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Using Flower eDNA Metabarcoding to Identify the Effects of Forest Structure and Microclimate on Flower-Visiting Arthropods

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ABSTRACT

Recording flower-associated taxa can be challenging in contexts where plant–arthropod interactions are limited, thereby constraining the assessment of their ecological responses. For example, forests typically provide fewer floral resources for pollinators than other ecosystems, such as grasslands, while understory microclimates influence the spatiotemporal dynamics of insect activity, further complicating their detection. In this study, we use environmental DNA (eDNA) to address these challenges and investigate the influence of forest microclimate, density, and tree composition on the diversity of flower-associated arthropods in a Swedish forest. We used two flowering plant species, *Fragaria vesca* and *Trifolium pratense*, as sentinel plants, translocating them to a mixed forest across 40 plots spanning a gradient of forest density and broadleaf tree dominance. The metabarcoding of flower eDNA documented a high diversity of arthropods with very specific communities in different forest plots. This high species turnover suggests either short eDNA persistence on flowers or unmeasured ecological factors structuring these communities. We found that forest structure, particularly light availability in broadleaf-dominated open plots, positively influenced species richness of arthropods detectable in flowers, while microclimate had a small impact. These effects varied between plant species, likely due to differences in flower visitor communities. Our study also offers significant methodological insights into using flower eDNA for detecting flower-associated taxa. We also emphasize the need for optimized sampling and DNA extraction processes to enhance the likelihood of successful amplification. We show that the number of flowers pooled in the same DNA extraction positively influences the number of taxa detected. By improving methods in flower eDNA sampling and analysis, future studies can more accurately assess the ecological interactions and conservation needs of forest environments and other ecosystems.

1 | Introduction

Arthropods play a fundamental role in the functioning and stability of ecosystems, contributing to pollination, nutrient cycling, decomposition, soil aeration, and the regulation of other species' populations through predation and parasitism (Fei et al. 2023; Kishore et al. 2024; McCary and Schmitz 2021; Wardhaugh 2015). Approximately 30% of arthropods visit flowers (Wardhaugh 2015) and may act as pollinators for both crops and wild plants. Moreover, arthropods are integral to numerous food webs, serving as prey for various species, and disruptions in their populations can cascade through the ecosystem (Thomann et al. 2013). This highlights the importance of understanding the factors influencing arthropod biodiversity and distribution, as their decline can significantly impact ecosystem processes (Potts et al. 2010).

Boreal forests are characterized by the dominance of coniferous trees, which provide the foundation for complex and diverse forest ecosystems (Kuuluvainen 2009). The low plant diversity of boreal forests does, however, lead to a limited diversity of arthropod species compared to other biomes (Esseen et al. 1997). Arthropod diversity in forested biomes is influenced by several biotic and abiotic factors, including tree diversity (Sobek et al. 2009), forest structure (Knuff et al. 2020), forest age (Jeffries et al. 2006), and microclimate (Seibold et al. 2016). Management decisions and silvicultural practices, crucial in forests managed for wood production, also affect insect diversity and abundance. For example, broadleaved stands support higher carabid beetle diversity than conifer stands (Fuller et al. 2008), greater canopy openness increases butterfly richness and abundance (Ohwaki et al. 2017), and leaving or creating dead wood in forest stands can have positive effects on the abundance and richness of saproxylic insects (Sandström et al. 2019).

Arthropod pollinators are essential for the reproduction of many forest plants. It is estimated that 90% of all flowering plant species are animal-pollinated (Tong et al. 2023). Yet, knowledge of boreal pollinator diversity is still limited relative to other taxonomic groups, such as plants or for pollinators from other biomes (Kevan et al. 1993). Similarly, the interactions of boreal pollinators with plants are still poorly documented (Díaz-Calafat et al. 2025). This is possibly because pollinators can be challenging to record in boreal forests (Beattie 1971). Most boreal pollinators are ectothermic arthropods and therefore depend on external temperature to conduct their activities (Heinrich 1973). Forests, and especially those that are structurally complex, create a dynamic mosaic of microclimatic conditions that can drive pollinator activity (Herrera 1997; Zhang et al. 2022), making pollinators detectable only during the moments in which these microclimatic conditions are suitable. For instance, insects may only be available for pollination during a short timespan when the sun is shining directly on the flowers (Beattie 1971; Bovee et al. 2021). This, in combination with generally low temperatures and the fact that many pollinators of understory forest plants belong to understudied groups such as non-syrphid flies (Elberling and Olesen 1999; Orford et al. 2015), makes detecting, recording, and identifying boreal pollinators challenging.

