken von Kümmel interagierten insgesamt 34 Hymenoptera und 87 Brachycera als potenzielle Bestäuberarten mit insgesamt 139 Pflanzentaxa. Die qualitativen Unterschiede der Pollengemeinschaft zwischen Brachycera und Hymenoptera unterstreichen die Komplementarität in der Blütenaffinität beider Insektengruppen. Intrasaisonale Analysen des Pflanzen-Bestäuber-Netzwerks von Kümmel zeigten das Potenzial dieser Kulturpflanze als wichtige Nahrungsquelle für Insektenarten außerhalb des Zeitraums vieler früh blühender Kulturpflanzenarten, mit einer Aktivitätsspitze im Spätsommer. Zuletzt, unterstrichen starke tageszeitliche Variationen in der Bestäubervielfalt, die Wichtigkeit der Beprobung von Blütenbesucher und somit potenzielle Bestäuber zu verschiedenen Tageszeiten, um komplexe Pflanzen-Bestäuber-Netzwerke vollständig darzustellen.

In **Kapitel 3** wurde das Pflanzen-Bestäuber-Netzwerk des Apfels (*Malus domestica* BORKH.) analysiert, einschließlich der komplexen Interaktionen zwischen den potenziellen Bestäubern des Apfels und weiterer besuchten Pflanzen. Insgesamt interagierten 35 Hymenoptera- und 66 Brachycera-Arten mit 194 Pflanzentaxa. Neben der Zielpflanzenart dominierten andere frühblühende Pflanzentaxa die Pflanzen-Bestäuber-Netzwerke, was die Bedeutung dieser Arten als Nahrungsquelle in frühblühenden Obstgärten unterstreicht. Die Zusammensetzung der Pollenfracht unterschied sich stärker bei Brachycera zwischen den Jahren höher als zwischen Brachycera und Hymenoptera vom gleichen Beprobungsjahr. Die Pflanzenphänologie könnte daher die Unterschiede zwischen der Netzwerkstruktur der Pflanzenbestäuber erklären.

Neben der Analyse von Pollenproben kann DNA metabarcoding auch zur allgemeinen Auswertung der Artenvielfalt von Hymenoptera und Brachycera in Mischproben eingesetzt werden. Trotz der weit verbreiteten Anwendung dieser Methode ist die Vergleichbarkeit zwischen dem DNA metabarcoding und einer reinen morphologischen Auswertung bisher

wenig untersucht worden, besonders bei Insekten. In **Kapitel 4** wurden Brachycera und Hymenoptera mit Malaise-Fallen auf Spinatfelder (*Spinacia oleracea* L.) gesammelt und mit Hilfe einem nicht-destruktiven DNA metabarcoding Ansatz sowie vier verschiedenen Kluster- und Filteransätze analysiert und getestet. Die Ergebnisse des DNA metabarcodings variierten stark in der Gesamtanzahl identifizierter Brachycera- und Hymenoptera-Arten, je nach gewähltem Ansatz. Anhand der Syrphidae als Beispielfamilie einer gutuntersuchten Brachycera-Familie diskutiere ich mögliche Gründe für die Diskrepanzen zwischen DNA metabarcoding und der morphologischen Identifizierungen.

Das **abschließende Diskussionskapitel** bringt die vorherigen Themenbereiche zusammen und diskutiert kapitelübergeifend die beträchtliche Vielfalt potenzieller Wildbestäuber innerhalb der Ordnungen Brachycera und Hymenoptera in zentraleuropäischen Agrarökosystemen. Obwohl in dieser Arbeit nicht direkt auf die Effizienz oder Effektivität der Bestäuber eingegangen worden ist, trägt die hohe Vielfalt an Bestäubern zur Widerstandsfähigkeit dieser Ökosystemfunktion in Agrarökosystemen bei. In Abschnitt 5.2 werden spezifische Stärken und Herausforderungen von DNA metabarcoding von Pflanzen-Bestäuber-Netzwerken und Massenproben behandelt und mögliche Lösungen zur Überwindung dieser methodischen Herausforderungen diskutiert. In Agrarökosystemen hat die sich mangelnde Anerkennung und Voreingenommenheit auf den bedeutenden Beitrag von Wildbestäubern auf die Entwicklung und Umsetzung von Schutzmaßnahmen ausgewirkt. Basierend auf den vorgestellten Ergebnissen der vorangegangenen Abschnitte füge ich auch Handlungsempfehlungen für diverse Akteure bei. Abschließend werden offene Fragen und mögliche, künftige Forschungsvorhaben behandelt, u.a. integrative Ansätze mit Fokus auf Insektenspuren auf Pflanzengewebe oder die Einbeziehung der Interaktionen zwischen Pflanzen und Bestäubern in großskalige (agro-)ökologischer Netzwerke. Beendet wird diese Arbeit mit der Zusammenstellung der wichtigsten Schlussfolgerungen und Erkenntnisse aller Kapitel.

Resumen

La polinización es la transferencia de granos de polen de las anteras al estigma de las plantas. Esta es una estrecha relación mutualista entre animales y plantas florales, que globalmente ha moldeado la diversidad de angiospermas. Particularmente la polinización por insectos ,se considera un servicio ecosistémico esencial para el ser humano, ya que muchas especies cultivadas dependen en gran medida de la polinización para una producción óptima de frutos y semillas. Sin embargo, la intensificación de la agricultura en el último siglo, ha creado un dilema entre una mayor necesidad de una polinización óptima y satisfactoria y acelarada expansión agrícola asociada con prácticas agrícolas intensivas y permiciosas, que contribuyen a un declive global de los polinizadores. La consecuencia esun suministro insuficiente de servicios de polinizadores silvestres y en contraste un mayor uso de polinizadores domesticados, principalmente la abeja melífera europea *Apis mellifera* (Linnaeus, 1758) y otras especies seleccionadas de abejas silvestres. La dependencia de una sola o pocas especies polinizadoras, también ha influido en lasubestimación y percepción sesgada de la diversidad de polinizadores potenciales, especialmente en los agroecosistemas. Entre los grupos de insectos y polinizadores más destacados de los agroecosistemas se encuentran las abejas y las avispas (Hymenoptera), así como las moscas (Diptera: Brachycera). Los polinizadores potenciales, interactúan no sólo con las especies de cultivo de interés, sino también con otras especies de plantas, que pueden ser analizadas como complejas interacciones planta-insecto en redes ecológicas bipartitas.

Estas redes y particularmente su estructura, pueden indicar la estabilidad de las interacciones planta-insecto como funciones del ecosistema y su resistencia a influencias externas y factores de estrés. Los métodos más comunes, para analizar las redes planta-polinizador, son los sondeos de visita a las flores - aunque no todos los visitantes de las flores sean polinizadores - o mediante la identificación morfológica de la composición de la carga de polen recogida por los especímenes de insectos. Sin embargo, la identificación morfológica del polen lleva mucho tiempo, requiere mucha experiencia y suele dar como resultado una resolución taxonómica inferior. El ADN metabarcoding, el análisis de una muestra mixta de ADN con secuenciación de próxima generación, puede superar algunas de estas limitaciones. En esta tesis, se utilizó un enfoque integrador que combinaba principalmente el ADN barcoding de la *subunidad I de la citocromo c oxidasa* (COI) para identificar los especímenes de insectos y ADN metabarcoding del espaciador transcrito interno 2 (ITS2) para el análisis de las cargas de polen, al tiempo que también identificaba morfológicamente de forma parcial

los especímenes de insectos y las cargas de polen para evaluar las redes de polinizadores. El primer objetivo planteado, fue estudiar las interacciones planta-polinizador de todos los Hymenoptera y Brachycera, excluyendo la abeja melífera europea, en dos especies de cultivos específicos en agroecosistemas, identificar las especies de plantas y polinizadores claves para la estabilidad de las redes y analizar la dinámica temporal de las redes planta-polinizador. Estudios son los primeros de su clase para estas dos especies de cultivos.

En el **capítulo 2**, se analizaron las redes planta-polinizador del alcaravea (*Carum carvi* L.) centrándose en los hymenopteros y brachyceros, incluyendo las complejas interacciones entre los polinizadores potenciales del alcaravea y otros taxones de plantas visitados por esos mismos insectos. En las redes planta-polinizador del alcaravea, un total de 34 Hymenoptera y 87 Brachycera polinizadores potenciales interactuaron con un total de 139 taxones de plantas. Además, las claras diferencias cualitativas en las cargas de polen entre Brachycera e Hymenoptera destacan la complementariedad en la afinidad floral de ambos grupos. Los análisis intraestacionales de la redes planta-polinizador del alcaravea, mostraron el potencial de este cultivo, como importante fuente de alimento para especies de insectos, fuera del periodo de muchas especies de cultivos de floración temprana, con un pico de actividad a finales de verano. Por último, las fuertes diferencias intradiarias en la diversidad potencial de polinizadores, destacan la importancia de recolectar insectos a diferentes horas del día, para poder recopilar las redes completas de plantas-polinizadores.

En el **capítulo 3**, se analizó la red planta-polinizador del manzano (*Malus domestica* BORKH.), incluyendo las complejas interacciones entre los polinizadores potenciales del manzano y las plantas que visitan. En total, 35 especies polinizadoras de Hymenoptera y 66 de Brachycera interactuaron con 194 taxones de plantas. Aparte de las especies de cultivo de interés, otros taxones de plantas de floración temprana dominaron las redes planta-polinizador, destacando la importancia de estas especies como fuente de alimento en los cultivos de floración temprana. Además, la diferencia en la composición de la carga de polen de Brachycera entre los años fue mayor que entre Brachycera e Hymenoptera recolectados en el 2017, lo que indica que la fenología de las plantas, podría ser uno de los factores clave, que impulsen las diferencias en la red y la estructura de los polinizadores de plantas.

Además de analizar muestras de polen, el ADN metabarcoding también se puede utilizar para evaluar la diversidad de Hymenoptera y Brachycera en muestras mixtas . A pesar del uso generalizado de esta metodología, hasta ahora se ha investigado relativamente poco,hasta qué punto las muestras examinadas con metabarcoding son comparables a las identificadas por medios morfológicos, sobre todo en lo que respecta a los insectos. En el **capítulo 4**, se

analizaron Brachycera e Hymenoptera recogidos con la trampa Malaise en campos de espinaca (*Spinacia oleracea* L.) utilizando un protocolo de metabarcoding no destructivo y cuatro estrategias diferentes de agrupación y filtrado. Dependiendo del enfoque seleccionado, los resultados de la metabarcoding con respecto a las especies de braciceros e himenópteros detectadas variaron fuertemente. Utilizando Syrphidae como familia de Brachycera ampliamente estudiada, discuto las posibles razones de las discrepancias entre el metabarcoding y las identificaciones morfológicas.

En el **capítulo final**, sintetizo y destaco la considerable diversidad de polinizadores silvestres dentro de los órdenes Brachycera e Hymenoptera. A pesar de que en este trabajo de investigación doctoral no me he centrado en la eficiencia o eficacia de los polinizadores, esta alta diversidad de polinizadores puede mejorar la resistencia de la polinización como componente clave de las funciones ecosistémicas en los agroecosistemas. Además, dado que la principal metodología de investigación empleada fue principalmente ADN metabarcoding, en la sección 5.2 discuto las principales limitaciones e impedimentos, a la hora de estudiar las redes de plantas-polinizadores y las muestras mixtas de insectos con ADN metabarcoding, junto con las posibles soluciones para abordar estos obstáculos metodológicos.

La falta de reconocimiento y los limitados estudios sobre la importante contribución de los polinizadores silvestres en los agroecosistemas, también ha tenido consecuencias en el desarrollo y la aplicación de medidas de conservación. Por lo tanto, y considerando los resultados presentados en las secciones anteriores, sugiero algunas recomendaciones para *stakeholders*. Además, de enunciar algunas preguntas abiertas y futuras ideas de investigación, que incluyen enfoques integrativos para estudiar las interacciones planta-polinizador, a partir de muestras centradas en plantas o el análisis de las interacciones planta-polinizador como parte de redes ecológicas en agroecosistemas. Por último, se expone una recopilación de las principales conclusiones y hallazgos clave de los últimos capítulos.

Abstract

Pollination, the transfer of pollen grains from the anthers to the stigma, is a tight mutualistic relationship between animals and flowering plants, which has globally shaped angiosperm diversity. Particularly pollination by insects is considered an essential ecosystem service for humans since many crop species depend highly on pollination for optimal fruit and seed production. However, agricultural intensification over the past century has created a dilemma between an increased need for successful and optimal pollination and an agricultural expansion associated with intensive farming practices contributing to a global pollinator decline. Therefore, a sufficient pollinator service supply has only been achieved by increased use of managed pollinators, mainly the European honeybee *Apis mellifera* (Linnaeus, 1758) and other selected wildbee species. In turn, this has led to a dependency on one species and an underestimation and skewed perception of the diversity of potential pollinators, particularly on agroecosystems. Among agroecosystems' most prominent insect groups and pollinators are bees and wasps (Hymenoptera), as well as flies (Diptera: Brachycera). These potential pollinators interact not only with the targeted plant species but also with many other plant species, which can be analyzed as complex plant-insect interactions in bipartite ecological plant-pollinator networks. These networks and their structure can indicate the stability of plant-insect interactions as ecosystem functions and their resilience to external influences and stressors. The most popular methods to analyze these plant-pollinator networks are flower visitation surveys, even though not all flower visitors are pollinators, or by morphologically identifying the pollen load composition collected from insect specimens. However, the morphological identification of pollen is time-consuming, requires a lot of expertise, and usually results in a lower taxonomic resolution. DNA metabarcoding, the analysis of a mixed DNA sample with Next Generation Sequencing, can overcome some of these limitations. In this thesis, I used an integrative approach combining primarily DNA barcoding of *cytochrome c oxidase subunit I* (COI) to identify the insect specimens and DNA metabarcoding of the *internal transcribed spacer 2* (ITS2) for the analysis of the pollen loads while also additionally morphologically identified partially the insect specimens and pollen loads to assess the plant-pollinator networks. The first aim of the present thesis was I) to study plant-pollinator interactions of all non-honeybee Hymenoptera and Brachycera of two targeted crop species in agroecosystems, II) identify the key plant and pollinator species for the networks' stability, and III) analyze potential temporal dynamics of the plant-pollinator networks. These studies are the first of its kind for these two crop species.

In **Chapter 2,** the plant-pollinator networks of caraway (*Carum carvi* L.) with a focus on non-honeybee Hymenoptera and Brachycera were analyzed, including the complex interactions between potential pollinators of caraway and other plant taxa visited by those same insects. In the plant-pollinator networks of caraway, a total of 34 Hymenoptera and 87 Brachycera potential pollinators interacted with a total of 139 plant taxa. Additionally, the distinct qualitative differences in the pollen loads between Brachycera and Hymenoptera highlight the complementarity in flower affinity of both groups. Intraseasonal analyses of the plant-pollinator network of caraway showed the potential of this crop as an important food source for insect species outside the period of many early-flowering crop species, with an activity peak in late summer. Finally, strong intraday differences in potential pollinator diversity emphasized the importance of collecting insects at different times of the day to compile complete complex plant-pollinator networks.

In **Chapter 3,** the plant-pollinator network of apple (*Malus domestica* BORKH.) was analyzed, including the complex interactions between the potential pollinators of apple and the plants they visit. In total, 35 Hymenoptera and 66 Brachycera pollinating species interacted with 194 plant taxa. Aside from the targeted crop species, other early-flowering plant taxa dominated the plant-pollinator networks, highlighting the importance of these species as food source in early-flowering orchards. Moreover, the difference in pollen load composition of Brachycera between the years was higher than between Brachycera and Hymenoptera 2017, which hints that plant phenology could potentially be one of the key drivers of differences in the plant-pollinator network and structure.

Aside from analyzing pollen samples, DNA metabarcoding can also be utilized to assess the diversity of Hymenoptera and Brachycera in bulk samples. Despite the prevalent use of this methodology, to what extent samples examined with metabarcoding are comparable to those identified through morphological means has been investigated relatively little so far, particularly regarding insects. In **Chapter 4**, Brachycera and Hymenoptera collected with Malaise trap in spinach fields (*Spinacia oleracea* L.) were analyzed and tested using a non-destructive DNA metabarcoding approach and four different clustering and filtering approaches. Depending on the selected approach, DNA metabarcoding results regarding detected brachyceran and hymenopteran species strongly varied. Using Syrphidae as an exemplar family of a well-studied Brachycera family, I discuss possible reasons for the discrepancies between DNA metabarcoding and the morphological identifications.

In the **final discussion chapter**, I provide concluding remarks highlighting the considerable diversity of potential wild pollinators within the orders Brachycera and Hymenoptera.

Despite that I did not focus on pollinator efficiency or effectiveness in this thesis, a diverse pollinator diversity can enhance the resilience of pollination as a key component of ecosystem functions in agroecosystems. Moreover, since the primary methodologies employed throughout the thesis was DNA metabarcoding, key strengths and impediments when studying plant-pollinator networks and bulk samples with DNA metabarcoding, along with potential solutions to address these methodological obstacles, will be discussed in section 5.2. A lack of recognition and bias in understanding the significant contribution of wild pollinators in agroecosystems has also impacted the development and implementation of conservation efforts. Therefore, based on the results presented in the preceding sections, I added some recommendations for various stakeholders. Furthermore, I will address open questions and potential gaps for future research ideas, which include integrative approaches to study plant-pollinator interactions from plant-targeted samples or the analysis of plant-pollinator interactions as a part of ecological networks in agroecosystems. Lastly, this thesis will conclude with a compilation of all chapters' main conclusions and key findings.

List of Publications

Publications as part of this thesis:

1. **Kilian, I. C.**, Swenson, S. J., Mengual, X., Gemeinholzer, B., Hamm, A., Wägele, J. W., & Peters, R. S. (2023). More complex than you think: Taxonomic and temporal patterns of plant–pollinator networks of caraway (Carum carvi L.). Molecular Ecology, 32, 3702–3717. DOI: 10.1111/mec.16943

2. **Kilian, I. C.**, Swenson, S. J., Peters, R. S., Gemeinholzer, B., Wägele, J. W., Hamm, A., & Mengual, X. (*in prep*). Think on flies when you eat an apple: Brachycera have more interactions than Hymenoptera in the plant-pollinator network of *Malus domestica* Borkh. (Rosaceae).

3. **Kilian, I. C.**, Kirse, A., Peters, R. S., Bourlat, S.J., Fonseca V.G., Wägele, J. W., Hamm, A., & Mengual, X. (*under review*). Maximizing metabarcoding precision: ASV clustered to OTUs and LULU filtering for enhanced species diversity analysis of bees, wasps (Hymenoptera), and flies (Diptera: Brachycera) with a non-destructive DNA metabarcoding approach. *Ecology and Evolution.*

Table of Content

Chapter 1.
General introduction

1.1. Pollination as an essential ecosystem service

Pollination by insects, i.e., the transfer of pollen grains from the anthers (male reproductive organ) to the stigma (female reproductive organ), resulting in the fertilization and production of seeds and fruits (Abrol, 2011), evolved at least 250 million years ago (Bao et al., 2019; Labandeira & Currano, 2013; Stephens et al., 2023). The relationship between insect-pollinated angiosperms and pollinating insects is a tight symbiotic relationship, whereby the insects are attracted to the numerous rewards provided by the flowers (such as pollen, nectar, oils, or perfumes), allowing the plants to spread their pollen attached to the insect from one flower to another (Abrol, 2011).

As one of the most crucial ecological processes, pollination is vital for the reproduction and survival of angiosperms (flowering plants) and is considered an essential ecosystem service for agricultural production and, consequently, food security (Abrol, 2011; Porto et al., 2020). Out of the 115 most important crop species worldwide, at least 87 crop species are dependent on insect pollination (Klein et al., 2007), with an estimated annual global value of crop pollination between US$195 to 657 billion (Lautenbach et al., 2012; Porto et al., 2020). Therefore, promoting pollination services can increase the productivity of many crops (Abrol, 2011). Optimized pollination service is a delicate balance between foraging behavior, the efficiency of transferring pollen, the interaction of the flower and the pollinators, and surrounding environmental factors (Rader et al., 2024).

Pollination decline, drivers, and consequences

Despite the essential role that insects play as pollinators, providing necessary goods and ecosystem services for both humans and flowering plants, there is concerning and undeniable evidence of a recent decline in their populations and diversity (Barendregt et al., 2022; Potts et al., 2010). Within Europe, an estimated 9.2 % of bees (Nieto, 2014) and 8.5 % of butterflies (Swaay et al., 2010) are threatened with extinction. In Germany, at least 48% of wildbees (Westrich et al., 2011) and 31% of hoverflies (Ssymank et al., 2011) are listed as endangered or extinct in the national Red List, the decline of hoverflies even in a faster pace than for bees (T. Zeegers et al., 2024). Indirect evidence of pollinator losses stems from studies analyzing insect decline, often including taxa known for their role as pollinators (Goulson, 2019; Hallmann et al., 2017).

Primary factors contributing to pollinator decline are a combination of various anthropogenic drivers, which can be condensed into the following: climate change, land-use change, and management intensity, followed often by the use of pesticides and genetically modified crop

species, pollinator management and pathogens, and finally, invasive alien species (Potts et al., 2010; Vanbergen & Initiative, 2013).

While climate change impacts not only the abundance and population of pollinators (I.-C. Chen et al., 2011) and plants (Chitu & Paltineanu, 2020), it also leads to a mismatch in the phenology of these groups, and therefore, the interaction between insects and plants is lost (Gérard et al., 2020; Hegland et al., 2009; Høye et al., 2013; Inouye, 2022; Memmott et al., 2007; Schweiger et al., 2008). Although the complete extent of this discrepancy remains unclear, initial studies have already identified an impact on the fitness of both the pollinators and plants (Hutchings et al., 2018; Schenk et al., 2018).

A global demand for food, fiber, water, and shelter for an increasing world population has caused a massive shift in land use and an intensification in agroecosystems (Foley et al., 2005; Robinson & Sutherland, 2002). These changes have caused the destruction, fragmentation, or degradation of semi-natural habitats, all vital habitats where pollinators nest and forage (Potts et al., 2010). The availability of floral resources (pollen and nectar) or nesting sites for pollinators can be altered and, consequently, endanger pollinators' population and diversity (Biesmeijer et al., 2006; McNeil et al., 2020; Persson et al., 2015; Requier et al., 2015).

Agricultural intensification is often accompanied by increased use of pesticides and herbizides, applied directly to the crop or frequently concentrated in pollen or nectar of adjacent wildflowers (Botías et al., 2015). Despite that pollinators are not the targeted group, the effects can vary, from changes in behavior (Clem et al., 2020; Easton & Goulson, 2013; O'Reilly & Stanley, 2023; D. B. Smith et al., 2020; Tasman et al., 2021), higher vulnerability towards pathogens (Di Prisco et al., 2013), a direct effect on the population (Alston et al., 2007; Rundlöf et al., 2015; Woodcock et al., 2016) or a mixed effect often amplified by other agrochemicals (Van Der Sluijs et al., 2013). Moreover, the exposure not only affects the first generation, but can also have lasting carryover effects on the next generations (Stuligross & Williams, 2021).

Regarding food production, a pollination decrease could compromise the quality or quantity of pollinating-dependent crops (Garratt et al., 2014; Hünicken et al., 2020; Klein et al., 2007). Numerous pollinator-dependent crop species also serve as a primary source of various micronutrients (i.e., vitamin A, folate, and iron) (Chaplin-Kramer et al., 2014). A lower consumption of these food sources caused by an increased production price may lead to an increase in preventable diseases that are associated with malnutrition, including cardiovascular diseases, diabetes, oesophageal cancer, and lung cancer (Bauer & Sue Wing,

2016; M. R. Smith et al., 2015). Aside from crop species, over 87% of wild flowering plants depend on animal pollination for their reproduction and fitness (Ollerton et al., 2011).

Ultimately, it is crucial to acknowledge that our comprehension and scope of pollinator decline is still limited to those being monitored or studied by experts (IPBES, 2019). Thus, the underlying severity and factors contributing to the decline of other understudied wild-pollinating insects remain unclear.

Brachycera and Hymenoptera are vital pollinators in agroecosystems

Land use and agricultural intensification have also resulted in greater dependence on proper pollination services. This dependency has, to some degree, been fulfilled by managed pollinators, with the European honeybee (*Apis mellifera* L.) being the most prevalent (Breeze et al., 2014). Honeybees have been promoted to a greater extent than wild pollinators due to their easiness of handling and managing. However, they are not necessarily more efficient than wild pollinators (Albano et al., 2009; Garibaldi et al., 2013; Jauker & Wolters, 2008; Lefebvre et al., 2019; Phillips et al., 2018; Rader et al., 2009; Viana et al., 2014). Recent studies have even emphasized the potential threat of honeybees to native wild pollinators, competing for the same floral resources, particularly when these resources are limited (Goulson, 2003; Goulson & Sparrow, 2009; Paini, 2004; Wojcik et al., 2018) or transferring diseases (Fürst et al., 2014). For the sake of simplicity, we will define from here on wild pollinators as all non-managed pollinator, and non-bee pollinators as all non-honeybee and wildbee pollinators.

Nonetheless, especially these native underestimated wild pollinators within Hymenoptera and Brachycera (Diptera) might play an important role, which, however, are still relatively unknown, despite being among the most speciose insect orders worldwide and a predominant group of pollinators (Forbes et al., 2018; Ollerton, 2017; Stork, 2018). These taxa are usually overlooked in pollination studies assumed to be less effective, consequently underestimating their pollination abilities and affecting furthermore conservation efforts based on those assessments (Ssymank et al., 2008).

The Order Hymenoptera with over 154,000 species encompasses, among others, wasps, sawflies, ants, and bees (Aguiar et al., 2013; Huber, 2017; Noort & Broad, 2024). Despite bees' global popularity (especially honeybees), they represent only a portion of potential hymenopteran pollinators (Ollerton, 2021). In recent years, there has been a noticeable shift in attention towards other non-bee hymenopterans for their significant role as pollinators (Çoruh & Çoruh, 2012; Ollerton, 2017; Rader et al., 2016; Requier et al., 2023a). As a case in point, a recent study by Borchardt et al. (2024) demonstrated that wasps can be as efficient

pollinators as other wildbee species in terms of pollen diversity and amount being transported.

Meanwhile, Brachycera (flies) are even further overlooked as pollinators and frequently underestimated, despite that over 55 brachyceran families are considered flower visitors, feeding on nectar, pollen or both (Larson et al., 2001; Raguso, 2020; Ssymank et al., 2008). Within Brachycera, just the family Syrphidae (commonly known as hoverflies or flower flies) has gained a little bit more attention concerning its essential contribution to pollination, especially in agroecosystems (Doyle et al., 2020; Innouye et al., 2015; Orford et al., 2015; Rader et al., 2020). Particularly in low temperatures, Brachycera is the main pollinator for many flowering plants (Doré et al., 2021; Howlett, 2012; Lefebvre et al., 2018; Tiusanen et al., 2016). This is particularly important in the face of climate driven shifts causing a mismatch between the flowering crop and the pollinator phenology, which is already the case in many apple orchards (Wyver et al., 2023).

Additionally, many non-honeybee hymenopteran and brachyceran species can also be crucial control agents of pest species (Brock et al., 2021; Kremen & Chaplin-Kramer, 2007; Pekas et al., 2020), essential in organic farming (Porcel et al., 2018). For example, numerous hoverflies feed on aphids during their larval phase, thus serving as crucial agents in regulating aphid population (Dunn et al., 2020). Likewise, many parasitoid wasps lay their eggs and develop in or on the host, which are often natural enemies, eventually killing it and influencing the population (Begg et al., 2017; Godfray, 1994; Jervis et al., 1993).

Additionally, the diversity and population of various wild pollinators are being employed as bioindicators, crucial tools to assess conservation efforts, due to the higher sensitivity to spatial and temporal changes (Birkhofer et al., 2018; M. Naeem et al., 2020). However, using a specific taxon as a bioindicator requires feasibility to be identified accurately by trained amateurs and to understand the precise environmental and ecological requirements of these species (Birkhofer et al., 2018). As many taxa do not meet this prerequisite, there have been more efforts to identify bioindicating species within Syrphidae and wildbees (Burgio & Sommaggio, 2007; Schindler et al., 2013).

1.2. DNA barcoding and metabarcoding

Identifying species is a crucial component of biodiversity assessments and, ultimately, conservation efforts. Historically, the identification has relied on unique intraspecific traits, making classifying specimens into different species possible. However, these assessments can be expensive, time-consuming, and sometimes lack taxonomic resolution, which also derives from a decline in taxonomic expertise (Chimeno et al., 2022; Piper et al., 2019; Souza et al.,

2016). These classic identification methods also have clear limitations for species where morphological traits are somewhat ambiguous or indistinguishable, for example, in cryptic species complexes (Jackson et al., 2014; Song et al., 2018) or understudied taxa without identification keys (Chimeno et al., 2022).

Advances in molecular techniques at the beginning of the 21st century have provided novel approaches for processing large sample numbers, addressing some previously mentioned obstacles, or supplementing the traditional methods (Hebert & Gregory, 2005). DNA barcoding takes advantage of the conserved homologous regions of a gene present in numerous species or groups of taxa. Which and how many DNA barcodes should be selected may differ depending on the studied organisms (Coissac et al., 2016; Freeland, 2017). For plants and fungi, a combination of nuclear and chloroplastic gene markers, such as *internal transcribed spacer* (ITS) and *maturaseK* (matK), are often selected DNA barcodes (Chase & Fay, 2009; S. Chen et al., 2010; Group, 2009). The most prevalent mitochondrial gene used as a DNA barcode for the animal kingdom is *Cytochrome c Oxidase subunit 1* (COI) (Hebert et al., 2003a).

The process of DNA barcoding involves extracting the targeted gene using standardized molecular steps consisting of DNA extraction, PCR amplification of the selected gene fragment with a (forward and reverse) primer set, and Sanger sequencing (Kress et al., 2015; Shokralla et al., 2014). The obtained DNA barcodes are then compared to reference databases such as the GBOL Reference Library (M. F. Geiger et al., 2016), BOLD (Ratnasingham & Hebert, 2007) or NCBI (Sayers et al., 2022) using alignment-based tools such as BLAST (Altschul et al., 1990; Camacho et al., 2009). These repositories provide reference sequences of accurately identified organisms by taxonomists and are ideally regularly curated (Pentinsaari et al., 2020). The reference sequence with the lowest genetic distance is then considered a valid match, and its taxonomy is assigned to the own sequence.

While DNA barcoding allows the analysis of individual specimens, the development of high-throughput sequencing (HTS) has enabled the possibility to analyze samples with multiple taxa, a process also known as DNA metabarcoding (Compson et al., 2020; Deiner et al., 2017). The analyzed samples can be either bulk tissue samples of whole organisms or environmental samples with traces of species DNA (eDNA) in soil (Kirse et al., 2021b), water (Aunins et al., 2023), sediments (Sinniger et al., 2016) or other materials (Liu et al., 2019). While the lab work, including DNA extraction and PCR amplification, is similar to DNA barcoding, they differ mainly in the final bioinformatic analysis of sequence reads summarized into either *Operative Taxonomic Units* (OTUs; Kopylova et al., 2016; Westcott & Schloss, 2015) or *Amplicon Sequence Variants* (ASVs; Porter & Hajibabaei, 2018) table, which can be used for further

analysis (Liu et al., 2019). These bioinformatic steps are as necessary as the laboratory protocol to obtain a precise species list as possible. Proper quality trimming, clustering, and denoising of these raw sequence reads can be the decisive step between finding false positives (species just identified via DNA metabarcoding) or false negatives (species just identified morphologically) in the sample. This is particularly important in agroecosystems, where using this methodology is crucial to detect invasive or pest species (Borrell et al., 2017; R. G. Young et al., 2021).

The use of DNA metabarcoding as a method to analyse plant-pollinator interactions has gained popularity in recent times (Bell et al., 2017; Pornon et al., 2016). In comparison to flower visitations surveys under the false assumption that flower visitors are also pollinators (King et al., 2013; Wardhaugh, 2015), or utilizing traditional morphological identification to identify pollen grains (palynology; Beattie, 1971; Erdtman, 1943), DNA metabarcoding of pollen achieves a higher taxonomic resolution (Bell et al., 2017) and is also more cost-efficient (Hawthorne et al., 2024a; Macgregor et al., 2019). This higher taxonomic resolution makes it possible to identify invisible plant-pollinator interactions that otherwise would not be found with traditional methods (Pornon et al., 2017) and a more proper interpretation of plant-pollinator network indices (Soares et al., 2017). The high taxonomic resolution is also a prerequisite to explore and understand the impact of ecosystem changes or anthropogenic impact on plant-pollinator interactions, from the lowest individual forager to the colony or species level (Bell et al., 2023).

1.3. Pollination ecology and plant-pollinator networks

Studying pollinators as key elements of mutualistic ecological networks of ecological communities has several advantages compared to studying the pollinators of focal plant species. Primarily, it is possible to analyze the contribution and stability of plant-insect interactions to ecosystem functions, as well as potential key components that can affect the adaptation or stability of these interactions (Bascompte & Jordano, 2007; Bascompte & Scheffer, 2023; Ings et al., 2008). Additionally, it enables comparing and assessing interactions over different spatiotemporal scales or resolution levels (Hemprich-Bennett et al., 2021; Pornon et al., 2017; Renaud et al., 2020).

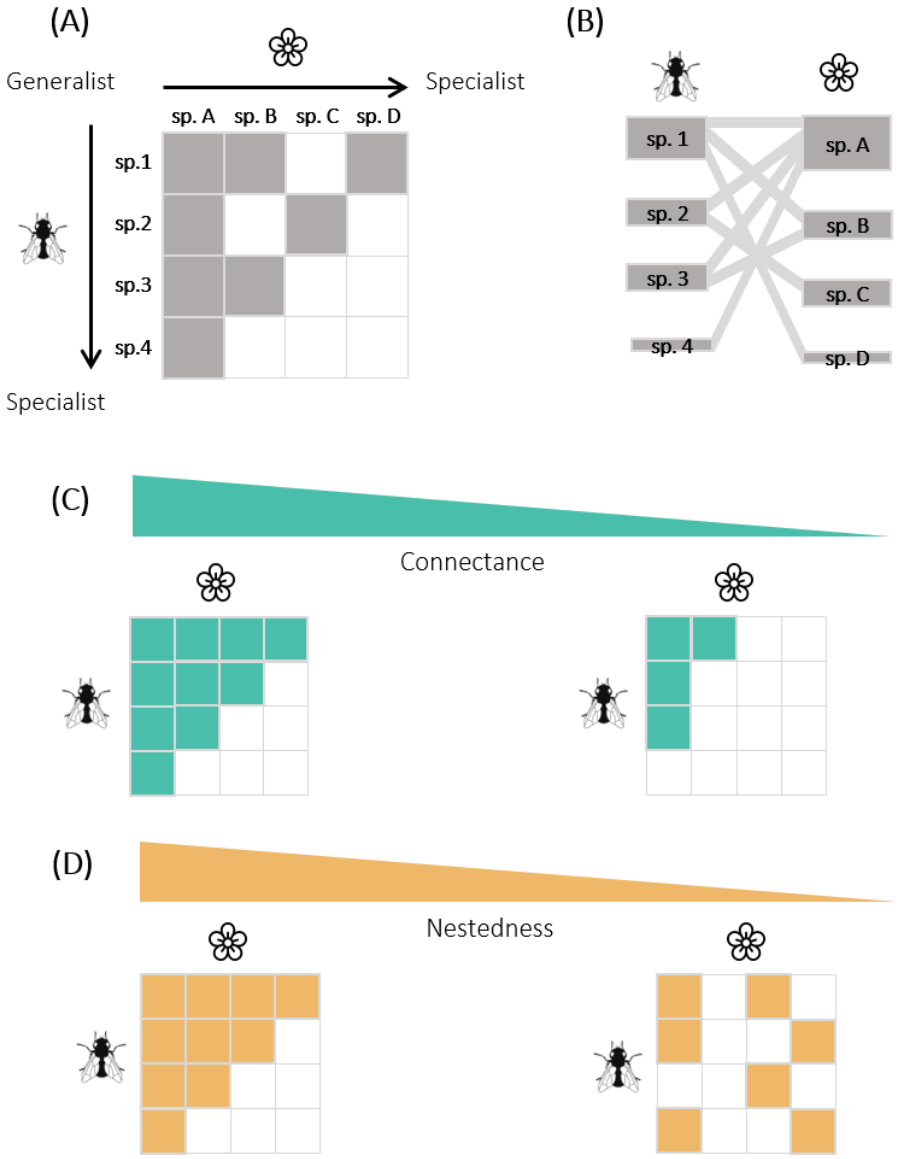

Figure 1: Typical plant-pollinator topology. Plant-pollinator networks can either be illustrated as a (A) matrix or as a (B) bipartite network. One of the most commonly used indices to compare plant-pollinator networks are (C) connectance and (D) nestedness. Connectance is the fraction of all possible links in a network level of nestedness of a network relates to the set of insects that interact with one plant set compared to another one (modified from Besson et al., 2019). The level of nestedness of a network describes to what extent sets of specialists interact with a set of generalists (Almeida-Neto et al., 2008; Bascompte et al., 2003).

