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ORIGINAL ARTICLE

Assessing flower-visiting arthropod diversity in apple orchards through metabarcoding of environmental DNA from flowers and visual census

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Abstract

Arthropods are essential in maintaining healthy and productive agricultural ecosystems. Agricultural crops such as apples are typically pollinated by domesticated honey bees, but wild bees and other arthropod flower visitors also contribute to pollination. Flower visitors can also be natural enemies of crop-pests or herbivores. Biodiversity is under pressure and knowledge of wildflower visitors is an important tool in designing orchards that can support high functional biodiversity. In our study, we assessed the diversity of arthropod flower visitors in four Danish apple orchards using both molecular and nonmolecular techniques to study arthropod communities in agricultural ecosystems. Arthropod DNA collected from apple flowers was analyzed using a DNA metabarcoding approach using the mitochondrial COI marker, while arthropod pollinators were recorded through visual assessment surveys. These complementary techniques resulted in a total of 19 arthropod taxa detected. Nonbee arthropods constituted a large proportion of arthropods detected by both methods (84%, 16 taxa). Metabarcoding detected 12 taxa and had 83% species resolution. Visual census recovered flower visiting groups to the order level (Coleoptera, Diptera, Hymenoptera and Lepidoptera) but not species level and also provided relative abundance data, which is not possible with molecular methods. We demonstrated that by utilizing both molecular and nonmolecular techniques to assess arthropod communities, we are able to obtain a broader overview of the arthropod fauna present. The methodology used and the outcome of this study can be used to inform and tailor suitable arthropod-pest management practices in orchards to increase crop yield and maintain healthy agricultural systems.

KEYWORDS
agricultural crops, biodiversity, environmental DNA, high-throughput sequencing, insects, molecular techniques

Physilia Ying Shi Chua and Lene Sigsgaard contributed equally and are joint last-authors.

1 | INTRODUCTION

Arthropod biodiversity in agricultural systems is crucial for maintaining health and resilience of agroecosystems (Angelella et al., 2021; McKerchar et al., 2020; Pardo & Borges, 2020; Rader et al., 2016). Biodiversity increases the overall system's stability towards changes in seasonal population fluctuations by optimizing pollination services (Fründ et al., 2013; Leonhardt et al., 2013). Pollinators and natural enemies of insect pests provide important ecosystem services (McCravy, 2018; Porcel et al., 2018; Saunders et al., 2016; Todd et al., 2020). In some groups such as Syrphids, arthropods can have more than one role in agricultural ecosystems where adults function as pollinators, and larvae are natural enemies of crop pests. Many of these species are currently facing multiple threats linked to anthropogenic activities such as land management, pesticides, and invasive species (García & Miñarro, 2014; McKerchar et al., 2020; Pardo & Borges, 2020). Species diversity, or more specifically complementarity, has been demonstrated to increase crop productivity and sustainability (Klein, 2011; McCravy, 2018; Nunes-Silva et al., 2020; Rader et al., 2016; Russo et al., 2015). Arthropod diversity and abundance are limited by the amount of noncrop flowering habitat size (hedgerows or flower strips) and pest management practices (Hambäck et al., 2021; Rader et al., 2020). Therefore, the conservation of semi-natural habitats in managed landscapes, such as apple orchards, is crucial for improving arthropod agroecosystem services (Herz et al., 2019; Joshi et al., 2016; Klein et al., 2007; Pardo & Borges, 2020). Apples are a perennial crop with 501 Kha cultivated in Europe and 1.5 Kha cultivated in 2020 in Denmark (EU Commission, 2021). It is highly pollinator-dependent, and most studies of apple pollinators have been focused on Hymenopteran species (Földesi et al., 2020; Pardo & Borges, 2020; Weekers et al., 2022). However, apple orchard fauna includes a broad diversity of arthropod pollinators like Coleoptera, Diptera, and Lepidoptera, of which some are also natural enemies of insect pests (Földesi et al., 2020; Herz et al., 2019; Lucas et al., 2018; Rader et al., 2016). Apples are a high-value crop and yields can be severely reduced by insect pests.

Visual assessments of pollinators provide data on pollinator groups. This relies on the observer's knowledge of capturing and identifying arthropod species and can be labor-intensive (Evans & Kitson, 2020; Russo et al., 2015). As insect activity is influenced by weather conditions (Rader et al., 2016; Ramírez & Davenport, 2013) and Danish spring weather is rather cold and windy, available periods for observations and resulting numbers of insects observed are limited. High-throughput sequencing (HTS) technologies have recently been used to study arthropod communities in managed ecosystems. One such approach, metabarcoding, consists of the amplification and detection of a specific sequence of interest from a mixture of environmental DNA (Evans & Kitson, 2020; Ruppert et al., 2019). This technique has many applications, including arthropod identification from different sources such as from bulk arthropod samples or from air (Polling et al., 2022; Roger et al., 2021; Yu et al., 2012). Metabarcoding of remnant arthropod DNA on plant samples is a relatively new tool that has just started to be used for plant-arthropod network research (Lowe et al., 2022; Ruppert et al., 2019; Thomsen & Sigsgaard, 2019). Compared to traditional nonmolecular techniques, the collection of samples used for metabarcoding is faster in the field. Molecular techniques such as metabarcoding can also potentially provide a more reliable overview of not only crop flower visitors but also arthropods present in the surroundings with better taxonomic resolutions and more broad results (Evans & Kitson, 2020; Ruppert et al., 2019). However, degraded DNA and inappropriate sample processing, such as sample contamination, might lead to skewed results (De Barba et al., 2014; Deagle et al., 2019). Moreover, this methodology is not appropriate for species abundance quantification studies (Evans & Kitson, 2020; Lamb et al., 2019; Todd et al., 2020). As arthropods are influenced by climatic and meteorological conditions, exhibit year-to-year and day-to-day variation, and can be too small, fast, and cryptic to be observed (Gibbs et al., 2017; Lucas et al., 2018), utilizing both molecular and nonmolecular techniques can provide a more complete overview of arthropods present in apple orchards.

For our study, we aimed to assess the diversity of flower-visiting arthropods in four Danish apple orchards and compare the outcomes of the following two techniques; (1) metabarcoding of arthropod DNA collected from apple flowers and (2) visual assessment surveys of all arthropod flower visitors present in study sites. Our results can be used for tailoring pest management strategies in orchards as well as conserving important arthropod species such as pollinators.

2 | MATERIAL AND METHODS

Arthropods were sampled in four apple orchards on Sealand, Denmark; one located 20 km north of Copenhagen (Frydenlund), two located 25 km and 37 km south-west of Copenhagen respectively (Kildebrønde, and Ventegodtgaard), and finally the Pometum located 16 km west of Copenhagen, belonging to University of Copenhagen (Figure 1a and see Table S1). Sites were separated by at least 9 km and located in an agricultural matrix. While Frydenlund and Kildebrønde were relatively large orchards with more than 20 rows of apples (more than 100 m wide), apple plots at the Pometum and Ventegodtgaard were only 7 and 10 rows wide, respectively (less than 40 m wide). Ventegodtgaard was managed organically while the other three followed integrated pest management (IPM). We used the term organic as eco-friendly farming managed according to the Danish state approval. In Denmark, IPM management permits the use of few insecticides, principally pyrethroids. Only Pometum orchard had a treatment with pyrethroid against winter moth larvae. The orchards had honey bee hives placed either within the field (Frydenlund and Kildebrønde) or in the surrounding crops (the Pometum and Ventegodtgaard).