Novel methodological approaches have been developed to overcome this issue, such as the use of motion-triggered high-definition cameras (Pegoraro et al. 2020) and environmental DNA (eDNA, Thomsen and Sigsgaard 2019), among others (see van Klink et al. 2022). eDNA refers to genetic material left by organisms that can be obtained directly from environmental samples (such as soil, water, air, or sediment) without the need to capture or observe the organism itself. This DNA can be sourced from cells, tissues, secretions, and excretions shed by organisms into their surroundings (Ruppert et al. 2019). Therefore, eDNA offers a noninvasive alternative to sampling flower visitors compared to other traditional sampling methods, which can also often detect species that might be missed by such traditional methods. For instance, eDNA metabarcoding is used to monitor endangered biodiversity without the need to capture individuals (Thomsen et al. 2012), as well as to detect invasive species (Thomas et al. 2020), study trophic interactions (D'Alessandro and Mariani 2021), and plant–animal interactions (Banerjee et al. 2022). More recently, in the field of pollination ecology, flower eDNA has been used to identify flower visitors that may potentially be pollinators, revealing how plants interact with bats (Edwards et al. 2019), birds (Newton et al. 2023), or arthropods (Thomsen and Sigsgaard 2019). However, despite the promising prospects of this molecular approach, many methodological questions remain unanswered, such as the drivers of flower eDNA persistence, whether some flower visitors leave more eDNA than others when foraging, and how pollinator visit frequency or length may impact eDNA yield (Thomsen and Sigsgaard 2019). As eDNA is typically found at low concentrations, it is important to develop protocols that ensure that enough genetic material is gathered in a way that accurately reflects ecological interactions.

Here, we used a flower eDNA metabarcoding approach to answer both ecological and methodological questions. First, we aimed to assess the community composition and diversity of pollinators and other flower-associated arthropods in boreal forests and to reveal the local environmental factors shaping these communities. Specifically, we quantified flower-associated arthropod diversity in two model plant species (red clover, *Trifolium pratense* L., and wild strawberry, *Fragaria vesca* L.) and then assessed how flower-associated arthropod diversity was affected by differences in forest density and overstory tree species composition, as well as by understory microclimate. Second, we investigated the effectiveness of different sampling designs in enhancing species detectability. Particularly, we addressed whether sequencing flowers individually or pooling them together before DNA extraction influences species detection. By addressing these questions, our research aims to provide comprehensive insights into how forests shape the diversity of forest flower-associated arthropods and improve eDNA metabarcoding methodologies for the assessment of interactions between plants and their potential pollinators.

2 | Methods

2.1 | Study Region and Design

The study took place in a mixed forest in southern Sweden (56°17′ N, 13°58′ E). There, the dominating conifer species was spruce (*Picea abies* H. Karst, 60.7%), and the dominating broadleaf species was birch (*Betula pendula* Roth and *B. pubescens*

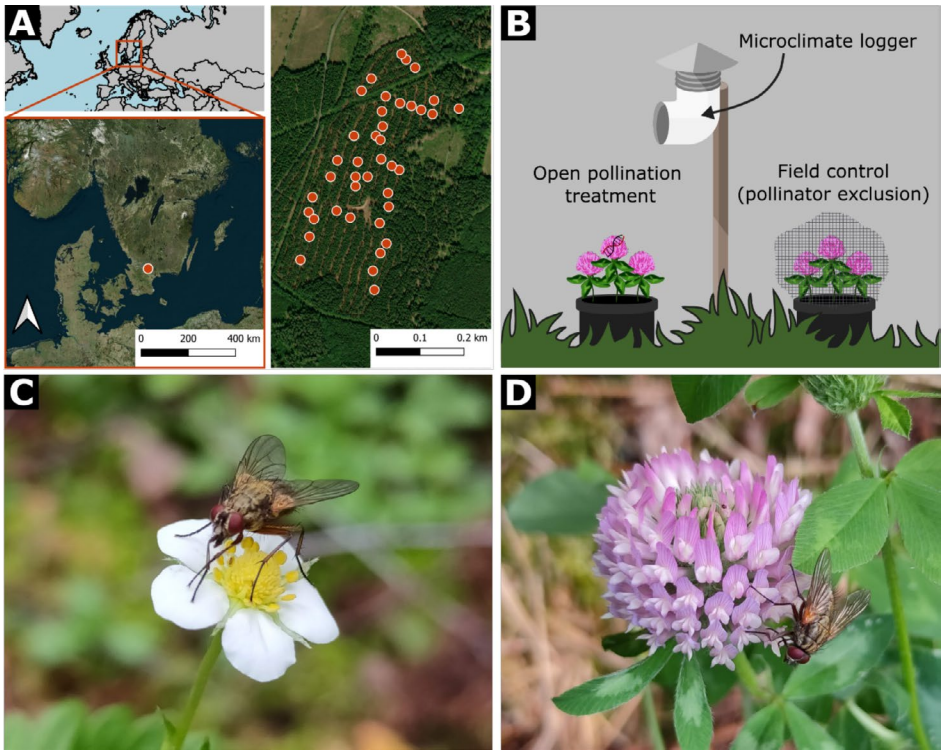

FIGURE 1 | (A) Location of the experimental forest and the 40 plots where measurements and samples were taken (red dots) (B) Experimental setup. In the center of each plot, a microclimate station measuring understory temperature was installed, and two pots each of planted *Trifolium pratense* and *Fragaria vesca* were placed, one of which was covered with a 0.3 mm mesh to avoid pollinator visitation (field control), and the other was uncovered and thus available to all pollinators (open pollination treatment). (C) A flower of *Fragaria vesca* and (D) An inflorescence of *Trifolium pratense*, both visited by flies.