Plant-pollinator networks are usually two-mode networks represented by either bipartite graphs or a matrix (Bascompte et al., 2003; Namin et al., 2022). The nodes on each side of the network are either the plant taxa (primary level species) or the insect taxa (secondary level species). The relationship between the node types is usually represented by connection links, where the interaction's strength reflects the interaction's frequency (Bascompte et al., 2003; Jordano, 1987; Jordano et al., 2006). While there are over 26 highly correlated network

indices that describe and help to examine the network structure under different ecological scenarios, mainly the distribution of links amongst species, connectance, and nestedness, all frequently used in the literature (Dormann et al., 2009), were used in Chapter 2 and 3.
Connectance is defined as the fraction of all possible links in a network (Dormann et al., 2009; Dunne et al., 2002). The level of nestedness of a network describes the extent of interaction among sets of specialist and generalist species (Almeida-Neto et al., 2008; Bascompte et al., 2003). Generally, plant-pollinator networks tend to be highly nested, meaning that specialist species usually interact with most generalist species (Bascompte et al., 2003; Vázquez & Aizen, 2003). The main characteristic of a nested network is a dominant core of generalist plant and insect species that interact with each other, making the network more robust against external influences that could potentially lead to extinction (Memmott et al., 2004). While climate variables impact pollinator richness and taxonomic composition of plant-pollinator networks, anthropogenic pressures cause a predominance of generalist over specialist plant and insect species (Doré et al., 2021). Additionally, in Chapter 3, the level of Generalism was calculated. Generalism is defined as the mean number of potential pollinator species per plant species (Bersier et al., 2002). It was calculated by the number of potential pollinators divided by the number of plant species in each network.
In terms of temporality, most of the studies on plant-pollinator networks provide a static snapshot of plant-pollinator networks. However, this constrains the ability to interpret the ecological and evolutionary mechanisms that shape those networks to some degree. Community dynamics and turnovers are highly different depending on whether the networks are being studied at the narrowest (days, weeks, months) or broadest (decades or centuries) temporal scales (CaraDonna et al., 2021).
Finally, plant-pollinator network is a very general term for several different network types: depending on the emphasis either placed on visitation frequency or pollinator effectiveness, the networks can be differentiated between visitation networks (i.e. the number of interactions between the pollinator and the flower), pollinator effectiveness or pollen transport networks (i.e. the amount and types of pollen transported by the pollinator) or pollinator importance networks (i.e. the amount and types of pollen deposited by the pollinator) (Ballantyne et al., 2017). The focus of this thesis was on analyzing pollen transport networks, which provides directly more attention to the pollinators´ perpective in the network (Bosch et al., 2009). However, for the sake of clarity and consistency, the term plant-pollinator network will be used in place of pollen transport network.

1.4. Agroecosystems as study systems

Agroecosystems present a unique and challenging environment due to their interplay between ecological and economic factors (Pert et al., 2013). Unlike natural ecosystems, they are intentionally designed and managed to optimize agricultural production (food, fiber, and other products). They are characterized by a unique complexity of interactions between the cultivated crop, the soil, as well as pests and beneficial organisms (e.g., pollinators) (Jeanneret et al., 2021). In comparison to natural ecosystems, the artificial selection and cultivation of specific plant and animal species creates complex dependencies. While the primary purpose of agroecosystems is to provide food and other secondary products for human life, it holds also the potential for friction between the different stakeholders, including the farmers, consumers, policymakers, and agribusinesses (Burkle et al., 2017; Tscharntke et al., 2012).

Table 1: worldwide production and value of the targeted crop species in 2022 (*FAOSTAT*, 2020).

Crop species	Area harvested [ha]	Yield [100 g/ha]	Value [billion USD]
Apple	4,825,729	198,594	84
Caraway*	2,315,212	11,882	3.9
Spinach	937,829	353,117	20

* Caraway is included in the FAOSTAT database as an item with other medicinal and spice plants

To study the potential wild pollinator diversity of Brachycera and Hymenoptera in agricultural ecosystems I chose apple (*Malus domestica*; Rosaceae), caraway (*Carum carvi L.:* Apiaceae), and spinach (*Spinacia oleracea* L.; Amaranthaceae) as the study crop species. They differ in flower morphology, phenology, and economic value.

Apple is the most ubiquitous temperate fruit with a high economic value (Ramírez & Davenport, 2013). It is highly dependent on cross-pollination between plant individuals of the same cultivar and the flowering period is usually in the spring (Broothaerts et al., 2004; Dennis, 2003). In case of pollination exclusion, deficits in fruit set and seed numbers can reach up to 75% and 56%, respectively (Garratt et al., 2013).

Caraway is an annual or biennial cultivated medical and spice plant. Due to the flower structure and arrangement, nectar and pollen are easily available for short-tongued generalist pollinators (d'Albore, 1986; McGregor, 1976). Pollination exclusion can reduce the (seed) yield by up to 40% (Bouwmeester & Smid, 1995; Toivonen et al., 2022).

The last selected crops species is spinach. While it is usually harvested before the flowering period, and thus not a primary attractant for flowering-visiting insects, there is a local concern

regarding the means to enhance the diversity of brachyceran and hymenopteran species through initiatives such as the implementation of flowering strips in cultivation (Meyhöfer et al., 2008). Hence, although pollination exclusion is not a concern, a variety of brachyceran and hymenopteran species have the potential to serve as beneficial pest biocontrol agents.

1.5. Aims and structure of this thesis

The recent and extensive worldwide pollinator decline has garnered renewed attention toward understudied or unknown wild pollinators, which are important ecosystem service provider and pivotal for angiosperm diversity. Additionally, methodological developments in DNA metabarcoding has allowed to get a deeper understanding of plant-pollinator networks in comparison to palynology. Therefore, we analyzed and focused on the potential of non-honeybee Hymenoptera and Brachycera as pollinators and how they interact in a plant-pollinator network with the targeted crop species (caraway and apple). In doing so, we also examined the floral affinity of these pollinators towards other species.

In **Chapter 2**, the plant-pollinator network of non-honeybee Hymenoptera and Brachycera collected in caraway fields over one year, during and after the flowering period of caraway is being assessed. All possible wild pollinators of caraway and the complex plant-pollinator network they are embedded are being presented. This was possible by combining DNA metabarcoding and the morphological identifications of the pollen loads carried by the potential pollinators and DNA barcoding and morphological identification of the insect specimens. Additionally, the intraday and intraseasonal variability (temporal pattern) of the plant-pollinator networks as well as flower affinity between Brachycera and Hymenptera was analyzed and compared.

In **Chapter 3**, the plant-pollinator networks of non-honeybee Hymenoptera and Brachycera collected in apple orchards over two years, before, during and after the flowering period of apple were analysed. After showing in Chapter 2 that it was possible to uncover a higher number of plant-pollinator interactions just by DNA metabarcoding the pollen loads and they correspond overall to the morphological identification at family level, we used in this chapter just metabarcoding of pollen loads combined with DNA barcoding and morphological identification of the insect specimens to analze the plant-polliantor networks. The focus of this chapter was on interannual differences in pollinator diversity and plant-pollinator interactions.

In recent years, DNA metabarcoding has evolved into a widely used technique for bioassessments, especially for the analysis of bulk samples. However, the evaluation of established methods is key and essential to understand possible limitations. Specially, there

remains a paucity of studies examining potential disparities between the traditional morphological identification based on taxonomic traits and DNA metabarcoding of bulk samples.

In **Chapter 4**, we analyzed the overlap between the morphological identification and a non-destructive DNA metabarcoding approach of Brachycera and Hymenoptera in bulk samples collected with Malaise traps on spinach fields (*Spinacia oleracea* L.). We focused especially on the in-silico approach of clustering and filtering approaches to have the closest match between morphological identification and DNA metabarcoding in terms of species abundance and species communities.

Chapter 2.

More complex than you think: Taxonomic and temporal patterns of plant-pollinator networks of caraway (*Carum carvi* L.)

This chapter is published in *Molecular Ecoloy* (open access; CC BY 4.0) as followed:

Kilian, I. C., Swenson, S. J., Mengual, X., Gemeinholzer, B., Hamm, A., Wägele, J. W., & Peters, R. S. (2023). More complex than you think: Taxonomic and temporal patterns of plant–pollinator networks of caraway (*Carum carvi* L.). *Molecular Ecology*, 32, 3702–3717. https://doi.org/10.1111/mec.16943.

2.1. Summary

Pollination by insects is a crucial ecosystem service to maintain angiosperm diversity and is particularly relevant for food production in agroecosystems (Klein et al., 2007; Ollerton et al., 2011). Moreover, most studies on this topic usually target well-studied taxa such as honeybees or bumblebees. As a result, the potential of other wild pollinating species is partially excluded or ignored, limiting the understanding of the real contribution of wild pollinators in agroecosystems and their drivers (Howlett et al., 2021; Rader et al., 2020). *Carum carvi* L. (caraway; Apiaceae), an annual or biennial cultivated medicinal and spice plant, was used here as a case study. Despite that pollination exclusion reduces seed production by up to 40% in caraway plants, current knowledge on the potential of wild pollinators is still minimal (Bouwmeester & Smid, 1995; Stelter, 2014; Van Roon & Bleijenberg, 1964).

One of the main goals of Chapter 2 was to analyze the plant-pollinator networks of caraway. Instead of exclusively using palynological identification of pollen samples, we used additional DNA metabarcoding to study the plant-pollinator interactions. A significant advantage of metabarcoding is the higher taxonomic resolution and efficiency than palynology analysis (Macgregor et al., 2019; Pornon et al., 2016). In this chapter, we used an integrative approach to analyze the potential pollinators of caraway by identifying morphologically and DNA barcoding the insect specimens, as well as identifying morphologically and DNA metabarcoding the pollen loads carried by the insect specimens.

In Chapter 2, based on the plant-pollinator networks of caraway, the aims we focused on were,

(i) to identify potential pollinators within the taxa of Brachycera and non-honeybee Hymenoptera, including variations in pollinator diversity between the different sampling intervals (intraday differences),

(ii) to analyze the leading qualitative differences between the network of Brachycera and Hymenoptera,

(iii) to examine potential intraseasonal differences in the plant-pollinator network of caraway, focusing on differences during and after the flowering period.

Out of 1,021 insect specimens collected, we identified 121 species that carried caraway pollen and, therefore, could be potential pollinators of caraway. Among these were 87 Brachycera and 34 Hymenoptera species encompassing numerous non-syrphid Brachycera and non-bee Hymenoptera species. Many of these potential pollinators have been described as flower visitors in the past or have been overseen in pollination studies. Particularly outstanding was the crucial role of *Athalia rosae* (Linnaeus, 1758) (Tenthredinidae, turnip sawfly) as a key player in the plant-pollinator network of Hymenoptera. The larva of this species is rather known as a common pest of Brassicaceae crop species (Oishi et al., 1993), showcasing the close ties in agroecosystems between crops and insect species. Notably, around one-fourth of the potential pollinators were collected exclusively during a single sampling interval out of possible three throughout the day. Therefore, a thorough sampling is essential to ensure a comprehensive representation of all potential pollinating species.

Overall, these caraway pollinators interacted with 139 plant taxa of different taxonomic levels. These plants include many species of the flowering strip present in one of the two sampling areas, in addition to other crop species present in the surrounding landscape. Surprisingly, we also identified some wind-pollinated plant species in the pollen loads, such as *Urtica dioica* L. or several Pinaeceae (pine) species. Both methodologies identified these species, which ruled out a methodological bias. Flower visitors have already been observed on most of these plant species. Therefore, we assume they actually visited the flowers or got in contact with the pollen in their environment (Ssymank & Gilbert, 1993; Taylor, 2009). The plant-pollinator networks of Brachycera and Hymenoptera differed in their structure, based principally on the differences in the number of interactions and the number of insect and plant species involved. The plant communities visited by these two pollinator groups also varied significantly, showcasing differences in flower affinity: Hymenopterans showed a preference for Fabaceae and Boraginaceae, which are also adapted to pollination by Hymenopterans, while Brachycerans showed a preference for Apiaceae (Faegri & Pijl, 1979; Sedivy et al., 2013). Apiaceae often include generalist plant species that are characterized by their umbellate flowers, which usually attract a high diversity of pollinators due to the superficial nectaries or are also used as a resting place (Niemirski & Zych, 2011; Zych et al., 2007).

Despite the assumption that the caraway plant-pollinator network's complexity decreases after the main flowering period of caraway, we found that many insect species also carried

caraway pollen after this period. This suggests that the late caraway flowers, like other umbellifers, remain an essential food source for pollinating species. Additionally, since caraway blossoms outside the main flowering period of many orchards and other crop species in the temperate region, farmers could cultivate it as an ecologically beneficial mass-blooming crop (Thomson, 1978; Zych et al., 2019). In conclusion, the results presented in this chapter show that an integrative approach combining DNA metabarcoding and barcoding, as well as morphological identifications, is a viable and effective methodology to analyze plant-pollinator networks of a targeted crop species and identify the dynamic and structure of the network.

Personal contribution

The study was conceptualized by Ximo Mengual, Ralph Peters, and Andrée Hamm. I did the fieldwork sampling the specimens. The caraway fields were set up by Hanna Blum and Markus Weber at Campus Klein-Altendorf (University of Bonn). I helped to weed the fields during the spring. I prepared the pollen samples for morphological identification and prepared the specimens (including tissue harvesting and pining) with the support of Katharina Geiger. Primarily, I morphologically identified the insect specimens with specific support from Ximo Mengual for Syrphidae and Ralph Peters for parasitoid wasps. I took the voucher pictures of the pollen samples with some support from Katharina Geiger and a student helper. The morphological identification of the pollen samples was carried out by the *Fachzentrum für Bienen und Imkerei (DLR)*. Stephanie Swenson and I did the lab work to extract and analyze the DNA of the pooled pollen samples. Stephanie Swenson curated bioinformatically the raw data from the DNA metabarcoding of pollen samples and submitted the ASVs to NCBI SRA. The DNA Barcoding of the insect specimens was done at the LIB – Museum Koenig Bonn. I combined and curated all datasets, analyzed the data, prepared the figures using my own R® scripts, and refined all the figures in Inkscape®. I interpreted all the results, and Ximo Mengual and Ralph Peters helped validate them. I wrote the first draft of the manuscript, which all co-authors helped to review and edit.

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More complex than you think: Taxonomic and temporal patterns of plant–pollinator networks of caraway (*Carum carvi* L.)

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Abstract

Caraway (*Carum carvi* L.) is a crop species that is gaining in importance in Europe, especially as a condiment and medicinal plant. Here, we present the plant–pollinator network of caraway in a central European agricultural landscape, focusing on two diverse potential pollinator taxa, Diptera: Brachycera (= true flies) and Hymenoptera (sawflies, bees, and wasps). We specifically studied qualitative differences in interactions between the two insect taxa as well as the intraday and intraseasonal variability of the network. Insect and pollen plant species determination was done via morphological identification and DNA (meta)barcoding. In total, 121 species representing 33 families of Hymenoptera and Brachycera were found to carry caraway pollen. These taxa included many nonhoneybee and nonhoverfly species, showing a wide taxonomic breadth of potential pollinators and a higher network complexity than previously anticipated. There are distinct qualitative differences between Brachycera and Hymenoptera networks, suggesting complementary roles of both taxa in the pollination of native and crop plants. Strong intraday differences in potential pollinator diversity make it necessary to collect insects and pollen at different times of the day to compile complete plant–pollinator networks. Intraseasonal analyses of the plant–pollinator network of caraway show the potential of caraway as an important food source for insect species with an activity peak in late summer.

KEYWORDS
bipartite networks, Brachycera, DNA barcoding, DNA metabarcoding, Hymenoptera, pollination

1 | INTRODUCTION

Insects are critical for a variety of ecosystem services necessary for the health of the planet. Pollination is among the most important ecosystem services insects provide and is pivotal for angiosperm biodiversity (Klein et al., 2007; Ollerton et al., 2011). A large number of publications have been devoted to well-studied taxa such as honeybees, bumblebees and solitary bees, but very few publications have focused on nonbee Hymenoptera or nonsyrphid Brachycera despite these taxa being pollinators for at least 105 crop species

(Howlett et al., 2021; Ollerton, 2017; Rader et al., 2020). Some recent studies have highlighted the importance of nonbee taxa for pollination, such as dipterans being the dominant pollinators at higher altitudes and latitudes (Lefebvre et al., 2019; Tiusanen et al., 2016), and for several crop species (Orford et al., 2015; Rader et al., 2016). In addition, there is evidence that dipterans are more resilient to stressors such as land-use change in comparison to managed and wild bees (Rader et al., 2016) and are as efficient as honeybees (Rader et al., 2009).

Most studies of pollination by Diptera have focused on one family (Syrphidae) despite many other taxa frequently being listed as flower visitors (Orford et al., 2015; Ssymank et al., 2008). The understudied taxa are often excluded or deliberately ignored, which leads to the erroneous assumption that they do not play an important role in pollination (Larson et al., 2001; Rader et al., 2016, 2020) or miss the fact that some species can be pollinators as well as pest or weed control agents (Dunn et al., 2020; Moerkens et al., 2021; Rizza et al., 1988; Sheppard et al., 1995). Consequently, many governmental programmes to enhance pollinator diversity have been designed and developed for well-studied taxa, neglecting the importance of including and safeguarding the less-studied taxa (Orford et al., 2015; Rader et al., 2020). A deeper knowledge of plant–pollinator interactions is required to understand the underlying multifactorial processes causing the worldwide pollinator decline and the effects of landscape changes from agricultural activity (Arstingstall et al., 2021). At the same time methods that protect beneficial insects while also protecting the economic interests of farmers necessitate further investigation.

Providing flower strips or fallow land in the vicinity of crops has been shown to be successful at attracting pollinators (Batáry et al., 2015; Feltham et al., 2015; Garibaldi et al., 2016) as well as natural enemies of crop pests (Cahenzli et al., 2019; Tschumi et al., 2015). However, the cost of seed stock for flower strips that do not provide an income source as well as a delayed increase of crop production for several years often make them unattractive to farmers (Christmann et al., 2021). An approach that would devote small plots of ecologically beneficial mass-blooming crops or "magnet-species" (Thomson, 1978; Zych et al., 2007) in the vicinity of fields and crops could be a potential alternative or addition to flower strips to enhance the populations and diversity of agriculturally beneficial insects while providing an additional income source (Christmann et al., 2021).

Carum carvi L. (caraway) is an annual or biennial cultivated medical and spice plant with a worldwide increasing market (Stelter, 2014; Van Roon & Bleijenberg, 1964). It belongs to the family Apiaceae and has characteristic yellowish white protandrous flowers arranged in compound umbels (d'Albore, 1986; McGregor, 1976) which require insect pollination to transport the pollen to the stigma. Pollination exclusion can reduce seed yield up to 40% (Bouwmeester & Smid, 1995; Toivonen et al., 2022). In Central Europe, the main flowering period of caraway has a duration of 25–30 days (Németh et al., 1997) between late May and early July (Langenberger & Davis, 2002). After the main flowering period, there is sometimes a second flowering period in autumn, but to a lesser extent (Hegi, 1926). Nectar and pollen are easily available and they constitute a valuable source of protein and carbohydrates for many potential pollinators (d'Albore, 1986; Langenberger & Davis, 2002; McGregor, 1976; Toivonen et al., 2022). Syrphidae and other flower-visiting Brachycera that provide both economically important services of pollination and pest control (at their larval stage in the case of syrphids) are known to be important pollinators of caraway and related Apiaceae species (Colley & Luna, 2000; Lamborn & Ollerton, 2000; Pérez-Bañón et al., 2007; Toivonen et al., 2022; Wojciechowicz-Żytko, 2019; Zych, 2002, 2007; Zych et al., 2014, 2019). These characteristics make caraway a possible crop to be added to an agricultural system to attract beneficial insects while offering an additional economic resource for growers.

In the present study, we analysed the astounding complexity of the plant–pollinator network of caraway in a central European agricultural landscape, targeting taxon-specific roles and, so-far neglected, temporal patterns within the network. The two prevailing approaches to analyse plant–pollinator networks are observing the interaction between the flower and a potential pollinator (Classen et al., 2020; Toivonen et al., 2022) or morphological identification of pollen loads (Beattie, 1971; Erdtman, 2013). While both methodologies are widely used, they often underestimate the number of plant species visited, leading to an underestimation of the total number of interactions (Jędrzejewska-Szmek & Zych, 2013; Macgregor et al., 2019). Furthermore, many described plant–pollinator networks are static records, not covering temporal shifts of species and interactions (Burkle & Alarcón, 2011; CaraDonna et al., 2017, 2021; CaraDonna & Waser, 2020). To gain a better estimate of plant species visited we implemented morphological identification as well as DNA metabarcoding for pollen determination. DNA metabarcoding is an emerging molecular tool that has the potential for more rapid pollen identifications with higher taxonomic resolution than possible with traditional morphology (Bell et al., 2017; Macgregor et al., 2019; Sickel et al., 2015). Previous studies utilizing DNA metabarcoding to investigate plant–pollinator networks have resulted in more complex networks when compared to those based on observational data alone (Michelot-Antalik et al., 2021; Pornon et al., 2017). In addition, the data contradicted the previous assumption that many pollinating species are specialists (Arstingstall et al., 2021). Still, many DNA metabarcoding studies target only those flower-visiting species with high pollen loads and do not cover the whole diversity of potential pollinators (Arstingstall et al., 2021; Bänsch et al., 2020; Bell et al., 2017; Cornman et al., 2015; Keller et al., 2015).

In this study, we aim to address the following questions: (i) Which Hymenoptera and Brachycera are involved in the plant–pollinator network of caraway? (ii) What are the qualitative differences in the plant–pollinator networks of Hymenoptera and Brachycera? (iii) What is the intraseasonal pattern of the plant–pollinator network?

(iv) Are there any intraday differences in potential pollinator diversity of caraway?

2 | MATERIALS AND METHODS

2.1 | Study site

Specimens were collected in 2016 at the agricultural research station Campus Klein-Altendorf. We collected on two plots, one with a flower strip (mixture "Blühende Landschaft Süd"; list of plant species in Table S1) (50°37′0.7″N, 7°0′4.19″E) and one without a flowering strip (50°37′15.86″N, 6°59′15.43″E). Plots were 1 km apart. The caraway plot with flowering strips had a length of 110 × 9 m (990 m^2) including the flowering strip (110 × 3 m; 330 m^2). The plot without flower strips had a length of 150 × 9 m (1350 m^2). The plots were surrounded predominantly by winter barley and wheat and flowering orchards (cherry, apple). By sampling both areas we wanted to attract as many potential caraway pollinators as possible.

The caraway (bi-annual variety "Sprinter"; N. L. Chrestensen) and flower strip were sown on April 8, 2016, seeding was carried out on both fields at a row spacing of 50 cm and the sowing rate was set at 10 kg ha^{-1}. Field emergence on April 23, 2016 in the plot with flowering strips was 77% and the plot without flowering strips had a field emergence of 91%. For weed control, a pre-emergent Aclonifen herbicide was applied on both fields once at 3 L ha^{-1} on April 14. To keep the plots weed-free during the vegetation period, additional weed control was performed with a roller and hand hoe.

2.2 | Collection of potential pollinators

During the caraway flowering period (July 5–25, 2016), sampling took place on all rain-free days, resulting in 10 collection days in total. After the flowering period, we sampled an additional 5 days (August 13 to September 1, 2016) after a 15-day gap to establish a boundary between the interactions during and after the main flowering time (Table S2).

Both caraway plots were sampled for 30 min each at three different time intervals to sample variation of insect activity: 10–12 h (interval I), 12–14 h (interval II) and 14–16 h (interval III). To avoid time bias in our sampling of the two caraway fields, we alternated the order, starting with a different caraway plot each day.

Specimens of Brachycera and nonhoneybee Hymenoptera were collected by hand-netting or direct collection into sampling vials along a 110 × 3-m transect, either (i) in the caraway fields on caraway flowers during the flowering period or (ii) in the border area of the caraway fields and the flowering strip on the respective flowers after the flowering period. Formicidae were excluded from the sampling based on the results by Zych et al. (2014).

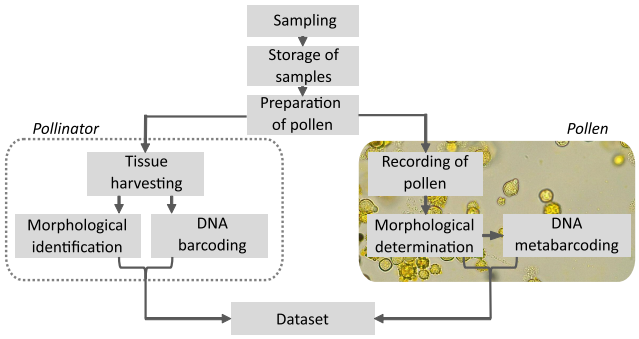

FIGURE 1 Visual summary of the methods applied to detect potential pollinators of caraway.

After sampling, specimens were stored dry individually at −20°C (Figure 1).

2.3 | Identification of potential pollinators

In the laboratory, all insect specimens were identified based on external morphology (Table S3 for keys used). For those insect specimens which could not be morphologically identified to the species level, we followed a DNA barcoding reverse-taxonomy approach (Morinière et al., 2019) using molecular sequences of the mitochondrial cytochrome *c* oxidase subunit I gene (*COI*; Hebert et al., 2003) (Figure 1).

2.4 | DNA barcoding of Brachycera and Hymenoptera

DNA was extracted from one to three legs per specimen in larger taxa (>5 mm long) or using a nondestructive protocol for lysis extraction of the whole specimen in small taxa (<5 mm long) (Gilbert et al., 2007) with the BioSprint96 magnetic bead extractor (Qiagen). Subsequently, specimens were pinned, mounted, or kept in ethanol, and labelled. Voucher specimens are deposited at the Zoologisches Forschungsmuseum Alexander Koenig (ZFMK, Leibniz Institute for the Analysis of Biodiversity Change). PCR (polymerase chain reaction)_ amplification followed the Canadian Centre for DNA Barcoding (CCBC) protocol for *COI* amplification with the primer pair HCO2198-JJ (AWACTTCVGGRTGVCCAAAR AATCA) and LCO1490-JJ (CHACWAAYCATAAAGATATYGG) (Astrin & Stüben, 2008). PCR products were sequenced at the Beijing Genomics Institute (BGI; https://en.genomics.cn/).

Sanger sequences were imported into Geneious version 7.1.9 (Kearse et al., 2012) and prepared with the Laboratory Information Management System plug-in (LIMS; Biomatters). Reverse and forward sequences were trimmed, filtered and de novo assembled. Assembled *COI* sequences were inspected and manually corrected if necessary. Sequences were submitted to GenBank (OQ611071

- OQ611458) and the GBOL reference database (GBOL DNA Barcode Reference Library 2022; Geiger et al., 2016). Sequences were then searched in BOLD (https://www.boldsystems.org/; Ratnasingham & Hebert, 2007). If all matches in BOLD with >99% similarity resulted in the same species name, this species name was used.

2.5 | Preparation and morphological identification of pollen loads

Pollen was collected by swabbing the insect specimens with a lentil-sized piece of Kaisers phenol-free glycerol gelatin (Carl Roth), with a focus on the areas where the pollen was present in the greatest concentration. Then, the glycerol gelatin fragment was mounted on a slide over a 55°C heating plate and covered with a cover slip (Figure 1) (Beattie, 1971). Morphological identification of their pollen load was done (i) for all specimens per species when the number of specimens per species was five or fewer per plot (data from both plots were pooled later, see below), (ii) from five specimens when the number of specimens per species was 6–50, ensuring that all intraday intervals were covered, and (iii) 10% of specimens when the number of specimens per species was >50 individuals, optimizing for intraseasonal collection dates. Slide-mounted pollen samples were then photographed to serve as a voucher, as the downstream method of DNA metabarcoding is destructive, and then identified with a microscope (Olympus, 400–1000× magnification) using the keys of von der Ohe and von der Ohe (2007) and the pollen reference collection of the Specialized Centre for Bees and Beekeeping in Mayen, Germany (www.bienenkunde.rlp.de) (Figure 1). "Types" represent taxonomic units that cannot be identified further to lower taxonomic levels and may contain several species or even genera.

2.6 | DNA metabarcoding of pollen loads

Following morphological identification, pollen slides were used in DNA metabarcoding. To maximize pollen content (and therefore higher DNA content) and reduce cost, all slides from a particular insect species (one to six) were combined in a single 2-mL SafeSeal microcentrifuge tube (Sarstedt) to create a species- and plot-specific, but not specimen- or time-specific sample with a total number of 206 samples. To minimize cross-contamination, the slides were wiped externally with Molecular BioProducts DNA AWAY before gently removing the coverslips with a sterile scalpel blade. Then, the glycerol gelatin sample was removed from the slide with the same sterile scalpel. In most cases removal of the coverslip and obtaining the gelatin sample required little effort, but in several instances, the slides were heated at 50°C for 5 s, and in a few cases, specimen samples were discarded due to glass fragmentation.

Following the sample creation, 1 g of 1.4-mm ceramic beads was added to the 2-mL tube and DNA was extracted with a Nucleomag 96 Plant Kit (Macherey Nagel). All reagents were used at 25% of the factory protocol, except for elution buffer MC6. Prior to lysis incubation lysis buffer MC1 and 5 µL Proteinase K (10 mg mL^{-1}) were added and the sample was homogenized for 2.5 min on a Mixer Mill MM 400 (Retsch) at 30 Hz, then incubated at 65°C for 60 min after which, 5 µL RnaseA (10 mg mL^{-1}) was added and incubated at room temperature (20 ± 2°C) for 30 min. Following all other protocol steps, 35 µL of elution buffer MC6 was added and incubated at 55°C for 5 min to remove residual ethanol, then 25 µL was removed for further processing and 2 µL for DNA quantification with a Qubit 4 fluorometer (Thermo Fisher Scientific).

Polymerase chain reaction was performed with three replicates per sample, with the addition of two DNA extraction-negative controls and two PCR-negative controls to evaluate contamination, and two positive controls. Amplification was performed with an adaptation of the Canadian Centre for Barcoding Platinum Taq Protocol (Ivanova et al., 2007) with the addition of 0.25 µL BSA (bovine serum albumin; 0.01 mg mL^{-1}) and 1.25 µL of 50% DMSO (dimethyl sulphoxide) in a total reaction volume of 12.5 µL. Universal plant-specific ITS2 primers were used: forward: ITS-3p62plF1, ACBTR GTGTGAATTGCAGRATC and reverse: ITS-4unR1, TCCTCCGCTTA TTKATATGC (Kolter & Gemeinholzer, 2021b). PCR cycling conditions were 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 45 s and a final extension of 72°C for 10 min. Following PCR cycling, the three replicates were combined by the addition of 5 µL of each replicate for a total volume of 15 µL and purified with Thermo Scientific Exonuclease 1. The pooled replicates of nonindexed PCR products were sent to LGC Genomics for sequencing on a MiSeq (2 × 300 bp) after an additional 12 PCR cycles, three cycles: 15 s 96°C, 30 s 50°C, 90 s 70°C, followed by nine cycles: 15 s 96°C, 30 s 58°C, 90 s 70°C with MyTaq Red Mix polymerase (Bioline BIO-25044).

Sequencing data were processed with usearch (Edgar, 2010) and dada2 (Callahan et al., 2016) in R (R Core Team, 2021). Sequencing primers were trimmed and quality filtered with a maximum expected error of 1.0 in usearch. Dada2 was then used for error learning, denoising by the error profile (pseudo pooling) and merging of reads. Chimeras were removed with uchime3. The resulting amplicon sequence variants (ASVs) were identified by implementation of the SINTAX algorithm (Edgar, 2016) using the PLANiTS database (Banchi et al., 2020) and submitted to NCBI SRA (Accession nos.: PRJNA935259 and PRJNA935270). The resulting ASVs with fewer than five reads per sample, as well as fungal contaminants, were discarded. Taxa that do not occur in Germany and were probably present due to laboratory contamination were removed from further analysis. Taxa with ambiguous species-level identifications, due to lack of coverage in the identification reference database, were given genus-level identifications.

2.7 | Curation of the data set

The data initially kept separately by plot (i.e., plot with and without flower strip) were pooled into two data sets: one based on

TABLE 1 Network indices of the five different networks analysed.

	Data set	Connectance	Nestedness	Links per species
General network	2	0.05	2.39	2.84
Brachycera network	2	0.05	2.97	2.18
Hymenoptera network	2	0.16	7.93	3.83
Network during flowering period	1	0.13	6.32	2.34
Network after flowering period	1	0.17	11.25	1.68

Note: For data set definitions, see main text.

morphological identification of pollen only (data set 1) and one based on the morphological identification combined with the DNA metabarcoding identifications of pollen (data set 2). Both data sets therefore differ in terms of identified plant/pollen species but include the same data on potential pollinators (i.e., insect species). Data set 1 is semiquantitative (i.e., includes the number of samples containing the respective interaction). Data set 2 was converted into a single presence/absence data set (qualitative data set). Since presence/absence data sets can over-accentuate interactions with rare plant taxa, we excluded plant species involved in less than 1% of the total number of interactions from the analysis, following Lucas et al. (2018).

2.8 | Terminology, statistical analysis, plant–pollinator networks and indices

We constructed bipartite networks composed of two node divisions (insect and pollen species) connected by a link defined as an interaction between the plant and a potential pollinator (Dormann et al., 2009).

All bipartite plant–pollinator networks analyses were carried out in R (version 1.4.) (R Core Team, 2021), using the function *plotweb* of the *bipartite* package for the network analyses and using the function *networklevel* for network indices (Dormann et al., 2008). We created five different plant–pollinator networks: (i) a network with all potential hymenopteran and brachyceran pollinating species of caraway, (ii) a plant–pollinator network with only brachyceran species, and (iii) with only hymenopteran species ((i–iii) based on the qualitative data set 2), and two intraseasonal networks, (iv) during and (v) after the main flowering period (based on the semiquantitative data set 1).

For the description of the main differences between the plant–pollinator networks, we calculated the Connectance (*C*), Nestedness (*N*) and the mean number of links per species for each network. *C* is defined as the number of links in proportion to all possible links (Dormann et al., 2009; Dunne et al., 2002). The overall *N* of a network (in this case 0 being highly nested) describes the specialization asymmetry, that is the proportion between specialists and generalists in the network (Bascompte & Jordano, 2007; Dormann et al., 2008, 2009). Since the morphological identification of pollen grains was only possible at genus, type or family levels in most cases, some plant species in data set 2 may be represented in several nodes.