Arthropods were sampled in four different distances from the margin of the orchard (side of orchard where flowers strip was sown): row 1 (first row of apple trees 0 m from the margin, at the edge of the orchard), row 3, row 5, and row 10. These rows are approximately 5, 10, and 25 m from the margin of the orchard, respectively (Figure 1b).

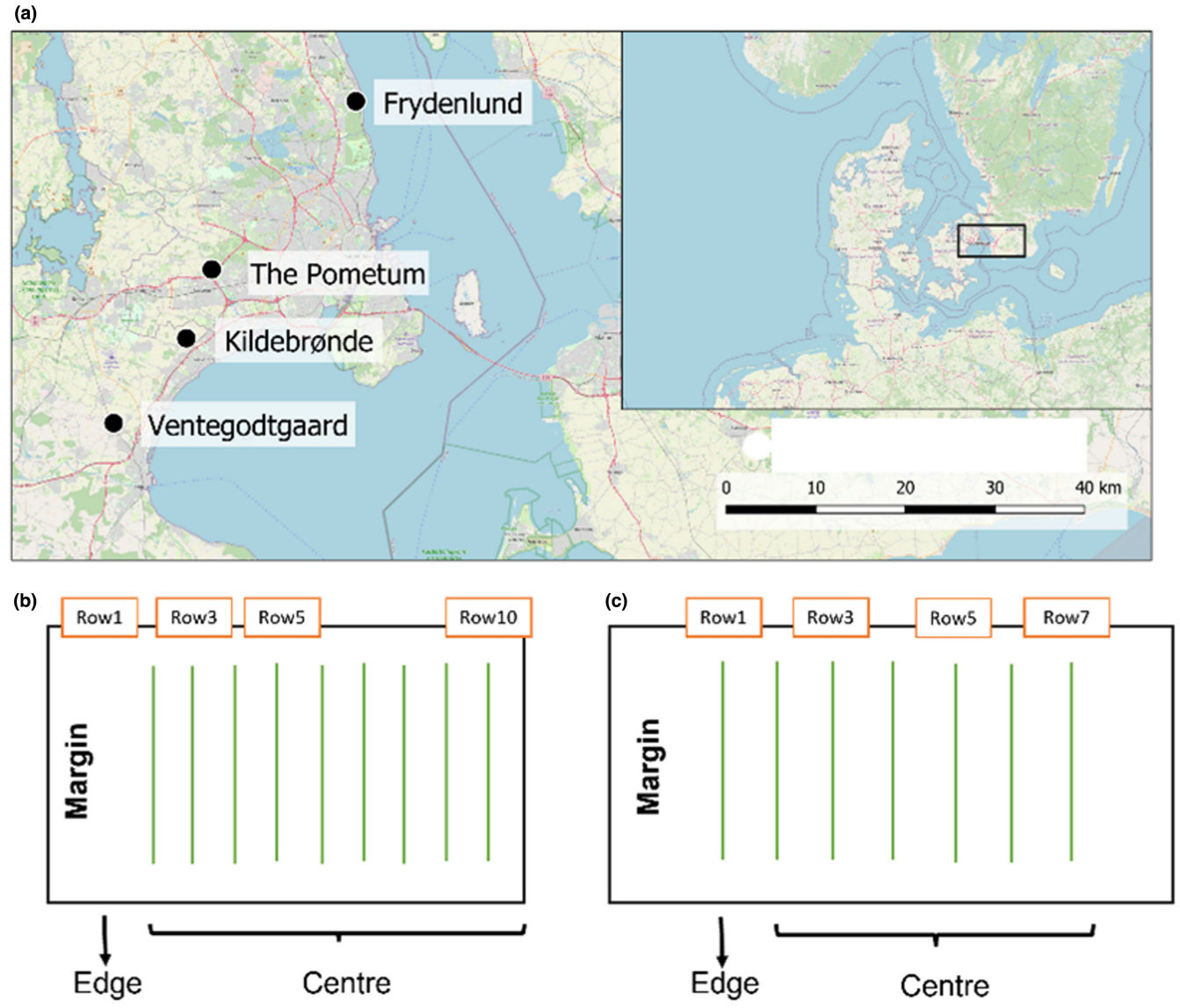

FIGURE 1 (a) Location of the four apple orchards where apple flowers ($n = 60$) and arthropods ($n = 279$) were collected, (b) rows disposition at Frydenlund, Kildebrønde, and Ventegodtgaard, and (c) rows disposition at the Pometum

In the smallest orchard, at the Pometum, there was more distance to the flower strip as there was a 10 m grass strip. In addition, tree rows in the Pometum were wider apart so the last row in this orchard was row 7 and was also approximately 25 m away from the margin, facing a strip of grass followed by a pear orchard (Figure 1C). Finally, in Ventegodtgaard, row 10 was the final row before a hedgerow (Figure 1b). The vegetation surrounding the orchards differed in species richness and abundance across the orchards. Frydenlund margin had eight different flower species present when sampled. Pometum had twelve flower species and pear trees flowering near the apples. On the other hand, Ventegodtgaard and Kildebrønde only had one species of flower and the margin was mainly composed of grass (See Table S2). Sampling was conducted when the percentage of open apple flower buds was between 50 and 90%. This occurred in late May 2020 for two weeks until the end of the apple flowering period.

2.1 | Metabarcoding of apple flowers

We followed the methods from Thomsen and Sigsgaard (2019) to analyze the environmental DNA (eDNA) present in the sampled apple flowers. In the morning, five individual apple flowers were collected in rows 1, 5, and 7 or 10 of each orchard. In comparison to Thomsen and Sigsgaard (2019) who collected 56 flowers, we collected a total of 60 flowers picked individually and stored in separate sterile plastic tubes (50 ml, Thermo Scientific). Collection was done

using single-use sterile nitrile gloves to avoid contamination. Flower samples were stored at −20°C prior to DNA extraction.