Ehrh., 35.6%), although other tree species also occurred at a lower frequency (*Alnus* sp., 1.9%, *Quercus* sp., 1.7%, *Carpinus betulus* L., 0.2%). The most common understory flowering plants were the foundational species *Vaccinium myrtillus* L. and *V. vitis-idaea* L., as well as other species such as *Calluna vulgaris* (L.) Hull, *Melampyrum sylvaticum* L., *M. pratense* L., and *Solidago virgaurea* L. Within this forest stand, we established 40 circular plots, each with a 10-m radius (Figure 1A). These plots were distributed along two gradients: forest density estimated by the basal area of trees (ranging from 11 to 37 $m^2 \cdot ha^{-1}$, with a mean ± SD of 19.9 ± 5.6) and tree species composition (shifting from spruce-dominated to birch-dominated basal area). Measurements and samples were collected within these plots.

2.2 | Overstory and Microclimate Measurements

In the center of each plot, we established a microclimate station that recorded air temperatures at approximately 1.2 m height using HOBO Pendant MX Water Temperature Data Loggers (Onset Computer Corp., Bourne, MA, USA). Temperatures were recorded every 15 min. Loggers were shielded from direct sun in a well-ventilated plastic radiation shield, as done in Díaz-Calafat et al. (2023). We inventoried all trees within a 10 m radius of each microclimate station, recording tree species identity and diameter at breast height (1.3 m, DBH). Basal area was calculated from these data, as well as the percentage of broadleaved trees. Additionally, to estimate canopy openness, we took hemispherical pictures from the center of each plot with a Nikon D5300 camera with a fish-eye lens and using a tripod. Pictures were taken in summer, from the top of the microclimate station at approximately 1.5 m height. Canopy openness was calculated as in ter Steege (2018).

2.3 | Studied Flowering Plant Species

To standardize the studied flowering plants and avoid any confounding effects of the soil characteristics and nutrient concentrations in leaves or flowers, we planted and translocated plants from outside the forest. Two different flowering plant species were chosen for this: wild strawberry (*F. vesca* var. *semperflorens* Jamin; Rosaceae) and red clover (*T. pratense*; Fabaceae, wild unimproved variety). These particular species were selected as model plants because they are both native to Sweden and commonly found in southern boreal forest landscapes, including the area where we conducted our study. Moreover, their diverging morphology (i.e., open vs. enclosed flowers) may make them available to different sets of pollinators, and this particular variety of *F. vesca* produces flowers throughout the growing season. Both plant species are also commercially grown, which facilitated procurement. *T. pratense* seeds and *F. vesca* seedlings were grown in a greenhouse in spring 2021 until seedlings reached approximately 5 cm tall. Seedlings were then transplanted to 20 cm diameter pots and moved to an open-air garden. Potted plants were placed in a sunny spot to induce flowering and were monitored daily. Once they developed flower buds, but before any flowers opened, the whole pot was covered with a mesh bag

(mesh size *ca*. 0.3 mm, manually measured) to avoid insect visitation prior to translocation to the forest. Plants were checked for the presence of insects, including between the leaves, before meshing.

Once plants started to flower, the pots were moved into our forest stand. Pots were placed in holes in the ground to avoid them drying out or tipping over. Two pots of each flowering plant species were placed in each plot, as close as possible to the microclimate station (i.e., total sample size: $n = 80$ *F. vesca* and 80 *T. pratense* plants in total). In the field, the mesh bag was removed from one pot of each plant–species pair, whereas the other pot remained meshed (Figure 1B). This paired design allowed us to control for false-positive detections of insects by serving as a field control for airborne eDNA and potential insect DNA present on the plant before the start of the experiment, for example, from the outdoor greenhouse. *F. vesca* plants were placed in the forest at the beginning of June 2022, and *T. pratense* in mid-July 2022.

2.4 | Flower Collection

Fragaria vesca flowers (Figure 1C) and flower heads of *T. pratense* (Figure 1D) were collected throughout the flowering period, starting on the 17th of June 2022, and then twice to three times a week until the 29th of July 2022. All pots in all plots were sampled during each sampling session, collecting flowers from both the field control and open-pollinated plants of the same species each time. Each flowering plant was sampled on the same day by cutting a flower/inflorescence using scissors previously sterilized in a 12% bleach solution. Nitrile gloves were changed after collecting each sample. Occasionally, more than one flower per plant and plot was collected per collecting session, which resulted in different number of flowers per plot. Flowering phenology of *F. vesca* and *T. pratense* only overlapped during one collection session (see Table S1 for details on phenology and sample collection). Flowers were stored in individual sterile vials and placed directly on ice until they could be stored in a freezer at −20°C in the lab.

Flowers were divided into two groups to address different questions. In the first group, in order to assess the impact of sampling intensity on species richness across our forest density and overstory composition gradients, the flowers collected in the three densest and the three most open plots based on canopy openness values were processed individually. In the second group, for the rest of the plots, all unmeshed flowers were pooled per plot and species. This allowed us to simultaneously address biodiversity patterns of arthropod flower visitors throughout the forest, as well as how different DNA extraction methodologies may influence species detection. Finally, field controls (i.e., plants that remained always meshed) were pooled based on their date of collection for each plant species, regardless of the plot they originated from.