To analyse the differences in interactions between Hymenoptera and Brachycera, we used a permutational multivariate analysis of variance (MANOVA) with the Jaccard similarity index (function *adonis* in the R package vegan; Oksanen et al., 2016), using 9999 permutations and Jaccard similarity index. The differences were also plotted as a nonmetric multidimensional scaling (nMDS) with the function *metaMDS* and ellipses were generated with the function VeganCovEllipse (package vegn: Oksanen et al., 2016). We also generated a UpsetR-plot (function upset in the R package UpsetR; Conway et al., 2017) to study the key intervals to sample the highest number of potential pollinators of caraway.

3 | RESULTS

We collected 1021 insect specimens (844 brachycerans and 177 hymenopterans). These specimens represent 121 species from 33 families (87 Brachycera taxa from 20 families and 34 Hymenoptera taxa from 12 families) (Figure S1). In total, 707 specimens were identified morphologically (559 Brachycera and 148 Hymenoptera) and 516 specimens (331 Brachycera and 185 Hymenoptera) were identified via DNA barcoding following the reverse-taxonomy approach. Of the 1021 specimens collected, 457 were selected for analyses of their pollen load, representing all collected insect species. Common species included *Melanostoma mellinum* (Linnaeus, 1758) (Syrphidae; 11.57% of all specimens), *Sphaerophoria scripta* (Linnaeus, 1758) (Syrphidae; 9.08% of all specimens), *Eristalis arbustorum* (Linnaeus, 1758) (Syrphidae; 6.92% of all specimens), *Lucilia silvarum* (Meigen, 1826) (Calliphoridae; 6.49% of all specimens), *Melanostoma scalare* (Fabricius, 1794) (Syrphidae; 4.65% of all specimens), *Episyrphus balteatus* (De Geer, 1776) (Syrphidae; 4.11% of all specimens) and *Athalia rosae* (Linnaeus, 1758) (Tenthredinidae; 3.46% of all specimens). Links were recorded by plant identification via DNA metabarcoding in 79 insect species, by the morphological pollen identification in eight species or by both approaches in 34 species.

A total of 457 pollen loads were identified morphologically and later pooled into 206 DNA metabarcoding samples. Of these, 17

metabarcoding samples (seven taken from Brachycera and 10 from Hymenoptera) did not yield an adequate quantity of DNA and were excluded, resulting in 189 samples used in further analyses.

3.1 | Plant–pollinator network of caraway

The plant–pollinator network of caraway included 121 potential pollinator species and 139 plant taxa of different taxonomic levels. Overall, we found 859 links, from which 199 links were identified only by pollen morphology, 617 links were recorded only by DNA metabarcoding of pollen samples and 43 links were recorded by both methodologies.

The mean number of links per insect species was 2.48 (hymenopterans = 3.8 links per species; brachycerans = 2.18) (Figure S2 and Table 1). Thirteen insect species (11 brachycerans and two hymenopterans) carried only caraway pollen. The key plant node (i.e., plant species with the highest number of interactions) is naturally *Carum carvi* L. (121 interactions = 18.4% of the total number of interactions), followed by *Urtica dioica* L. (33 interactions = 3.75%) and *Borago officinalis* L. (32 interactions = 3.6%) (Figure S2). Of the 50 plant species present in the flower strip (Table S1), 31 were present in the network. The network included 18 crop plants, which were either a component of the flower strip (12 species) or cultivated in the surrounding area (six species).

3.2 | Differences in Hymenoptera and Brachycera links

Within the plant–pollinator network of Brachycera, 87 insect species from 20 families and 96 plant taxa were involved, and we identified 399 links. Eight of the 10 species with the highest number of interactions belong to Syrphidae. *Eristalis nemorum* (Linnaeus, 1758) (Syrphidae) is the species with the highest number of interactions (18 interactions), followed by *Episyrphus balteatus* (De Geer, 1776), *Syritta pipiens* (Linnaeus, 1758) and *Syrphus vitripennis* (Meigen, 1822) (all Syrphidae; 12 interactions each) (Figure 2 and Figure S3).

Within the plant–pollinator network of Hymenoptera, 34 insect species from 12 families and 86 plant taxa were involved, with 460 links. *Bombus terrestris* (Linnaeus, 1758) (Apidae; 41 interactions) was the species with the highest number of interactions, followed by *Athalia rosae* (Linnaeus, 1758) (Tenthredinidae; 38 interactions), *Bombus pascuorum* (Scopoli, 1763) (Apidae; 26 interactions), *Lasioglossum pauxillum* (Schenck, 1853) (Halictidae; 25 interactions) and *Lasioglossum calceatum* (Scopoli, 1763) (Halictidae; 20 interactions) (Figure 3 and Figure S4). The plant–pollinator network of Hymenoptera had a higher connectance ($C = 0.14$) and nestedness ($N = 8.13$) than the pollination network for Brachycera ($C = 0.04$ and $N = 2.57$) (Table 1).

We found a significant difference in pollen load composition between Hymenoptera and Brachycera ($F = 5.4567$, $R^2 = .10402$, $p = .003$*) (Figure 4). Besides caraway as the key plant node for Hymenoptera, *Lotus corniculatus* L. (Fabaceae) and *Trifolium repens* L. (Fabaceae) (27 interactions each), *Centaurea cyanus* L. (Asteraceae; 24 interactions) and *Borago officinalis* L. (Boraginaceae; 22 interactions) were the important plant species in the network. *L. corniculatus* L. and *C. cyanus* L. were present in the flower strip.

For Brachycera, *Urtica dioica* L. (Urticaceae; 31 interactions), *Matricaria/Achillea* sp. (Asteraceae; 21 interactions), *Daucus carota* L. (Apiaceae; 20 interactions) and *Bellis* sp. (Asteraceae; 16 interactions) were other important plants in the network. Of these species only *D. carota* was present in the flower strip.

3.3 | Intraseasonal pattern of the plant–pollination network of caraway

We sampled a total of 15 days, 10 days during and 5 days after the main flowering period of caraway. During the main flowering period of caraway, 30 potential pollinators of caraway were present; 17 of these taxa were not present after the flowering period and 13 potential pollinators remained. *Sphaerophoria rueppellii* (Wiedemann, 1830) and *Pipizella* sp. (both Syrphidae) carried caraway pollen only in the period after the main caraway flowering period (Figure 5).

Moreover, we observed an overall decrease in the number of plant taxa (from 46 plant taxa to 38) and a shift in plant species composition (six plant species being only present after the flowering period) involved in the plant–pollination network between during and after the caraway flowering period. At the structural level, we observed an increase in the total number of interactions ($C = 0.13$ during and 0.17 after the flowering period) as well as an increase in the proportion of generalists to specialists ($N = 6.32$ during and 10.74 after the flowering period) (Figure 5 and Table 1).

3.4 | Intraday differences of potential pollinator diversity

In total, 75 out of the 121 potential pollinators species of caraway (61.98%) were found during the first time interval (10–12 AM), 70 (57.85%) during the second time interval (12–14 AM) and 78 (64.46%) during the last time interval (14–16 AM). Only 38 of the 121 potential pollinators (31.4%) were present in all three time intervals, whereas 59 insect species (48.76%) were reported exclusively in one of the three time intervals (Figure 6).

4 | DISCUSSION

To our knowledge, the present survey is the first study characterizing the complexity of the plant–pollinator network of caraway in an agro-ecosystem. With 121 species identified as potential caraway pollinators interacting with 139 plant taxa via 859 links, this network contains the largest number of species of any similar study of agro-ecosystems to date. Compared to previous studies on potential

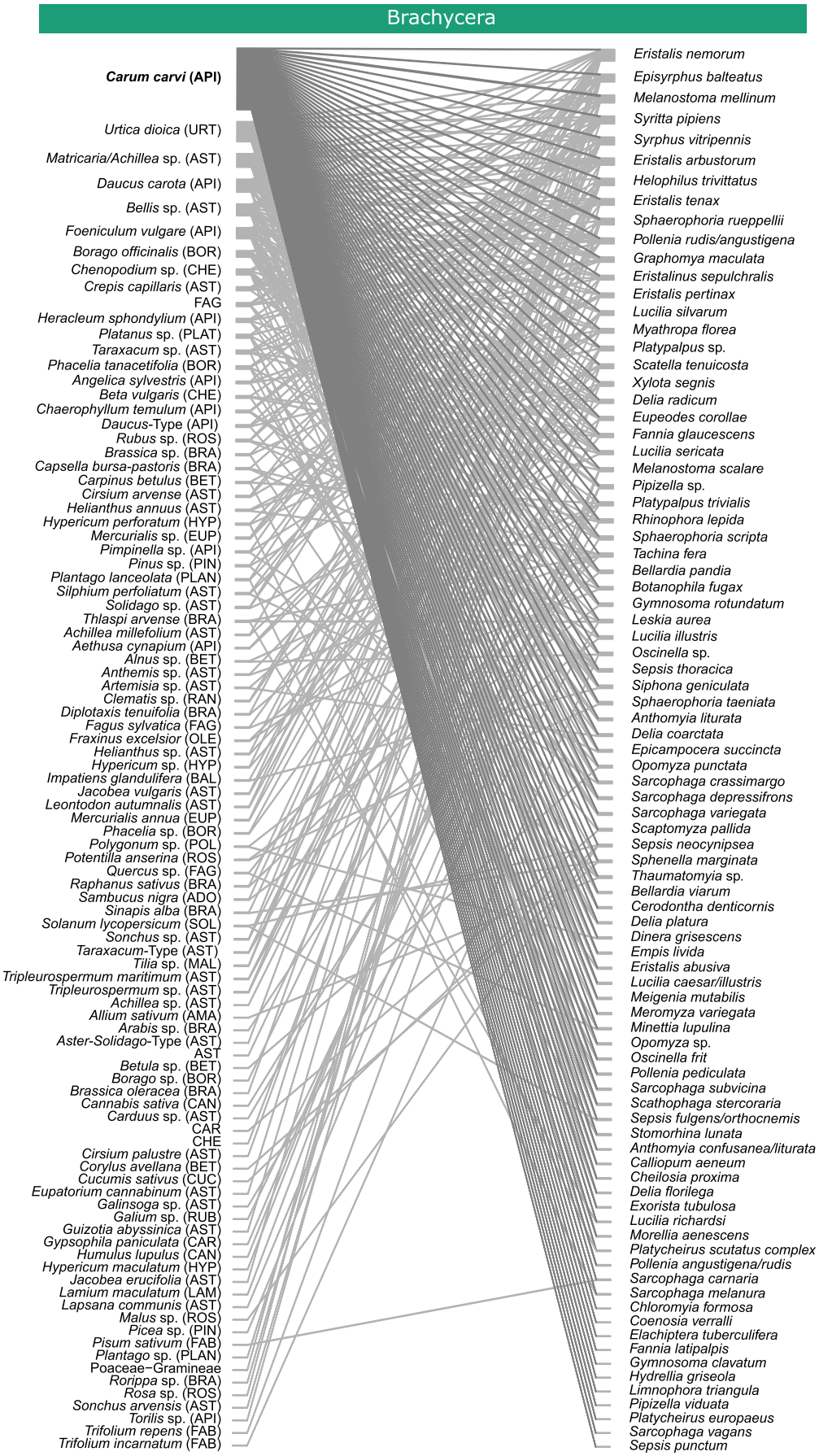
Brachycera
Carum carvi (API)
Urtica dioica (URT)
Matricaria/Achillea sp. (AST)
Daucus carota (API)
Bellis sp. (AST)
Foeniculum vulgare (API)
Borago officinalis (BOR)
Chenopodium sp. (CHE)
Crepis capillaris (AST)
FAG
Heracleum sphondylium (API)
Platanus sp. (PLAT)
Taraxacum sp. (AST)
Phacelia tanacetifolia (BOR)
Angelica sylvestris (API)
Beta vulgaris (CHE)
Chaerophyllum temulum (API)
Daucus-Type (API)
Rubus sp. (ROS)
Brassica sp. (BRA)
Capsella bursa-pastoris (BRA)
Carpinus betulus (BET)
Cirsium arvense (AST)
Helianthus annuus (AST)
Hypericum perforatum (HYP)
Mercurialis sp. (EUP)
Pimpinella sp. (API)
Pinus sp. (PIN)
Plantago lanceolata (PLAN)
Silphium perfoliatum (AST)
Solidago sp. (AST)
Thlaspi arvense (BRA)
Achillea millefolium (AST)
Aethusa cynapium (API)
Alnus sp. (BET)
Anthemis sp. (AST)
Artemisia sp. (AST)
Clematis sp. (RAN)
Diplotaxis tenuifolia (BRA)
Fagus sylvatica (FAG)
Fraxinus excelsior (OLE)
Helianthus sp. (AST)
Hypericum sp. (HYP)
Impatiens glandulifera (BAL)
Jacobea vulgaris (AST)
Leontodon autumnalis (AST)
Mercurialis annua (EUP)
Phacelia sp. (BOR)
Polygonum sp. (POL)
Potentilla anserina (ROS)
Quercus sp. (FAG)
Raphanus sativus (BRA)
Sambucus nigra (ADO)
Sinapis alba (BRA)
Solanum lycopersicum (SOL)
Sonchus sp. (AST)
Taraxacum-Type (AST)
Tilia sp. (MAL)
Tripleurospermum maritimum (AST)
Tripleurospermum sp. (AST)
Achillea sp. (AST)
Allium sativum (AMA)
Arabis sp. (BRA)
Aster-Solidago-Type (AST)
AST
Betula sp. (BET)
Borago sp. (BOR)
Brassica oleracea (BRA)
Cannabis sativa (CAN)
Carduus sp. (AST)
CAR
CHE
Cirsium palustre (AST)
Corylus avellana (BET)
Cucumis sativus (CUC)
Eupatorium cannabinum (AST)
Galinsoga sp. (AST)
Galium sp. (RUB)
Guizotia abyssinica (AST)
Gypsophila paniculata (CAR)
Humulus lupulus (CAN)
Hypericum maculatum (HYP)
Jacobea erucifolia (AST)
Lamium maculatum (LAM)
Lapsana communis (AST)
Malus sp. (ROS)
Picea sp. (PIN)
Pisum sativum (FAB)
Plantago sp. (PLAN)
Poaceae−Gramineae
Rorippa sp. (BRA)
Rosa sp. (ROS)
Sonchus arvensis (AST)
Torilis sp. (API)
Trifolium repens (FAB)
Trifolium incarnatum (FAB)
Eristalis nemorum
Episyrphus balteatus
Melanostoma mellinum
Syritta pipiens
Syrphus vitripennis
Eristalis arbustorum
Helophilus trivittatus
Eristalis tenax
Sphaerophoria rueppellii
Pollenia rudis/angustigena
Graphomya maculata
Eristalinus sepulchralis
Eristalis pertinax
Lucilia silvarum
Myathropa florea
Platypalpus sp.
Scatella tenuicosta
Xylota segnis
Delia radicum
Eupeodes corollae
Fannia glaucescens
Lucilia sericata
Melanostoma scalare
Pipizella sp.
Platypalpus trivialis
Rhinophora lepida
Sphaerophoria scripta
Tachina fera
Bellardia pandia
Botanophila fugax
Gymnosoma rotundatum
Leskia aurea
Lucilia illustris
Oscinella sp.
Sepsis thoracica
Siphona geniculata
Sphaerophoria taeniata
Anthomyia liturata
Delia coarctata
Epicampocera succincta
Opomyza punctata
Sarcophaga crassimargo
Sarcophaga depressifrons
Sarcophaga variegata
Scaptomyza pallida
Sepsis neocynipsea
Sphenella marginata
Thaumatomyia sp.
Bellardia viarum
Cerodontha denticornis
Delia platura
Dinera grisescens
Empis livida
Eristalis abusiva
Lucilia caesar/illustris
Meigenia mutabilis
Meromyza variegata
Minettia lupulina
Opomyza sp.
Oscinella frit
Pollenia pediculata
Sarcophaga subvicina
Scathophaga stercoraria
Sepsis fulgens/orthocnemis
Stomorhina lunata
Anthomyia confusanea/liturata
Calliopum aeneum
Cheilosia proxima
Delia florilega
Exorista tubulosa
Lucilia richardsi
Morellia aenescens
Platycheirus scutatus complex
Pollenia angustigena/rudis
Sarcophaga carnaria
Sarcophaga melanura
Chloromyia formosa
Coenosia verralli
Elachiptera tuberculifera
Fannia latipalpis
Gymnosoma clavatum
Hydrellia griseola
Limnophora triangula
Pipizella viduata
Platycheirus europaeus
Sarcophaga vagans
Sepsis punctum

FIGURE 2 Bipartite network of potential Brachycera pollinators (right), based on data set 2 (qualitative data set based on the morphological identification and DNA metabarcoding of pollen loads). The height of the nodes (insect and pollen) indicates the number of links connected directly to that taxon. ADO, Adoxaceae; AMA, Amaryllidaceae; ANA, Anacardiaceae; API, Apiaceae; AST, Asteraceae; BAL, Balsaminaceae; BET, Betulaceae; BOR, Boraginaceae; BRA, Brassicaceae; CAN, Cannabaceae; CAR, Caryophyllaceae; CHE, Chenopodiaceae; CUC, Cucurbitaceae; EUP, Euphorbiaceae; FAB, Fabaceae; FAG, Fagaceae; HYP, Hypericaceae; LAM, Lamiaceae; MAL, Malvaceae; OLE, Oleaceae; ONA, Onagraceae; PIN, Pinaceae; PLAN, Plantaginaceae; PLAT, Platanaceae; POA, Poaceae; POL, Polygonaceae; RAN, Ranunculaceae; ROS, Rosaceae; RUB, Rubiaceae; SOL, Solanaceae; URT, Urticaceae.

pollinators of caraway (Bouwmeester & Smid, 1995; d'Albore, 1986), we found a much higher diversity of potential pollinators, in particular featuring many species of Brachycera. This aligns with the results by Toivonen et al. (2022), which provided evidence of higher flower visiting rates by Brachycera on caraway than of bee species combined.

The overall plant–pollinator network with all species had an overall low nestedness and connectance, which usually makes networks more prone to external disturbances (Bascompte et al., 2003). However, when calculating these network indices, we encountered some methodological issues. DNA metabarcoding of pollen samples was able to generate plant species lists while the morphological identification of the pollen grains was, in most cases, possible only to types (i.e., a group of related plant species with morphologically indistinguishable pollen) or to the family or genus level. When merging the data sets, we might artificially inflate the number of plant species in the network, with some species potentially being listed as species and as part of a type, family or genus. This cannot be avoided when combining both methods; still, we consider our approach as the best way to cover all actual interactions. Alternatively, for example when considering only species-level plant identifications, we would probably severely underestimate the actual number of taxa and links. For the sake of completeness and reference, we provide a network based only on plant species in Figure S5. Accordingly, we need to be aware that the numbers of plant taxa used in the present paper might not be the exact plant species number. An analysis with this network would result in the same number of links per species, but a considerably higher connectance and nestedness value. Despite having the same average number of links per species as the one with all identified pollen taxa, the overall decrease in the total number of links would increase automatically the weight of each link, resulting in a higher connectance. While one possible solution to combine the morphologically and genetically identified pollen could have been to adapt the total number of plants identified via DNA metabarcoding to the number of species identified morphologically, as suggested by Jędrzejewska-Szmek and Zych (2013), this would have resulted in the loss of over two-thirds of the possible interactions. Therefore, we think the approach used in this study is the best approximation currently possible.

We found a higher number of brachyceran species than hymenopterans carrying caraway pollen, although hymenopterans had overall more links per species than brachycerans. The plant–pollinator network of Brachycera had low connectance and nestedness caused by a high number of species with a low number of interactions. On the other hand, the plant–pollinator network of Hymenoptera had fewer insect species but more links per species. These results align with previous studies (Phillips et al., 2018; Rader et al., 2011). Based on these numbers, hymenopterans might appear as more effective pollinators, but the higher abundance of brachycerans as flower visitors compared to hymenopterans (this study; Garibaldi et al., 2013; Garratt et al., 2014; Innouye et al., 2015; Rader et al., 2016), their higher resilience to land-use changes in comparison with bees (Rader et al., 2016; Ricketts et al., 2008), their ability to carry pollen to greater distances, and their potential additional ecosystem service as biocontrol agents (Dunn et al., 2020) make them equally important ecosystem service providers. Moreover, it has been pointed out that pollen transport and diversity in a species do not correlate with pollination effectiveness (King et al., 2013; Popic et al., 2013). Further studies are required to assess the difference in the effectiveness of hymenopteran and brachyceran potential pollinators, despite some studies on other Apiaceae species already noting a high effectiveness of Brachycera (Niemirski & Zych, 2011; Pérez-Bañón et al., 2007; Zych, 2007; Zych et al., 2014).

Brachyceran species with a high number of interactions were mainly anthropophilic syrphid species with a general preference for white or yellow umbels (Innouye et al., 2015; Speight, 2018). Nonetheless, the high number of generalized syrphid species may be attributed to the occurrence of short-term specialized feeding bouts between individuals of the same species (Lucas et al., 2018). Eleven syrphid species found in our study are aphidophagous in their larval stage and therefore are highly suitable as biocontrol agents (Dunn et al., 2020; Moerkens et al., 2021; Nelson et al., 2012; Tenhumberg & Poehling, 1995), which implies that caraway is not only an attracting resource for pollinators but also for the adult stages of natural enemies of crop pests.

Within Hymenoptera, as expected, Apidae species and other wild bees presented the highest number of interactions, except for two sawflies species, *Athalia rosae* (Linnaeus, 1758) (Tenthredinidae, turnip sawfly) and *Tenthredo notha* (Klug, 1817), indicating the importance of sawflies as potential generalist pollinators of caraway and other Apiaceae (Lamborn & Ollerton, 2000). *Athalia rosae* had the highest number of links of all Hymenoptera species in the network, but the larva of this species is known as a common pest of Brassicaceae crop species (Oishi et al., 1993), particularly of oilseed rape, *Brassica napus* subsp. *napus*, one of the most common oil crops throughout Europe (Woźniak et al., 2019). In addition to detecting non-Apidae species with high numbers of interactions, we also found two species, *Lasioglossum pauperatum* (Halicitidae: Brullé, 1832) and *Anthidium strigatum* (Megachilidae; Panzer, 1805), as potential caraway pollinators that are listed in the German Red List as severely endangered and prewarning list, respectively (Haupt et al., 2009). This shows the potential of caraway as a relevant food source for some endangered species.

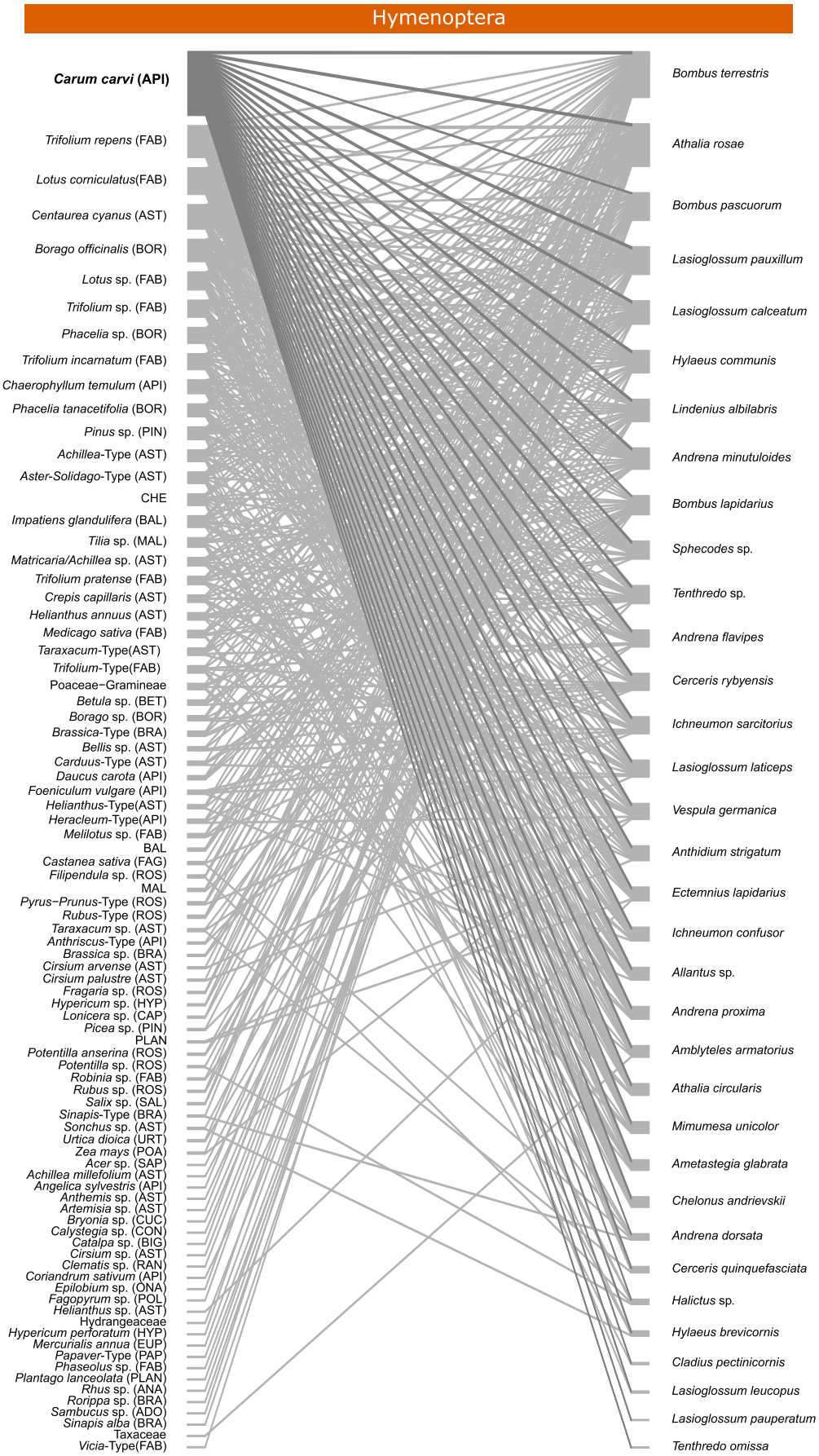
Hymenoptera
Carum carvi (API)
Trifolium repens (FAB)
Lotus corniculatus(FAB)
Centaurea cyanus (AST)
Borago officinalis (BOR)
Lotus sp. (FAB)
Trifolium sp. (FAB)
Phacelia sp. (BOR)
Trifolium incarnatum (FAB)
Chaerophyllum temulum (API)
Phacelia tanacetifolia (BOR)
Pinus sp. (PIN)
Achillea-Type (AST)
Aster-Solidago-Type (AST)
CHE
Impatiens glandulifera (BAL)
Tilia sp. (MAL)
Matricaria/Achillea sp. (AST)
Trifolium pratense (FAB)
Crepis capillaris (AST)
Helianthus annuus (AST)
Medicago sativa (FAB)
Taraxacum-Type(AST)
Trifolium-Type(FAB)
Poaceae−Gramineae
Betula sp. (BET)
Borago sp. (BOR)
Brassica-Type (BRA)
Bellis sp. (AST)
Carduus-Type (AST)
Daucus carota (API)
Foeniculum vulgare (API)
Helianthus-Type(AST)
Heracleum-Type(API)
Melilotus sp. (FAB)
BAL
Castanea sativa (FAG)
Filipendula sp. (ROS)
MAL
Pyrus−Prunus-Type (ROS)
Rubus-Type (ROS)
Taraxacum sp. (AST)
Anthriscus-Type (API)
Brassica sp. (BRA)
Cirsium arvense (AST)
Cirsium palustre (AST)
Fragaria sp. (ROS)
Hypericum sp. (HYP)
Lonicera sp. (CAP)
Picea sp. (PIN)
PLAN
Potentilla anserina (ROS)
Potentilla sp. (ROS)
Robinia sp. (FAB)
Rubus sp. (ROS)
Salix sp. (SAL)
Sinapis-Type (BRA)
Sonchus sp. (AST)
Urtica dioica (URT)
Zea mays (POA)
Acer sp. (SAP)
Achillea millefolium (AST)
Angelica sylvestris (API)
Anthemis sp. (AST)
Artemisia sp. (AST)
Bryonia sp. (CUC)
Calystegia sp. (CON)
Catalpa sp. (BIG)
Cirsium sp. (AST)
Clematis sp. (RAN)
Coriandrum sativum (API)
Epilobium sp. (ONA)
Fagopyrum sp. (POL)
Helianthus sp. (AST)
Hydrangeaceae
Hypericum perforatum (HYP)
Mercurialis annua (EUP)
Papaver-Type (PAP)
Phaseolus sp. (FAB)
Plantago lanceolata (PLAN)
Rhus sp. (ANA)
Rorippa sp. (BRA)
Sambucus sp. (ADO)
Sinapis alba (BRA)
Taxaceae
Vicia-Type(FAB)
Bombus terrestris
Athalia rosae
Bombus pascuorum
Lasioglossum pauxillum
Lasioglossum calceatum
Hylaeus communis
Lindenius albilabris
Andrena minutuloides
Bombus lapidarius
Sphecodes sp.
Tenthredo sp.
Andrena flavipes
Cerceris rybyensis
Ichneumon sarcitorius
Lasioglossum laticeps
Vespula germanica
Anthidium strigatum
Ectemnius lapidarius
Ichneumon confusor
Allantus sp.
Andrena proxima
Amblyteles armatorius
Athalia circularis
Mimumesa unicolor
Ametastegia glabrata
Chelonus andrievskii
Andrena dorsata
Cerceris quinquefasciata
Halictus sp.
Hylaeus brevicornis
Cladius pectinicornis
Lasioglossum leucopus
Lasioglossum pauperatum
Tenthredo omissa

FIGURE 3 Bipartite network of potential Hymenoptera pollinators (right), based on data set 2 (qualitative data set based on the morphological identification and DNA metabarcoding of pollen loads). The height of the nodes (insect and pollen) indicates the number of links connected directly to that taxon. ADO, Adoxaceae; ANA, Anacardiaceae; API, Apiaceae; AST, Asteraceae; BAL, Balsaminaceae; BET, Betulaceae; BIG, Bignoniaceae; BOR, Boraginaceae; BRA, Brassicaceae; CAP, Caprifoliaceae; CHE, Chenopodiaceae; CON, Convolvulaceae; CUC, Cucurbitaceae; EUP, Euphorbiaceae; FAB, Fabaceae; FAG, Fagaceae; HYP, Hypericaceae; MAL, Malvaceae; ONA, Onagraceae; PAP, Papaveraceae; PIN, Pinaceae; PLAN, Plantaginaceae; PLAT, Platanaceae; POA, Poaceae; POL, Polygonaceae; RAN, Ranunculaceae; ROS, Rosaceae; SAL, Salicaceae; SAP, Sapindaceae; URT, Urticaceae.

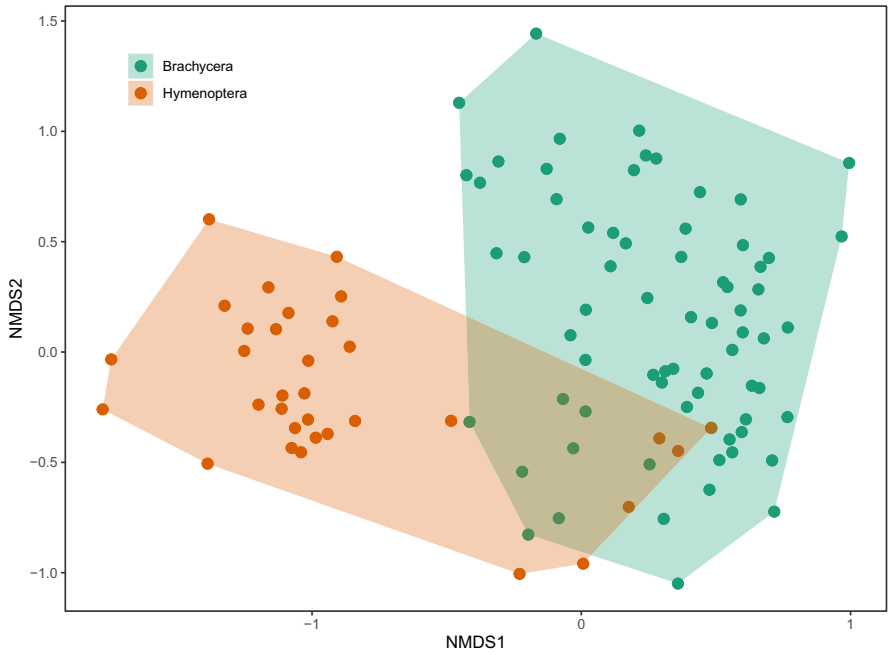

FIGURE 4 Nonmetric multidimensional scaling (NMDS; stress value = 0.19) ordination showing the interaction differences of Brachycera (green) and Hymenoptera species (orange) based on a presence/absence matrix of pollen taxa (data set 2; for data set definition, see main text). Polygons according to Brachycera (green) or Hymenoptera (orange).

We corroborate previous studies showing significant differences in flower affinity between Hymenoptera and Brachycera (Lowe et al., 2022). Hymenopterans showed a preference for Fabaceae and Boraginaceae flowers, which are highly adapted to Hymenoptera pollination (Faegri & van der Pijl, 1979; Sedivy et al., 2013; Westerkamp, 1996; Wood et al., 2021) and have restricted access to nectar, making long-tongued bees more suited for retrieving nectar rewards (Jeiter et al., 2020). Brachycerans prefer flowers with mainly white, sometimes yellow, umbrella-like inflorescences that provide pollen and exposed nectar throughout the flowering period, in addition to a resting place (Woodcock et al., 2013). Apiaceae have generally been considered nonspecialized in pollination biology, but studies have shown an increased visitation by dipterans (Niemirski & Zych, 2011; Wojciechowicz-Żytko, 2019; Zych, 2007; Zych et al., 2014, 2019). The composite flowers of Asteraceae also provide a large surface area, and sometimes shelter, as well as easy access to floral resources shown to be preferentially exploited by dipterans (Branquart & Hemptinne, 2000; Morales & Köhler, 2008). Stinging nettle, *Urtica dioica* L., is a primarily wind-pollinated species with highly reduced and inconspicuous flowers and is generally not considered of great interest to pollinating insects. However, pollination of *U. dioica* by insects is known to occur (Taylor, 2009) and it is known to provide important habitat for beneficial insects, including predatory and parasitoid flies and parasitoid wasps that can also serve as pollinators (Alhmedi et al., 2007; James et al., 2015). These two factors, combined with its prevalence in the study area, could in part explain the high abundance. In addition, *U. dioica* was in peak or moderate pollen flight through the duration of the study (2016 Archive; Stiftung Deutscher Polleninformationsdienst; https://www.pollenstiftung.de) and was probably ubiquitous in the air and on surfaces through the study area where insects could pick up pollen grains during contact with these surfaces. We therefore believe that the presence of *U. dioica* found from metabarcoding is not a false positive caused by sample or laboratory contamination, but a real occurrence in the environment.

The presence of three other wind-pollinated taxa both in the morphological and metabarcoding data (*Betula* spp., *Picea* spp. and *Pinus* spp.) and one insect-pollinated taxon (*Salix* spp.) is surprising, as pollen production occurs in early spring, well before our collection dates. The occurrence of wind-pollinated taxa is probably a result of persistence on surfaces in the environment, contact with nest provisions by nesting bees or by active collection of species which depend on anemophilous pollen (Ssymank & Gilbert, 1993). The presence of *Salix* spp. is best explained by contact with nest provisions. Some species of *Salix* have later flowering times in Germany (May–July), but these are present only in subalpine and alpine regions which are far distant from the study site.