2.1.1 | DNA extraction

DNA extraction was carried out at the Department of Plant and Environmental Science laboratories, University of Copenhagen. The experiment was performed in a PCR (polymerase chain reaction)-free laboratory to prevent contamination. DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit and protocol in a flow hood. First, the whole apple flower was transferred to a 2-ml Eppendorf tube prior to DNA extraction. Lysis was performed by adding 900 µl of a cell lysis solution (ATL buffer) and 100 µl of proteinase K. Samples were disrupted in the TissueLyser II for 2 min at 30 Hz and incubated at 56°C with agitation in a rotor for 3 h. Samples were vortexed for 10 s before transferring 800 µl of the lysis mixture to a new 2-ml Eppendorf tube. About 800 µl of lysis buffer (AL buffer) was added and the mixture was mixed thoroughly by vortexing before incubation at 56°C for 10 min; 800 µl of absolute ethanol was added to the mixture, followed by vortexing before adding the mixture to the spin columns. The mixture was spun through the membrane filter over three rounds (700 µl per round) with 1.5 min of centrifugation at 8000 rpm after each round. The flow-through was discarded every round. The Spin columns were washed by adding 600 µl of wash buffer (AW1) and centrifuged for 1.5 min at 8000 rpm, followed by adding 600 µl of AW2, and centrifuged for 3.5 min at 14,000 rpm. Each spin column was transferred to a new 2-ml Safe-lock Eppendorf tube and DNA was eluted in 2 × 60 µl AE buffer with a 15 min incubation step at 37°C before centrifugation (1.5 min at 10,000 rpm). One extraction blank was included at the beginning of the process to test for possible contamination during the procedure. DNA extracts from apple flowers collected from the same row, but different apple trees in the same orchard, were pooled according to the DNA concentration and 260/280 ratio, measured by Microvolume spectrophotometer (mySPEC, VWR) (See Table S3). This resulted in a total of 36 pooled DNA extracts and one extraction blank stored at −20°C prior to further analysis.

2.1.2 | PCR amplification

All DNA extracts including the extraction blank were sent to *AllGenetics & Biology SL* (www.allgenetics.eu) for PCR amplification and sequencing. For eDNA flower metabarcoding, a 157 bp fragment of the COI genomic region was amplified using the primers ZBJ-ArtF1c (5′ AGA TAT TGG AAC WTT ATA TTT TAT TTT TGG 3′) and ZBJ-ArtR2c (5′ WAC TAA TCA ATT WCC AAA TCC 3′) (Thomsen & Sigsgaard, 2019; Zeale et al., 2011). Three PCR replicates were generated for each sample. Polymerase chain reactions for each sample were carried out in a final volume of 12.5 µl, containing 1.25 µl of template DNA, 0.25 µM of the primers, 6.25 µl of Supreme NZYTaq 2× Green Master Mix (NZYTech), CES 1×, and ultrapure water up to 12.5 µl. The PCR was incubated as follows: an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 49°C for 45 s, 72°C for 45 s, and a final extension step at 72°C for 7 min. The oligonucleotide indices that are required for multiplexing different libraries in the same sequencing pool were attached in a second PCR round with identical conditions but using only five PCR cycles and 60°C as the annealing temperature. A PCR blank that contained 1.25 µl of ultrapure water instead of DNA (BPCR) was included to check for contamination during PCR library preparation. Additionally, PhiX Control v3 (Illumina) was used as a control library for Illumina sequencing runs. The PCR libraries were run on 2% agarose gels stained with GreenSafe (NZYTech) and imaged under UV light to verify the library size (227pb). Polymerase chain reaction libraries were purified using the Mag-Bind RXNPure Plus magnetic beads (Omega Biotek), following the instructions provided by the manufacturer. Polymerase chain reaction libraries were pooled together in equimolar amounts. Pooled PCR libraries were purified using the Mag-Bind RXNPure Plus magnetic beads (Omega Biotek). The pooled PCR libraries were sequenced in 3/16 of an Illumina MiSeq PE300 run.

2.1.3 | Data analysis

Illumina paired-end raw files consist of forward (R1) and reverse (R2) reads sorted by PCR libraries and quality scores. The indices and sequencing Illumina adapters were trimmed during the demultiplexing step. Any remaining Illumina adapters were removed using the software CUTADAPT (Martin, 2011). The resulting trimmed sequences were used for further analysis. The analysis of the trimmed sequences was carried out using the OBITools package, which allows sorting and filtering of sequences based on the taxonomy (Boyer et al., 2016). A total of 36 samples, one extraction blank (sample 37), and one PCR blank (BPCR) were included in the dataset. Forward and reverse reads were first merged (ILLUMINAPAIREDEND) and unaligned sequence records were removed with an alignment score below 40 (OBIGREP). Reads were dereplicated into unique sequences by OBIUNIQ. Sequences with only a single copy (singletons) and shorter than 100 bp were removed by OBIGREP. Amplification and sequencing errors generated during PCR and sequencing were identified and cleaned with OBICLEAN, using a threshold ratio of 5% (De Barba et al., 2014). For Taxonomic assignment, the EMBL reference database (Deiner et al., 2017) was built through ecoPCR, as it is one of the main public databases used for taxonomic assignment of *COI* insect sequences (Meiklejohn et al., 2019). Taxonomic assignment for Zeale primers (Zeale et al., 2011) was performed using the ECOTAG program, which compares each sequence of the dataset to the created taxonomic database (Boyer et al., 2016). Post-OBITools sequence filtering and merging of the taxonomic assignments were carried out with R-studio 1.4.1103 (R Core Team, 2020). Following the analysis detailed in Chua et al. (2021), only sequences

that fulfilled the following criteria were kept: (i) matched 98% of our reference database, (ii) had a minimum of three reads of each taxon observed within each PCR replicate (See Table S4), and (iii) occurring in at least two out of three PCR replicates of a sample (Ficetola et al., 2015; Rasmussen, Nielsen, et al., 2021) (see Table S5). These criteria were followed to minimize the risk of false positives. No sequences were found in the extraction blank and PCR blank when checking for possible contamination. Only taxa that belonged to the arthropod phylum were kept (See Table S6). Sequences from the final dataset after filtering were also matched to the Barcode of Life Data System (BOLD) to check for discrepancies. For mismatches between the EMBL and BOLD database, we carried out the following: (i) BOLD identification was kept if it was at a lower taxonomic resolution than EMBL and (ii) for mismatches at the species-level, we reclassified the taxa to genus level if neither or both species can be found in Denmark (see Table S6). The resulting output was grouped according to four different orders: Blattodea (BT), Coleoptera (CP), Diptera (DI), and Lepidoptera (LP) (see Table S7).

2.2 | Visual census

The data from the visual assessment were generated as part of the Beespoke project, where the effect of arthropod flower visitor diversity and abundance was assessed as a function of distance from a flower strip. The visual assessment protocol was adapted from Westphal et al. (2008). An observer noted all the arthropod flower visitors 2.5 meters on each side of the observer (covering two rows of apple trees) at each of the four different distances previously established from the margin of the orchard. The type and number of arthropods were recorded for five minutes in each observational transect walk. Arthropods were identified to morpho-groups: Bumblebee (BB), Coleoptera (CP), Diptera (DI), honey bee (HB), Lepidoptera (LP), Syrphid (SY), and wild bee (WB). This classification was established following previous studies on typical pollinators found in apple orchards (Ramírez & Davenport, 2013). We did not sort Hymenoptera to family level but instead sorted them according to honey bees, wild bees, and bumblebees as foraging habits and activity differ within the same family (Delaplane et al., 2000; Földesi et al., 2020; Gardner & Ascher, 2006; Pardo & Borges, 2020). Transects were performed at each orchard during two different times of the day: mornings (between 9:00 and 12:00) and afternoons (between 13:00 and 16:00). We sampled twice in Kildebrønde, the Pometum, and Ventegodtgaard, once in the morning and once in the afternoon. We sampled thrice in Frydenlund, once in the morning, and twice in the afternoon. Surveys were only carried out when wind speed was not exceeding 7 m/sec and a threshold temperature of 10°C on sunny days and 15°C on overcast days (Ramírez & Davenport, 2013). These factors decided when to select the days and times for sampling (using regional weather forecast from Danish Meteorological Institute).