2.5 | DNA Filtration and Extraction

Prior to DNA extraction, DNA was filtered as in Kirtane et al. (2022) in a laminar flow hood. Flowers were placed in a sterilized 500 mL glass jar filled with milliQ water and shaken vigorously for 10 s to suspend the eDNA from the flowers in the water. Then, all the contents from the jar were pre-filtered using a 3 mm nylon mesh. This removed the flowers and other large debris, as well as any possible small insects that could have been hidden in the flowers. Finally, this water was filtered through a 0.8-μm track-etched polycarbonate membrane (Whatman, Pittsburg, PA) to capture the eDNA on the filter. This filtration step was performed with a vacuum filtration funnel connected to a built-in vacuum port in a fume hood. If a filter became clogged before all liquid could be filtered (as was the case for some samples from plots where many flowers were pooled together, especially because of the pollen in *F. vesca*), the remaining water was filtered onto a second filter. In such cases, filters were incubated separately for lysis, but the resulting lysates were pooled and homogenized, and the same volume as used for single-filter samples was used for DNA extraction. Every ten samples, a negative control using only milliQ water was run through a filter. The negative controls were extracted separately but pooled after eDNA extraction. Jars, funnels, other equipment, and the working area were sterilized using a bleach solution prepared to *ca*. 1% sodium hypochlorite before processing each sample.

After filtration, the filters were transferred into 2 mL Eppendorf tubes using sterile forceps and stored at −20°C until DNA extraction. Filters were inserted in the tubes in such a way that the side of the membrane that contained the DNA was facing inward and therefore was in maximum contact with the reagents added during DNA extraction.

We extracted the DNA from the polycarbonate filters with the Qiagen DNeasy Blood and Tissue kit following the manufacturer's instructions. DNA lysis was done through incubation with 180 μL buffer ATL and 20 μL proteinase K for 3 h at 56°C with constant shaking at 300 rpm, as in Thomsen and Sigsgaard (2019). Then, the lysate was transferred to a microcentrifuge tube to follow the rest of the manufacturer's protocol, with the exception of the final elution step, where we split the 200 μL elution volume into two steps of 100 μL to maximize DNA recovery. Negative controls, as well as samples that were split during filtration (due to clogging) were pooled after elution.

2.6 | PCR Protocol

We amplified a mini-barcode within the Cytochrome Oxidase I (COI) gene as a genetic barcode marker using the ZBJ-ArtF1c and ZBJ-ArtR2c primers (Zeale et al. 2011). These primers produce an amplicon of approximately 157 bp and perform well for amplifying degraded DNA while still maintaining the ability to resolve taxa to species level. These primers have also previously been successful in the amplification of arthropod eDNA from flowers (Thomsen and Sigsgaard 2019). Primers were ordered with Illumina adapters attached to them but separated by three, five, or seven random nucleotides to increase sequence diversity and increase sequence throughput (Wu et al. 2015). PCR reactions were carried out in volumes of 25 μL consisting of 3 μL of template DNA, 12.3 μL of ddH_2O, 1 μL of each primer (10 μM), and 7.7 μL of AmpliTaq Gold 360 Master Mix (Applied Biosystems; catalog no. 4398881). Thermocycler parameters were 95°C for 10 min, 55 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 1 min, and a final elongation of 72°C

for 7 min. PCR reactions were carried out in duplicates to increase the diversity detected. In total, we included three negative controls across two PCR plates, where the DNA template was replaced by H_2O. All PCR products were verified on a 2% agarose gel stained with GelRed. PCR replicates were pooled by sample for sequencing.

2.7 | Library Building and Sequencing

Library building and Illumina sequencing were performed by Macrogen (Amsterdam). Libraries were constructed using the Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2, following the 16S Metagenomic Sequencing Library Preparation Part #15044223 Rev. B. A total of 171 libraries were built, one for each plot with pooled flowers (34 for *F. vesca* and 32 for *T. pratense*), one for each individual flower in the most dense and most open forest plots (49 for *F. vesca* and 42 for *T. pratense*), one for each field control (six for *F. vesca* and four for *T. pratense*), three PCR blanks, and one extraction negative control. To verify the size of PCR-enriched fragments and the quantity of prepared libraries, template size distributions were assessed using an Agilent Technologies 2100 Bioanalyzer with a DNA 1000 chip, and libraries were quantified by qPCR according to the Illumina qPCR Quantification Protocol Guide. Libraries were pooled in equimolar concentrations of 150 pM and then sequenced on Illumina NovaSeq (150 bp pair-end sequencing).

2.8 | Bioinformatics

Sequences were demultiplexed based on library dual unique indices. The nfcore/ampliseq bioinformatics pipeline V2.8 (Straub et al. 2024) was used to evaluate sequence quality, trimming reads, and to infer Amplicon Sequence Variants (ASVs). Namely, FastQC (Andrews 2010) performed a series of quality checks to evaluate metrics such as per base sequence quality, per sequence quality scores, GC content, sequence length distribution, and the presence of adapter contamination. Cutadapt (Martin 2011) was used for primer trimming and DADA2 (Callahan et al. 2016) for ASV inference (including expected error filtering, denoising, merging and chimaera removal). Default options were used. This produced sequences at mostly two peaks, one at 149 bp and one at 157 bp. The latter peak corresponded to the expected fragment length of the arthropod COI region amplified by the Zeale et al. (2011) primers; the former resulted from the co-amplification of a *F. vesca* chloroplast sequence. As very little length variation is expected in COI sequences, as COI is a protein coding gene, we applied a length filtration to all sequences with a length window of 157 ± 6 bp. In total, the sequencing yielded 608 million reads, with 26 million reads passing quality filtering. Length filtration removed 22.25% of all reads. Despite this taking away some sequencing depth, the remaining sequences were sufficient for all samples (Figure S1).