Our results show temporal shifts of pollinator networks during and after the flowering period of caraway. Caraway continued to

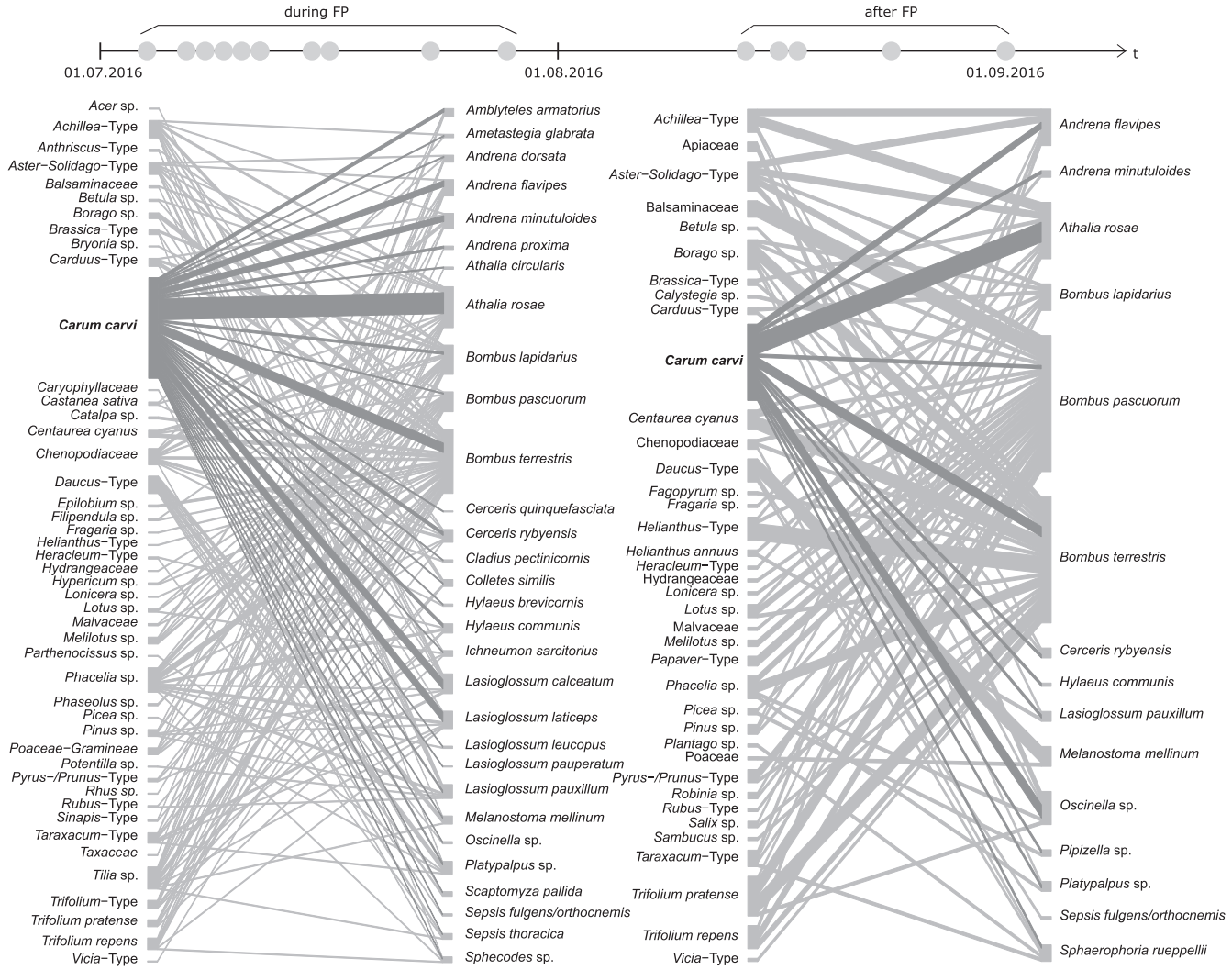

FIGURE 5 Semiquantitative bipartite network during and after the main flowering period (FP) of caraway, based on data set 1 (semiquantitative data set based on the morphological identification of pollen loads). Plant taxa on the left side, potential pollinators on the right side. The width of the link indicates the total number of samples analysed containing the respective link. Timeline on the top illustrates the number of sampling days during and after the flowering period (FP).

flower after the main flowering period but to a much lesser extent. Thirteen of the 32 potential pollinators of caraway were present after the main flowering period and carried caraway pollen. This suggests that for these species the late caraway flowers as well as other late flowering umbellifers, present after the main flowering periods of flowering plants in general, might be an important food resource and therefore a good candidate for farmers to support pollinators (Zych et al., 2007).

The increment in connectance and nestedness after the flowering period is caused by different factors: over half of the insect species are no longer present, the number of carried pollen species is declining, and we observe an increase in links and abundance of a few *Bombus* species. Despite having thoroughly sampled over the flowering period of caraway, around 50% of the collected species would have been missing if the sampling had not taken place at three time intervals per day. The activity levels of Brachycera and Hymenoptera are susceptible to weather conditions (e.g., temperature, wind or cloudiness) and therefore it was anticipated that some potential pollinators of caraway will not be present over the whole day (Innouye et al., 2015; Koul et al., 1993; Willmer, 1983). Therefore, the strong intraday differences in potential pollinator diversity make it necessary to collect insects and pollen at different times of the day to compile complete plant–pollinator networks.

By combining the results of both pollen identification methods, we were able to distinguish over four times more interactions than with the morphological identification of the slide-mounted pollen alone, and 1.3 times more interactions than with the pollen identification via DNA metabarcoding alone. While DNA metabarcoding has an overall higher species identification resolution than morphological identification, its accuracy is limited by the quality of the database (Meiklejohn et al., 2019; Michelot-Antalik et al., 2021), and by the barcode marker used (Kolter & Gemeinholzer, 2021a). Also, possible cross-contamination can produce false positives, and PCR bias has the potential to produce both false positives and negatives. In our study,

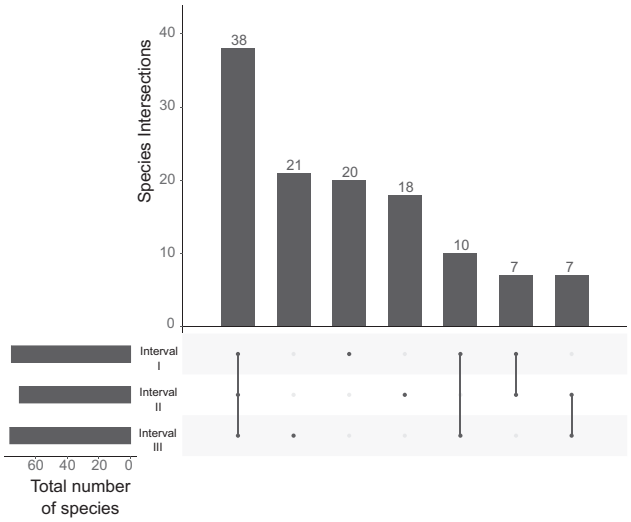

FIGURE 6 UpsetR-plot of species intersection during time intervals I (10–12 AM), interval II (12 AM to 2 PM) and interval III (2–4 PM), based on data set 2 (for data set definition, see main text). The horizontal bars indicate the total number of potential pollinator species during one of the three different time intervals. Vertical bars indicate the number of species shared between intervals (connected black dots) or exclusive to one of the intervals (single black dots).

we benefited from a well-curated database for our region and incorporated best practice protocols of sterile techniques and a dense system of negative controls to account for contamination as well as positive controls to confirm methods. Unfortunately, there is no definitive way to account for false negatives. DNA metabarcoding is a developing method and several factors could have influenced our results. Our samples for the most part comprised very low pollen loads (<5000 pollen grains) that could be highly prone to false positives (Alberdi et al., 2018). In addition, cross-contamination in the field and laboratory processes can be mitigated but are impossible to eliminate, and some scrutiny needs to be applied to the results.

We also observed a great difference in the abundances of the species, ranging from a single specimen in 83 species up to 108 specimens in *Athalia rosae*. Further sampling over multiple years, with a higher number of specimens per species, would be necessary to get the full picture of the plant–pollinator network of caraway and other possible hidden links. This multiyear sampling would also account for possible interannual and spatial variations of caraway, which could influence the patterns of the plant–pollinator network of caraway.

5 | CONCLUSIONS

Our results highlight the unexpected complexity of the studied network and the high diversity of nonhoneybee Hymenoptera and Brachycera species involved as potential caraway pollinators. Furthermore, we observe significant network differences over the course of the year, as well as strong qualitative differences between main potential pollinator taxa. We emphasize the importance of caraway as a food source outside the peak flowering periods of other crops and natural plants for pollinating insect communities. Moreover, we highlight the potential of caraway as a complement to flowering strips and other biodiversity-fostering methods in agricultural areas, providing farmers with an additional source of income. We also argue that upscaling this type of study to cover intraseasonal and intraday variation as well as all main pollinator species is crucial to obtain complete data. Implementing beneficial and evaluating beneficial as well as detrimental measures will rely on this comprehensive understanding of the plant–pollinator networks in agro-ecosystems.

AUTHOR CONTRIBUTIONS

ICK, SJS, XM, AH and RSP designed the study. ICK collected the samples. SJS conducted the laboratory part for pollen analysis. ICK processed and analysed the data. SJS, XM, BG, AH, JWW and RSP prepared, contributed to and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

Sequencing data generated and analysed in this study are publicly available through the NCBI SRA (Accession nos. PRJNA935259 and PRJNA935270) for the pollen analyses via metabarcoding and in GenBank (DOI: 10.5883/DS-POLLCARA: OQ611071 - OQ611458) for insect barcoding. Metadata and data sets are publicly available in Mendeley data (DOI: 10.17632/3xghzfvfmj.2).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Chapter 3.

Think on flies when you eat an apple: Brachycera have more interactions than Hymenoptera in the plant-pollinator network of *Malus domestica* Borkh. (Rosaceae)

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3.1. Summary

Pollination is an essential ecosystem service provided by insects and a demand for the production of at least 75% of the most important and highly pollination-dependent crop species worldwide (Garibaldi et al., 2009; Klein et al., 2007; Ollerton et al., 2011). *Malus domestica* Borkh. (apple; Rosaceae) is one of the most economically important fruit orchards worldwide, with a annual production of about 87.5 megatons (*FAOSTAT*, 2020). Apple flowers are self-incompatible and need a successful cross-pollination between the same cultivars to achieve the highest possible yield (Free, 1964; McGregor, 1976; Westwood, 1988). Due to climate change, apple orchards and other early flowering crop species could risk an asynchrony between flowering and insect phenology (Wyver et al., 2023). Nonetheless, existing knowledge on apple orchard pollination has primarily focused on wildbees and managed species (particularly honeybees), given limited visibility and attention to the importance of other wild pollinators (particularly Dipterans) as potential pollinators (Barahona-Segovia et al., 2023; Rader et al., 2016; Rosa García & Miñarro, 2014). Moreover, these studies have relied primarily on observational data, leaving the question open of whether all those species were also pollinating (i.e., transporting the pollen) or just visiting the flowers. In this chapter, we applied a similar methodology as in Chapter 2 to assess the plant-pollinator networks of apple orchards with a minor deviation. Instead of morphologically identifying the pollen loads combined with DNA metabarcoding, we solely utilized metabarcoding.

In Chapter 3, based on the plant-pollinator networks of apple, the aims were to:

(1) to identify the potential pollinators of apple, with a focus on Brachycera and Hymenoptera,
(2) to analyze the potential interannual differences in brachyceran apple pollinators,
(3) to determine the key generalist taxa crucial for the network's stability and
(4) to describe differences between the Brachycera and Hymenoptera networks.

Of the 233 insect species transporting pollen, 103 species (66 Brachycera and 37 Hymenoptera) were identified as potential apple pollinators. Aside from being pollinators of

apple, 25 species additionally carried *Prunus*-pollen (e.g., from cherries or plum trees), indicating the relevancy as potentially pollinators for other early flowering orchards. The plant-pollinator network of Hymenoptera was primarily dominated by mining bees (Andrenidae) and wildbees, except for a few parasitoid wasps and sawflies. Within the plant-pollinator networks of Brachycera, several species of Syrphidae and Anthomyiidae played an essential role as generalist species within the networks. Surprisingly, five Brachyceran species were only found to be transporting pollen in one of the two sampled years, displaying the high variability in the pollinator diversity between both years. While the efficiency of selected wildbees can be higher than that of Brachycera (Boyle & Philogène, 1983), Brachycera are more tolerant of lower temperatures, exemplified by the abundance and activity of these pollinators in arctic and alpine regions (Lefebvre et al., 2019; Tiusanen et al., 2016). Additionally, they are more tolerant to land-use changes due to a high variability in nesting sites and floral resources (Rader et al., 2016; Ricketts et al., 2008). Aside from apple pollen, all the potential pollinators of apple visited also other 194 plant species. Just five of these plant species were present in a flowering strip established in one of the sampling areas. Dandelion (*Taraxacum* spp.), daisies (*Bellis perennis),* and other early flowering wild plant species were generalist plant species in the networks. Despite often being labeled as weed species, they can be essential nectar and pollen providers in the spring when other flowering species or strips are not flowering yet (Lisek & Sas-Paszt, 2015). Finally, the plant-pollinator network structure and interactions between Brachycera and Hymenoptera were closer in comparison to the interannual Brachycera networks. Plant phenology could be the leading driver defining in this case the plant-pollinator networks` structure (Nicholls & Altieri, 2013). In conclusion, the results presented in this chapter demonstated the high diversity of potential pollinators of apple even when solely focusing on Brachycera and Hymenoptera. Moreover, early-flowering plant species are key taxa to maintain the structure of the networks, while plant phenology and diversity shape additionally the plant-pollinator networks.

Personal contribution

The study was conceptualized by Ximo Mengual, Ralph Peters, and Andrée Hamm. I prepared the pollen samples for morphological identification and prepared the specimens (including tissue harvesting and pining) with the support of Katharina Geiger. Primarily, I morphologically identified the insect specimens with specific support from Ximo Mengual for Syrphidae and Ralph Peters for parasitoid wasps. Additionally, a few specimens were

identified by external taxonomists. I took the voucher pictures of the pollen samples with some support from Katharina Geiger and a student helper. Stephanie Swenson and I did the lab work to extract and analyze the DNA of the pooled pollen samples. Stephanie Swenson curated bioinformatically the raw data from the DNA metabarcoding of pollen samples and submitted the ASVs to NCBI SRA. The DNA Barcoding of the insect specimens was done at the LIB – Museum Koenig Bonn. I combined and curated all, analyzed the data, prepared the figures using my own R® scripts, and refined all the figures in Inkscape®. I interpreted all results, and Ximo Mengual and Ralph Peters helped validate them. I wrote the first draft of the manuscript, which all co-authors helped to review and edit.

Think on flies when you eat an apple: Brachycera have more interactions than Hymenoptera in the plant-pollinator network of *Malus domestica* Borkh. (Rosaceae)

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Keywords: DNA metabarcoding, DNA barcoding, non-bee pollinators, pollination, Diptera, Hymenoptera

Abstract

Pollination by insects is an essential ecosystem service crucial for many crop species, such as apple. Because of its worldwide economic importance and high dependence on pollination by insects, previous studies on apple pollinator diversity and efficiency have focused predominantly on wildbees or other well-studied taxa. Moreover, flies have rarely been considered as pollinators or previous studies have instead identified only a selection of flower-visitors due to methodology limitations. Here, we present the plant-pollinator network of apple with a focus on Brachycera (Diptera) and Hymenoptera. By analyzing via DNA metabarcoding the pollen loads attached to the potential pollinators, we studied the qualitative differences in the plant-pollinator interactions of Brachycera and Hymenoptera sampled in two years. We found in total 35 potential hymenopteran pollinators and 66 potential brachyceran pollinators of apple interacting with 194 plant taxa. The potential pollinators of apple include many non-syrphid Brachycera and some non-bee Hymenoptera, which also interacted with other early-flowering orchards and plants that can supplement the floral resource requirements of potential pollinators. Overall, the difference in pollen load composition of Brachycera between the years was lower than between Hymenoptera and Brachyera. Our results indicate that a significant number of species are potential pollinators of apple and underscore the importance of conserving a large diversity of potential

pollinators within Hymenoptera and Brachycera, together with the need to take a closer look at the ecology and efficiency of those taxa in future studies. This is crucial to secure and increase proper pollination services for apple and other early-flowering orchard species.

Introduction

Pollination is a crucial ecosystem service provided by insects in agroecosystems. Worldwide, at least 75% of the most important crop species are highly dependent on constant and efficient pollination (Garibaldi et al., 2009; Klein et al., 2007; Ollerton et al., 2011). One of the most economically essential orchard crops worldwide are apples (*Malus domestica* Borkh.; Rosaceae), composed of multiple cultivars. The worldwide production of apple comprises 87.5 megatons. In Germany, apple plantations cover around 34 thousand hectares of land and are therefore a substantial component of agricultural landscapes and diversity (*FAOSTAT*, 2020). Apple flowers are self-incompatible and require a successful cross-pollination by insects between the same cultivars to harvest in profitable quantities (Free, 1964; Garratt et al., 2014; McGregor, 1976; Westwood, 1988). The pollination dependency level also differs significantly between cultivars (Garratt et al., 2021). Moreover, weather conditions heavily influence apple pollination due to its status as an early-flowering plant susceptible to significant temperature fluctuations, with an average blossoming duration of approximately nine days in spring (McGregor, 1976). Deficits in pollination in apple orchards may cause a deficiency in fruit set of at least 41 % and loss of yields worldwide in the million dollar (Garratt et al., 2014; Hünicken et al., 2021; Leonhardt et al., 2013; Olhnuud et al., 2022).

From a farmer's perspective, these high dependencies for a successful and effective pollination in a short period of the year have caused a demand for managed pollinating species. Honeybees (*Apis mellifera* Linnaeus, 1758; Hymenoptera) are most frequently used for this purpose, although they are not even the most efficient pollinators compared to other bee species (Bernauer et al., 2022; Delaplane et al., 2000; Eeraerts et al., 2020; Weekers et al., 2022). While Hymenoptera are the most predominant pollinators of apple, other insect groups such as Coleoptera, Diptera, and Lepidoptera are also important apple pollinators and are usually neglected in pollination studies (Barahona-Segovia et al., 2023; Boyle & Philogène, 1983; Burns & Stanley, 2022; Orford et al., 2015; Pardo & Borges, 2020; Rader et al., 2020; Roquer-Beni et al., 2022). Recent studies have even shown that successful apple pollination can only be achieved by having a higher diversity of pollinating species during the flowering period (Barahona-Segovia et al., 2023; Blitzer et al., 2016; Földesi et al., 2016; Garibaldi et al., 2013; Mallinger & Gratton, 2015; Olhnuud et al., 2022; Russo et al., 2015). Despite this,

research focus on pollinators of apple is still mostly restricted to honeybees and wildbees, while the potential of other wild pollinators, especially non-bee hymenopterans, is rarely addressed and therefore underestimated (Barahona-Segovia et al., 2023; Földesi et al., 2016; Gamonal Gomez et al., 2023; Mupepele et al., 2023; Rader et al., 2016).

Globally, a variety of external drivers are affecting apple pollination and could impact global apple production in the near future. Particularly in early-flowering crop species like apples, global warming is causing, among others, an asynchrony between the apple flowering period and bee phenology (Wyver et al., 2023). In addition, the massive decline in pollinating insect species diversity and biomass could also mean a decline in apple production worldwide (Garratt et al., 2014; Hallmann et al., 2017). Therefore, there is a need to look deeper into the diversity of non-bee pollinators and their ecology to secure more effective pollination services in the future (Garratt et al., 2014; Rader et al., 2016; Wyver et al., 2023).

Plant-pollinator network analysis facilitates studying the interactions between potential pollinators and other plant species and is ideal for these purposes. Compared to the study of the pollinators of focal plant species, plant-pollinator network studies can help to understand underlying ecosystem functions and the stability of the interactions (Bennett et al., 2018; Briggs et al., 2019). However, in many pollinator assessments, there is usually a main misinterpretation of the difference between flower-visitors and pollinators: while all the insects visiting a flower are flower-visitors, just the species transporting the pollen from one flower to another can be considered pollinator (King et al., 2013). Therefore, while studies on the plant-pollinator network of apple are not rare (Barahona-Segovia et al., 2023; Blitzer et al., 2016; Boyle & Philogène, 1983; Mupepele et al., 2023; Ramírez & Davenport, 2013), the methods mainly used for these assessments continue to be surveys on insect visits or sampling along a transect in the orchard, under the assumption that flower-visitors are also pollinating insects. While visit surveys are a more affordable method than an animal-centered approach based on investigating pollen loads, it is sensitive to sampling effort (Baksay et al., 2022). It can underestimate the number of interactions between the plant and the pollinator compared to advanced methods such as DNA metabarcoding (Pornon et al., 2017).

DNA metabarcoding using Next Generation Sequencing (NGS) to analyze mixed DNA samples, is a powerful method used to study plant-pollinator interactions by, e.g., analyzing the pollen samples collected from pollinators (Lucas et al., 2018; Macgregor et al., 2019; Pornon et al., 2017). In contrast to conventional approaches utilizing light microscopy to analyze pollen samples, metabarcoding allows for a more extensive identification of plant taxa, including a higher number of species-level identifications, which allows for the identification of a higher number of interactions (Kilian et al., 2023; Pornon et al., 2017). Moreover, it is also the

prerequisite to explore and understand the impact of ecosystem changes on the structure of plant-pollinator networks (Bell et al., 2023; Pornon et al., 2017).

Here, we present the first plant-pollinator network study of apple in a central European agricultural landscape, targeting the taxon-specific roles. By characterizing the attached pollen loads of the insects via DNA metabarcoding, we aim to investigate the potential pollinators of apple based on the analysis of a plant-pollinator network, addressing additionally the following questions: (1) which are the potential pollinators of apple? (2) what are the potential interannual differences in plant-pollinator interactions of Brachycera? (3) Which are the key plant species many apple pollinators share? (4) are there interaction differences between Brachycera and Hymenoptera?

Material and Methods

Study sites

The sampling of Hymenoptera and Brachycera was conducted on two transects. The first one was located on a conventionally farmed apple orchard with flowering strips (list of plants in supplements and planted in 2014) at the agricultural research station Campus Klein-Altendorf (50°37’23.2"N 6°59'21.4"E) managed by the Service Center for Rural Areas of Rhineland-Palatinate (DLR Rheinpfalz), Germany. Ten flower boxes (80 x 17,5 cm) with early-flowering native plant species (e.g., *Primula vulgaris*, *Campanula* sp., *Erica x darleyensis*) were placed along the transect during the blooming period of the apple orchards until the flowering strips started to bloom. The transect (82 m long) was surrounded by other early-flowering orchards (pears and cherry trees).

The second transect (137 m long) was located on an organically farmed apple orchard without flowering strips in the farm of the family Nachwey (50°35'46.0"N 7°02'48.6"E) and surrounded mainly by cereals and maize. The distance between both areas was around 5 km, therefore, beyond most pollinating species' movement range (Rader et al., 2011). The blossom development of the apple trees was tracked using the BBCH scale, which, based on a decimal code, describes the phenological growth stages of crop species (U. Meier et al., 1994).

Sampling and morphological identification of potential pollinators

We sampled during the apple blossoming (BBCH: 60-69) on all rain-free days and every two weeks before (BBCH: ≤ 59) and after (BBCH: ≥ 70) the flowering period of the apple orchards

from March to September 2016 and 2017, for a total of 39 days on both years (Table S2; Meier et al., 1994). Both locations were sampled for 30 min each sampling day at three different time intervals to sample intraday variation in insect and interaction diversity: 10-12h, 12-14h, and 14-16h. To avoid time bias in the sampling design, we alternated the order of the locations, starting with a different location each day.

Brachycera and non-honeybee Hymenoptera were collected by hand-netting or direct collection from the flowers into sampling vials along the transects, either (i) from the apple flowers or (ii) from the flowering strips or flowering plants in the transect after the blossoming period of apple. Within Hymenoptera, Formicidae were excluded from the study, as they are better known as biocontrol agents in apple orchards instead of pollinators (J. Cross et al., 2015; Miñarro et al., 2010). In addition, honeybees were excluded from the study as their role and efficacy for apple orchards are already well-established (Bernauer et al., 2022; Delaplane et al., 2000; Eeraerts et al., 2020; Weekers et al., 2022). After sampling, specimens were stored dry individually at -20°C. Each specimen was identified based on morphological characteristics (information about used identification keys can be found in the supplements). For specimens that could not be accurately identified with external characters, we followed a reverse-taxonomy approach via DNA barcoding using either three legs or the whole specimen (Morinière et al., 2019) to obtain molecular sequences of the mitochondrial cytochrome c oxidase subunit I gene (COI; Hebert et al., 2003). A detailed description of the DNA extraction and barcoding protocol for the insects is described in Kilian et al. (2023). Voucher specimens are deposited at the Museum Koenig Bonn (Leibniz Institute for the Analysis of Biodiversity Change).

Preparation and pooling of pollen load samples

The pollen attached to the specimens was collected by swabbing the insects with a lentil-sized piece of Kaisers phenol-free glycerol gelatin (Carl Roth), focusing on the body areas where the pollen was present. Afterward, the fragment of glycerol gelatin was mounted on a slide cover over a 55°C heating plate and covered with a cover slip (Beattie, 1971). Mounted samples were photographed to serve as vouchers, as the downstream method of DNA metabarcoding is destructive. To maximize the pollen content and DNA content while minimizing expenses, 1-6 slides obtained from a specific insect species and location were merged into a single metabarcoding sample. For each location, (i) if fewer than 5 specimens of a species were present, all pollen loads were pooled into a sample; (ii) if there were 5-50 specimens of a species, five pollen loads were pooled, ensuring that all intraday intervals were

covered, or (iii) if there were > 50 specimens present, 10% of the samples were pooled, optimizing for intraday intervals and sampling dates. Therefore, the pooled pollen samples for DNA metabarcoding, while being species-, plot-, and year-specific, are not specimen-specific.

Identification of pollen loads via DNA metabarcoding

The laboratory protocols followed the same procedures for DNA extraction, PCR amplification, and Illumina MiSeq (2 x 300 bp) sequencing are described in Kilian et al. (2023). DNA was extracted from pollen slides (single or polled) with Nucleomag 96 Plant Kit (Macherey Nagel), and all reagents were used at 25% of the factory protocol except for the elution buffer MC6 (35 µL, with 25 µL removed after magnetic bead incubation to remove residual ethanol). Sample homogenization occurred for 2.5 min on a Mixer Mill MM 400 (Retsch) at 30 Hz after adding MC1 and 5 µL Proteinase K (10 mg mL^{-1}).

We used the internal transcribed spacer 2 (ITS2) region of the nuclear ribosomal DNA as DNA barcode for its ability to provide species level identifications due to its extensive representation in public DNA sequence repositories and its proven success in identification across a wide breadth of plant taxa (S. Chen et al., 2010, 2010; Han et al., 2013; Kolter & Gemeinholzer, 2021b; Yao et al., 2010) as well as its success in identification of the family Rosaceae in general and the genus *Malus* Mill. in particular (Pang et al., 2011). PCR was performed in three replicates with the plant specific ITS2 primers ITS-3p62plF1 (forward; ACBTRGTGTGAATTGCAGRATC) and ITS-4unR1 (reverse; TCCTCCGCTTATTKATATGC) (Kolter & Gemeinholzer, 2021a). PCR cycling conditions were 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 45 s, and a final extension of 72°C for 10 min. 5 µL of each three PCR replicates were for a total volume of 15 µL and purified with Thermo Scientific Exonuclease 1. The pooled replicates of nonindexed PCR products were sent to LGC Genomics for sequencing on a MiSeq (2 × 300 bp) after an additional 12 PCR cycles, three cycles: 15 s 96°C, 30 s 50°C, 90 s 70°C, followed by nine cycles: 15 s 96°C, 30 s 58°C, 90 s 70°C with MyTaq Red Mix polymerase (Bioline BIO-25044).

The sequencing data was also processed using the same procedure as found in Kilian et al. (2023) and implemented in R (R Core Team, 2021). Sequencing primers were trimmed and quality filtered with a maximum expected error of 1.0 in USEARCH. DADA2 was used for error learning, denoising by the error profile (pseudo pooling), merging of reads, and chimera removal (Callahan et al., 2016). Resulting amplicon sequence variants (ASVs) were implemented with the SINTAX algorithm (Edgar, 2016) using the PLANiTS database (Banchi

et al., 2020). Raw data has been deposited to NCBI SRA. ASVs with fewer than five reads per sample and fungal reads were removed from further analysis, as well as species that do not occur in Germany (either in the wild or in gardens). Taxa with ambiguous species-level identifications were only identified at genus level. Finally, metabarcoding samples of the same species, year, and location were combined and converted to a binary dataset (presence-absence).

Terminology, statistical analysis, plant-pollinator networks and indices

To properly analyze the interactions of apple pollinators with plants, we constructed bipartite networks composed of two node divisions (insect species versus plant/pollen species) connected by a link defined as the interaction between both parties (Dormann et al., 2009).

Species level identification of *Malus domestica* Borkh. based on DNA barcodes is complicated because of the rapid evolution driven by hybridization. Furthermore, the single apple species native to central Europe, *Malus sylvestris* (L.) Mill., is known to hybridize with *Malus domestica*, although it is extremely rare in the environment (Spengler, 2019; Wagner et al., 2014). Therefore, we refer to apple pollen as *Malus* spp. (Rosacea). For each plant-pollinator network of apple per insect order (Brachycera and Hymenoptera) and year (2016 and 2017), we filtered those insect species carrying pollen of *Malus* spp. and defined them as potential apple pollinators. We filtered the potential pollinators per year to analyze the differences in interactions per year. We used the tidyverse package for data wrangling (Wickham et al., 2019).

The bipartite networks and indices were calculated in R (v. 2023.03.1) (R Core Team, 2021) with the function *plotweb* and function *networklevel*, respectively (*bipartite* package; Dormann et al., 2008). To describe the main differences between the networks, we compared the total and average number of links per species, the number of plant and insect nodes, Connectance (C), Nestedness (N), and the level of generalism in the network. C is defined as the number of links in proportion to the overall possible links (Dormann et al., 2009; Dunne et al., 2002). N describes the specialization asymmetry, therefore, the proportion of interactions between specialists (species carrying pollen of one plant taxa) and generalists (species carrying pollen from more than one plant taxa) (Bascompte & Jordano, 2007; Dormann et al., 2008, 2009). Generalism is defined as the mean number of potential pollinator species – that is, the species that carry pollen – per plant species (Bersier et al., 2002). It was calculated by the number of potential pollinators divided by the number of plant species in each network.

Finally, to visualize and test the differences between the interactions of brachyceran and hymenopteran apple pollinators, we performed a Principal Coordinate Analysis (PCoA) and a permutational multivariate analysis of variance (PERMANOVA). We started by analyzing a binary Jaccard dissimilarity matrix of pollen occurrence between years and insect species with the function *vegdist* (R package vegan; Oksanen et al., 2016). The prerequisite of a homogeneous dispersion for a PCoA, as well as the analysis itself, was tested with the *betadisper* function (vegan package). The PERMANOVA was analyzed with the Jaccard dissimilarity matrix as a dependent variable and the different insect orders by years as independent variables using 9.999 permutations (function *adonis* in the R package vegan).

Results

In 2016, of the total 1.366 collected specimens, 91 brachyceran species (of 26 families) were selected to analyze the pollen loads, representing all collected pollen-carrying insect species. In 2017, of the total 1680 specimens collected, 91 brachyceran species (of 26 families) and 76 hymenopteran species (of 14 families) were selected for the analysis of the pollen loads, representing all pollen-carrying insect species in the samples. Altogether, we identified a total of 233 species transporting pollen.

A total of 987 pollen loads (519 from 2016 and 468 from 2017) were pooled into 443 DNA metabarcoding samples: 376 from Hymenoptera and 612 from Brachycera. Five metabarcoding samples were pooled pollen loads from both study areas. However, since we are not considering any spatial differences here, they were used for further analysis as they do not affect the final results. Contrarily, 21 other metabarcoding samples were excluded from further analysis since they were cross-contaminated, as well as all the metabarcoding samples of Hymenoptera from 2016 (84 samples in total), as the high level of cross contamination between several samples led to low confidence in the entire sequencing run. Therefore, they were excluded from further analysis, which ended with 338 metabarcoding samples.

From the remaining 338 DNA metabarcoding samples, 258 plant taxa (of 55 families) were molecularly identified. 159 plant taxa (of 43 families) were identified in the samples from Brachycera 2016 (DNA metabarcoding samples = n = 139), 149 plant species (of 41 families) in the samples of Brachycera 2017 (n = 113), and 123 plant taxa (of 32 families) in the sample of Hymenoptera 2017 (n = 86).

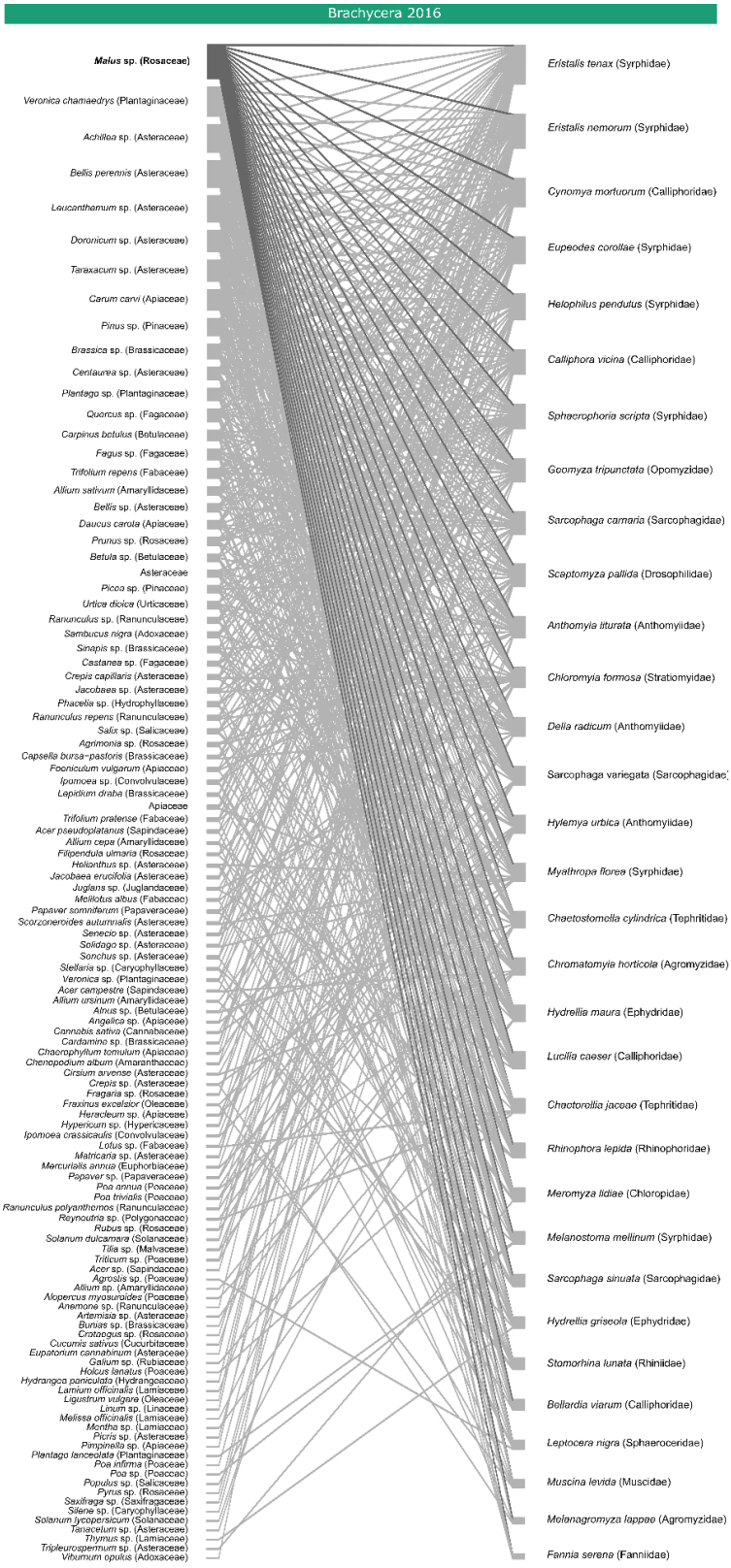

Figure 2: Bipartite network of the potential brachyceran pollinators (right) sampled in 2016 and their respective interaction to plant taxa identified via DNA metabarcoding of pollen loads carried by the insects. The thickness of the node (insect and pollen) correlates with the number of links, and the plant or insect taxa are listed from the taxon with the highest number of links (top) to the taxon with the lowest numbers of links (bottom).