2.3 | Statistical analysis

For metabarcoding, statistical analysis was carried out using the R package *vegan* (Oksanen et al., 2020). Only taxonomic units assigned to Arthropoda and present in Denmark were considered (see Table S8). Sequence counts were analyzed in two different ways: presence/absence of each insect taxa measured using the frequency of occurrence (Fo) and relative read abundance (RRA) obtained by proportional summaries of counts (Deagle et al., 2019). Read counts were transformed into RRA data using the *decostand* function from R package *vegan* (Oksanen et al., 2020). Summaries based on occurrence data (Fo) are more sensitive to rare taxa and pooled samples than RRA (Deagle et al., 2019). Thus, results were discussed using both RRA and Fo values. We used order as a taxonomic unit for metabarcoding diversity analysis to compare results from both methodologies.

For visual assessment, the sampling effort was different across the orchards. Due to meteorological conditions and orchard location, the four orchards were sampled on a different number of days. Honey bee (HB) data were not included in the arthropod richness analysis as it occurs in Denmark only as a managed species (Rasmussen, Dupont, et al., 2021). We considered morpho-groups as taxonomic units for arthropod composition analysis. The effect of honey bee hives on wild pollinators was studied. Orchards with honey bee hives placed within the field were considered as orchards with close honey bee hives (Kildebrønde and Frydenlund), while those with hives found in the surrounding crops were established as orchards with distant hives (Ventegodtgaard and the Pometum). Wild pollinators were defined as nonhoney bee insects. Chi-squared test was used to test for dependency between the variables, while the type of relationship was measured by an ODDS ratio and risk estimate from R package *fmsb* (Nakazawa, 2021).

To avoid biased results due to the small sample size and sampling effort, rarefaction tests were carried out for both the molecular and nonmolecular analyses to assess sampling completeness and the relationship between arthropod richness and type of orchard (Gotelli & Chao, 2013; Russo et al., 2015). We used arthropod order and the orchard identity to compare both the molecular and nonmolecular methodologies. Arthropod communities were compared using 95% confidence intervals (Chao1 estimator) of the rarefaction curves and extrapolation of Hill numbers (species richness [q = 0]). Differences across the expected diversities are significant when 95% confidence intervals do not overlap (Chao et al., 2014). The R package *iNEXT* (Chao et al., 2014; Hsieh et al., 2016) from R version 4.0.3, based on the bootstrap method, was used to assess the uncertainty of the proposed sample completeness measure. Orchard (Frydenlund, Kildebrønde, the Pometum, and Ventegodtgaard) was used as a sampling effort unit. For the molecular analysis, only occurrence data (Fo) were used as it represented the presence and absence data of the different genera in the orchards.

3 | RESULTS

3.1 | Metabarcoding

From our metabarcoding data, we generated 1,322,923 reads and 5273 sequences matched to the EMBL database (See Table S4). However, 4918 sequences (1,147,682 reads) were removed due to either unsuccessful PCR amplification or filtering parameters (see Table S4). The final dataset consisted of 355 sequences (175,241 reads) with a minimum percentage identity of 98% to at least one taxon in the EMBL reference database after taxonomic assignment (see Table S5). One hundred and twenty-nine of these sequences belonged to an oomycete (Peronosporaceae family). After comparing with BOLD reference database and merging the sample replicates (see Table S6), the final dataset consisted of 12 insect taxa and 143,838 high-quality reads (See Tables S7 and S8). The taxonomic resolution was 17% at genus level (2 taxa) and 83% at species level (10 taxa) (Table 1). Due to ambiguity in two of the taxa identified from both databases and their status in Denmark (DanBIF Secretariat, 2021; Naturbasen, 2021), we corrected the following: (i) matches to *Lonchoptera uniseta/L. bifurcata* were changed to *Lonchoptera*, and (ii) matches to *Yponomeuta padella/Y. malinellus* were changed to *Yponomeuta*.

3.1.1 | Arthropod composition

The composition of arthropods found in the apple orchards differed based on the metabarcoding analysis used: frequency of occurrence (Fo) or relative read abundance (RRA) (see Table S9). Based on Fo analysis, the occurrence of Diptera (DI) in apple orchards was the highest as compared to the other orders at 44% (Figure 2a), whereas based on RRA analysis, Lepidoptera (LP) had the highest read counts at 49% (Figure 2b). The least common arthropod groups were Blattodea (BT) and Coleoptera (CP) for both analyses (Figure 2a, b).

3.1.2 | Arthropod richness

Sampling coverage (SC) indicated sample adequacy at Frydenlund, Kildebrønde, and the Pometum, but with values below the estimated total (SC < 0.95) in Ventegodtgaard (See Table S10). Thus, estimated values were used to compare arthropod richness (q = 0) across sites. Rarefaction curves showed that there were significant differences in arthropod richness (nonsuperimposed confidence intervals) between Frydenlund (Sestimated = 2) and Ventegodtgaard (Sestimated = 4.47) (see Figure S1). All arthropod orders were detected in Ventegodtgaard. Blattodea and Coleoptera were not detected in Frydenlund, Blattodea was not detected in Kildebrønde, and Diptera was not detected in the Pometum (See Table S9).

3.2 | Visual census

3.2.1 | Arthropod composition

A total of 279 individuals within 7 taxa were identified through visual census. The most abundant arthropods were honey bees (47%), followed by Diptera (38%) (Figure 3). The least abundant arthropods detected were bumblebees and Syrphidae (5% each), Coleoptera (3%), wild bees (2%), and Lepidoptera (0.7%). Nonbee pollinators constituted 47% of total individuals recorded.

3.2.2 | Arthropod richness

Sampling coverage (SC) indicated sample adequacy at Frydenlund and Ventegodtgaard, but with values below the estimated total (SC < 0.95) in Kildebrønde and the Pometum (See Table S12). Thus, estimated values were used to compare arthropod richness (q = 0) across sites. Rarefaction curves showed that there were no significant differences in arthropod richness between the orchards (See Figure S2). All arthropod morpho-groups were detected in Ventegodtgaard. Lepidoptera was not detected in Frydenlund, Syrphidae and Lepidoptera were not detected in the Pometum orchard, and Coleoptera was not detected in Kildebrønde (see Table S11). Regarding the arthropod abundance in the orchards, we detected a lower number of individuals in the Pometum (n = 42) as compared to the other orchards (Kildebrønde n = 85, Frydenlund n = 84, Ventegodtgaard n = 68) (see Table S11). However, this changes when we removed honey bees from the analysis (n = 149). Fewer wild pollinators were recorded in Kildebrønde (n = 20) and the Pometum (n = 33) as compared to Frydenlund (n = 47) and Ventegodtgaard (n = 49) (see Table S13). Honey bees were more common in orchards with hives inside the orchard compared to orchards with hives placed outside the orchard (>100 m away) (Odds ratio estimate, CI = 2.63–7.56, p = 1.193e-08, estimate = 4.46), where wild pollinators were observed twice as often as honeybees (Risk ratio estimate, CI = 1.43–2.13, p = 1.193e-08, estimate = 1.75).