We used Swarm v3 (Mahé et al. 2015) for single linkage clustering of the ASVs. We set the local clustering threshold to $d = 1$, which is very conservative and prevents overclustering. A dual approach was taken for taxonomic assignment. First, BOLDigger (Buchner and Leese 2020) was used to assign sequences to species using the JAMP pipeline. For the cases where sequences could not be matched to species, the Sintax function in the VSEARCH tool (Rognes et al. 2016) was used to obtain probabilistic assignments at different taxonomic levels, using the MIDORI2 vGB259 database (Leray et al. 2022) as a reference and applying an 80% confidence threshold. Finally, the cases that showed a disagreement between the taxonomic classification of BOLDigger and Sintax were manually explored, and a consensus taxonomic classification was assigned at each taxonomic level possible. Then, we merged ASVs with the same taxonomic assignment at the species level, or at the genus level if the species level assignment is missing.

Sequences with no phylum assignment were removed, as well as non-arthropod sequences and phytophagous arthropods that typically do not contribute to pollination (e.g., Aphididae). Sequences found in the lab controls (i.e., blanks with only milliQ water) were removed from the data as well. No sequences were found in the PCR blanks or the extraction negative control. We subtracted, from all samples, twice the highest number of reads for the taxa found in the field controls (i.e., plants that were permanently meshed to avoid pollinator visits).

2.9 | Data Analyses

All statistical analyses were performed with R 4.3.3 (R Core Team 2024).

2.9.1 | Field Control Contamination

In order to assess eDNA contamination and its potential increase over time, we summarized the sequences from the field controls (i.e., meshed plants where insect pollination was not possible) across the different sampling occasions.

2.9.2 | Rarefaction Curves

Sample-based rarefaction curves were calculated for the three most dense and three most open forest plots for each flower species, using the individually collected flowers as independent samples. This analysis was based on the taxonomy-clustered ASVs. To obtain an estimation of the number of flower-associated taxa for each species and in each forest context (dense/open), asymptotic diversity estimates and their standard errors were calculated using the *iNEXT* function in the "iNEXT" V3 R package (Hsieh et al. 2022). Standard errors were calculated based on the bootstrapping of 999 replicates.

2.9.3 | Richness Pattern Analyses

To analyze the effect of environmental factors on flower-visitor richness, we used Generalized Additive Mixed Models (GAMMs) with Poisson error distribution and log link for *F. vesca* and *T. pratense* separately with the *gamm4* function in the "gamm4" V0.2–6 package (Wood and Scheipl 2020). The number of detected taxa was used as a response variable. The models included a bidimensional smoother term with forest density

(as either basal area or canopy openness) and the percentage of broadleaf trees. Then, as parametric covariates, we used the number of flowers pooled for sequencing, whether the plot belonged to the rarefaction or pooling group, and the abundance of flowers of plants growing naturally in the plot. The best model for each plant species was selected based on likelihood ratio tests with the function *buildgamm* in the "buildmer" V2.11 package (Voeten 2023). The residuals of the models were checked and evaluated for overdispersion using the "performance" V0.13.0 R package (Lüdecke et al. 2021).

Additionally, General Linear Models (GLMs) were fitted starting with full models including microclimate, the number of sequenced flowers, whether the plot belonged to the rarefaction or pooling group, and the abundance of wild flowers. The mean, minimum, and maximum microclimatic temperatures were calculated for the extent of the experiment and used in different models, as these temperature variables were strongly correlated with each other. The best model was selected based on likelihood ratio tests with the function *drop1* from the "stats" V4.4.2 R package. Similarly, models were evaluated and overdispersion assessed using the "performance" package.

3 | Results

3.1 | Field Control Contamination

Two taxa were detected for each plant species in the field controls (i.e., meshed plants). On *F. vesca*, these were *Aphidius tarsalis* (Hymenoptera: Braconidae) and *Bradysia* sp. (Diptera: Sciaridae), and on *T. pratense*, *Peripsocus subfasciatus* (Psocoptera: Peripsocidae) and an unidentified Eupodidae (Arachnida). There was no increase in detected diversity over time. Namely, no arthropod sequences were found for the first two collection dates on *F. vesca*. *Bradysia* sp. was found in the third collecting session, and *A. tarsalis* in the fourth, followed by two more collecting sessions in which no arthropod eDNA was found on the field controls. In *T. pratense*, no sequences were found in the first collecting session, *P. subfasciatus* was found in the third session, and unidentified Eupodidae in the fourth and fifth sessions.