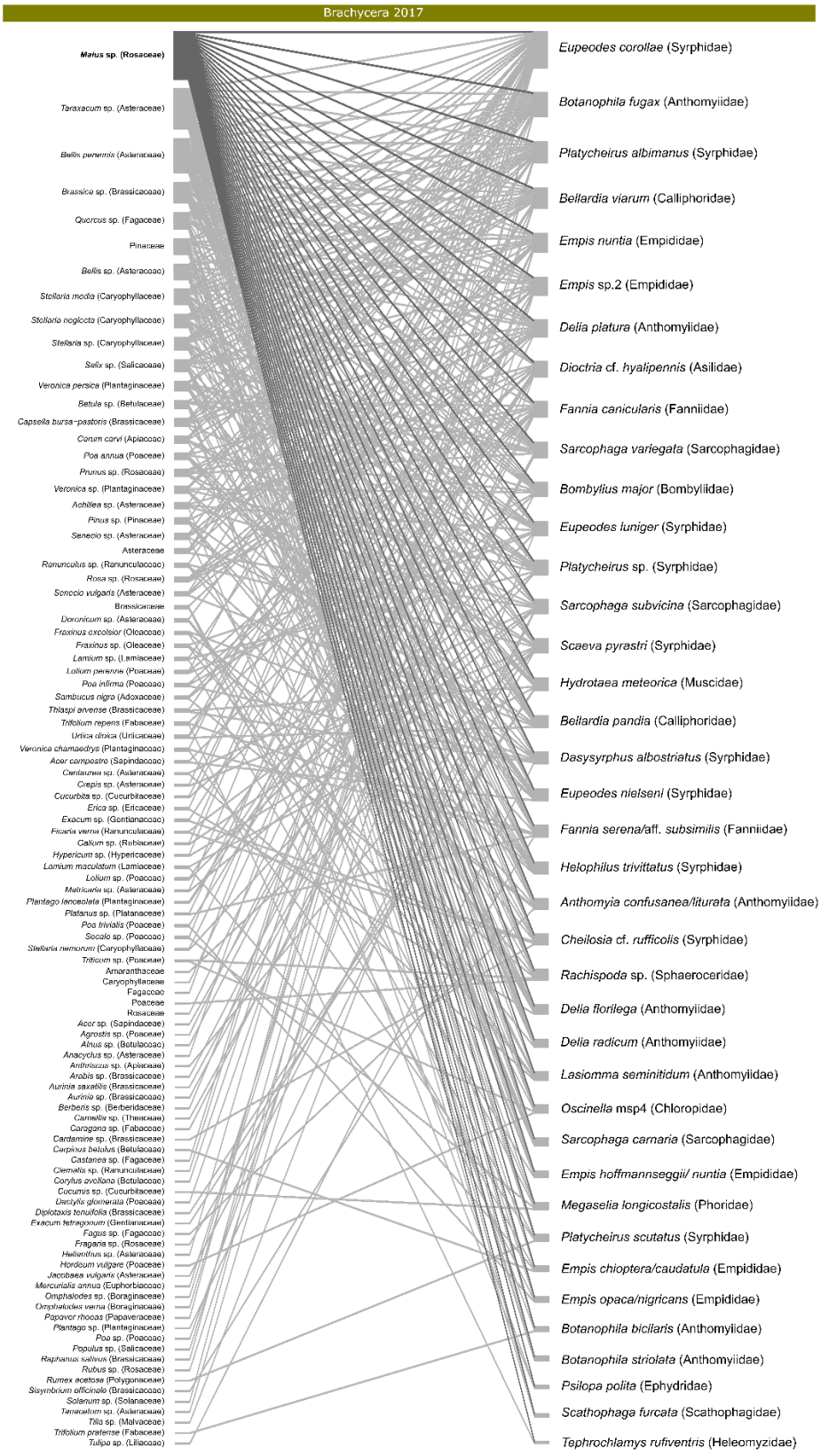

Figure 3: Bipartite network of the potential brachyceran pollinators (right) sampled in 2017 and their respective interaction to plant taxa identified via DNA metabarcoding of pollen loads carried by the insects. The thickness of the node (insect and pollen) correlates with the number of links, and the plant or insect taxa are listed from the taxon with the highest number of links (top) to the taxon with the lowest numbers of links (bottom).

Plant-pollinator networks of apple

Among the 233 insect species transporting pollen, 103 species carried *Malus* spp. pollen and interacted with additionally 194 plant taxa. Of the plant taxa identified via DNA metabarcoding of the pollen loads, only 5 species were present in the flowering strips (*Carum carvi* L., *Daucus carota* L., *Pastinaca sativa* L., *Trifolium pretense* L., and *Veronica chamaedrys* L.). In both of the recorded years, a total of 66 different brachyceran species were identified as potential pollinators of apple: 32 species (of 16 families) in 2016 and 39 species (of 15 families) in 2017. Five brachyceran species, namely *Bellardia viarum* (Robineau-Desvoidy, 1830), *Delia radicum* (Linnaeus, 1758), *Eupeodes corollae* (Fabricius, 1794), *Sarcophaga carnaria* (Linnaeus, 1758), and *Sarcophaga variegata* (Scopoli, 1763), were identified as potential pollinator of apple on both years (Fig. 1 and 2). Within the Hymenoptera, a total of 37 species (of 8 families) were identified as potential pollinators of apple from 2017 samples (table 1) (Fig.3).

From the insect species carrying apple pollen, 25 species (10 Hymenoptera and 15 Brachycera) also carried pollen from *Prunus* sp. (including cherries and plums), and three species, namely *Andrena dorsata* (Kirby, 1802), *Andrena fulva* (Eversmann, 1852), and *Calliphora vicina* (Robineau-Desvoidy, 1830)., also carried pollen of *Pyrus* sp. (pears), together with pollen from apple and *Prunus* sp.

Table 2: Network indices and key information of the plant-pollinator network of apple-Brachycera 2016, apple-Brachycera 2017, and apple-Hymenoptera 2017. Number of plant nodes are the number of plant taxa identified from the pollen loads via DNA metabarcoding, while the number of insect nodes are the number of potential pollinating species. For further information concerning the indeces, see main text section.

	Brachycera 2016	**Brachycera 2017**	**Hymenoptera 2017**
Total number of links	554	397	360
Avg. Links per species	3.82	2.84	2.71
No. plant nodes	113	101	96
No. plant families	35	34	29
No. insect nodes	32	39	37
No. insect families	16	15	8
Connectance	0.15	0.1	0.1
Nestedness	20.74	11.43	9.52
Generalism	0.28	0.39	0.39

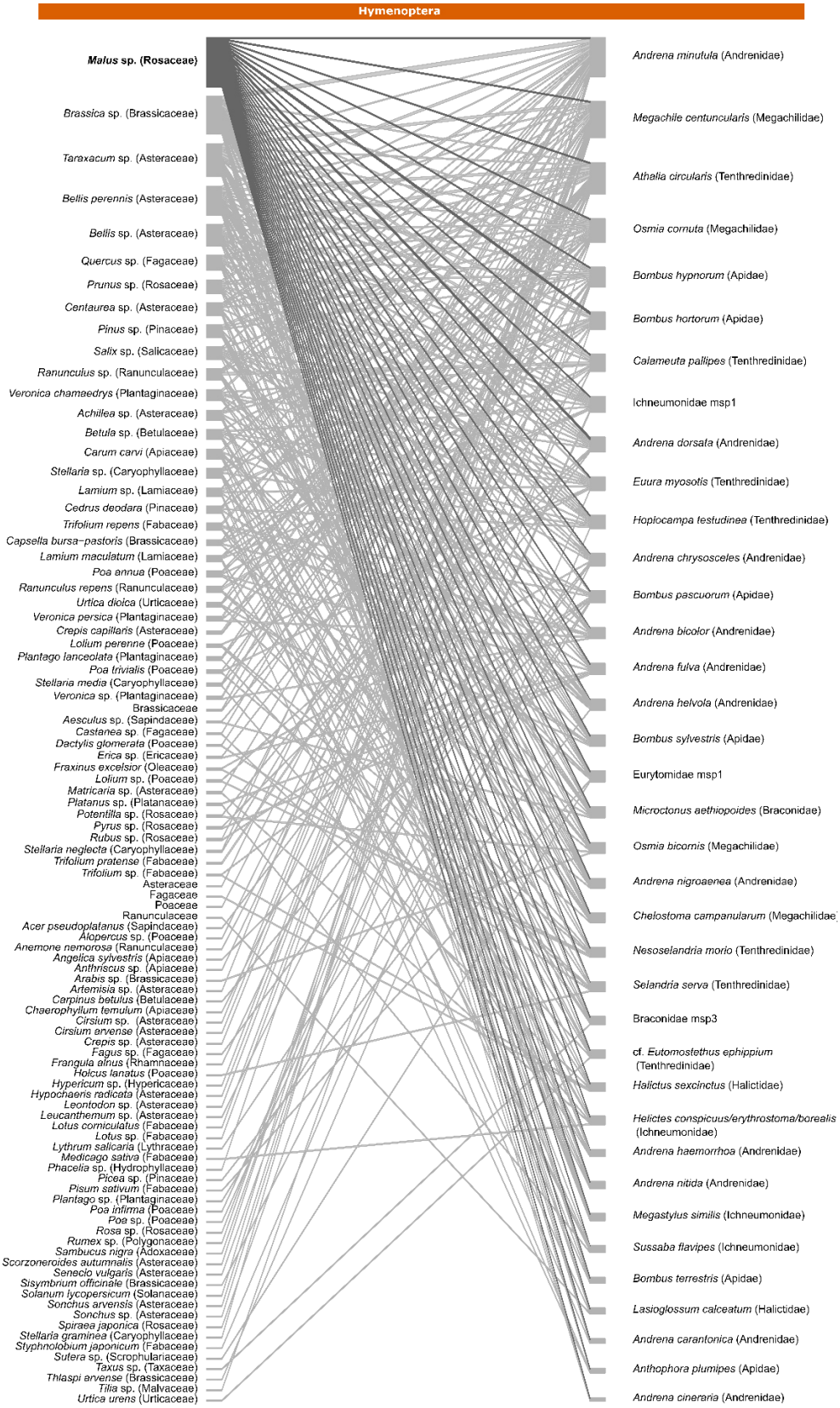

Figure 4: Bipartite network of the potential hymenopteran pollinators (right) sampled in 2017 and their respective interaction to plant taxa identified via DNA metabarcoding of pollen loads carried by the insects. The thickness of the node (insect and pollen) correlates with the number of links, and the plant or insect taxa are listed from the taxon with the highest number of links (top) to the taxon with the lowest numbers of links (bottom).

The plant-pollinator network with the highest level of connectance and nestedness was the network for Brachycera sampled in 2016 (table 1, fig.1). With a connectance of 0.153 and a nestedness of 20.74; it involves a total of 554 links (with an average of 3.82) of 113 plant species (of 35 families) with 32 brachyceran species (of 16 families). The plant-pollinator networks of Brachycera sampled in 2017 had a total of 397 interactions (with an average of 2.84) of 101 plant species (of 34 families) with 39 brachyceran species (of 15 families), with a nestedness of 11.43 as well as the connectance of 0.1 (table 1, fig.2). Finally, the plant-pollinator network of Hymenoptera sampled in 2017 had a total of 360 interactions (with an average of 2.71) of 96 plant species (of 29 families) with 37 hymenopteran species (of 8 families), with a nestedness of 9.52 and a connectance of 0.1 (table 1, fig.3).

The level of pollinator generalism across both Hymenoptera and Brachycera from 2017 was consistent, while it was slightly lower in Brachycera from 2016 (see Table 1). Among the Brachycera species collected in 2016, *Eristalis tenax* (Linnaeus, 1758) had the most interactions within the network, with a total of 37, followed by *Eristalis nemorum* (Linnaeus, 1758) with 32, and *Cynomya mortuorum* (Linnaeus, 1761) with 27. *Fannia serena* (Fallén, 1825) had with only 5 the lowest number of interactions. In the plant-pollinator network of Brachycera collected in 2017, *E. corollae* had the most interactions (30), followed by *Botanophila fugax* (Meigen, 1826) with 20, and *Platycheirus albimanus* (Fabricius, 1781) with

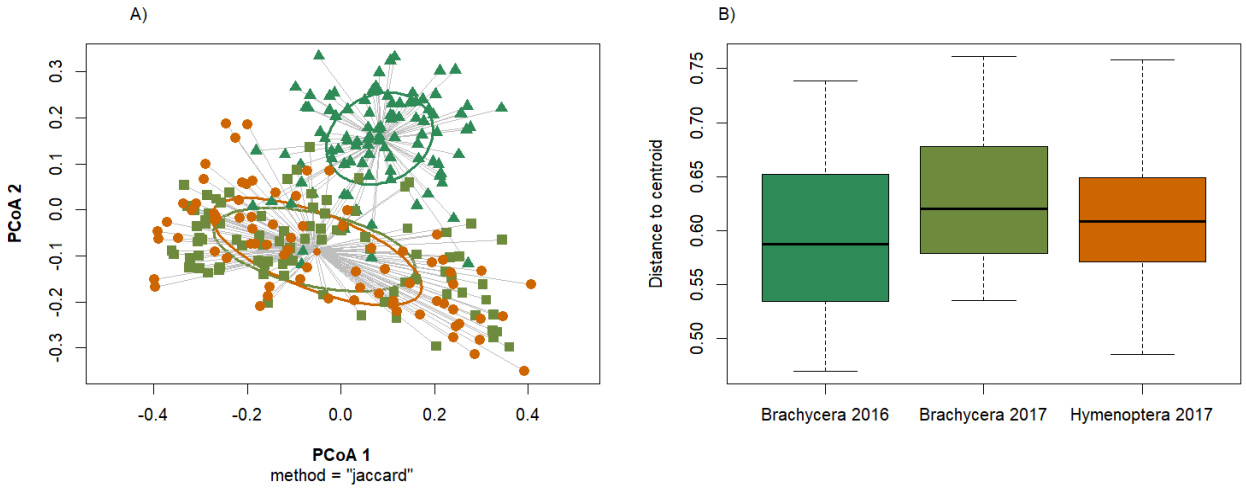

Figure 4: (A) Principal Coordinate Analysis (PCoA) based on a Jaccard dissimilarity matrix of the differences in insect-plant interactions (based on the pollen community) for brachyceran apple pollinators collected in 2016 (n = 32 species) and 2017 (n = 37 species), as well as hymenopteran apple pollinators collected in 2017 (n=35 species). (B) The group dispersion plot indicates homogeneity of pollen community composition.

18. *Tephrochlamys rufiventris* (Meigen, 1830) had with just 2 the lowest number of interactions. Within the plant-pollinator network of Hymenoptera specimens collected in 2017, *Megachile centuncularis* (Linnaeus, 1758) had the most interactions (25), followed by *Andrena minutula* (Kirby, 1802) with 24, and *Athalia circularis* (Klug, 1815) with 22. The species with the lowest number of interactions was *Andrena cineraria* (Linnaeus, 1758), with only three recorded interactions.

The PCoA indicated a higher overlap between the composition of pollen loads (and therefore the plant-pollinator interactions) between Brachycera collected in 2017 with Hymenoptera collected in 2017, rather than with Brachycera collected in 2016 (Fig. 4A). Among the plant species with the highest number of interactions within the brachyceran network of 2016 (excluding *Malus* sp.) was *Veronica chamaedrys* (Plantaginaceae) with 27, followed by *Achillea* sp. (Asteraceae) with 26, and *Bellis perennis* and *Leucanthemum* spp. (both Asteraceae) with 25. Moreover, 32 plant taxa have a single link within this network. In the brachyceran network of 2017, apart from apple pollen, *Taraxacum* spp. (Asteraceae) showed the most interactions with 31, followed by *Bellis perennis* (Asteraceae) with 26, and *Brassica* spp. (Brassicaceae) with 17. In this network, 48 plant taxa were connected only once. Within the Hymenoptera network of 2017, aside from *Malus* spp. and similarly to the brachyceran network of 2016, the most interconnected plant species was *Brassica* spp. with 25 interactions, followed by *Taraxacum* spp. with 22, and *B. perennis* with 21. A total of 51 plant taxa in this network had a single interaction. Despite visual similarities in the PCoA, the pollen composition of Brachycera collected on both years and Hymenoptera is significantly different (PERMANOVA, $F = 7.7239$, $p < 0.001$). Also, the betadisper-test indicated a heterogeneous dispersion of the samples of each dataset ($F = 7.5621$, $p < 0.001$) (Fig. 4B). This suggested that the differences between the groups were due to the distance of the samples to their centroid in the PCoA.

Discussion

This study provides evidence of the high diversity of potential pollinators of apple, with 66 species of Brachycera and 35 species of Hymenoptera. Recorded potential pollinators of the plant-pollinator network of apple interacted with 194 other plant taxa, many of them being other orchard or crop species. Although we did not consider pollinator effectiveness in this study, previous studies on this topic have already shown that a higher richness of pollinating taxa can improve apple pollination (Blitzer et al., 2016; Mallinger & Gratton, 2015). The high diversity of potential pollinators of apple and particularly the high number of Brachycera is consistent with the findings of Gamonal Gomez et al. (2023) and Barahona-Segovia et al.

(2023), who implemented different methodologies. In the first work, environmental DNA metabarcoding was used to detect the presence of the insect on apple flowers, and in the second work, insect visits on apple flowers were recorded. Our results mirror those of these two other studies as they reveal that more species of flies than Hymenoptera (also considering wildbees) are significant flower-visitors of apple orchards. Additionally, our survey aligns with previous studies on apple orchards, highlighting the vital contribution of wild pollinators (Rosa García & Miñarro, 2014). Our study provides additional evidence for flies transporting pollen and, therefore, are not just flower-visitors but also potential apple pollinators.

Despite the importance of Brachycera for the pollination of apple confirmed here, there are just a handful of studies acknowledging it, the recent most important ones are mentioned above. This pattern of undermining or ignoring the potential of Brachycera, particularly the importance of non-syrphid pollinators for many crop species, has just been addressed in this century (Orford et al., 2015; Ssymank et al., 2008). Many families of brachycera, including Muscidae and Scatophagidae, have bristles that can trap pollen, similar to some bee species (Skevington & Dang, 2002). In this study, the Brachycera species with the highest number of interactions belonged to Syrphidae, highlighting here, among others, *Eristalis tenax*. Syrphidae are among the most studied pollinators, many species being as efficient as wildbees (Hodgkiss et al., 2018; Orford et al., 2015). Syrphidae are not just essential pollinators, but many species start as predatory larvae and are, therefore, particularly interesting in integrated pest management (Dunn et al., 2020). *Eristalis tenax* is a cosmopolitan bee-mimicry syrphid species that has already been reported as an important pollinator of many crop species (Howlett & Gee, 2019). Moreover, *Delia radicum* (Anthomyiidae) feeds on nectar and, therefore, can be found in the plant-pollinator network. Nonetheless, this species is also known as a pest for cruciferous crops (Nilsson et al., 2011). This also shows the dual roles of many species in agroecosystems, similar to *Athalia rosae* (Tenthredinidae) for caraway (Kilian et al., 2023).

Within Hymenoptera, we had a higher number of wildbee species, especially Andrenidae. Andrenidae is not just among the wildbee families which carry the highest number of pollen grains in apple orchards and, therefore, are considered an essential pollinator of *Malus domestica* (Boyle & Philogène, 1983; Campbell et al., 2017) but are also among the species with the highest number of interactions in the networks. Furthermore, *Andrena* species are also commonly known to be more resilient towards lower temperatures (Herrera et al., 2023), a likely condition for orchards with an early flowering phenology. Despite the high numbers of wildbees in the network, we have also reported some unique insect groups as

potential pollinators of apple, which are usually not being recognized as potential pollinators, likely due to their natural history. For instance, parasitic wasps of the families Eurytomidae, Braconidae, and Ichenumonidae are in the network. Members of these families, besides being potential pollinators, parasitize a wide range of pest species, in apple orchards species like moths, aphids, or leaf midges (J. V. Cross et al., 1999; Dib et al., 2012; Fernández-Triana et al., 2009; Mates et al., 2012).

Of the 30 plant taxa in the flowering strip, five species were also present in the plant-pollinator network. Although it suggests that the pollinator did not visited the flowering strips, it is more likely 1.) due to the asynchrony between the flowering of the flowering strip and apple blooming, 2.) due to perennial flowering strip aging and potential plant community changes (De Cauwer et al., 2005) or 3.) the surrounding wild flowering plants were more attractive to potential pollinators (Kowalska et al., 2023). Aside from the few plant taxa present in the original flowering strips and also being present in the networks, we remark the presence in the networks of dandelion (*Taraxacum* spp.), *Bellis perennis,* and other early blooming wild plant species, which are also pervasive in agroecosystems. While particularly *Taraxacum* spp. is known as a weed plant in apple orchards (Lisek & Sas-Paszt, 2015; Mia et al., 2021), it can provide wild pollinators with nectar and pollen during early spring when apple blossoming has not started yet (Rosa García & Miñarro, 2014). Therefore, we highlight the potential of these species to attract potential pollinators of apple flowers and complement established flowering strips (Campbell et al., 2017) or be potential plant candidates for flowering strips, as in the case of *Bellis perennis* (Pfiffner et al., 2019). These flowering strips, in addition to other landscape structures, e.g., hedges, are also essential after the flowering period of the apple orchards (Mupepele et al., 2023) or any other early-flowering plants (Campbell et al., 2017), alongside the landscape`s influences including the surrounding management types (Barahona-Segovia et al., 2023).

This is particularly important in the context of phenological asynchrony between apple crops and pollinators, caused predominantly by climate change (Wyver et al., 2023). This mismatch does not only affect the fitness of specialized pollinators (Kőrösi et al., 2018) but also a heavy reliance on managed pollinators for farmers (Wyver et al., 2023), which cannot wholly compensate for wild pollination (Blitzer et al., 2016; Rader et al., 2016). The phenological synchrony and, therefore, pollination function can be ensured with high levels of pollinator diversity (Bartomeus et al., 2013). Here, similarly to bumblebees, Brachycera pollinators could help to fill in the gap as they are overall more tolerant for lower temperatures, as illustrated by their abundance and importance as pollinators in arctic and alpine regions (Boyle & Philogène, 1983; Doré et al., 2021; Howlett, 2012; Lefebvre et al., 2018; Orford et al.,

2015; Tiusanen et al., 2016). Additionally, Brachycera are more resilient to land-use changes in comparison to bees (Rader et al., 2016; Ricketts et al., 2008) and can transport pollen over larger distances as they are not reliant on nesting sites (Larson et al., 2001; Rader et al., 2011). Finally, despite hymenopteran apple pollinators having the ability to transport higher quantities of pollen at a species level, they do not differ significantly in the efficiency compared with Brachycera species (Boyle & Philogène, 1983) since the efficiency is not necessarily linked to any morphological trait (Roquer-Beni et al., 2022).

The plant-pollinator networks presented here have a similar structure to other networks assessed by metabarcoding of pollen samples (Arstingstall et al., 2021; Kilian et al., 2023). Although the Brachycera and Hymenoptera networks of 2017 had different nodes, the overall structure was more similar between them than between the interannual Brachycera networks. This may indicate that the effect of plant phenology and interannual differences in plant abundance is here not only one of the main drivers of the structure of the plant-pollinator networks but could also attract and enhance the overall pollinator diversity (Nicholls & Altieri, 2013).

The high level of complexity in the network is also associated with the higher taxonomic resolution resulting from DNA metabarcoding of the pollen samples compared with the affordable identification level using traditional methods such as palynology. Even though it is possible to identify a more significant number of interactions with DNA metabarcoding of pollen samples (Kilian et al., 2023; Pornon et al., 2017) and it provides a more detailed diet breadth (e.g., polylecty vs. oligolecty) than observational data (Arstingstall et al., 2021), this methodology still has some inherent limitations when analyzing pollen samples. Notably, the selection of barcode markers and primers (Kolter & Gemeinholzer, 2021a) and the quality of reference databases (Kolter & Gemeinholzer, 2021b) can have their own taxonomic detection biases. However, we believe that the choice of ITS2 as a DNA barcode and the primers used are the optimal choices for this study system. Additionally, the analysis of the abundance of different taxa in pollen mixtures and, therefore, the strength of interactions in the network are among the most limiting factors, even though there is a strong correlation between the amount of pollen and the number of sequences for some plant taxa (Baksay et al., 2022).

Nonetheless, advances in DNA metabarcoding techniques have opened new perspectives in the analysis of potential pollinators of apple. Current developments in the analysis of eDNA on flowers have improved the assessments of potential flower-visitors of apple, improving the detection of nocturnal pollinators (Gamonal Gomez et al., 2023), for instance. The use of metabarcoding for pollen samples combined with eDNA from the flowers could provide an interesting insight into the dynamics between the plant and the potential pollinators, since it

describes the pollen transport and interaction networks tentatively. This could provide a more detail inside into the dynamics of plant-insect interactions of the potential pollinators of apple and improve current agri-environmental schemes to protect and improve pollination services.

Conclusion

Our results shed light on the diversity of potential pollinators of apple, including many non-syrphid Brachycera and some non-wildbee Hymenoptera. While previous studies showing the diversity of potential pollinators of apple were primarily based on interaction surveys of observations, we showed here the actual diversity of potential pollinators of apple by analyzing the pollen loads with DNA metabarcoding. The potential pollinators included many brachyceran species with potential dual roles as pollinator and pest control species or also as pollinator and pest species. Additionally, the potential pollinators of apple were nested in a complex network of interactions with other plant species, including early-flowering orchards and wild plants. The plant-pollinator interactions differed more between the years than between Brachycera and Hymenoptera, suggesting the importance of some generalist wild plant species, which are also the backbone of the plant-pollinator networks. This study proves the importance of including all Brachycera and Hymenoptera species in future assessments of apple pollination. Otherwise, getting an accurate representation of the complex network will not be possible. This is essential to get a better picture of how future environmental factors could affect the pollination service, for apple orchards particularly a plant-pollinator asynchrony caused by climate change, and to understand the impact and improve current measures to enhance biodiversity in apple production worldwide.

Supplementary information

Table S1: List of plant species present in the flowering strips.

Plant species	
Achillea millefolium agg.	*Origanum vulgare* L.
Campanula patula L.	*Pastinaca sativa* L.
Campanula rapunculus L.	*Picris hieracioides* L.
Carum carvi L.	*Pimpinella* major (L.) Huds.
Centaurea jacea L.	*Primula veris* L.
Daucus carota L.	*Prunella vulgaris* L.
Galium album MILL.	*Salvia pratensis* L.
Galium verum L.	*Sanguisorba minor* SCOP.
Geranium pratense L.	*Scabiosa columbaria* L.
Knautia arvensis (L.) Coult.	*Thymus pulegioides* L.
Leontodon hispidus L.	*Tragopodon pratensis* L.
Leucanthemum sp. MILL.	*Trifolium pretense* L.
Lotus corniculatus agg.	*Trifolium dubium* Sibth.
Malva moschata L.	*Veronica chamaedrys* L.
Onobrychis viciifolia SCOP.	*Vicia cracca* L.

Table S2: Sampling dates before, during and after the flowering period of apple with their corresponding phenological development stage based on the BBCH-scale of apple orchards (U. Meier et al., 1994).

year	Sampling date	BBCH - scale
2016	10.03.2016	50
	17.03.2016	52
	02.04.2016	53
	03.04.2016	53
	04.04.2016	53
	12.04.2016	54
	20.04.2016	56 - 57
	02.05.2016	59
	06.05.2016	61-64
	07.05.2016	62-63
	08.05.2016	64
	09.05.2016	65
	19.05.2016	67
	31.05.2016	70
	09.06.2016	71
	05.07.2016	72
	09.07.2016	72-73
	19.07.2016	74
	25.08.2016	>70
	31.08.2016	>70
	08.09.2016	>70
2017	09.04.2017	31-59
	14.04.2017	31-61
	20.04.2017	53-63
	21.04.2017	54-61
	24.04.2017	54-65
	26.04.2017	54-65
	27.04.2017	54-65
	29.04.2017	54-65
	09.05.2017	57-69
	17.05.2017	61-71

	01.06.2017	71-72
	16.06.2017	73-74
	30.06.2017	72-75
	19.07.2017	75-79
	02.08.2017	79-85
	10.09.2017	87

Table S3: List of literature references used for the identification of Hymenoptera and Brachycera.

Order	**Targeted taxa**	**References**
Brachycera	Family overview	(Oosterbroek, 2006; Stresemann & Klausnitzer, 2011)
	Stratiomyidae	(Reemer, 2014; Rozkošný, 2000)
	Syrphidae	(Bartsch & Binkiewicz, 2009a, 2009b; Haarto & Ståhls, 2014; Van Veen, 2010)
	Tachinidae	(Tschorsnig, 1994; Van Emden, 1954; T. W. P. Zeegers, 1992)
	Tephritidae	(White, 1988)
	Other families	(Stresemann & Klausnitzer, 2011)
Hymenoptera	Family overview	(Goulet et al., 1993)
	"wild bees"	(Amiet et al., 1999, 2001, 2012; Bellmann, 1995; Gokcezade et al., 2010; Müller et al., 1997; Scheuchl & Schmid-Egger, 2000; Schmid-Egger & Scheuchl, 1997)
	Pompilidae	(Wolf, 1992)
	Sphecidae	(Bitsch, 1992; Schmidt, 2000)
	Tenthredinidae	(D. R. Smith, 1971, 1979)

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Chapter 4.

Maximizing metabarcoding precision to enhance species diversity analysis of Hymenoptera and Brachycera with a non-destructive DNA metabarcoding approach

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Kilian, I. C., Kirse, A., Peters, R. S., Bourlat, S. J., Fonseca V. G., Wägele W. J., Hamm, A. & Mengual, X. (*under review*). Maximizing metabarcoding precision: ASV clustered to OTUs and LULU filtering for enhanced species diversity analysis of bees, wasps (Hymenoptera), and flies (Diptera: Brachycera) with a non-destructive DNA metabarcoding approach. *Ecology and Evolution*.

4.1. Summary

Monitoring of insect diversity is crucial and necessary in the face of a global decline to address and understand and mitigate potential drivers (Hallmann et al., 2017; Van Klink et al., 2020). Reliable and standardized methods allow the collection of valuable information on insect community compositions and dynamics for these purposes. Malaise trap is a well-established method to assess flying arthropods, particularly Diptera and Hymenoptera (Hallmann et al., 2017; Matthews & Matthews, 1971; Skvarla et al., 2021). However, sorting and morphological identifying specimens and species-rich bulk samples can be also challenging and time-consuming (Chimeno et al., 2022; Piper et al., 2019; Souza et al., 2016). DNA metabarcoding can overcome some of these challenges by avoiding the individual specimen processing (as in barcoding) and has become, therefore, a widely method to analyze this type of samples (deWaard et al., 2019; Huang et al., 2022; Wägele et al., 2022). In particular, non-destructive approaches can preserve the sample as a morphological voucher for further analysis, if necessary (Hausmann, Segerer, et al., 2020). Sequences are usually clustered into *Operative Taxonomic Units* (OTUs), which are a cluster of sequences with a fixed similarity threshold (Kopylova et al., 2016; Westcott & Schloss, 2015), or *Amplicon Sequence Variants* (ASVs), which are OTUs with zero genetic distances to infer putative species from metabarcoding data (Porter & Hajibabaei, 2018). In order to mitigate the possible occurrence of erroneous clusters in an analysis, multiple post-clustering algorithms have been developed (Olesen et al., 2017; Palmer et al., 2018). The LULU algorithm has the advantage of being independent of reference databases and of integrating read abundance as a premise (Frøslev et al., 2017). To what extent samples examined with metabarcoding are comparable to morphologically identified samples has been investigated relatively little so far, particularly in the realm of insects (Kirse et al., 2023; Mata et al., 2021; Remmel et al., 2024; Zenker et al., 2016). Moreover, previous studies comparing metabarcoding with morphological identification

have often been based on mock communities to facilitate the comparison, which does not usually correspond to the diversity's complexity of an actual sample (Marquina et al., 2019).

In Chapter 4, using exemplarily adult Brachycera (and particularly Syrphidae) and Hymenoptera collected with Malaise traps on spinach fields, we compared morphological identified specimens with a non-destructive metabarcoding approach coupled with four different clustering and filtering approaches: (1) ASVs clustered in OTUs at 97% cutoff and LULU-filtered using default settings at 84% minimum match, (2) or using at 96% minimum match, (3) ASVs directly LULU-filtered using the default settings at 84% minimum match, (4) or at 96% minimum match.

At least for Brachycera, ASVs clustered into OTUs followed by LULU using a 96% minimum match (OTU96) was the best combination to get the closest result to morphological identification in terms of species number. However, we also found many false positives, where species were detected using DNA metabarcoding but not morphologically, and false negatives, where morphologically identified species were not detected using DNA metabarcoding. Using Syrphidae as an exemplarily family of a well-studied Brachycera family, we found an overlap between 9 and 81%, depending on the approach. This is somewhat surprising since the morphological identification of syrphid species is not a serious challenge. The species present in the samples are also among the most common ones in Central-European agroecosystems and should, therefore, be well-represented in the reference databases (Bartsch, 2009b, 2009a; Bot & Van de Meutter, 2023; Van Veen, 2010). Syrphid false negatives may not have been detected due to the low number of specimens present in the sample (also known as biomass bias; Strutzenberger et al., 2024) or shared COI haplotypes (Dietz et al., 2023; Haarto & Ståhls, 2014; Locke & Skevington, 2013; A. D. Young et al., 2016), it is unclear how false positives could have originated. Since with DNA metabarcoding it is also possible to capture environmental DNA, it cannot be ruled out that a potential source for false positives might have been cross-contamination with other DNA traces in the sample, e.g., the gut content of predatory insect (Blösch, 2000; Gilbert, 2005; Kirse et al., 2023; Pickard, 1975; Reeves et al., 2018). Moreover, while DNA mini-barcodes (313 bp instead of 658 bp long) are commonly used in metabarcoding, they might not provide enough genetic information for precise species identification. For Hymenoptera, while OTU96 detected the highest number of species, it was still considerably lower than the one reached via morphological identification. While there are probably several reasons for the absence of so many Hymenopteran species, the most probable ones are the failure of the universal primer to amplify Hymenoptera, also known as primer bias (Brandon-Mong et al., 2015; Elbrecht et al., 2019; Yu et al., 2012) and the different levels of sclerotization of the specimens

within this order (Erdozain et al., 2019; Kirse et al., 2023; Marquina et al., 2019; Zizka et al., 2018).