3.3 | Comparison between metabarcoding and visual census

Using metabarcoding, we did not detect any Hymenoptera or Syrphidae. However, we detected other orders such as Blattodea that were not observed in the visual census. The arthropods detected from visual census were all adult pollinators that visited apple flowers (Figure 3). Coleoptera, Diptera, and Lepidoptera were detected using both metabarcoding and visual census (Table 2). Ventegodtgaard was the only orchard where all three orders were detected using both methods. While visual census recorded adult Lepidoptera, metabarcoding results would have been traces of immatures, based on the life cycle of species identified. Arthropod presence differed

TABLE 1 Arthropod composition found across all orchards through environmental DNA (eDNA) flower metabarcoding with the COI Zeale primers

Taxon assignment	Order	Family name	Genus name	Final taxa	Resolution final taxa	Stage	Functional group	Contribution to pollination
1	B	Blattodea	*Periplaneta*	*Periplaneta americana*	S	A	Omnivore	1[a]
1	C	Coccinellidae	*Harmonia*	*Harmonia axyridis*	S	A	Predator	1[b,c]
1	C	Nitidulidae	*Brassicogethes*	*Brassicogethes aeneus*	S	A	Herbivore	0[d]
1	D	Anthomyiidae	*Delia*	*Delia platura*	S	A	Herbivore	1[e]
2	D	Drosophilidae	*Scaptomyza*	*(Scaptomyza pallida)*	S	A	Herbivore	1[f]
3	D	Lonchopteridae	*Lonchoptera*		G	A	Herbivore, adults feed on nectar	1[g]
1	D	Muscidae	*Musca*	*Musca domestica*	S	A	Scavenger, Coprophage	1[h]
1	L	Geometridae	*Operophtera*	*Operophtera brumata*	S	L	Herbivore	0[i,j]
1	L	Geometridae	*Operophtera*	*Operophtera fagata*	S	L	Herbivore	0[i,j]
1	L	Tortricidae	*Pandemis*	*Pandemis cerasana*	S	L	Herbivore	0[j]
3	L	Yponomeutidae	*Yponomeuta*		G	L	Herbivore	0[k,l]
1	L	Yponomeutidae	*Yponomeuta*	*Yponomeuta cagnagella*	S	L	Herbivore	0[l]

Note: Taxon assignment: 1 = final taxon stays the same, 2 = final taxon changed to another species in the same order indicated by (), 3 = final taxon moved up a taxonomic level due to species not found in Denmark; changes based on Naturbasen (2021) and DanBIF Secretariat (2021). Order: B = Blattodea, C = coleoptera, D = Diptera and L = lepidoptera. Resolution final taxa: Final level at which taxa is assigned to S = species or G = genus or O = order. Life stage according to time of year sampling was done: A = adult, L = larva. Functional group: Role of arthropod in apple orchards during the specific life stage. Contribution to pollination: 0 = none (they do not act as a pollinator), 1 = some (they act as pollinators or based on data from related species, have the potential to act as pollinators).

[a]Macgregor and Scott-Brown (2020).

[b]Bertrand et al. (2019).

[c]Steenberg and Harding (2009).

[d]Sutter and Albrecht (2016).

[e]Cook et al. (2020).

[f]Pitkin et al. (2019).

[g]Stalker (1956).

[h]Ramírez and Davenport (2013).

[i]Holliday (1977).

[j]Svensson et al. (1999).

[k]CFIA (2006).

[l]Turner et al. (2010).

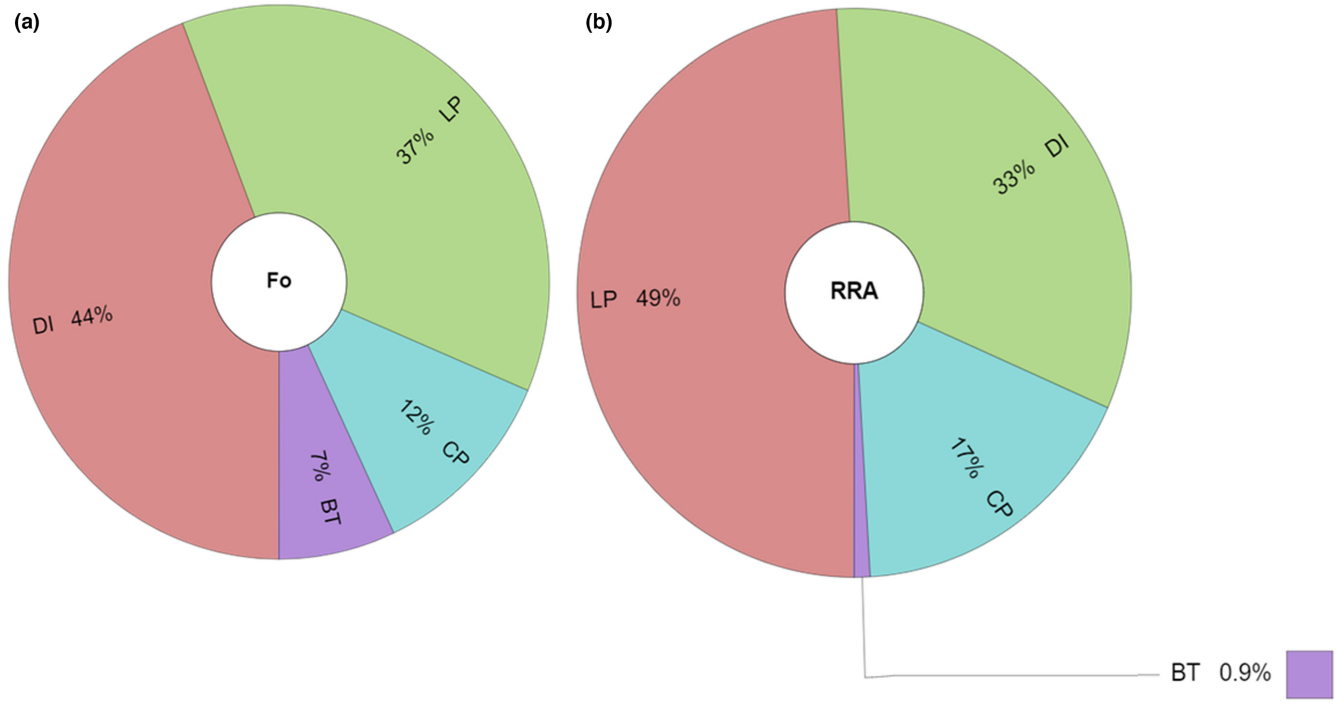

FIGURE 2 Krona chart showing the percentage of four different arthropod orders identified from COI metabarcoding of flower samples collected in the four orchards based on (a) frequency of occurrence (Fo) and (b) relative read abundance (RRA)