3.2 | Arthropods From Flower eDNA

After removal of low-quality reads, short-length reads, chimeras, plant-derived contaminations, and clustering at a 97% similarity level, a total of 23,703,978 high-quality reads were obtained. On average, each sample produced 134,681 reads. A total of 92 arthropod taxa were identified through flower eDNA (Table S2): 34 on *F. vesca* and 64 on *T. pratense*. Six species were found in the flowers of both plant species (Table S3). Of the 40 plots sampled, 23 yielded amplifiable eDNA in *F. vesca* and 28 in *T. pratense*. The most frequently identified species were *Entomobrya nivalis* L. (Collembola: Entomobryidae), *Epinotia tedella* (Clerck, 1759) (Lepidoptera: Tortricidae), and *Chamobates borealis* (Trägårdh, 1902) (Acari: Oribatida). The maximum number of taxa for a plot was nine in *T. pratense* and six in *F. vesca*. Notably, 12 *F. vesca* plots contained only a single taxon, as did seven *T. pratense* plots. The mean number of taxa per plot was 1.03 for *F. vesca* and 2.05 for *T. pratense* (Figure 2). Of the 49 *F. vesca* flowers that were sequenced individually, only 10 amplified arthropod DNA, and of the 42 individual flowers from *T. pratense*, 25 amplified DNA. The microclimatic temperatures during the experiment ranged between 4.83°C and 33.6°C (mean ± SD = 16.7°C ± 0.2°C).

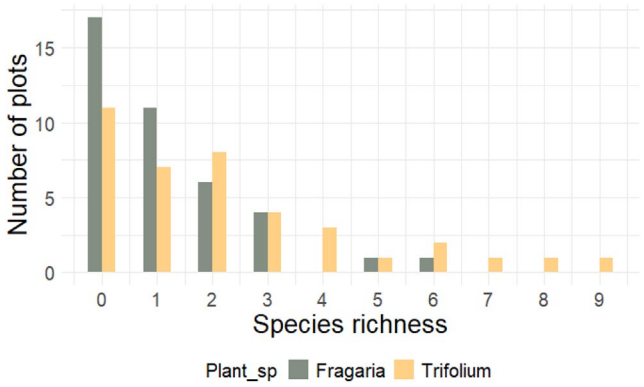

FIGURE 2 | Number of plots in which different arthropod species richness (ranging from 0 to 9) was detected for *F. vesca* and *T. pratense*.

3.3 | Estimated Number of Arthropods Per Flower

Based on the asymptotic diversity estimates extrapolated from single flowers, the expected number of flower-associated arthropods in *F. vesca* and in *T. pratense* across all plots was 24.40 ± 15.39 and 341 ± 154.38, respectively. Specifically, in the plots with the highest canopy openness, 24.50 ± 10.44 species were expected for *F. vesca* and 67 ± 27.22 for *T. pratense*, while in the plots with the lowest canopy openness, 12.5 ± 5.88 species were expected for *F. vesca* and 140.26 ± 63.44 for *T. pratense* (Figure 3).

3.4 | Effects of Forest Structural Characteristics and Microclimate on Arthropod Richness

In *F. vesca* flowers, there was a negative effect of forest density (as basal area) and a positive effect of the proportion of broadleaves on the detected species richness of flower visitors (GAMM, df = 2, chi-sq = 9.28, $p = 0.009$, $R^2_{adj} = 0.28$), with the highest richness in open broadleaved plots (Figure 4). The abundance of flowers that occurred naturally in the plot had a significant negative effect on the number of species detected through eDNA on our focal *F. vesca* plants (GAMM, $Z = -2.4$, $p = 0.017$). Plots that had their flowers sequenced separately (i.e., for the asymptotic diversity estimates) and pooled after taxonomic annotation, also showed a significantly higher number of flower-associated species (GAMM, $Z = 2.3$, $p = 0.019$).

In *T. pratense*, there was no significant effect of forest density (as basal area nor canopy openness) and percentage of broadleaves on flower visitor richness (GAMM, df = 2, chi-sq = 4.6, $p = 0.103$), and neither was there any effect of the abundance of naturally occurring flowers in the environment (GAMM, $Z = -1.6$, $p = 0.112$). Sequencing flowers individually positively affected the number of species detected (GAMM, $Z = 3.6$, $p < 0.001$), as well as the number of flowers pooled before DNA extraction (GAMM, $Z = 3.7$, $p < 0.001$; Table 1).