Despite these limitations, we argued that metabarcoding is a powerful and effective method for insect monitoring. We showed possible pathways to enhance metabarcoding results by adapting and modifying the bioinformatic pipeline. This vital step in the analysis is often neglected since it requires a certain level of bioinformatic expertise, which potential end-users do not always have or is still not part of the service repertoire of metabarcoding service providers (Liu et al., 2019). Nonetheless, it is essential to understand the current limitations and explore options to overcome them, mainly when aiming for the long-term utilization of metabarcoding for bioassessments or when cross-validation with mock communities or morphological identifications is unfeasible.

Personal contribution

The study was conceptualized by Ximo Mengual, Ralph Peters, and Vera Fonseca. Vera Fonseca took care of acquiring funds to do the genetic analysis. I set up the Malaise traps with the help of a student and a technician. I emptied the samples and sorted them. I counted and morphologically identified all the specimens, with some support from Ximo Mengual for Syrphidae and Ralph Peters for parasitoid wasps. Ameli Kirse and Hendrik Giebner did the laboratory work on metabarcoding. Ameli Kirse bioinformatically curated the raw data and did the cluster and filtering analysis. I combined and curated the datasets, analyzed the data, prepared the figures using R® scripts written by myself, as well as with BioRender®. Some figures were refined in Inkscape®. I interpreted all results, and Ximo Mengual, Ralph Peters, and Ameli Kirse helped validating them. I wrote the first draft of the manuscript, which all co-authors helped to review and edit.

Maximizing metabarcoding precision: ASV clustered to OTUs and LULU filtering for enhanced species diversity analysis of bees, wasps (Hymenoptera), and flies (Diptera: Brachycera) with a non-destructive DNA metabarcoding approach

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Abstract

In recent years, DNA metabarcoding has been used for a more efficient assessment of bulk samples. However, there remains a paucity of studies examining potential disparities in species identification methodologies. Here, we explore the outcomes of diverse clustering and filtering techniques on data from a non-destructive metabarcoding approach, compared to species-level morphological identification of Brachycera (Diptera) and Hymenoptera. The study evaluated four distinct approaches, namely clustering to ASVs or ASVs clustered to OTUs coupled with subsequent filtering using the LULU algorithm at 84% and 96% minimum match. Depending on the selected approach, DNA metabarcoding results strongly varied in terms of detected molecular units blasted to brachyceran and hymenopteran species. Using Syrphidae as an exemplary family, we found an overlap ranging from 9% - 81% between the morphological identification and the different clustering and filtering approaches. For Brachycera, ASVs clustered into OTUs followed by LULU using a 96% minimum match (OTU96) inferred the number of molecular units closest to the number of morphologically identified species. For Hymenoptera, while OTU96 also yielded the highest number of

molecular units, it was still considerably low compared to the number of morphologically identified species. Our results show that metabarcoding methodology needs to be significantly improved to be applied to Hymenoptera. Conversely, for Brachycera, we acknowledge the promise of employing a non-destructive metabarcoding approach, incorporating ASV clustering into OTUs and filtering with LULU, to derive dependable species lists. Such lists hold significant potential for applications in biomonitoring, conservation efforts, and other related fields.

Introduction

In view of a worldwide insect decline (Van Klink et al., 2020), large-scale biomonitoring initiatives on the basis of standardized protocols are more important than ever. Malaise trapping is a well-established method to collect flying insects, and they have been extensively utilized in various local (M. Geiger et al., 2016; Hallmann et al., 2017) and global biodiversity assessment initiates (e.g. Global Malaise Program; https://biodiversitygenomics.net/projects/gmp/). Malaise traps are non-attractant, static interception traps, which consist essentially of an open-fronted tent with a trapping device attached to the inner highest corner of the tent (Henderson & Southwood, 2016; Muirhead-Thompson, 1991; Townes, 1962). Diptera and Hymenoptera are usually the most specimen and species-rich insect taxa found in Malaise trap catches (Matthews & Matthews, 1971; Skvarla et al., 2021). Despite their significance, many contemporary studies utilizing Malaise trap samples often lack detailed species-level information (Hallmann et al., 2017).

DNA metabarcoding is frequently utilized for assessing arthropod diversity from bulk samples (deWaard et al., 2019; Huang et al., 2022; Wägele et al., 2022). It is capable of identifying thousands of specimens in parallel by analyzing with high-throughput sequencing (HTS) (Taberlet et al., 2012). A common practice in DNA metabarcoding to yield high DNA quantities involves homogenizing the entire sample (Beng et al., 2016; Gibson et al., 2015). However, by homogenizing the sample, undetected and rare species are irreversibly destroyed, thereby hindering a subsequent morphological identification (Carew et al., 2018; Kirse et al., 2023). Our knowledge of flying insect diversity in Central Europe and beyond, particularly in Diptera and Hymenoptera, remains limited, highlighting the importance of preserving morphological vouchers (see, e.g., Hausmann et al., 2020). Therefore, new developments in DNA extraction protocols for metabarcoding are shifting towards non-destructive extraction methods, such as DNA extraction directly from incubated lysis buffers (Batovska et al., 2021; Carew et al., 2018; Kirse et al., 2021a, 2023; Morinière et al., 2016;

Zizka et al., 2018) as well as from preservative ethanol (Kirse et al., 2023; Zenker et al., 2020). To infer putative species from metabarcoding raw data of Malaise trap samples (or other bulk samples), sequences can be either clustered into Operational Taxonomic Units (OTUs, also known as Molecular Operational Units or MOTUs) or into Amplicon Sequence Variants (ASVs). OTUs are clustered sequences based on a fixed similarity threshold (Kopylova et al., 2016; Westcott & Schloss, 2015), while ASVs are zero radius OTUs, encompassing only sequences that exhibit zero genetic distance from any other sequence in the dataset (Porter & Hajibabaei, 2018). Although different terminologies may be used in practice for the Amplicon Sequence Variants, such as ASV, zero radius OTUs (zOTU), and ESV (Exact Sequence Variant), they essentially refer to similar methods (Antich et al., 2021; Nearing et al., 2018). For simplicity, we will use the term ASV hereafter.

OTUs come with two major limitations. Firstly, while OTUs are often used as a proxy for species (Porter & Hajibabaei, 2018), if closely related species exhibit only limited variation in the barcode region and the clustering threshold is not appropriately chosen, it can artificially reduce the number of species detected. Secondly, OTUs are only valid within the dataset they have been created in, meaning comparison across datasets is only feasible when the data is being combined and reanalyzed. In contrast, ASV tables can be compared across datasets, because the ASV approach clusters sequences without a threshold and infers groups already based on a single nucleotide difference (Callahan et al., 2017). Several studies have demonstrated that ASVs often represent the true ecological situation and diversity patterns as well or even more accurately than OTUs (Callahan et al., 2016; Joos et al., 2020; Porter & Hajibabaei, 2018, 2020). However, taxa exhibiting high levels of intraspecific variation are prone to be represented by multiple ASVs, thereby artificially inflating the number of detected putative species (Callahan et al., 2017). In addition, both OTUs and ASVs can generate artificial clusters due to sequencing errors, leading to discrepancies in the number of actual species present in the sample (Koeppel & Wu, 2013; Schloss & Westcott, 2011). To mitigate the occurrence of erroneous molecular units, algorithms have been developed for post-clustering curation of resulting OTU- and ASV-tables such as AMPtk (Palmer et al., 2018), dbOTU3 (Olesen et al., 2017), or LULU (Frøslev et al., 2017). LULU assesses the pattern of sequences present in lower counts, often arising from sequencing or PCR artifacts, to curate the list and filter out misleading ASVs or OTUs. A major advantage of LULU is its independence from a reference database, as it integrates read abundance with the degree of minimum match (sequence similarity). Minimum_match is one of the user-selected parameters representing the minimum threshold difference between sequences from the cluster for considering any OTU as an error. After analyzing the initial OTU or ASV table with the LULU algorithm, a new

OTU table is constructed. Some studies have already shown that abundance filtering alone may lead to over-filtering, resulting in an underestimation of overall OTU diversity, since OTUs with low read counts could be erroneously filtered out (Callahan et al., 2016; Frøslev et al., 2017).

Comparative studies between species identified through clustering DNA metabarcoding data and those identified morphologically are still relatively scarce (Beentjes et al., 2019; Huo et al., 2020; Topstad et al., 2021), particularly within the realm of insects (Kirse et al., 2023; Mata et al., 2021; Remmel et al., 2024; Zenker et al., 2016). Moreover, such comparisons often rely on mock communities to facilitate analysis. While mock communities serve as a robust tool for systematically comparing different methodologies (Iwaszkiewicz-Eggebrecht et al., 2023; Nielsen et al., 2019), studies involving bulk samples (e.g., Malaise trap samples) may yield different outcomes due to the high complexity of samples (Marquina et al., 2019). To our knowledge, no study has yet evaluated the outcomes of different clustering and filtering approaches based on ASVs or OTUs, and filtered with LULU at different minimum_match settings, against the results from morphological identification of species across diverse flying insect taxa. In this study, we compared the overlap between species identification with a non-destructive DNA metabarcoding approach coupled with four different clustering and filtering approaches with the morphological identification of adult Brachycera and Hymenoptera from bulk samples collected with Malaise traps.

Material and Methods

Study area and sample collection

Study sites were located in the area of Borken, north-western Germany (51.807765 N/6.832369 E in 2016; 51.810295 N/6.830871 E in 2017). The area is dominated by agricultural fields with maize, spinach, and other non-flowering plants (Meyhöfer et al., 2008) (Fig. 1A). For the collection of the

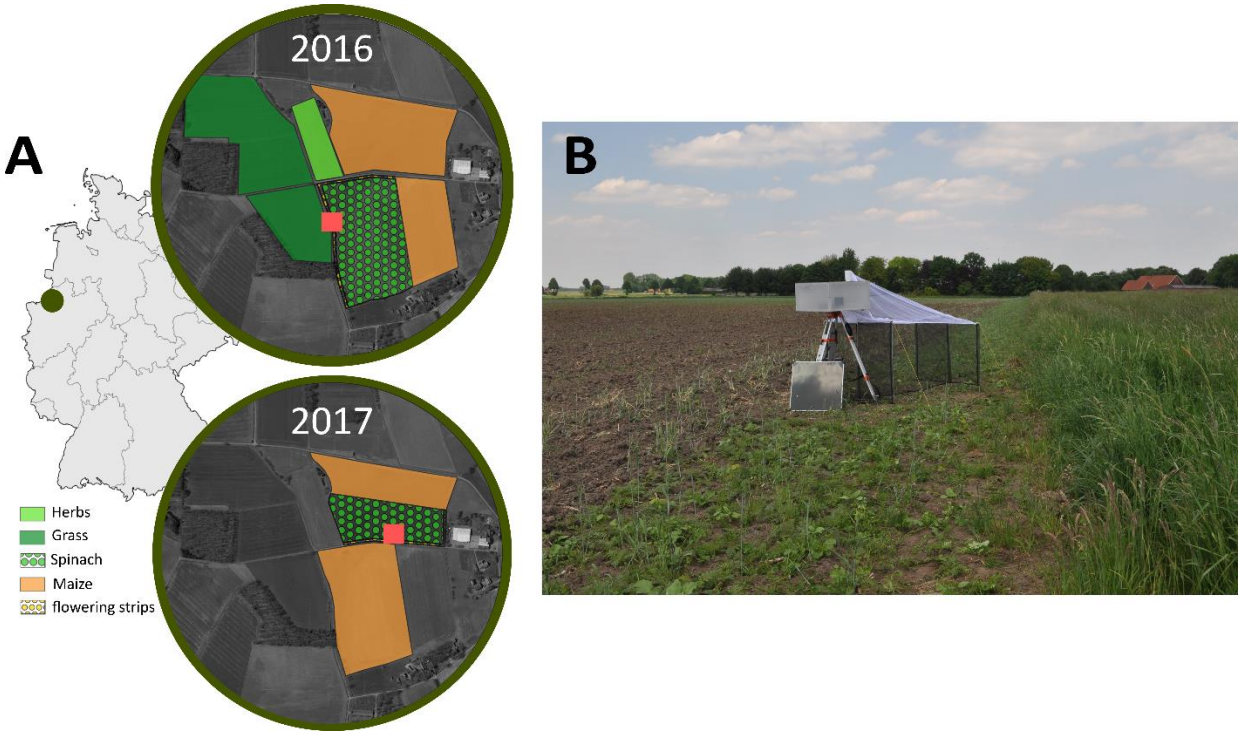

Figure 1: (A) Map with the location of the (B) Multisampler attached to a Malaise trap (in red) located in 2016 and 2017 in spinach fields with flowering strips.

Brachycera and Hymenoptera specimens, an automated multi-sampler unit attached to a commercial Townes-style Malaise trap (Kirse et al., 2024; Wägele et al., 2022) was set up on spinach fields with flowering strips in 2016 and 2017 (Fig. 1B). Spinach (*Spinacia oleracea* L.) is the most important field-grown vegetable in the area of Borken, with an annual harvest of around 34,000 tons. This constitutes approximately half of the total harvested in Germany, with the majority being processed into frozen food (FAOSTAT, 2023; Frerichs & Daum, 2021). Since spinach is usually harvested before the flowering period, and thus not a primary attractant for flowering-visiting insects, there is a local interest in increasing biodiversity in spinach-dominated areas through the implementation of flower strips. Malaise traps were positioned directly at the border between the spinach field and the flowering strips with 1000 ml collection bottles filled with 96% ethanol. Following retrieval from the field, the samples were kept in 96% ethanol and stored at room temperature. The study presented here is based on two bulk samples, collected during two periods: from August 24th to 31st 2016 and from July 4th to 11th 2017, respectively.

Morphological identification

Adult specimens assigned to Hymenoptera and Brachycera from both bulk samples were counted and identified to the species or morphospecies level based on morphological

characters (Table S1). The highly diverse superfamily Ichneumonoidea (Hymenoptera) were excluded from the analysis due to a lack of specific expertise within our team, and the identification to morphospecies based solely on external features can be inadequate (Horstmann, 2002; Veijalainen et al., 2011). The sorted samples are deposited as vouchers at the Museum Koenig Bonn (Leibniz Institute for the Analysis of Biodiversity Change).

DNA extraction and analysis

DNA extraction was carried out following a modified protocol from Aljanabi & Martinez (1997) (Vesterinen et al., 2016). Initially, the ethanol in the bulk sample was decanted from the bottles using the MICROFIL®V Filter (White Gridded 0.45 µm-Dia 47 mm & 100 ml Funnel Sterilized) equipped with a 0.45 µm filter membrane to retain small individuals and body parts. The remaining insects were dried for 10 min. Due to the high biomass of both bulk samples and to ensure thorough contact of all the specimens with the extraction buffer, we divided each sample into four equal subsamples (Fig. S1). Subsequently, each subsample was mixed with 50 ml of extraction buffer (0.4 M NaCl, 10 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, and 2% SDS of the final concentration). Additionally, 400 µg Proteinase K per ml of lysis buffer was added to each subsample. Subsamples were then incubated for digestion overnight at 52 °C on an orbital shaker set at 200 rpm. After digestion, the lysis solution from each subsample was evenly divided into three 50 ml falcon tubes, resulting in three replicates per subsample (24 samples in total, two samples x four subsamples x three replicates; Fig. S1). The lysate was filtered using MICROFIL®V Filter (White Gridded 0.45 µm-Dia 47 mm & 100 ml Funnel Sterilized) equipped with a 0.45 µm filter membrane to filter out the insects. After this second filtering step, we once again pooled the 24 samples into 6 extraction triplicates (3 for each sample; Fig. S1), which were processed separately throughout the remaining protocol and kept separate until the bioinformatic analysis. In the subsequent step, each tube received an additional 1.12-fold amount of lysis solution containing 6 M NaCl. The tubes were then vortexed for 30 seconds before being centrifuged for 30 min at 4,700 rpm. The supernatant was carefully transferred to new tubes, to which an equal amount of isopropanol was added. The solution was gently mixed by inverting the tubes upside down a few times before placing them at -20 °C for one hour. Following this, the tubes were centrifuged at 4 °C and 4,700 rpm for 60 min. The solution was carefully decanted and 2 ml of -20 °C 70% EtOH was added to the remaining pellet. The tubes were centrifuged at 4 °C and 4,700 rpm for 15 min. Subsequently, the supernatant was discarded and tubes with the remaining pellet were left to dry overnight at room temperature. Afterwards, pellets in each tube were dissolved in 1 ml

of sterile H_2O at room temperature for four hours. DNA extracts were quantified using the Quantus Fluorometer (Promega) and stored at -20 °C until further processing.

Library preparation strategy

Library preparation was conducted following a two-step PCR approach (Bourlat et al., 2016; Fonseca & Lallias, 2016). The first PCR (amplicon PCR, PCR1) was carried out using amplicon-specific primers with Illumina adapter overhangs and the second (index PCR, PCR2) allowed the incorporation of Illumina index adapters (Bourlat et al., 2016). The 313 bp long mitochondrial COI region of interest was amplified using the forward primer mlCOIintF (5'-GGWACWGGWTGAACWGTWTAYCCYCC-3') (Leray et al., 2013) and the reverse primer jgHCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Leray et al., 2013), yielding a suitable fragment size for both performing with higher success rates than other primer sets (e.g., LCO1490/HCO2198) in NGS applications (Leray et al., 2013).

Approximately 10 ng of template DNA was used for all PCR reactions. For the amplicon PCR, the here used mastermix consisted of 7.5 µl Q5 Hot Start High-Fidelity 2X Master Mix (New England BioLabs), 1 µl Sigma H_2O, 0.5 µl of forward Primer, 0.5 µl of reverse primer, 0.5 µl Bovine Serum Albumin (Thermoscientific) and 1 µl template DNA, making up a total volume of 15 µl. The amplicon PCR was initialized by denaturation of 2 min at 98 °C, which was followed by 20 cycles with 40 sec at 98 °C, 40 sec at 45 °C, 30 sec at 72 °C and a final extension of 3 min at 72 °C. PCR1 products were purified with HT ExoSAP-ITTM (Applied Biosystems) by adding 4 µl of HT ExoSAP-ITTM to each sample. Following the manufacturer's protocol, samples were incubated for 15 min at 37 °C, followed by 15 min at 80 °C before being cooled down for 5 min at 4 °C. For the index PCR, 8 µl of purified PCR1 products was used. The purified PCR products were therefore split into two PCR tubes. Each tube contained 12.5 µl Q5 Hot Start High-Fidelity 2X MasterMix (New England BioLabs), 3 µl Sigma H_2O, 1.2 µl of forward primer, 1.2 µl of reverse primer and 8 µl purified PCR1 product. Again, an initial denaturation step of 2 min at 98 °C was applied, followed by 20 cycles with 40 sec at 98 °C, 30 sec at 55 °C, 30 sec at 72 °C and a final extension of 3 min at 72 °C. PCR2 products were visualized by gel electrophoresis and purified using the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's instructions. All final purified amplicons (PCR2) were quantified using the Quantus Fluorometer (Promega) and diluted to the same concentration (3 ng/µl) before pooling. The resulting purified amplicon pools were sequenced on an Illumina Miseq (2x 300 bp) sequencing platform at Liverpool University's Centre for Genomic Research (UCGR, Liverpool). The raw data have been deposited at the Genbank SRA archive under accession number PRJNA1105927.

High throughput sequencing data analysis

An initial quality check was carried out at the UCGR. The raw fastq files were trimmed for the presence of Illumina adapter sequences using Cutadapt (v. 1.2.1) (Martin, 2011). Additionally, sequences were trimmed using Sickle (v. 1.200) with a minimum window quality score of 20. Reads shorter than 20 bp were removed after trimming. Additionally, demultiplexing was carried out by the sequencing company.

The raw fastq files were trimmed for the presence of COI primers using Cutadapt (v. 1.18) using the following settings: maximum error rate (-e): 0.1, minimum overlap (-O): 20, and minimum sequence length (-m): 50. Only sequences containing both forward and reverse primers were kept for further analyses. Subsequently, filtered and trimmed raw reads without the primer pairs were uploaded to QIIME2(v. 2022.2) (Bolyen et al., 2019). In an initial filtering step all forward reads were truncated to 269 bp and reverse reads to 274 bp. Further analysis steps including paired-read merging, quality filtering, and denoising were conducted with the implemented DADA2 version (Callahan et al., 2016).

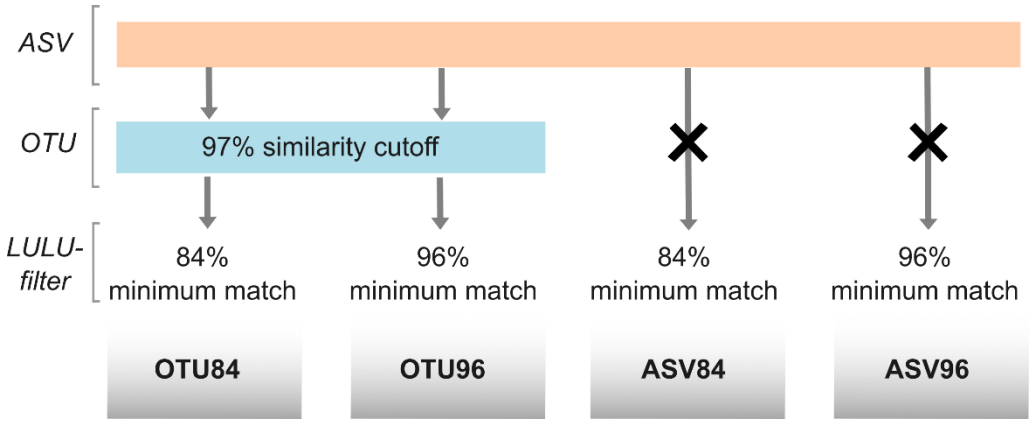

Figure 2: Schematic differences between the four different clustering approaches: ASVs were either clustered in OTUs at 97% cutoff and LULU-filtered using default settings at 84% minimum match (OTU84) or using a 96% minimum match (OTU96), or ASVs were just directly LULU-filtered using the default settings at 84% minimum match (ASV84) or (4) at 96% cutoff (ASV96).

We then started four different clustering and filtering approaches starting either with ASVs or ASVs clustered to OTUs at a 97% similarity cutoff. Initially we used a BLAST search of ASV and OTU representative sequences, respectively, against each other using BLASTN (v. 2.9.0) with the following settings: "query coverage high-scoring sequence pair percent" (−qcov_hsp_perc) was set to 80 and minimum percent identity (−perc_identity) was set to 84

(default setting). To filter for erroneous sequences, the post-clustering filter algorithm LULU (v. 0.1.0) (Frøslev et al., 2017) was applied either directly to the ASVs or OTUs dataset with the (1) minimum match set at 84% (from here on referred to as ASV84 or OTU84) or (2) minimum match at 96% (ASV96 or OTU96) (Fig. 2). We used the default value of the minimum_match parameter in LULU (84%) and selected another higher value (96%) — "*a higher value is recommended for markers with little variation and/or few expected PCR and sequencing errors*" (Frøslev et al., 2017). Then, for each dataset, the number of sequences found in the negative controls were subtracted from the according OTUs or ASVs. For the data analysis, we aggregated the total number of reads per molecular unit from each extraction triplicate per sample (Fig. S1) and converted into a binary present-absence dataset. Finally, the taxonomic assignment was carried out against the BOLD database (https://www.boldsystems.org) using BOLDigger (access date: 06.03.2023 ; Buchner & Leese, 2020), including early-release and private records. The output list was filtered using the JAMP-Pipeline method implemented in BOLDIGGER (Buchner & Leese, 2020). In detail, assignments to different taxonomic levels were conducted according to the following similarity thresholds: 98% species, 95% genus, 90% family, 85% order, <85% class. For instance, for a 96% hit, the species-level assignment will be discarded and genus-level information will be used as the lowest taxonomic level.

We manually checked the output list for possible synonyms (same species with different scientific names) to facilitate comparisons between the different methods. Moreover, we computed Shannon's index (H') (*vegan* package; Dixon, 2003) and Pielou's measure of species evenness (E) (*chemodiv* package; Petrén et al., 2023) based on the morphological dataset to gain a better understanding of both bulk samples. To compare morphology with the different clustering and filtering approaches, we focused on two different species diversity components: 1) species richness, defined as the number of identified molecular units, morphospecies, or species (in the case of Syrphidae), and 2) species composition (applied only for Syrphidae), representing the species community of Syrphidae identified using the different methodologies. For the analysis of the Syrphidae species composition, we used the Jaccard dissimilarity index (J) (*vegan* package, Oksanen et al., 2016). All the species diversity and composition analyses were performed in R (v1.4) with the *tidyverse* package (Wickham et al., 2019), while the heatmaps were additionally generated using the *cowplot* package (Wilke, 2024) combining data from both samples.

Results

Morphological identification

In the 2016 sample, we identified a total of 839 brachycerans and 533 hymenopterans (H' = 3.23, E = 0.694) sorted into 71 and 36 morphospecies respectively, belonging to 29 Brachycera and 17 Hymenoptera families. In the 2017 sample, we identified a total of 1,189 Brachycera and 813 Hymenoptera specimens (H' = 3.39, E = 0.693) sorted into 75 and 59 morphospecies respectively, belonging to 31 Brachycera and 22 Hymenoptera families. Combining both sampling years, we identified a total of 114 species of Brachycera (35 families) and 85 species of Hymenoptera (27 families) (Table 1).

In 2016, Drosphilidae was the most abundant family in Brachycera (225 specimens), followed by Syrphidae (144 specimens), while Tenthredinidae (56 specimens) and Proctotrupidae (14 specimens) were the most abundant among Hymenoptera. In 2017, Anthomyiidae was the most abundant family of Brachycera (493 specimens) followed by Hybotidae (107 specimens), while Apidae (187 specimens, of which 110 specimens were identified as *Bombus lucorum* (Linnaeus, 1761)) and Tenthredinidae (62 specimens) had the highest number of individuals for Hymenoptera. Regarding morphospecies richness, Syrphidae (12 species) and Tenthredinidae (11 morphospecies) were the most diverse families in 2016, whereas Pteromalidae (12 morphospecies), Tachinidae (11 morphospecies) and Syrphidae (11 species) exhibited the highest morphospecies richness in 2017.

Concerning the species diversity of Syrphidae, the most abundant species in both years was *Melanostoma mellinum* (Linnaeus, 1758): 109 specimens in 2016 (75.7% of the total number of hoverflies), while 45 specimens in 2017 (43.7% of the total number of hoverflies). Additionally, 4 syrphid species in 2016 and 5 syrphid species in 2017 were singletons, i.e., each species represented by just one specimen (Table S2).

DNA metabarcoding assessment

DNA metabarcoding directly from the lysis buffer with all four analysis approaches recovered between 180,010 and 189,562 reads from the bulk sample of 2016. Specifically, OTU84 generated 180,010 reads, ASV84 produced 181,983 reads, OTU96 resulted in 189,562, and ASV96 yielded 180,010 reads. Similarly, for the bulk sample of 2017, the DNA metabarcoding approach retrieved between 244,564 to 244,501 reads. OTU84 and ASV84 both generated 244,586 reads, OTU96 produced 244,501 reads, and ASV96 resulted in 244,586 reads.

Table 1: Final list of Hymenoptera (excluding Ichneumonoidea) and Brachycera diversity identified via DNA-metabarcoding applying four different clustering approaches compared to the morphologically identified diversity: 1) LULU-filtered ASVs at a minimum match of 84% (default settings; ASV84); 2) LULU-filtered ASVs at a minimum match of 96% (ASV96); 3) ASVs clustered to OTUs at 97% similarity cutoff and LULU-filtered at a minimum match of 84% (default settings; OTU84); and 4) ASVs clustered to OTUs at 97% similarity cutoff and LULU-filtered at a minimum match of 96% (OTU96). Blasted molecular units refer to molecular units identified with a name after blasting.

Sample	**Taxa**	**Unit**	**ASV84**	**ASV96**	**OTU84**	**OTU96**		**Morphology**
2016	**Brachycera**	Reads	54,116	62,519	62,519	54,499		
		Family	11	10	10	24	Family	29
		Genera	13	14	14	66		
		Molecular unit (MU)	15	16	16	97		
		Blasted MU	15	16	16	80	Morphospecies	71
	Hymenoptera	Reads	7	7	7	1,162		
		Family	1	1	1	2	Family	17
		Genera	1	1	1	5		
		Molecular unit (MU)	1	1	1	13		
		Blasted MU	1	1	1	11	Morphospecies	36
	Syrphidae	Reads	14,602	31,266	31,266	18,433		
		Genera	3	5	5	10	Genera	6
		Molecular unit (MU)	3	5	5	11		

		Blasted MU	3	5	5	11	Species	12
2017	**Brachycera**	Reads	51,311	47,415	47,415	46,781		
		Family	11	10	10	31	Family	31
		Genera	14	15	15	64		
		Molecular unit (MU)	16	18	18	119		
		Blasted MU	16	17	17	96	Morphospecies	75
	Hymenoptera	Reads	644	746	746	746		
		Family	3	3	3	3	Family	22
		Genera	4	4	4	4		
		Molecular unit (MU)	5	5	5	5		
		Blasted MU	5	5	5	5	Morphospecies	59
	Syrphidae	Reads	26,827	28,449	28,449	29,075		
		Genera	3	5	5	11	Genera	8
		Molecular unit (MU)	3	5	5	16		
		Blasted MU	3	5	5	15	Species	11
Both years combined	**Brachycera**	Reads	105,427	109,934	109,934	101,280		
		Family	16	15	15	34	Family	35
		Genera	20	21	21	95		
		Blasted MU	23	24	24	144	Morphospecies	114
	Hymenoptera	Reads	651	753	753	1,908		
		Family	3	3	3	3	Family	27
		Genera	4	4	4	8		

	Blasted MU	5	5	5	15	Morphospecies	85
	Reads	41,429	59,715	59,715	47,508		
Syrphidae	Genera	4	6	6	14	Genera	12
	Blasted MU	6	10	10	26	Species	21

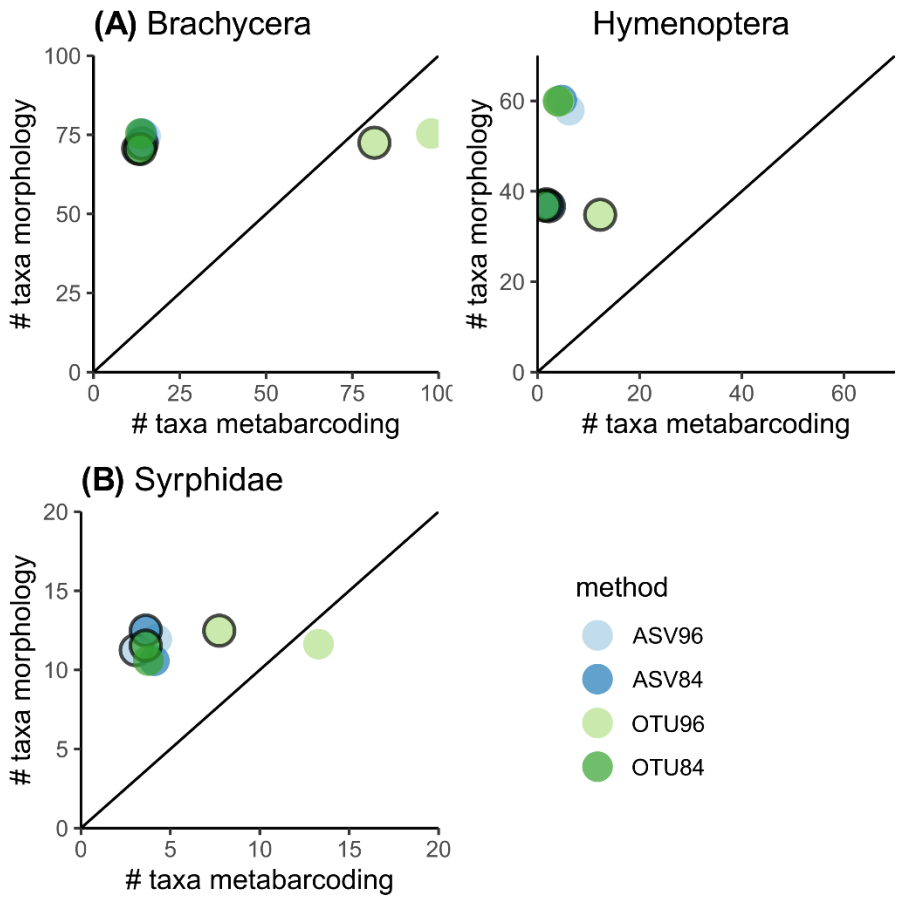

Figure 3: Comparison between the number of (A) hymenopteran and brachyceran taxa and (B) Syrphidae species detected with morphological identification and DNA metabarcoding with four different clustering approaches across two samples of 2016 and 2017. ASVs were either directly LULU-filtered at 96% minimum match (ASV96) or using the standard setting at 84% minimum match (ASV84), or ASVS were firstly clustered to OTUs (at 97% similarity cutoff) and afterwards LULU-filtered at 96% minimum match (OTU96) or using the standard setting at 84% minimum match (OTU84). The solid line represents a 1:1 relationship. Points with black borders represent the sample of 2016, and points without border represent the sample of 2017.

In 2016, we detected between 15 to 97 Brachyceran molecular units depending on clustering and filtering method, corresponding to 15 to 80 species across 11 to 24 families. For the Hymenoptera, we detected 1 to 13 Hymenoptera molecular units matching 1 to 11 species across 1 to 2 families (Table 1). Similarly, in 2017, we detected between 16 to 119 Brachyceran molecular units matching 16 to 96 species across 11 to 30 families. Hymenoptera exhibited 5 molecular units corresponding to 5 different taxa across 5 families using all 4 different clustering methods. Overall, OTU96 detected the highest number of reads and species in both orders (Table 1).

Table 2: Jaccard dissimilarity index (J) of Syrphidae between the different samples based on morphological data (morpho) and DNA metabarcoding with four different clustering and filtering approaches, based on a binary dataset. The closer the value is to 0, the higher the similarity is between the methods. OTU84 = ASVs clustered in OTUs at 97% similarity cutoff and LULU filtered using the standard settings at 84% minimum match, ASV84 = ASVs and LULU filtered using standard settings at 84% minimum match, ASV96 = ASV and LULU filtered using a 96% minimum match, OTU96 = ASV clustered to OTUs at 97% similarity cutoff and LULU filtered using a 96% minimum match.

Comparison	**2016 (J)**	**2017 (J)**
OTU84 : morpho	0.69	0.5
ASV84 : morpho	1	0.4
ASV96 : morpho	1	0.4
OTU96 : morpho	0.67	0.4

Comparison of morphological identification and metabarcoding

DNA metabarcoding revealed different numbers of putative species (molecular units) in Hymenoptera and Brachycera depending on which of the four different clustering and filtering approaches was used (Fig. 3). For Brachycera across both sampling years, ASV84, ASV96, and OTU84 underestimated the number of the species identified using morphology (in Fig. 3, point above the 1:1 crossline), while OTU96 was the closest though notably overestimating the number of species (in Fig. 3, points below the 1:1 crossline). Among the brachyceran families identified by adult morphology, nine were not found by any of the clustering and filtering approaches, namely Dryomizidae, Ephydridae, Heleomyzidae, Lonchaeidae, Rhinophoridae, Stratiomyidae, Tephritidae, Therevidae and Xylomyidae (Fig. 4 and Fig. 5). Contrary, three families were not identified using morphology in the sample, but with DNA metabarcoding only: Sciomyzidae was detected through all four clustering and filtering approaches, while Milichiidae and Polleniidae were identified specifically with OTU96. The pattern of underestimation of brachyceran species richness, evident across all clustering and filtering approaches, except for OTU96, is also apparent for Syrphidae. Although a slight underestimation of species number persists in the sample of 2016, OTU96 exhibited the closest species number to those identified using morphological characters (i.e., the closest to the 1:1 crossline) (Fig. 3).