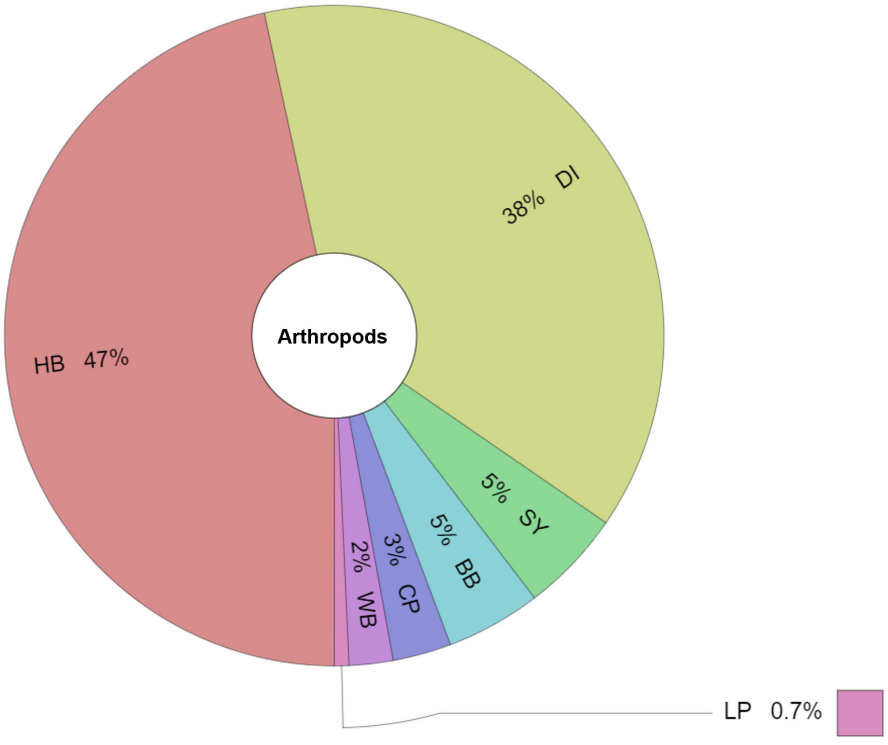

FIGURE 3 Krona chart showing the percentage of arthropod morpho-groups recorded by visual census in the four Danish apple orchards

between orchards (Frydenlund, Kildebrønde, the Pometum, and Ventegodtgaard) and methodologies (metabarcoding and visual census) in terms of the orders detected (Table 2). Ventegodtgaard had the highest arthropod richness for both methodologies. Based on metabarcoding, arthropod richness in Frydenlund was the lowest as compared to the other orchards, whereas, based on visual census, the Pometum had the lowest arthropod richness.

4 | DISCUSSION

We demonstrated in our study the reproducibility of using environmental DNA (eDNA) shed from arthropods on flowers to detect arthropod visitors in apple orchards (Thomsen & Sigsgaard, 2019). We further showed the complementarity of using metabarcoding and visual census to improve the current knowledge of arthropod

TABLE 2 Arthropod composition found in the four Danish orchards with metabarcoding (frequency of occurrence (Fo)) and visual census

	Frydenlund		Kildebrønde		The Pometum		Ventegodtgaard	
Arthropod	*Fo*	*Visual*	*Fo*	*Visual*	*Fo*	*Visual*	*Fo*	*Visual*
Diptera (DI)	4	41	8	12	0	27	7	26
Coleoptera (CP)	0	1	1	0	3	1	1	6
Lepidoptera (LP)	4	0	3	1	4	0	5	1
Arthropod richness (Sestimated)	2	5.98	3	6.9	3	4.97	4.47	6.88

Note: Arthropod richness is shown by the Sestimated by rarefaction analysis results from both methodologies.

communities visiting flowers in apple orchards. Metabarcoding detected DNA traces of several arthropod herbivores, some of which are apple pests. It also detected other flower-visitors, which may be more night-active and hence missed by visual census. As flowers for metabarcoding were collected in the morning, it could explain why we did not manage to detect any day-active Hymenoptera traces, whereas from our visual census surveys, we found that pollinator communities were mainly represented by Hymenoptera and Diptera. Below, we highlight some challenges regarding sample collection, analysis, and arthropod lifestyle to consider when using either technique for future studies.

4.1 | Arthropods identified in apple orchards

From our metabarcoding results, we detected DNA from some common apple flower visitors including some species from Diptera (e.g., *Musca domestica*) and Coleoptera (e.g., *Harmonia axyridis*). Additionally, we detected Blattodea that has not commonly been reported from apple orchards (García & Miñarro, 2014; Hambäck et al., 2021; Rader et al., 2020). These differences might be driven by the detection methods utilized, as traditional assessments used in other arthropod assessment studies in orchards might miss cryptic or nocturnal fauna (Deiner et al., 2017; Evans & Kitson, 2020; Geppert et al., 2020; Russo et al., 2015). As compared to a similar flower eDNA metabarcoding study where a broader diversity of arthropods was identified (135 taxa) (Thomsen & Sigsgaard, 2019), the lower diversity of arthropods detected in our study (12 taxa) is likely due to us sampling in an agricultural landscape, with the sampling period being limited to the flowering of apple trees during a cold spring. There were also many differences in the experimental settings between studies (Leonhardt et al., 2013; Marquina et al., 2019), where (i) their sampling season was in August when considerably higher temperatures are reached and therefore more arthropod visitors are present compared to spring (Gibbs et al., 2017; Rader et al., 2016; Ramírez & Davenport, 2013), (ii) they collected seven flower species, which would have attracted more flower visitors instead of just one flower species collected in our study (apple flowers) (Pardo & Borges, 2020; Quinet et al., 2016; Samnegård et al., 2019), and (iii) the use of two primers in their research instead of one (Mitochondrial COI (Zeale et al., 2011) and ribosomal 16S (Elbrecht et al., 2016)). This could also be the reason why we did not recover any sequences belonging to Hymenoptera and Syrphidae, as the COI primer that we used is a generic primer and could have failed to amplify DNA from these taxa (Lamb et al., 2019; Marquina et al., 2019; Rasmussen, Nielsen, et al., 2021). The amount of eDNA left by this group might have also not been enough to be amplified (Barnes & Turner, 2016). However, some studies such as Brandon-Mong et al. (2015) have shown that it is possible to detect Hymenoptera using these primers. But this was not observed from our dataset (Lamb et al., 2019; Marquina et al., 2019; Rasmussen, Nielsen, et al., 2021). The Zeale primer also nonselectively amplifies DNA fragments from other taxonomic groups other than arthropods (De Barba et al., 2014; Lamb et al., 2019; Marquina et al., 2019; Rasmussen, Nielsen, et al., 2021; Thines & Choi, 2016), which is demonstrated by the presence of an oomycete plant pathogen found in our data (Peronosporaceae). This suggests that the Zeale primers could potentially be used to identify mildew infections in apple orchards due to conserved regions shared between insects and oomycetes (Marquina et al., 2019; Rasmussen, Nielsen, et al., 2021). The analyses of metabarcoding data can also be limited by issues regarding incomplete DNA reference databases used for taxonomic identification (Deiner et al., 2017; Rasmussen, Nielsen, et al., 2021; Schenk et al., 2019). This is shown in our study, where some sequences were assigned to different taxa depending on the database used (Deiner et al., 2017; Rasmussen, Nielsen, et al., 2021; Ruppert et al., 2019; Schenk et al., 2019). Some sequences were assigned to *Periplaneta americana*, a species that has not previously been documented in Danish apple orchards (DanBIF Secretariat, 2021) except as an occasional pest in bakeries (Jensen, 1993). This raised the issue of possible contamination during the workflow that was not measured by the extraction blanks (Deiner et al., 2017). Nevertheless, both databases used provided high similarity matches for this species. As this is a cold-intolerant species (Tsuji & Mizino, 1973), the presence of *P. americana* could be attributed to individuals found in drainage systems near orchards (Bao, 1997; Evangelista et al., 2013), but we cannot ascertain that these species live outdoors in apple orchards. Even though eDNA metabarcoding can complement traditional methods by revealing species not previously recorded (Macgregor & Scott-Brown, 2020), we need to take into account species ecology when detecting species that have not been previously recorded. Hence, further studies with larger sample sizes would be needed to confirm this result.