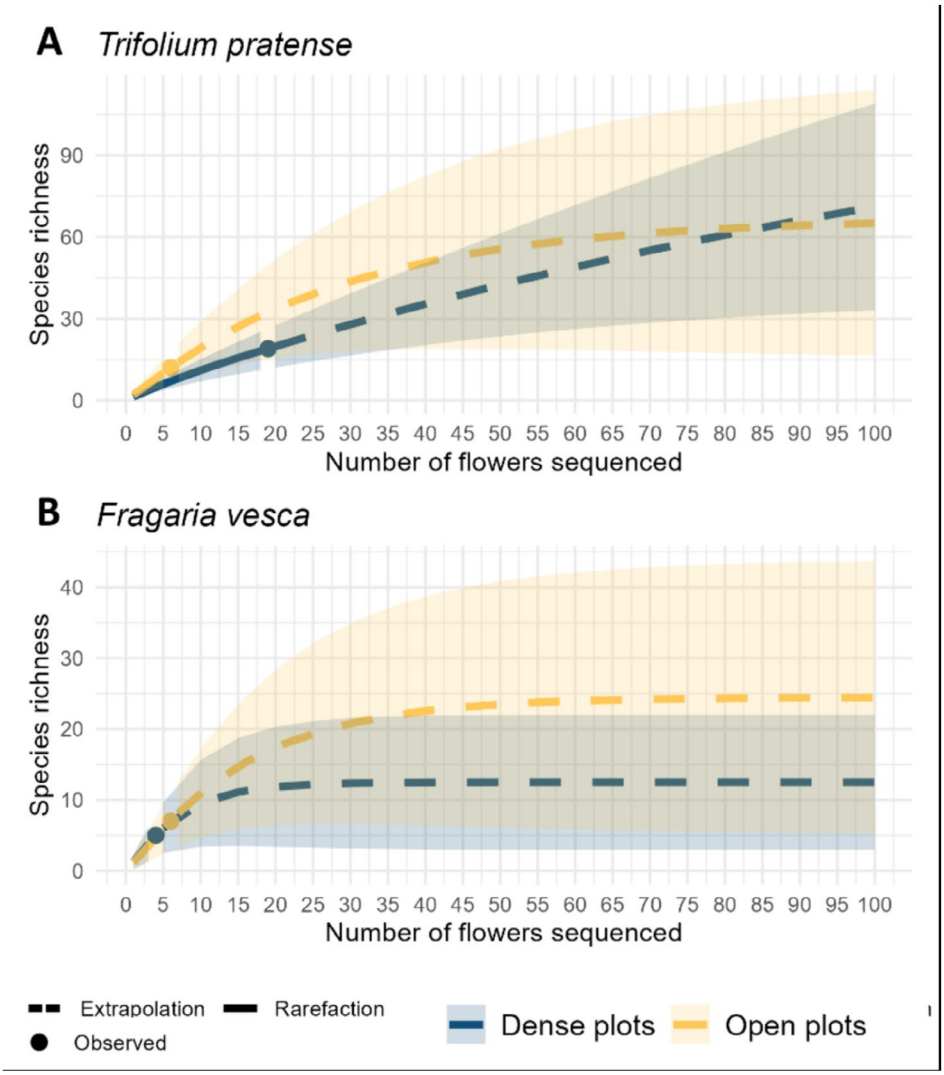

FIGURE 3 | Cumulative (interpolated and extrapolated) number of flower-associated arthropods detected through the sequencing of flower eDNA from individual flowers of *Fragaria vesca* (A) and *Trifolium pratense* (B) in different types of forest plots (dense and open). Note that a different number of flowers was used between plant species and forest plot type.

Neither minimum, mean, nor maximum microclimatic temperature had an effect on the richness of arthropods visiting flowers of *T. pratense* or *F. vesca* according to the GLMs. Finally, in both the GLMs for *F. vesca* and *T. pratense*, the effects of the number of flowers pooled before DNA extraction and of the wild flowers naturally occurring in each plot were similar to those described for the GAMs (Table 2).

4 | Discussion

Our study revealed a relatively high diversity of flower-associated arthropods to *F. vesca* and *T. pratense* in a Swedish boreal forest, with very specific communities in different samples and with many species only recorded once. We found that forest structure had an effect on the species richness of flower-associated arthropods and that microclimate seems to play only a small role, if any, as a driver of the observed patterns. Moreover, these effects may be plant species-dependent. Here, we offer some methodological insights into the use of flower eDNA to detect flower-associated taxa and answer relevant ecological questions.

4.1 | Comparison With Other Flower eDNA Studies

Whereas in one Swedish forest we detected 92 taxa of arthropods that visited two flowering plant species, a study from approximately the same latitude in Denmark (Thomsen and Sigsgaard 2019) detected 216 arthropod taxa using the same COI primers but sampling a wider range of flowering species within a 2000 ha area that consisted of both grasslands and young forests. The difference between these ecosystems, as well as the diverging number and diversity of sequenced flowers between studies, makes a comparison between the number of taxa found difficult. Grasslands not only have a greater diversity of flowers than forests, which may attract a wider range of flower visitors, but also their high flower abundance may be able to sustain a more diverse pollinator community

(Fründ et al. 2010). On the other hand, another Danish study only found 12 taxa after sequencing eDNA from 60 flowers in an apple orchard (Gamonal Gomez et al. 2023), suggesting that habitat type and flower diversity influence arthropod richness. While we cannot fully separate methodological limitations from ecological realities, our study was conducted in boreal forest habitats where insect activity is known to be relatively low, particularly under cool, shaded conditions. Previous studies have reported low pollinator visitation rates in similar environments (Barrett and Helenurm 1987; Beattie 1971; Kevan et al. 1993), which supports the interpretation that the low arthropod richness may reflect genuine scarcity rather than a detection artifact. Nevertheless, methodological differences should be considered when comparing different studies. For example, our approach involved filtering water used to wash flowers, potentially diluting the final DNA concentration, whereas Thomsen and Sigsgaard (2019) and Gamonal Gomez et al. (2023) extracted DNA directly from flowers. Moreover, eDNA methods have inherent limitations, such as sensitivity to low DNA concentrations and stochastic amplification, that may also contribute to reduced taxa detection.