For Hymenoptera, all clustering and filtering approaches substantially underestimated the species number identified by morphological characters (in Fig. 3, point above the 1:1

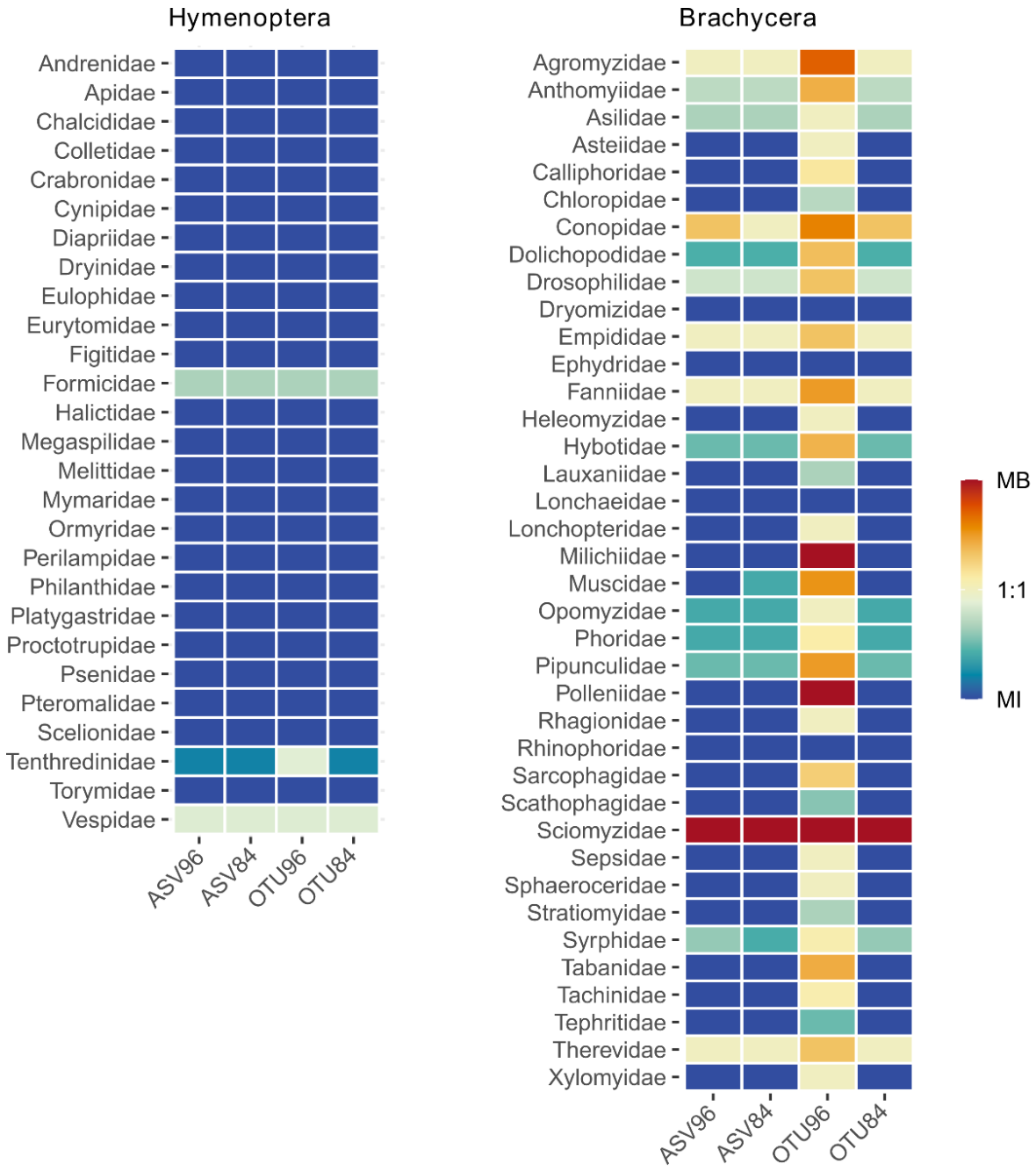

Figure 4: Comparison of the ratio between four different bioinformatic approaches (ASV filtered with LULU using a 96% (ASV96) or 84% minimum match (default setting; ASV84), ASV clustered to OTUS at 97% similarity cutoff and LULU curated at 96% (OTU96) or at 84% minimum match (OTU84)) and the morphological identification of Hymenoptera and Brachycera. Morphological and metabarcoding identification could have either identified the same number of taxa (1:1) or inclined to identify more taxa with metabarcoding (MB, red) or via morphological identification (MI, blue).

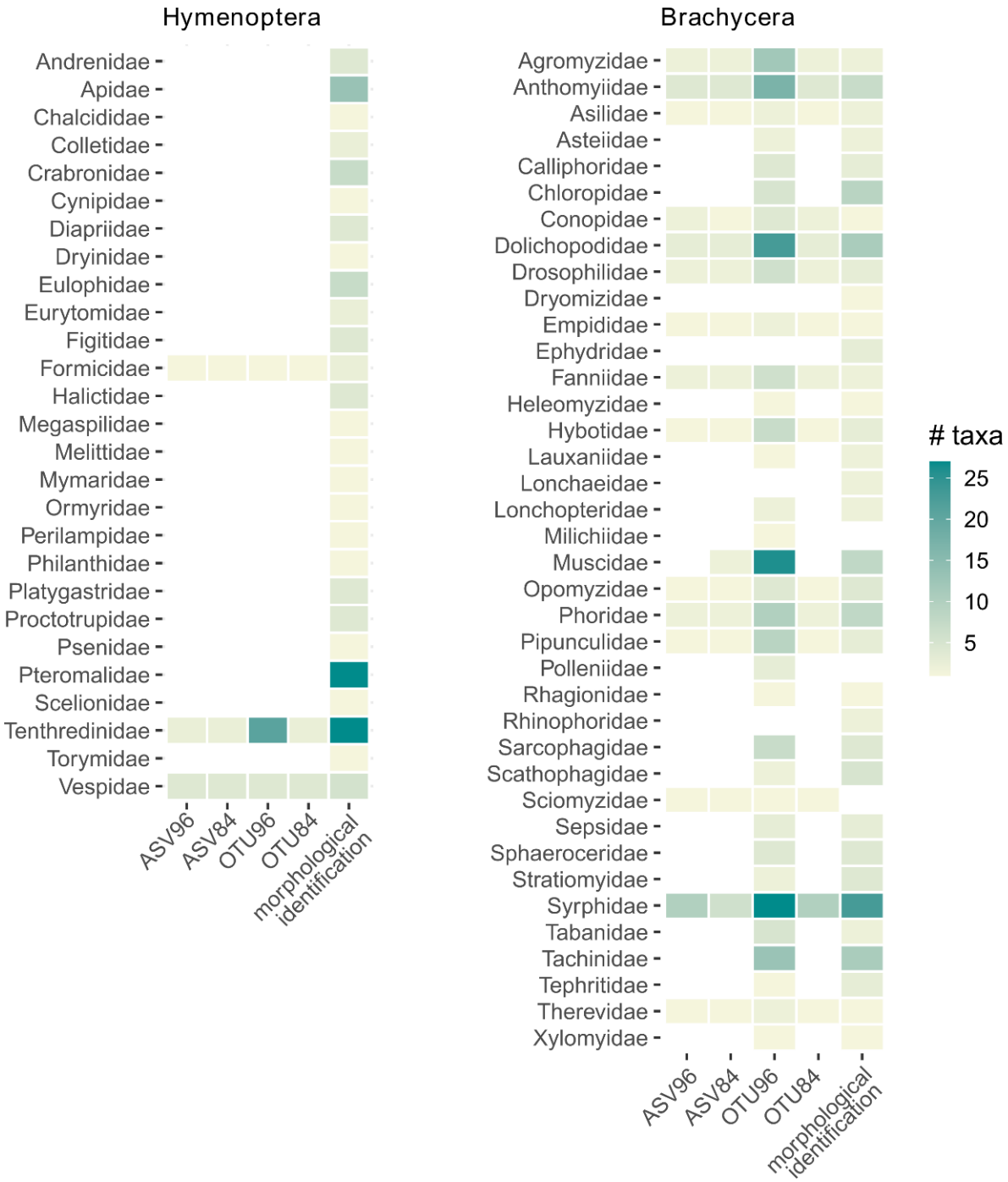

Figure 5: Heatmap comparing the number of hymenopteran and brachyceran species identified morphologically and via DNA metabarcoding either by LULU-curated ASV using a 96% (ASV96) or 84% minimum match (default setting; ASV84), or ASV clustered to OTUS at 97% similarity cutoff and LULU curated at 96% (OTU96) or at 84% minimum match (OTU84), of two samples collected in 2016 and 2017.

crossline), with OTU96 providing the closest estimation of species numbers in both samples (Fig. 3). Among the taxa identified by morphology, *Athalia rosae* (Linnaeus, 1758) (Tenthredinidae) and *Lasius niger* (Linnaeus, 1758) (Formicidae) were also identified using DNA metabarcoding (Fig. 4 and 5). While Vespidae was also identified using both DNA metabarcoding and morphology, the two species of this family retrieved by DNA metabarcoding were not found morphologically. Moreover, 25 additional families identified morphologically were not determined by any of the clustering and filtering approaches (Fig. 4 and 5).

In terms of similarities in syrphid species composition, OTU96 showed the highest similarity with morphology for both samples (J = 0.67 in 2016 and J = 0.4 in 2017). The same highest Jaccard value was found for the 2017 sample with ASV84 and ASV96 (J=0.4) (Table 2). *Melanostoma mellinum*, as the most abundant species identified with morphology in both years, was detected with all four clustering and filtering approaches (Table S2). However, none of the syrphid singletons from 2016 were found using DNA metabarcoding. In contrast, in the 2017 sample, 4 out of the 6 singletons were identified with OTU84. Lastly, 9 syrphid species (7 in 2016 and 2 in 2017) identified using morphology were not detected by DNA metabarcoding (Table S2). Contrary, different metabarcoding approaches detected 13 syrphid species (6 in 2016 and 7 in 2017) that were not found in the morphology study, being OTU96 (in the 2016 sample) and OTU84 (in the 2017 sample) the metabarcoding approaches with the highest number of syrphid species detected that were not present in the morphological study (6 each) (Table S2).

Discussion

To our knowledge, this study represents the first comparative analysis of species-level diversity in Malaise trap samples (focusing specifically on Brachycera and Hymenoptera) comparing the diversity assessments from traditional adult morphology with those obtained through a non-destructive DNA metabarcoding approach. Our survey places particular emphasis on testing various clustering and filtering methods, combining ASVs, OTUs, and LULU-curation. Among the four different clustering and filtering approaches tested here, OTU96 emerges as the method that better reflects species richness identified by morphological characters and closely approximates species composition for Brachycera, specifically in Syrphidae. This alignment between DNA metabarcoding and morphological identification for Brachycera mirrors the findings from similar studies on arthropod diversity

in Malaise trap samples (Remmel et al., 2024) and freshwater invertebrates (Beentjes et al., 2019; Cahill et al., 2018).

Previous research has extensively examined the advantages and disadvantages of using OTU and ASV clustering, primarily in the context of microbiome assessments (Barnes et al., 2020; Chiarello et al., 2022) but also for arthropods (Giebner et al., 2020; Porter & Hajibabaei, 2020). Some authors argue that ASVs exhibit a clear superiority over OTUs in their ability to identify a larger number of distinct taxa (Giebner et al., 2020; Porter & Hajibabaei, 2020). However, other studies suggest that ASVs may lead to an overestimation of diversity due to high levels of intraspecific diversity of the sampled taxa, often combined with a high degree of artificially introduced sequences (e.g., PCR artifacts) (Andújar et al., 2021; Brandt et al., 2021). In contrast, while OTUs mitigate the impact of sequencing noise, they often achieve this by clustering similar interspecific sequences together into a single OTU, which can artificially lower the assessed diversity and is, therefore, a more conservative approach. For this particular dataset we advocate for an optimal approach of non-destructive sample analysis, followed by a clustering intraspecific ASVs into interspecific OTUs. This process involves grouping closely related sequences from the same species (intraspecific ASVs) together within larger clusters representing different species (interspecific OTUs). By adopting this method, we aim to accurately represent genetic variation within species while providing a comprehensive understanding of overall diversity.

LULU curation at 96% minimum match, compared to the default setting of 84%, enables the reduction of intraspecific ASVs erroneously considered/assigned to different species, ultimately leading to an estimated species number that aligns more closely with morphological identification. Despite the close resemblance in the estimated number of species between OTU96 and the morphological identification, disparities in species number and composition persist (Fig. 4 and Table 2). These variations primarily stem from either false positives, where species were detected using DNA metabarcoding but not morphologically, or false negatives, where species were identified morphologically but not detected using DNA metabarcoding. Potential causes for false positives or negatives include cross-contamination, shared haplotypes of COI , morphological misidentification, and inaccuracies in reference databases. Cross-contamination often arises from the analysis of DNA traces, e.g. gut content of predatory arthropods (Kirse et al., 2023; Iwaszkiewicz-Eggebrecht et al., 2023; Lynggaard et al., 2019; Reeves et al., 2018). While analyzing trophic interactions can enhance the analysis, it cannot be quantified until which degree it may lead to an overestimation of species richness and a distortion of species composition compared to morphology. Also, some species (here i.e., *Melanostoma* species) may share haplotypes between species when analyzing the

COI gene fragment (Haarto & Ståhls, 2014). While LULU curation partially addresses this issue, it does not account for variations in haplotype proportions (Brandt et al., 2021). Furthermore, morphological identification of highly diverse groups like Diptera can also be difficult (Huang et al., 2022), leading to misidentifications due to the lack of differences in morphological characters (here i.e., females of certain *Platycheirus* and *Sphaerophoria* species). The presence of cryptic species within species complexes can contribute to these false negatives and/or positives. Lastly, erroneous species identifications can result from inaccuracies in reference databases caused by misleading vouchers. Indeed, conducting a thorough validation of the dataset before performing any diversity analysis as presented by Remmel et al. (2024) by cross-checking the species list with occurrences in the GBIF (Global Biodiversity Information Facility; Telenius, 2011) or GBOL databases (German Barcode of Life; M. F. Geiger et al., 2016), as well as a double-check by taxonomists can mitigate some of the misleading results. However, it depends again directly on the quality of further databases and availability of taxonomists, which can be challenging for many understudied insect taxa. The first and direct consequence of the mismatches, false positives or negatives, are an under- or overestimation of diversity. In cases where morphological data are lacking for comparison, this can result in inaccurate biodiversity assessments, leading to e.g. ineffective conservation plans (Ficetola et al., 2016).

Overall, no single approach stood out as optimal for analyzing Hymenoptera diversity. The notably low number of Hymenoptera OTUs found cannot be solely attributed to the clustering and filtering approaches, as even the unfiltered ASV dataset already exhibited a surprisingly low number of ASVs assigned to Hymenoptera. This suggests that false negative errors likely occurred during sample processing in the laboratory. There are multiple potential explanations for the absence of many hymenopteran species. Firstly, the lack of larger and more common Hymenoptera species (e.g., *Bombus* species with 190 specimens in the sample of 2017), also evidently from the study by Remmel et al. (2024), could potentially be attributed to a primer bias. Primer bias, which refers to the failure of universal primers to amplify certain taxa, has been documented across various taxa (Clark et al., 2020; Piñol et al., 2015), but is particularly noticeable in Hymenoptera samples (Brandon-Mong et al., 2015; Elbrecht et al., 2019; Yu et al., 2012). This limitation hampers the comprehensive assessment of Hymenoptera diversity when using one primer alone, as it excludes many important species, including numerous important pollinators (Kilian et al., 2023). Secondly, biomass bias could have directly influenced the species richness of Hymenoptera detected via DNA metabarcoding. This bias arises because species represented by a limited number of specimens or those that are generally smaller in size may yield lower quantities of DNA

(Elbrecht et al., 2019; Erdozain et al., 2019). This could explain why nine brachyceran families represented by a low number of specimens and small in size were not identified at all with any of the clustering and filtering approaches, although recent studies show that a non-destructive approach can counteract this bias (Marquina et al., 2019). Furthermore, different degrees of sclerotization among the different taxa may impact the quantity of extracted DNA, particularly when using a non-destructive approach as in our study (Erdozain et al., 2019; Kirse et al., 2023; Marquina et al., 2019; Zizka et al., 2018). While size-sorting and a destructive extraction method may potentially increase the amount of extracted DNA (and theoretically the number of identified species), they also present significant drawbacks. These methods do not alleviate the issues related to primer and mass biases, as discussed earlier. Furthermore, they prevent the possibility of re-checking voucher specimen after metabarcoding — a critical step for validation and verification in biodiversity studies (Remmel et al., 2024). Therefore, the non-destructive extraction method has the significant benefit of preserving vouchers, enabling subsequent taxonomic analysis. This preservation is particularly crucial in the largely understudied taxa of Diptera and Hymenoptera, often referred to as "Dark Taxa" (Chimeno et al., 2022; Hausmann, Krogmann, et al., 2020). In the current context, despite advancements in DNA metabarcoding, applications of species-level data in Hymenoptera still heavily rely on results obtained through morphological identification rather than solely on metabarcoding outcomes.

In the particular case of the family Syrphidae, our exemplary family in the present survey, the high dissimilar community between the DNA metabarcoding and morphology is unexpected. Cross-contamination and shared COI haplotypes can be a source of mismatch between the two approaches, as syrphids are frequent prey of predaceous arthropods such as Diptera and Hymenoptera (Blösch, 2000; Gilbert, 2005; Pickard, 1975) and certain genera have species with shared COI haplotypes (Dietz et al., 2023; Haarto & Ståhls, 2014; Locke & Skevington, 2013; A. D. Young et al., 2016). But the COI haplotype is not common among all the syrphid genera and would not explain all the cases of discrepancies, e.g., the detection of *Episyrphus balteatus*, *Eristalis tenax* or *Helophilus pendulus* in the OTU96 from 2016. Should the Syrphidae include taxa or species groups characterized by minimal interspecific differentiation, the aggregation of multiple species within a single OTU/ASV becomes a likely outcome. Still, this would not explain the detection of genera using metabarcoding that were not present among the morphological species such as *Dasysyrphus* or *Episyrphus* in the 2016 sample. The interspecific divergence in the subfamily Syrphinae exhibits significant overlap with the intraspecific divergence distribution, thereby negating the presence of a general DNA barcoding gap for hover flies as a group (Kurt Jordaens et al., 2015; R. Meier et al., 2008); this

overlap is not as frequent in the subfamily Eristalinae as in Syrphinae, but it exists. *Eristalis tenax* or *Helophilus pendulus* belong to Eristalinae, and although *Episyrphus balteatus* is a Syrphinae, it is the single species of the genus occurring in Europe. Thus, the detection of *E. balteatus* in the OTU96 from 2016 implies not only a species not found in the morphological survey, but a genus not studied morphologically. We must point out that all the hover flies identified morphologically, as well as those identified only by metabarcoding, have reference sequences in BOLD; hence, the lack of a reference barcode in the database cannot explain the observed discrepancies.

In addition, although morphological misidentification is likely to occur, Syrphidae is a well-studied flower-visitor group with several good identification tools for northern and central Europe (Bartsch, 2009b, 2009a; Bot & Van de Meutter, 2019, 2023; Speight & Sarthou, 2017; Van Veen, 2010) and the collected species in 2016 and 2017 (Table S2) do not represent a serious challenge in their morphological identification, with the exception of some females or partially destroyed specimens. Inaccuracies in reference databases are very likely to occur and we cannot rule it out completely, although in a minor percentage for Syrphidae as the community of syrphid researchers helped to build a well-curated database for GBOL and other parts of the world.

The biomass bias with unique organisms or single species showing lower detection rates (Strutzenberger et al., 2024) could be argued as the absence of certain species identified morphologically in any DNA metabarcoding approach, such as *Triglyphus primus* from 2016 or *Eupeodes luniger* from 2017, but not for the non-detection of other species with more specimens, i.e., *Platycheirus* species other than *P. clypeatus* (Table S2). But this biomass bias cannot explain the relatively high number of species detected by DNA metabarcoding that were not found in our morphological survey, especially as the detected species are mostly medium-to-large-sized syrphids, i.e., *Eristalis tenax*, *Dasysyrphus tricinctus*, *Helophilus pendulus* or *Syrphus ribesii*. Based on our experience, and corroborated by the GBOL database, these species show a barcoding gap with the nearest neighbor species (in other words, the intra- and the interspecific p-distance do not overlap) and they are very common and abundant species in Central Europe, which makes the overlooking by us in the morphology survey unlikely.

Our findings mirror other works where morphologically identified species are compared with metabarcoding species results (Remmel et al., 2024; with J=0.5 for Syrphidae). Thus, the high Jaccard dissimilarity index calculated for Syrphidae in the present study might be explained by other reasons, such as the accuracy of the DNA mini-barcode of 313 bp in length used in

metabarcoding to identify species. While DNA mini-barcodes are commonly used in metabarcoding, there is a concern about whether they might affect the accuracy of identifying syrphid species, especially when compared to full-length 658 bp COI barcodes, as shorter sequences may not provide enough genetic information for precise species identification, at least for some species. Many studies comparing DNA mini-barcodes and morphology are based on mock communities (Aylagas et al., 2016; Baloğlu et al., 2021; Govender et al., 2022), which may not fully capture the complexities present in real-world scenarios. This suggests that the observed mismatch could be influenced by factors unique to natural environments, such as the presence of closely related species and environmental variables affecting DNA extraction and amplification. Srivathsan et al. (2018), for example, found no difference in the number of species between full-length DNA barcodes (658 bp) and the 313-bp fragments, although they did not state if the species composition was highly similar or not. Identifying syrphid species based on full-length DNA barcodes accurately can be challenging due to various factors such as genetic variability within species and incomplete reference databases, as mentioned. This raises the question of whether DNA mini-barcodes exacerbate this issue or if it is inherent to metabarcoding techniques in general. Without a better explanation, it seems that the detection of these species not present in our morphological study may be due to cross-contamination.

Despite the current limitations in DNA metabarcoding, we also highlight the potential of a non-destructive DNA metabarcoding approach for uncovering e.g. cryptic diversity in highly diverse groups, which morphologically can still be very challenging. We emphasize the potential of enhancing DNA metabarcoding results not only through improvements in the DNA extraction and PCR processes, but also by refining the final assessment through appropriate bioinformatic analysis, particularly focusing on the clustering and filtering approaches. This is especially important in studies where the metabarcoding analysis cannot be cross validated with morphology.

Conclusion

While DNA metabarcoding has become a valuable tool for insect biomonitoring assessments, there are still many limitations that require attention, especially during the in silico stage where the impact of clustering and filtering approaches is significant. Our study highlights that for Brachycera clustering ASVs into OTUs at 97% cutoff and subsequently applying LULU-filtering at a 96% minimum match yields the most interesting results, with the number of detected species closely approximating the diversity identified using morphology when

using a non-destructive DNA protocol, although these total numbers may be misleading due to the high number of false positives and false negatives which end up with low Jaccard dissimilarity index (see the results of the species composition with Syrphidae, Tables 2 and S2). We advocate for a species composition analysis whenever is possible. However, for Hymenoptera, the same approach resulted in considerably different estimate compared to diversity assessed via morphological identification, likely due to the presence of false negatives introduced during the laboratory processing of the samples, mainly driven by primer bias. Despite the variations in results and resolution observed for Brachycera and Hymenoptera, and the persistent limitations in the application of this methodology, we recognize the potential of achieving high species-level resolution with a non-destructive DNA metabarcoding approach. This approach not only retains for future analyses, but preserves the entire sample as a voucher. This preservation is particularly valuable for large-scale monitoring programs utilizing DNA metabarcoding as a standard methodology. Addressing these limitations and optimizing protocols will enhance the reliability and accuracy of DNA metabarcoding for insect biomonitoring in the future.

Supplementary information

Table S1: Publications with identification keys used in the morphological survey of the samples.

Taxa	**Identification keys**
Hymenoptera	Amiet et al., 2012; Benson, 1951, 1958; Bitsch, 1992; Gokcezade et al., 2010; Goulet et al., 1993; Prous et al., 2019; Schmid-Egger & Scheuchl, 1997, 1997; Stresemann & Klausnitzer, 2011; Witt, 1998
Brachycera	Drake, 1993; Gregor et al., 2016; Naglis, 2012; Oosterbroek, 2006; Rozkosny & Frantisek, 2004; Stresemann & Klausnitzer, 2011; Tschorsnig, 1994; Van Emden, 1954; Zeegers, 1992
Syrphidae	Bartsch, 2009a, 2009b; Haarto & Ståhls, 2014; Van Veen, 2010

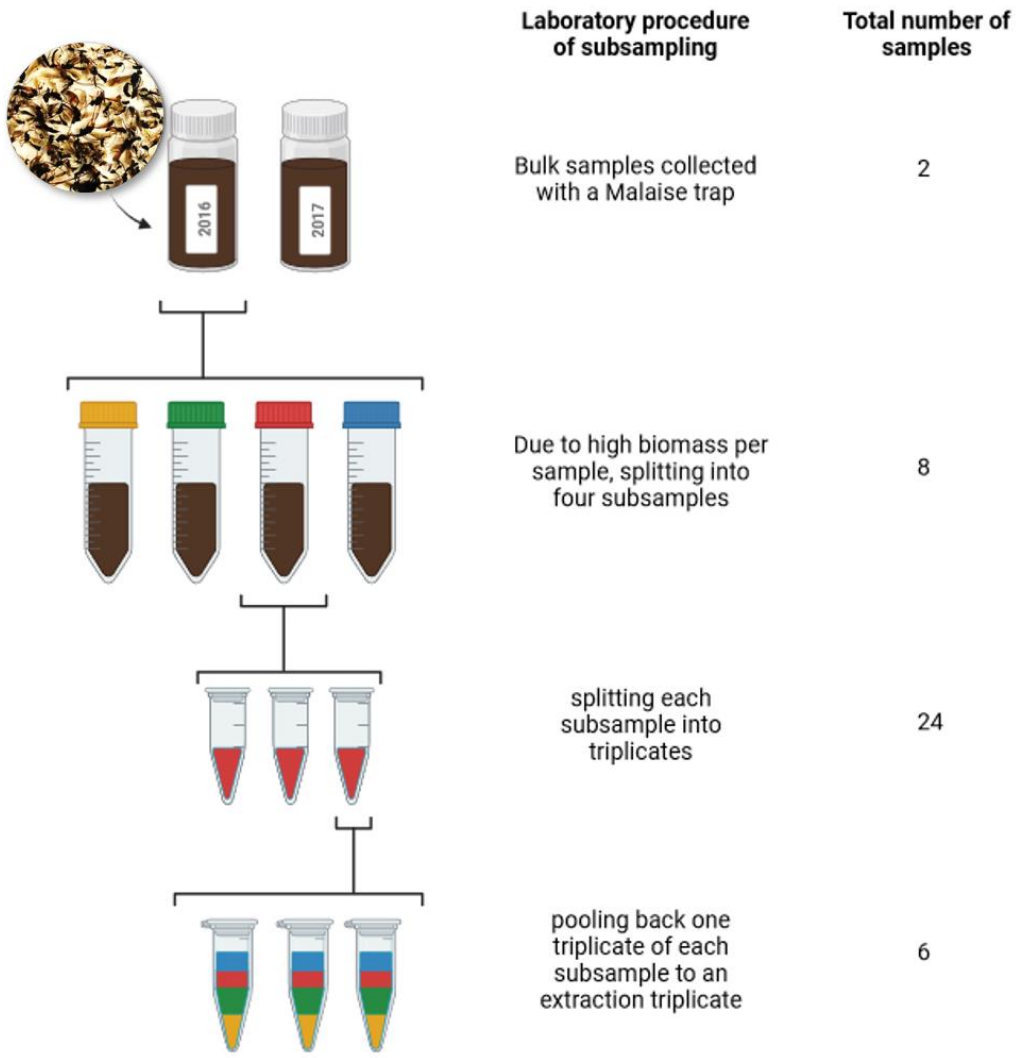

Figure S1: Experimental setup for DNA extraction. The specimens of two Malaise trap bulk samples were split into four equal subsamples after drying. Each subsample was mixed with an extraction buffer. After digestion, the lysis solution was split into three replicates per subsample, which were pooled back together to three extraction replicates after the second filtering process. For a more detailed description, see Material and Methods section. Created with BioRender.com.

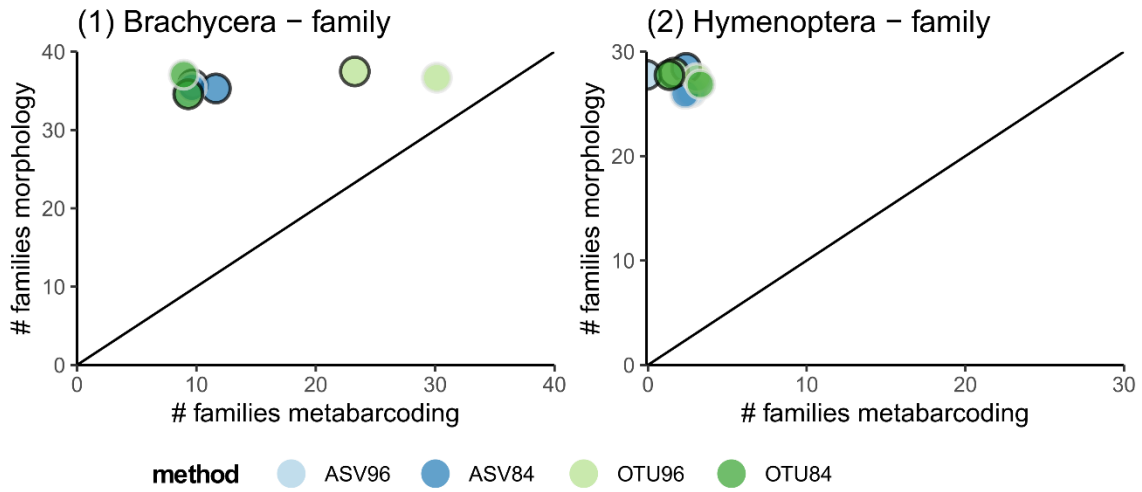

Figure S2: Comparison between number of families (1) of Brachycera and (2) of Hymenoptera identified with morphological identification and DNA metabarcoding with four different clustering approaches across both samples of 2016 and 2017. ASVs were either clustered in OTUs at 97% similarity cutoff and LULU-filtered using 1.) standard settings at 84% minimum match (OTU84) or 2.) using a 96% minimum match (OTU96), or ASVs were just directly LULU-filtered using the standard settings at 3.) 84% minimum match (ASV84) or 4.) at 96% minimum match (ASV96). The solid line represents a 1:1 relationship. Points with black borders represent the sample of 2016, and points with grey borders represent the sample of 2017.

Table S2: Final list of Syrphidae diversity identified via DNA-metabarcoding applying four different clustering and filtering approaches (qualitative data) and morphological identification (quantitative data). ASV84 = LULU-curated ASVs using standard settings at 84% minimum match, ASV96 = LULU-curated ASV using a 96% minimum match, OTU84 = ASVs clustered in OTUs at 97% similarity cutoff and LULU filtered using the standard settings at 84% minimum match, OTU96 = ASV clustered to OTUs at 97% similarity threshold and LULU filtered using a 96% minimum match.

	2016					**2017**				
Syrphid species	ASV84	ASV96	OTU84	OTU96	Morph.*	ASV84	ASV96	OTU84	OTU96	Morph.*
Dasysyrphus tricinctus	0	0	0	1	0	0	0	0	0	0
Episyrphus balteatus	0	0	0	1	0	0	0	1	0	18
Eristalinus sepulchralis	0	0	0	0	0	0	0	1	0	1
Eristalis tenax	1	1	1	1	0	1	1	1	1	0
Eristalis arbustorum	0	0	0	0	0	0	0	1	0	1
Eristalis intricaria	0	0	0	0	0	0	0	1	0	1
Eupeodes corollae	0	0	0	0	0	0	0	1	0	0
Eupeodes sp.	0	0	0	0	0	0	0	1	0	0
Eupeodes luniger	0	0	0	0	0	0	0	0	0	2
Helophilus pendulus	0	1	1	1	0	0	0	0	0	0
Helophilus trivittatus	0	0	0	0	0	0	0	1	0	1
Fagisyrphus cinctus	0	0	0	1	0	0	0	0	0	0
Melanostoma mellinum	1	1	1	1	109	1	1	1	1	45
Melanostoma sp.	0	0	0	1	0	0	0	0	0	0
Melanostoma scalare	0	0	0	0	5	0	0	0	0	0
Paragus sp.	0	0	0	1	1	0	0	1	0	0
Platycheirus clypeatus	1	1	1	1	3	0	1	1	1	0
Platycheirus sp.	0	0	0	0	1	0	0	0	0	0
Platycheirus albimanus	0	0	0	0	3	0	0	0	0	0

Platycheirus angustatus	0	0	0	0	1	0	0	0	0	0
Platycheirus europaeus	0	0	0	0	13	0	0	0	0	0
Platycheirus inmaculatus	0	0	0	0	3	0	0	0	0	0
Rhingia campestris	0	0	0	1	1	0	0	0	0	0
Scaeva pyrastri	0	0	0	0	0	0	0	1	0	0
Sphaerophoria scripta	0	1	1	1	3	0	1	1	1	15
Sphaerophoria sp.	0	0	0	0	0	0	0	0	0	16
Sphaerophoria taeniata	0	0	0	0	0	0	0	0	0	1
Syrphus ribesii	0	0	0	0	0	1	1	1	1	0
Syrphus vitripennis	0	0	0	0	0	0	0	1	0	2
Triglyphus primus	0	0	0	0	1	0	0	0	0	0
syrphidae molecular unit	0	0	0	0	0	0	0	1	0	0

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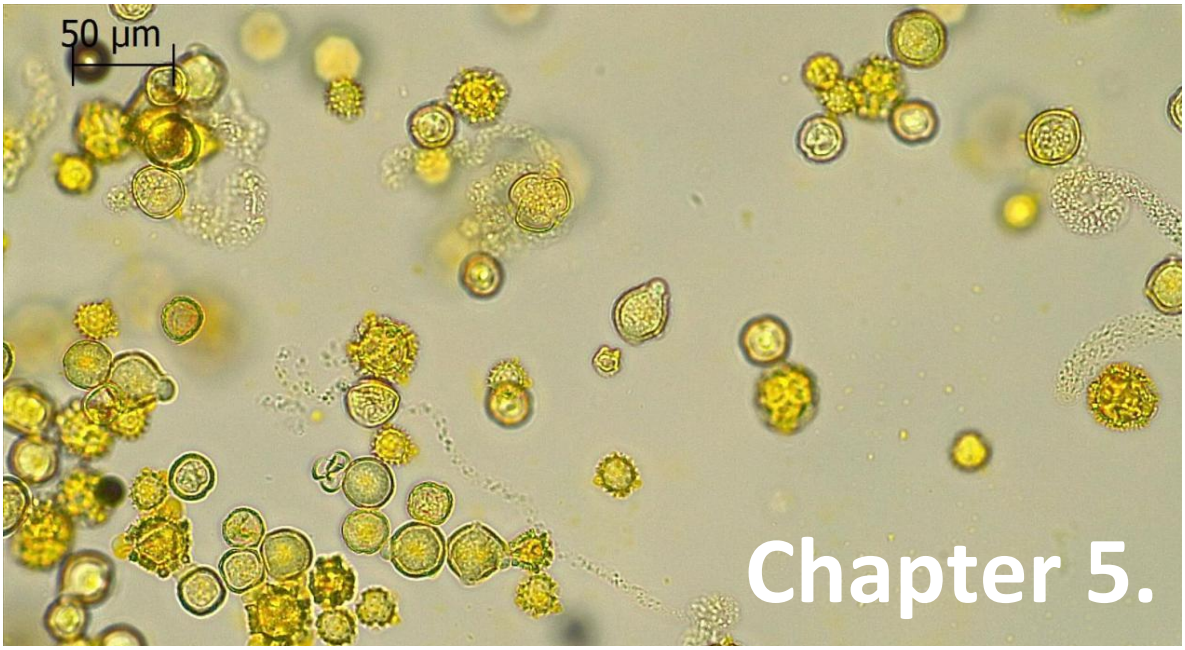

Chapter 5.