The study of pollinators in orchards has mainly been focused on Hymenoptera, which is considered the most effective pollinator order (Dunn et al., 2020; Fründ et al., 2013; Pardo & Borges, 2020; Rader et al., 2020; Ramírez & Davenport, 2013). However, pollination

in apple orchards includes a wider range of wild insects such as Coleoptera, Diptera, and Lepidoptera (Alford et al., 2003; Földesi et al., 2020; Lucas et al., 2018; Rader et al., 2016). The findings from our visual census showed that although non-Hymenoptera pollinators were abundant in apple orchards (47%), Hymenoptera was the most abundant order observed (53%). This could be due to honey bee hives being placed within or near apple orchards to increase the success rate of pollination, explaining their high presence in our study (47%) (Corbet et al., 1991). Some challenges of using visual census for arthropod diversity assessment include long sampling time and expert knowledge in accurately identifying arthropods (Evans & Kitson, 2020), and sampling days being dependent on the weather (Ramírez & Davenport, 2013). Additionally, some of the most important crop pollinators such as bumblebees, hoverflies, and wild bees can be small, cryptic, fast, and difficult to identify through visual census (Corbet et al., 1991; Dunn et al., 2020; Leonhardt et al., 2013; Orford et al., 2015; Rader et al., 2020; Rasmussen, Dupont, et al., 2021). This could lead to an underestimation of pollinator presence and diversity assessed using visual census (Lefebvre et al., 2017; Rader et al., 2016).

Arthropod richness did not differ significantly across orchards between the metabarcoding and visual census results, although some differences did emerge between orchards. The Pometum orchard flowered later than the other three orchards and was sampled about 10 days later, so the absence of some arthropod groups might reflect the later flowering. Interactions among flower visitors and suitable additional resources also play a role in shaping arthropod communities (Delaplane et al., 2000; Delpon et al., 2019; Klein, 2011; Rasmussen, Dupont, et al., 2021; Valido et al., 2019; Weekers et al., 2022), where high densities of honey bees have been demonstrated to detrimentally affect wild pollinator populations in the orchards as an effect of competitive exclusion (Angelella et al., 2021; Klein, 2011; Rasmussen, Dupont, et al., 2021; Valido et al., 2019). This is also reflected in our study where we observed lower wild pollinators densities in the two orchards with honey bee hives placed inside the orchard (Frydenlund and Kildebrønde). Ventegodtgaard had the highest, though not significantly so, arthropod richness according to both methodologies as compared to Frydenlund and Kildebrønde. Hence in Ventegodtgaard, as showed by other studies in organic fields (Ahrenfeldt et al., 2019; Gomiero et al., 2011; Samnegård et al., 2019), organic management practice may have resulted in a higher arthropod presence.

4.2 | Comparison between metabarcoding and visual census

All arthropod orders detected with both methodologies were previously recorded in apple orchards (Garcés & Soto, 2000; Rader et al., 2020). Coleoptera, Diptera, and Lepidoptera were common across both methodologies. While metabarcoding detected DNA traces from herbivorous Lepidopterans, which are pests of apples during the larval stage when sampling was carried out, visual census showed few adult Lepidopteran species visiting the flowers. Even though only 60 flowers were collected, metabarcoding provided us with an insight into flower-visiting arthropod biodiversity. Apple orchards represent one of the most diverse cultivated agroecosystems with over 1000 arthropod species recorded (Šťastná & Psota, 2013; Szentkiralyi & Kozar, 1991). While apple pests are well known and described (Blommers, 1994), less is known about the communities of natural enemies and pollinators and how to augment them (Gomiero et al., 2011; Herz et al., 2019; Samnegård et al., 2019). The flowering season in Denmark is characterized by variable spring conditions, often windy and cold, which negatively affects arthropod activities (Gibbs et al., 2017; Ramírez & Davenport, 2013). While we selected sampling dates when minimum criteria for weather were met, a larger sampling effort may be needed this far north to detect certain groups such as hoverflies (Syrphidae) or wild bees (Apidae), which are more active in warm, sunny weather (Ball & Morris, 2015; Corbet et al., 1991; Fründ et al., 2013; Orford et al., 2015; Pardo & Borges, 2020). Here, metabarcoding can be extremely useful and allows us to study arthropod flower visitors' presence regardless of foraging periods. The arthropods identified using metabarcoding and visual census included arthropods with different roles in the ecosystem (pollinator, pest, or predator) and both nocturnal and diurnal arthropods. Metabarcoding also does not distinguish between developmental stages. This was shown through the detection of Lepidopteran species on some flowers that were observed to have larvae and frass presence when sampling took place. In contrast, visual census data were limited to observing adult arthropods, many of which are pollinators (CFIA, 2006; Deiner et al., 2017; Rader et al., 2020; Taylor, 2019; Turner et al., 2010).

Metabarcoding can provide species-level taxonomic identification as compared with traditional approaches (Evans & Kitson, 2020; Ruppert et al., 2019). Metabarcoding can also be a useful tool to study nocturnal or cryptic flower visitors that can be difficult to observe using visual census (Barnes & Turner, 2016; Deiner et al., 2017; Ruppert et al., 2019). Examples are Diptera and Lepidoptera, which might be present in the flowers early in the morning or during the night (Elberling & Olesen, 1999; Nunes-Silva et al., 2020; Rader et al., 2016, 2020; Ssymank et al., 2008). Thus, the higher presence of Lepidoptera (37–49%) and Diptera (33%–44%) detected with metabarcoding might be due to DNA traces being more recent and less degraded than other groups of arthropods when sampled in the morning (CFIA, 2006; Woodcock et al., 2014); or in the case of Lepidopteran larval traces, it could be due to frass left on flowers or shed skins (Feinstein, 2004). However, issues such as primer biases and incomplete DNA reference database used for taxonomic assignment probably led to the underrepresentation of typical pollinator taxa such as Hymenoptera and Syrphidae (Lamb et al., 2019; Marquina et al., 2019; Rasmussen, Nielsen, et al., 2021). Conservative filtering strategies used to minimize the risk of false positives could also have led to missing out on true detections (Barnes & Turner, 2016). Another consideration is that metabarcoding is very

sensitive and could have detected DNA traces transported from other locations by air (Clare et al., 2022; Deiner et al., 2017; Klepke et al., 2022; Lamb et al., 2019; Lynggaard et al., 2022; Roger et al., 2021; Todd et al., 2020), which may in turn lead to misrepresentation of flower visitor diversity (Barnes & Turner, 2016). Thus, more studies are needed to determine the exact sources of arthropod DNA found on flowers.