General discussion & conclusions

5.1. Complexity of plant-pollinator networks in agroecosystems and why pollinator diversity matters

Despite the global importance of pollination to angiosperm diversity and crucial for food production, the potential of wild pollinators (defined here as all non-managed pollinators) has been largely neglected or it is still unknown (Klein et al., 2007; Larson et al., 2001; Ollerton et al., 2011; Orford et al., 2015; Ssymank et al., 2008). Results from this thesis show a particularly high number of less studied brachyceran and non-bee hymenopteran pollinating species of caraway and apple, many of them even potential pollinating both crop species. Earlier research on pollination of those crop species compiled for the most part, in the case of caraway, no more than a list of pollinators or, as for apples, plant-pollinator networks based primarily on flower-visiting surveys (Barahona-Segovia et al., 2023; Bouwmeester & Smid, 1995; Toivonen et al., 2022). Despite flower-visitation being a poor proxy for pollination as many species visit flowers without transporting intraspecific pollen, it is still commonly used in pollination ecology studies (King et al., 2013; Theodorou et al., 2017; Wardhaugh, 2015). For this thesis, increased accuracy could be achieved by using an integrative approach with DNA barcoding of the insect specimens and metabarcoding of the pollen loads. This approach has the potential not only to identify potential pollinators of crop species in agroecosystems, but also to reveal the complex interactions with other plant species present in fields or in the surrounding landscape (Chapter 2 and 3). These interactions also changed over time, showcasing temporal dynamics of plant-pollinator networks. Additionally, key pollinating species for both crop species were identified, which are responsible to some degree for the resilience and stability of the network's structure. In the discussion sections of Chapters 2 and 3, some more detailed information is given for those crucial pollinating species. To avoid redundancy in the following discussion, going on, I want to focus on the overall high taxonomic diversity of Brachycera and Hymenoptera potential pollinators, and their potential implications and consequences for the stability of ecosystems functions and particularly pollination.

Following the concept of functional redundancy, where multiple species perform a similar function in an ecosystem (Blüthgen & Klein, 2011; S. Naeem, 1998), it could be assumed that a higher diversity of pollinators could lead to a redundancy of individual pollinating species. Current studies show that even with a high pollinator diversity (particularly communities or functional groups), pollinating species not necessarily share the same ecological requirements or utilize overlapping resources, but can share or complement each other niches. Therefore, this niche complementarity increases the pollination service by reducing interspecific overlaps in flower visitations or pollination, and can help to mitigate changing

environmental conditions (Albrecht Matthias et al., 2012; Brittain et al., 2013; Cantwell-Jones et al., 2023; Fründ et al., 2013; S. Naeem & Li, 1997). For example, flies often forage when bees or butterflies do not (Inouye et al., 2015), which is particular important for early flowering crop species such as apples (Ssymank et al., 2008). Moreover, phenological asynchronies between plants and pollinator could increase in the future due to climate change, which could be mitigated by enhancing the diversity of pollinators (Bartomeus et al., 2013). Nonetheless, functional redundancy can only secure pollination services up to a certain level; in the case of a sudden collaps of a functional group, given that they are usually tightly connected in plant-pollinator networks, the risk in mutualistic networks may also increase the vulnerability in the case of a sudden collapse (Lever et al., 2014).

Numerous wild-pollinators contribute also significantly not only as pollinators, but also provide other ecosystems services: Syrphidae (hoverflies), for example, contribute to crop pollination and their predatory larvae are natural biological control agents of certain pest species (Dunn et al., 2020; Lundin et al., 2013; Pekas et al., 2020). Aculeate (stinging) wasps also do not just contribute to pollination, but also to biocontrol, decomposition, as well as biological indicators, independent if they are social or non-social species (Brock et al., 2021). Directly linked conservation efforts are often ecosystem-service-based approaches with a focus on flowering food sources, particularly targeting the most efficient pollinating species, and ignoring other food sources or non-food related requirements (Iwasaki & Hogendoorn, 2021; Requier & Leonhardt, 2020; Wood et al., 2015; further details in section 5.3). However, strategies centered on ecosystem-based conservation efforts frequently overlook the most endangered pollinating species, as they proportionally contribute less to the overall pollination of mayor crops (Kleijn et al., 2015).

Pollinator diversity is also tightly connected to plant diversity (Biesmeijer et al., 2006; Ferreira et al., 2013; Ramos-Jiliberto et al., 2020). It is, therefore, not surprising that the high number of potential pollinators of apple and caraway also interacted with many other flowering plant species within the surrounding landscape and was not restricted to plant species within the sampling transect. Regarding nectar availability, attractiveness, floral morphology, and biochemical factors of the flowers (i.e., scent, color, and nutritional values of the nectar or pollen), the plant taxa diversity observed in the plant-pollinator networks differs significantly. These functional flower traits are crucial determinants in whether or not a flower will be visited, the level of flower fidelity and affinity, as well as the fitness and abundance of pollinators (Abrol, 2011; Junker et al., 2015; Van Rijn & Wäckers, 2016; Wäckers, 2004). Flowers with restricted access to the nectaries, the floral tissue that produces nectar, or sometimes also oils or scents, are usually pollinated by long-tongued bees and other

insects (Faegri & Pijl, 1979; Sedivy et al., 2013; Wood et al., 2021), while flowers with easy access to floral resources are often visited by dipterans (Woodcock et al., 2013).

Exploring the effectiveness and efficiency of wild pollinators

The pollinators' "performance" is often defined as effectiveness and efficiency. Although there are numerous definitions for these two parameters, following the modular definition by Ne'eman et al. (2010), pollen deposition effectiveness can be defined as the pollinator's contribution to pollen deposition and measured as the number of pollen grains delivered by a pollinator to the stigma of a given flower. On the contrary, the pollen deposition efficiency of a pollinator can be defined "as the pollinator's contribution, by deposition of conspecific, compatible and viable pollen grains on the receptive target stigma in relation to the maximal possible female reproductive success (i.e., maximum seed set with no pollen limitation). Thus, pollen deposition efficiency refers to a measure that reflects whether a pollinator deposits enough pollen to achieve full seed set per flower" (Ne'eman et al., 2010).

The insect-targeted methodology used in Chapters 2 and 3 did not allow for a statement regarding the pollen deposition efficiency or effectiveness of the collected potential pollinators of apple and caraway. However, plant targeted sampling approaches, such as exclusion experiments, the analysis of fruit quality or fruit set (the proportion of a plant's flowers that develop into mature fruits or seeds), could provide evidence of the effectiveness of single insect species or communities (Boyle & Philogène, 1983; Garibaldi et al., 2013; Hünicken et al., 2021; Quinet et al., 2016; Webber et al., 2020). On the contrary, an efficiency assessment involves, among others, the evaluation of pollen quality, which include aspects like the viability, the presence of conspecific pollen grains in the pollen load, compatibility of pollen grains, as well as pollen surplus described as the maximum amount of pollen required for optimal seed formation (Jacquemart et al., 2006; Ne'eman et al., 2010; Razanajatovo et al., 2024). By now, other studies have already shown that wild pollinators can be as effective as managed pollinating species, particularly if wild pollinator populations can accomplish the visitation frequencies as high as or even higher than managed species (Rader et al., 2009), if they are bigger in size than managed species (Földesi et al., 2021) or have certain morphological traits, such as the level of hairiness (Stavert et al., 2016). In the context of climate change, the efficiency and effectiveness of pollinators is becoming an increasingly important topic to understand the plant reproductive consequences caused by changes in the

plant-pollinator network or the plant or pollinator communities (IPBES, 2019; Rafferty & Ives, 2013).

Beyond the plant or insect perspective: exploring the plant-pollinator networks

Examining the interactions between pollinators and flowering plants as mutualistic bipartite networks can help to get a deeper understanding of the stability of plant-insect interactions rather than solely focusing on targeted plant species (Bascompte & Jordano, 2007; Bascompte & Scheffer, 2023). Additionally, it allows to compare the network structure across various spatiotemporal scales and resolutions (Hemprich-Bennett et al., 2021; Memmott et al., 2004; Pornon et al., 2017; Renaud et al., 2020), leading to scale-dependent outcomes. Exemplarily, while at the landscape level, pollinator and plant diversity highly correlate (Ferreira et al., 2013; Ramos-Jiliberto et al., 2020), field-scale management is a better predictor of pollen-insect interactions when analyzing the plant-pollinator network. Consequently, while smaller-scale conservation efforts could provide better results in restoring the plant-pollinator network (Hall et al., 2022), large conservation efforts can help maintain plant and pollinator communities (see section 5.3).

Generalist plant and pollinator species, which represent the species with the highest number of links and also highlighted as such in Chapters 2 and 3, are considered the core of any plant-pollinator network, as they maintain the main network structure and functionality over time (Resasco et al., 2021; Zografou et al., 2020). Their ability to adapt and interact with a variety of plants and pollinators allows them to act as mediators in the face of environmental changes and disruptions (Blüthgen et al., 2006). This flexibility also allows generalists to occupy a broader range of habitats, making them less vulnerable to losing specific symbiotic partners (Resasco et al., 2021; Zografou et al., 2020). Consequently, losing abundant generalist plant species impacts the network's structure and enhances the sensibility to external changes and stressors. This loss can be managed if pollinator efficiency and diversity do not fluctuate simultaneously (Bain et al., 2022; Waser et al., 1996).

Even if single individuals of a pollinator species may show a high degree of specialization, the species could still be characterized as a generalist by aggregating the interactions of many intraspecific individuals (Araújo et al., 2021; Brosi, 2016). This pattern can often be observed when plant-pollinator networks are being analyzed at different organismal hierarchies, e.g. from individuals to communities (Pornon et al., 2017). Consequently, it particularly challenging to securely identify potential pollinating specialists of caraway and apple, since it requires a thorough sampling and analysis. Insufficient sampling of specimens, and

consequently the analysis of limited pollen samples, may lead to a distorted understanding of a species' level of specialization (Bosch et al., 2009; Dorado et al., 2011), what could be with the highly specialized species found in Chapter 2 and 3. Nonetheless, if there are true specialists identified, the high interdependence of specialist pollinators and plants can lead to a higher vulnerability in comparison to generalist species (Weiner et al., 2014). Contrarily, the influence of specialist species to the network structure is relatively minimal: the presence of specialist species generally do not alter significantly the overall robustness of plant-pollinator networks, particularly if specialist species are able to adapt their diets to changes in flowering sources (Bain et al., 2022; Fontaine et al., 2008; Gómez-Martínez et al., 2022; Zografou et al., 2020).

While plant-pollinator networks are commonly portrayed as a static snapshot over a specific time or space, their structure is far more complex and dynamic. By analyzing the interactions as a static snapshot, it is just possible to identify the general pattern of a network, missing the nuanced changes. Which underlying ecological process (e.g. from behavioural shifts of individuals specimens to dramatic community changes) is the targeted question is determined by the specificities of the spatiotemporal perspective through which they are analyzed (CaraDonna et al., 2021; Dupont et al., 2009). Therefore, temporal dynamics regarding intraday and intraseasonal shifts of plant-pollinator networks were more closely studied in Chapters 2 and 3. Over the years, despite the high variability in species and interaction composition, the network's general structure has remained constant, indicating that species can be replaced by topologically similar species (Dupont et al., 2009). Over a single day, changes in the availability of floral resources (e.g., pollen and nectar) and pollinators' activity can drive a turnover in interaction rewiring or species turnover (Nagano, 2023).

Especially in agroecosystems, which are characterized by intensive land use and fragmentation levels, the reduction of species richness of plants and pollinators adds to the vulnerability of plant-insect interactions (López-Vázquez et al., 2024; Morrison et al., 2020; Xiao et al., 2016). Nonetheless, the impact's extent of agricultural practices or other anthropogenic factors are always context-dependent and can vary across taxa (plants and pollinators) and regions (López-Vázquez et al., 2024).

5.2. Methodological strengths and limitations

The genomic revolution, with the rise of the analysis of genetic diversity via universal DNA barcodes, has fundamentally changed how we assess biodiversity. DNA barcoding and metabarcoding have become universal methods for detecting and monitoring species (Kestel

et al., 2022). Throughout this thesis, DNA barcoding and metabarcoding were implemented through various means: ranging from the identification of single insect specimens with DNA Barcoding (Chapters 2 and 3) to the analysis of mixed pollen samples (Chapters 2 and 3) and insect bulk samples (Chapter 4), both with DNA metabarcoding. The significant advantages and versatility of DNA barcoding and metabarcoding are showcased in the results presented in the Chapters before. The most important advantage is the increase in taxonomic resolution in the analysis of pollen samples and bulk samples. For instance, in the analysis of the plant-pollinator networks of caraway, this increased taxonomic resolution led to the identification of a greater number of plant-pollinator interactions, as seen in Chapter 2. These interactions would have remained unknown if only the morphological identification of the pollen loads would have been carried out.

Nonetheless, there were some methodological differences between Chapters 2 and 3, that need further attention: for the analysis of the plant-pollinator networks of caraway in Chapter 2, DNA metabarcoding was combined with the morphological identification of pollen loads. In contrast, the sole baseline data to analyze the plant-pollinator network of apple in Chapter 3 was the metabarcoded pollen samples. By combining both methodologies, it was possible to morphologically identify those plant species that may be challenging to be identified via DNA metabarcoding, due to a low number of pollen grains and, consequently, low DNA quantity. When merging the data, the number of plant taxa in the network might have been artificially inflated due to differences in taxonomic resolution between the methodologies. Here, the morphological identification of phenotypically similar but phylogenetically distant pollen types, as well as the identification up to family level was particularly challenging when combining with the DNA metabarcoding results. For example, the *Aster-Solidago* pollen type compiles *Aster* spp. and *Solidago* spp.. While both belong to the family of Asteraceae, they are currently polyphyletic and, therefore, not sister groups (Kang et al., 2024; Zhou et al., 2022). While a more conservative approach merging both datasets could have been performed following the approach from Jędrzejewska-Szmek & Zych (2013), it would have resulted in the loss of many insect-plant interactions. However, since the plant taxa identified with DNA metabarcoding and morphological identification matched at least at a higher taxonomic level, the plant-pollinator networks of apple, presented in Chapter 3, were then only analyzed by metabarcoding pollen loads. The differences in taxonomic resolution of morphological identification and DNA metabarcoding of pollen samples opens the question to what extent the plant-pollinator networks based on these two approaches generate comparable results and to what extant the network structure is otherwise the result of methodological biases.

The versatility of DNA metabarcoding is also showcased in Chapter 4 in the analysis of insect specimens from bulk samples. A common question remains to which extent metabarcoding results can be compared or aligned to morphological identifications of insect specimens to be able to conduct and interpret reliable bioassessment efforts with metabarcoding. Therefore, in Chapter 4, a combination of different bioinformatic pipelines with a non-destructive extraction protocol was tested to match the closest the morphological identification of Brachycera and Hymenoptera from bulk samples collected with Malaise traps on spinach fields. The use of a non-destructive protocol allows to keep the samples as a voucher, enabling later reexamination if necessary.

However, similarly to any emerging technique, there are also challenges and limitations associated with DNA metabarcoding that need to be considered when interpreting the results. These are also some the reasons why metabarcoding remains underutilized in agroecosystems (Compson et al., 2020; Kestel et al., 2022). For the analysis of mixed pollen and bulk samples, the lack of reliable quantitative or abundance data, the differences in quality and resolution of reference databases, and the correct selection of barcodes or primers are general limitations to consider when deriving conclusions from these analyses. Additionally, the lack of standardized protocols in DNA metabarcoding compared to the more established DNA barcoding hinders the comparison among similar studies. Differences in DNA extraction protocols, PCR reagents, sequencing protocols, and finally, the in-silico bioinformatic analysis are among the main components that can generate differing results (Bailet et al., 2020; Bohmann et al., 2022; Jeunen et al., 2019). To achieve standardization during DNA extraction, a universal modular DNA extraction method (Mu-DNA) could be a promising solution (Sellers et al., 2018). However, biases can be introduced also after the DNA extraction. A potential standardized solution for multiple steps using a modular framework to harmonize metabarcoding data was recently suggested by Arribas et al. (2022). This modular approach allows, in particular, flexibility for future methodological developments. While these two promising solutions are just a few of a long list of other methods, they all generally need further validation and testing.

An accurate identification at species level also depends highly on the proper selection of DNA barcodes and primers. For the analysis of arthropods and other animals, COI has been the standardized DNA barcode since the methodology was first introduced by Hebert et al. (2003), even though it may not always provide an adequate number of conserved regions, which are essential in amplicon-based metabarcoding (Deagle et al., 2014). Alternatives such as the ribosomal *16S* marker could be more appropriate depending on the application goals; however, the most extensive and most established reference databases for animals are still

based on COI (Deagle et al., 2014; Elbrecht et al., 2016). In agroecosystems, where the main goal is often to identify harmful insect pest species, multi-gene surveys (Cowart et al., 2015) and targeted primer sets could improve detection rates (Avalos et al., 2023). A proper selection of DNA barcodes to analyze plant material is sometimes even more challenging; While ITS2 still has the highest successful identification rate (S. Chen et al., 2010), a multi-gene approach combining this nuclear ribosomal with a plastid DNA barcode, such as *rbcL, matK, trnH-psbA or trnL-trnF* may enhance the detection and delimitation of plant species (Chase & Fay, 2009; S. Chen et al., 2010; CBOL Plant Working Group, 2009; Kolter & Gemeinholzer, 2021b). Still, ITS reference databases show the highest identification rate and, therefore, remain the preferred barcode to use, even though ITS plant reference databases are far from complete (Kolter & Gemeinholzer, 2021b). Consequently, conducting a study of plant-pollinator networks by metabarcoding the pollen loads, especially in remote areas, could be challenging, as flora in remote areas is typically not extensively studied and often missing in reference databases.

Moreover, the reference databases' scope, resolution, and quality can also impact the species identification rate (Cowart et al., 2015; Keck et al., 2023; Kolter & Gemeinholzer, 2021b). Taxonomic mislabelling, sequencing errors, sequence conflicts, taxonomic conflicts, low taxonomic resolution, missing taxa, and missing intraspecific variants are the most common obstacles (Keck et al., 2023). Proper curation of the databases with the removal or correction of false entries when necessary, the performance of multi-marker surveys, and the continuing addition of missing taxa could significantly improve the performance of databases. At least for insects, initiatives like GBOLIII: Dark Taxa can help to fill specific gaps in reference databases (Hausmann, Segerer, et al., 2020). Future developments in machine learning algorithms could also help to improve local databases, for example, by including spatial distribution data of targeted taxa (Kolter & Gemeinholzer, 2021b). Especially for the DNA metabarcoding of pollen from insects, developing local references with all surrounding flowering plant species is also a good alternative to improve taxonomic identification (Pornon et al., 2016). Nonetheless, many of these solutions are also associated with high management, curation and storage costs.

Another limitation between classical methodological approaches and DNA metabarcoding is the limited ability to gather abundance (quantitative) data from DNA metabarcoding. This was particularly relevant for the analysis of the pollen loads in Chapters 2 and 3, contributing to a limited interpretation of the interaction's strengths in the plant-pollinator networks. When analyzing plant-pollinator networks, the strength of the interaction is usually based on the interactions frequency which is often measured as visitation frequency (Bascompte et al.,

2006; Vázquez et al., 2007, 2012). Here, the underlying data to analyze the plant-pollinator network was a binary dataset of the presence-absence of a plant taxon, limiting the possibility of adding the pollen quantity. While some positive relationship between sequence count and flower-visiting frequency (Baksay et al., 2022; Pornon et al., 2016) or the number of pollen grains (Baksay et al., 2020) has been detected, it is still limited to certain plant species and therefore not universally applicable. Some of the possible solutions to get more accurate semi-quantitative data of pollen when applying DNA metabarcoding include the use of long-read technologies and limitation of amplification biases, such as shotgun metagenomics or minion sequencing (Lowe et al., 2022; Peel et al., 2019), as well as PCR-free genome-skimming (Lang et al., 2018). For the analysis of bulk samples, aside also from long-read technologies, pre-lab processing steps such as sieving (Elbrecht et al., 2017) or the use of mock communities as quantitative (Lamb et al., 2019) controls have been particularly compelling to get quantitative data. However, none of the methods mentioned before has yet led to a universal solution. However, quantitative data is a requirement in biodiversity monitoring and one of the biggest challenges when using metabarcoding for this purpose. By continuously improving the methodology, this issue could be overcome in the near future (Piper et al., 2019). Further developments and improvements in metabarcoding will also make this versatile methodology more accessible for researchers, governments, and NGOs (Compson et al., 2020; Hawthorne et al., 2024b; Macgregor et al., 2019).

5.3. Implications and recommendations for stakeholders

Considering the high levels of dependency on insect pollination of many crop species, mitigating the threats and safeguarding the diversity of pollinators and ecosystem services they provide is a critical matter that needs collective action and collaboration from diverse stakeholders. In agroecosystems, the recommendations and goals can be grouped into the following two categories: (1) support pollinators on agricultural landscapes and (2) enhance the scientific knowledge on pollinator diversity.

Support pollinators on agricultural landscapes

Farm management practices and the degree of intensification have a direct effect to the availability of qualitative foraging and nesting resources for pollinators (Kovács-Hostyánszki et al., 2017; Kremen et al., 2002; Potts et al., 2010); therefore, affecting directly and indirectly pollination services. Some farming systems and techniques can mitigate the negative impacts of intensified management to some degree. Yet, the quantity of research validating this

statement is derived primarily from studies of intensively or frequently studied pollinating species, such as wildbees or hoverflies. Studies on agricultural measures that can improve the conditions for other wild pollinators are rare (Davis et al., 2023). Nonetheless, under the assumption that wild pollinators identified in this study profit from alternative farming systems and techniques similar to prominent bee and syrphid pollinators, wild pollinators also benefit from agroecological principles and organic farming practices. While organic farming is restricted to the management type used by farmers, agroecological practices go beyond the field scale by considering the position, quality, and connectivity of the fields, as well as semi-natural or natural habitats at a landscape level (Jeanneret et al., 2021).

Generally, retaining or creating patches of natural vegetation helps to preserve local pollinator diversity (Cole et al., 2017; IPBES, 2019; Rahimi et al., 2021). However, given the limitations of retaining natural habitats and the potential financial burdens it may impose on farmers, a commonly implemented solution are so-called agri-environmental schemes (AES) (Batáry et al., 2015). Some of these schemes involve providing short-term payments to farmers in exchange for implementing prescribed environmental management practices to compensate for the farmer's loss (IPBES, 2019). Maximizing the potential benefits of the AES can be achieved by distributing them equally across a landscape, allowing an accumulation of their effect radius (Gill et al., 2016). Among the most popular AES is the establishment of flowering strips (Batáry et al., 2015), which are typically implemented along the edge of fields and are aimed to attract pollinators and provide secondary food sources after the main flowering period of the crop species (Ganser et al., 2018). Additionally, biocontrol properties for crop species have been identified, implying flowering strips also interrelate with parasitoids (Windsor et al., 2021). Flowering strips are usually composed of annual and perennial plant species: perennial plant species provide overwintering and nesting sites for insects (Ganser et al., 2019), while annual plant species are ecological focus areas that can be included in the usual crop rotation (Klatt et al., 2020). Generally, the plant species composition of the flowering strips determines the abundance and diversity of insect species being attracted (Albrecht et al., 2020; Kuppler et al., 2023; Ouvrard et al., 2018; N. M. Williams et al., 2015) and are particularly attractive for generalist and common pollinating species (Burkle et al., 2020). Nonetheless, the effect of flowering strips can significantly be enhanced when combined with other productive and non-productive measures, such as patches of natural vegetation, hedges, or organic crops (Gayer et al., 2021; Kremen et al., 2019; Sanchez et al., 2014; Von Königslöw et al., 2021; Wood et al., 2015), or diminished when e.g. pesticides are being used nearby (Fountain, 2022). Plant species like caraway or other medicinal plants could also function as "magnet-species" or ecologically beneficial mass-flowering crops

(Thomson, 1978; Zych, 2007), as discussed in Chapter 2. These plant species have the potential to increase the population of pollinators and other beneficial insects while also serving as an additional source of income for farmers, in contrast to flowering strips (Christmann et al., 2021). However, it is imperative to note that current AES may be inadequate to support non-honeybee pollinators (Dib et al., 2012; Pywell et al., 2005). A worldwide biased view toward bees as pollinators and little knowledge of the actual diversity of pollinators has caused a skewed development of AES and other conservation effort (Geldmann & González-Varo, 2018; Sivinski et al., 2011). Many AES usually focus on the provision of sufficient pollen and nectar; neglecting non-food related conditions that need equal consideration (Requier & Leonhardt, 2020; Wood et al., 2015). For instance, to efficiently enhance dipteran and other non-bee pollinating species, it is essential that current pollinator conservation strategies in agroecosystems not only consider food sources, but also the conditions needed during larval stages (Ramos-Jiliberto et al., 2020): wet organic material, dung or streams as habitat as well as other non-floral resources are substantial for many Diptera larvae (Davis et al., 2023; Raitif et al., 2019).

Farming practices can also have an effect on pollinator diversity. Organic farming does not only increase the overall species richness in agricultural landscapes (Tuck et al., 2014) but is particularly highly beneficial for many pollinator species (Gabriel & Tscharntke, 2007; Happe et al., 2018; Rosas-Ramos et al., 2020). This is primarily due to the exclusion of synthetic pesticides and mineral fertilizers that negatively impact the fitness of insects and greater variability in crop rotation and diversification (Beillouin et al., 2021; Bengtsson et al., 2005). While organic farming does not directly enhance pollination in apple orchards due to the limited timeframe where the service is being provided (Porcel et al., 2018), field size itself and the restoration of natural orchard edges have a cascading effect on pollination services (Hulsmans et al., 2023) by , exemplarily, increasing the biological control of apple orchards (Porcel et al., 2018).

On a more global scale, is the responsibility of policies and policy makers to develop best-management practices to ensure pollination services (Dicks et al., 2016; IPBES, 2019). Unfortunately, due to increased pressure on insect pollination in conjunction with a worldwide decline of pollinator species, policy measures targeting pollinator conservation in agroecosystems have only recently been brought to the forefront of the global agenda since the start of the 21st century. The International Pollinator Initiative, coordinated by the Food and Agriculture Organization (FAO), was among the first to address this issue (I. H. Williams, 2003). This first pioneering guidance has been since then further developed, among others, by the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services

(IPBES, 2019) and, most recently, the revised EU Pollinators Initiative from 2023 (European Commission, 2023) (Gemmill-Herren et al., 2021; Rose et al., 2016). Among the most pressing issues that still need to be improved are pesticide regulatory standards by, for example, promoting integrated pest management. Additionally, strategies to enhance wild pollinators' health through diversified farming systems and the conservation and restoration of semi-natural or natural habitats in agricultural and urban landscapes should be set up. Finally, it is vital to further develop long-term monitoring programs for pollination services, given their significance in understanding long-term stressors and their impact (Dicks et al., 2016; Hipólito et al., 2021; Stout & Dicks, 2022).

Research outlook

While this thesis showed the potential of many non-bee Hymenoptera and Brachycera to be pollinators of apple and caraway as two exemplarily different crop species, many questions still open that limit the interpretation of the results presented here. To give at least a preliminary assessment of the efficiency and effectiveness of the pollinators, it is necessary to get a deeper understanding of the ecology, phenology, and life-cycle dynamics of the potential pollinators since this information can directly affect the efficiency of conservation efforts. For example, for many dipteran pollinators, many floral management schemes can be insufficient to enhance dipteran pollinators (Davis et al., 2023). Additionally, there are substantial knowledge gaps regarding the conservation status and of many species, which are substantial to develop more efficient conservation and management efforts (Saunders et al., 2020).

The analysis of meta-networks, a combination or meta-analysis of plant-pollinator networks, could unravel underlying ecological processes at a landscape level as they provide new possibilities to map interactions across time or sites and identify the central nodes (Emer et al., 2018; Jordán, 2009). Central nodes can be understood as those key plant and insect species maintaining the structure of the networks and, when lost, have the most substantial detrimental effects on the whole system (Martín González et al., 2010). Especially when conservation efforts can just be targeted toward specific areas or periods in a mosaic landscapes, these measures could have their greatest positive impact by targeting these key plant-pollinator interactions (Devoto et al., 2014; Hall et al., 2022; Librán-Embid et al., 2024).

Despite the methodological limitations of DNA metabarcoding as outlined in section 5.2, DNA metabarcoding of pollen loads attached to Brachycera and Hymenoptera specimens allowed the identification of over two times more interactions than with traditional palynological

methods. Recent developments in the extraction and analysis of eDNA (environmental DNA) on flowers (Avalos et al., 2023; Banerjee et al., 2022; Johnson et al., 2023; Newton et al., 2023; Thomsen & Sigsgaard, 2019) or in combination with other conventional entomological sampling techniques, such as metabarcoding the pan trap water (Hawthorne et al., 2024b; Kestel et al., 2024) has paved new pathways away from an insect-targeted to a plant-targeted sampling (Evans & Kitson, 2020). For instance, Gamonal Gomez et al. (2023) detected eDNA of 12 out of 19 flower-visiting taxa on apple flowers, showcasing this method's current and future potential. Although there are still unresolved uncertainties surrounding the origin of the traces, the extent of visits required to leave genetic material on these flowers and whether these flower visitors also serve as pollinators may offer insight into potentially overlooked or rare interactions due to sampling restrains (Buxton et al., 2022; Macgregor & Scott-Brown, 2020; Requier et al., 2023b). Moreover, the study by Thomsen & Sigsgaard (2019) detecting DNA traces of at least 135 arthropod species from diverse ecological groups (e.g., pollinators, parasitoids, or predators) also revealed the potential to use eDNA approaches for biomonitoring through accurate analyses of large sample numbers. Challenges in assessing nocturnal pollinators could also be breached by analyzing eDNA on flowers. These nocturnal flower visitors are rarely addressed as important pollinators in crop production (Buxton et al., 2022) and have already been identified as significant contributor for apple production (Robertson et al. (2021).

Moreover, since insects are the primary pollen vectors of many flowering plants, the assessment of plant diversity targeting plant traces (e.g. pollen) extracted from the ethanol of Malaise traps harbors also has the potential to assess the plant diversity at a landscape level. While Malaise trap samples in combination with DNA metabarcoding can be utilized to determine the diversity of Brachycera and Hymenoptera as described in Chapter 4, when instead targeting plant traces, it is possible to effectively identify flowering species. This include plant species listed in the Red List or additionally pest species. By metabarcoding plant material from bulk samples could be much more efficient than relying on plant surveys alone (Swenson et al., 2022).

Finally, the combination of methods such as DNA metabarcoding of pollen loads, eDNA analysis, and inclusion of phylogenetic or functional traits as an integrative approach into higher complex ecological networks provide new opportunities to comprehend mutualistic and antagonistic interactions in agroecosystems such as pollination, predation, or herbivory (Allen et al., 2022; Banerjee et al., 2022; Evans & Kitson, 2020; Hawthorne et al., 2024b; Saunders et al., 2020). These complex ecological network analyses are a significant part of assessing ecosystem functioning and resilience to environmental changes and stressors

(Bohan et al., 2017; Derocles et al., 2018; Evans et al., 2016; Evans & Kitson, 2020). The analysis of ecological networks could additionally help to elaborate more efficient long-term monitoring schemes in more extensive spatial (from plant to landscape) and temporal scales to mitigate in the long-term insect decline (Petsopoulos et al., 2021; Xiao et al., 2016) while also enhancing the efficiency of current practices and guiding the future development of sustainable agricultural ecosystems (Allen et al., 2022).

Conclusions

1 DNA metabarcoding is a valuable method to analyze pollen samples retrieved from the pollen loads attached to Brachycera and Hymenoptera.

2 DNA metabarcoding of pollen samples does not only showcase a similar diversity than palynological identifications, but also determined plant taxa at a higher taxonomic level.

3 DNA metabarcoding of pollen loads is a valuable methodology to analyze plant-pollinator networks in temperate agroecosystems.

4 The diversity of potential brachyceran and hymenopteran pollinators of caraway and apple is much higher than previously studied, as it includes many non-syrphid Brachycera and non-wildbee Hymenoptera. Unlike previous studies, which relied on surveying plant-pollinator interactions under the assumption that flower-visitors are usually pollinators, the studies presented here were able to identify accurately specific species responsible for pollen transport.

5 The analysis of plant-pollinator networks with a focus on potential Brachycera and Hymenoptera as pollinators provides more ecological information than just analyzing the pollinator diversity of the targeted crop. Particularly the dynamics in plant-pollinator interactions along various temporal scales can be identified and outlined, as well niche complementarity between different insect taxa.

6 With the analysis of the plant-pollinator networks´ structure, it is possible to identify species essential to preserve the network architecture and the associated resilience.

7 DNA metabarcoding on pollen loads revealed temporal variation in plant taxa composition, specifically between Brachycera and Hymenoptera species sampled from caraway fields or between the years for species collected in apple orchards. These variations were likely attributed to differing flower affinity in the case of caraway pollinators or differing availability of flowering plants during the apple flowering period.

8 Although non-destructive DNA metabarcoding can improve the assessment of Brachycera diversity in bulk samples, challenges remain in analyzing Hymenoptera. For Hymenoptera, a proper diversity assessment still relays on morphological identifications.

9 Depending which bioinformatic pipeline combining clustering and filtering tools is being used in combination with a non-destructive extraction protocol, DNA metabarcoding of insect bulk samples can lead to substantially different diversity assessments. For Brachycera, a combination of ASVs clustered to OTUs inferred the closest the diversity identified with morphological traits.

10 Despite current limitations in DNA metabarcoding, it is a powerful method to assess plant-pollinator interactions.

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Additional publications & conference contributions

Non-scientific contributions:

Kilian, I.C. (2023). Los insectos y la polinización: un servicio ecosistémico vital. Catálisis Revista Digital. Vol 5. No 9. La importancia de los insectos para hoy y el futuro. 25-28. https://www.catalisisec.com/_files/ugd/780a0c_609b17445bd14c2cb628992e2c568019.pdf

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Additional peer-reviewed publications (co-) authored during the period of the thesis:

Motivans Švara, E., Ştefan, V., Sossai, E., Feldmann, R., Joy Aguilon, D., Bontsutsnaja, A., E-Vojtkó, A., **Kilian, I. C.**, Lang, P., Mõtlep, M., Prangel, E., Viljur, M.-L., Knight, T. M., & Neuenkamp, L. (2021). Effects of different types of low-intensity management on plant-pollinator interactions in Estonian grasslands. Ecology and Evolution, 11, 16909–16926. DOI: 10.1002/ece3.8325

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Conference Contributions:

Kilian, I. C., Swenson, S. J., Mengual, X., Gemeinholzer, B., Hamm, A. & Peters, R. S. (2022). Assessing the plant-pollinator of caraway (*Carum carvi* L.) via DNA (meta)barcoding [talk]. 36h Scandinavian Association for Pollination Ecology conference, Uppsala, 13 – 16 October 2022.

Kilian, I. C., Kurzrock, K., Swenson, S. J., Ssymank, A., Wägele, W.J., Hamm, A., Peters, R. S., Gemeinholzer, B., & Mengual, X. (2019). Assessing the role of Syrphidae as potential pollinators in agro-ecosystems vis DNA-Metabarcoding [talk]. 10th International Symposium on Syrphidae, Lesvos, 8 – 12 September 2019.

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