As metabarcoding of arthropod eDNA from flowers can also be applicable both in and outside of agricultural systems to study (agro) ecological networks including plant–animal interactions or the impacts of climate change in wild arthropod populations (Deiner et al., 2017; Liu et al., 2020; Lucas et al., 2018; Nordstrom et al., 2022), future studies should seek to optimize arthropod eDNA detection from flowers. To do so, the metabarcoding workflow can be tweaked such as using two or more primers, which will allow for adequate amplification of low-quality template DNA (Ficetola et al., 2015; Marquina et al., 2019; Thomsen & Sigsgaard, 2019; Zhan et al., 2014). This is consistent with other studies that support the need for multiprimer approaches to assess arthropod communities (Marquina et al., 2019; Thomsen & Sigsgaard, 2019). Future studies may also want to consider using longer COI primers specific to arthropods to increase taxonomic resolution (Aly, 2014; Salonna et al., 2021) or to use packages such as BOLDigger to access to private and early-release data for taxonomic assignment (Buchner & Leese, 2020). Increasing the number of biological and technical replicates, albeit at an increased cost and time, can also help to better detect rare and cryptic species (Deiner et al., 2017; Ruppert et al., 2019). As DNA traces in flowers may be short-lived, the inclusion of additional sampling in the afternoons may better capture traces of day-active flower visitors.

Visual census can provide a priori knowledge of the specific community being assessed, making it a valuable complementary tool to be used in conjunction with metabarcoding, and provides quantitative data (Ruppert et al., 2019). Even though metabarcoding reads can be converted into presence-absence data or relative read abundance (RRA), quantitative inferences of biomass are challenging as it depends on several well-documented variables. These include the starting DNA yield (larger species generate more reads), sample size (appropriate sample size to represent the sampled community), and primer selection (different primers can amplify different taxa within the same sample) (Blanckenhorn et al., 2016; Deagle et al., 2019; Lamb et al., 2019). Visual census, on the other hand, can be biased by risk of double-counting and is dependent on the expertise of the observer, whose presence could lead to an alteration of arthropod behaviors (Deiner et al., 2017; Todd et al., 2020). Hence, the utilization of either technique on its own has its limitations for arthropod diversity assessment, and forthcoming studies could consider employing both in unison to get a better overview. Additionally, the performances of other conventional nonmolecular means of studying arthropods in orchards such as malaise traps, sticky traps, or insect nets can be further investigated in comparison to metabarcoding of arthropod eDNA from flowers.

4.3 | Implications on orchard managements

Agricultural systems are facing several threats such as pollinator decline and climate change (García & Miñarro, 2014; McKerchar et al., 2020; Pardo & Borges, 2020). Having a high diversity of arthropods in these systems is seen as a way to overcome some of these threats (Dunn et al., 2020; Hambäck et al., 2021; Russo et al., 2015). Hence, the study of flower visitors is an important step towards preserving agricultural systems (Klein et al., 2007; Leonhardt et al., 2013; Porcel et al., 2018). Knowledge on beneficial and detrimental arthropods visitors to apple orchards is crucial for improving management techniques (Hambäck et al., 2021). We demonstrated in our study using both visual census and metabarcoding the existence and importance of wild nonbee pollinators such as Diptera, whose presence in apple orchards has been underestimated or even understudied in previous studies (Delaplane et al., 2000; Rader et al., 2020; Ramírez & Davenport, 2013). Further management practice should consider the trade-offs of some arthropods with dual roles in orchards depending on their life cycle. Interactions across groups of arthropod flower visitors, as well as nocturnal and diurnal ones, should also be taken into consideration for future management practices. Awareness about possible complementarity and competition among arthropods could be necessary to improve fruit yield and quality.

Our study detected the presence of two important apple pests, *Operophtera* and *Yponomeuta* moths, that feed on flowers, developing apple fruitlets, and buds and leaves which can result in high economic losses if not controlled (CFIA, 2006; Svensson et al., 1999; Turner et al., 2010). Within the orders Diptera and Coleoptera, there are both apple pests and natural enemies of pests. Adults from these two orders can also function as pollinators in plant species flowering later in the summer than apple (Brown et al., 2007; Clement et al., 2007; Compton & Key, 2000; Free, 1993; Kolcsár et al., 2016; Levesque & Burger, 1982; Orford et al., 2015; Rader et al., 2020). Arthropod predators naturally occur in apple orchards and their contribution to pest regulation reduces the need for insecticides to maintain apple production, yield, and quality (Dunn et al., 2020; Hambäck et al., 2021; Herz et al., 2019; Porcel et al., 2018). Results from this study confirmed the presence of the invasive ladybird *Harmonia axyridis*, a natural enemy of aphids and coccids, and established in Denmark (Steenberg & Harding, 2009). Additional resources such as flowers, alternative prey, and habitat are needed to ensure the presence of not only pollinators but also predators in managed ecosystems, such as apple orchards (Brown et al., 2007; Cahenzli et al., 2019; Jacobsen et al., 2022; Joshi et al., 2016; Pfiffner et al., 2019; Saunders et al., 2016). Thus, data provided in this study can assist farmers in selecting more efficient and specific pest control techniques while improving the surrounding landscape to attract certain beneficial arthropods that they know are visiting their orchards. Management practices (IPM, organic) orchard design (number of rows, field size), additional floral resources (heterogeneous landscape, apple varieties), and interactions among arthropods (density and location of honey bee hives) can affect arthropod

communities within the orchards (Campbell et al., 2017; Delaplane et al., 2000; Földesi et al., 2020; Jacobsen et al., 2022; Leonhardt et al., 2013; Pardo & Borges, 2020; Rasmussen, Dupont, et al., 2021; Russo et al., 2015). Future studies should include vegetation surveys of the landscape surrounding the orchards, as well as addressing the possible influence of management variables, such as orchard design, on arthropod activity.

5 | CONCLUSION

Our study provides the starting point for a more complete overview of the biodiversity of arthropod flower-visitors found in apple orchards. The combination of molecular and traditional nonmolecular techniques for arthropod assessment is complementary and can overcome some of the limitations inherent with using only one method. Going forward, we recommend the use of both molecular and nonmolecular approaches in the assessment of arthropod diversity if time and budget permits. Otherwise, utilizing a molecular approach such as metabarcoding with at least two primers can help to optimize arthropod detection. The outcomes of our study can support future management practices moving towards more resilient and environmentally friendly agricultural systems.

AUTHOR CONTRIBUTIONS

NGG, PYSC, and LS designed the research; NGG and LS collected samples; NGG, DHS, and AllGenetics performed laboratory work; NGG and PYSC did the bioinformatic analysis; NGG did the statistical analysis; NGG made the figures and wrote the paper with input from PYSC and LS. All authors contributed to the final version of the submitted manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Raw Illumina sequencing files trimmed files used for analyses, R and bioinformatics scripts used for data generation and .csv files used for statistical analyses are available at the DRYAD Digital Repository, https://doi.org/10.5061/dryad.w0vt4b8vk.

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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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