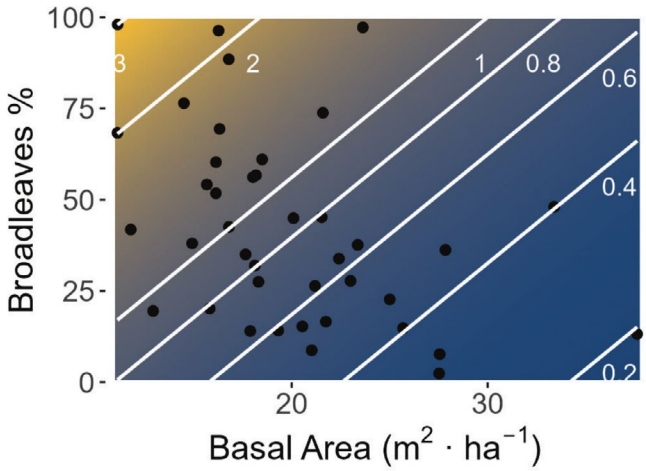

FIGURE 4 | Model predictions from a Generalized Additive Model for the number of flower-associated arthropod taxa amplified from *F. vesca* flower eDNA for our gradient of forest density and proportion of broadleaves. The numbers on the isolines represent the predicted number of arthropod taxa. The black dots show the forest density (in basal area) and percentage of broadleaves in each of the sampled plots.

We detected almost twice as many taxa on the flowers of *T. pratense* compared to *F. vesca*, in line with the findings of Newton et al. (2023), who observed that the highest diversity of arthropod taxa detected through flower eDNA was associated with large inflorescence flower types. Larger inflorescences may offer more rewards or be more visually attractive, thereby supporting a greater diversity and abundance of flower visitors, potentially increasing taxonomic richness. Other flower eDNA studies have focused on comparing this emerging method with conventional monitoring approaches, using insect nets (Avalos et al. 2024), traps (Kestel et al. 2023) or cameras (Kestel et al. 2023; Stothut et al. 2024), consistently reporting that eDNA outperforms traditional techniques.

4.2 | Diversity patterns in our study

The number and identity of taxa varied significantly between our flower species, which further stresses the importance of plant species identity for pollinator richness. For example, we found almost twice as many taxa in *T. pratense* compared to *F. vesca*, despite *T. pratense* being collected later in the season and over four sampling occasions instead of the six occasions used for *F. vesca*. Considering how simplified our study forest system is and that we used only two plant species, the arthropod diversity we detected can be regarded as relatively high. This is particularly notable when looking at the number of species from our asymptotic diversity estimates. However, these estimates are based on extrapolation from limited sampling and should be interpreted with caution. Asymptotic richness estimators can be sensitive to the presence of many rare taxa or singletons and may overestimate true diversity, particularly in datasets with low detection rates or uneven sampling effort. These projections do not reflect observed richness but rather represent a theoretical upper bound assuming continued sampling under similar conditions. Nonetheless, they provide a useful comparative

TABLE 1 | Full and final GAM models assessing the flower-associated arthropod richness of *Fragaria vesca* and *Trifolium pratense* as a function of the flower abundance naturally occurring in forest plots (Flower_abundance_plot), whether the sequenced flowers were pooled before DNA extraction or not (Flowers_pooled_Y_N), the number of flowers pooled before DNA extraction (Num_pooled_flowers), and forest structure. Note that all models included a bidimensional smoother with forest density and the share of broadleaves. Either basal area or canopy cover was used as a proxy for forest density. R^2_{adj} and AIC values are shown for the same models using each of these variables as forest density proxies.

			Basal area		Canopy openness	
Model	**Plant species**	**Covariates**	R^2_{adj}	**AIC**	R^2_{adj}	**AIC**
Full model	*Fragaria vesca*	Flower_abundance_plot + Flowers_pooled_Y_N + Num_pooled_flowers	0.32	113.8	0.25	114.1
Final model	*Fragaria vesca*	Flower_abundance_plot + Flowers_pooled_Y_N	0.28	119.4	0.20	119.7
Full model (= Final model)	*Trifolium pratense*	Flower_abundance_plot + Flowers_pooled_Y_N + Num_pooled_flowers	0.46	136.4	0.54	132.3

TABLE 2 | Full and final GLMs assessing the flower-associated arthropod richness of *Fragaria vesca* and *Trifolium pratense* as a function of (either mean, minimum or maximum) microclimatic temperature, the flower abundance naturally occurring in 40 forest plots (Flower_abundance_plot), whether the sequenced flowers were pooled before DNA extraction or not (Flowers_pooled_Y_N), the number of flowers pooled before DNA extraction (Num_pooled_flowers).

Model	Plant species	Microclimatic temperature	Covariates	R^2_{adj}	AIC
Full model	*Fragaria vesca*	Mean	Flower_abundance_plot + Flowers_pooled_Y_N + Num_pooled_flowers	0.35	115.3
Final model	*Fragaria vesca*	Mean	Flowers_pooled_Y_N + Num_pooled_flowers	0.33	114.5
Full model	*Fragaria vesca*	Min	Flower_abundance_plot + Flowers_pooled_Y_N + Num_pooled_flowers	0.30	118.2
Final model	*Fragaria vesca*	Min	Flowers_pooled_Y_N + Num_pooled_flowers	0.24	117.9
Full model	*Fragaria vesca*	Max	Flower_abundance_plot + Flowers_pooled_Y_N + Num_pooled_flowers	0.30	117.5
Final model	*Fragaria vesca*	Max	Flowers_pooled_Y_N + Num_pooled_flowers	0.26	117.0
Full model	*Trifolium pratense*	Mean	Flower_abundance_plot + Flowers_pooled_Y_N + Num_pooled_flowers	0.50	132.8
Final model	*Trifolium pratense*	Mean	Flowers_pooled_Y_N + Num_pooled_flowers	0.47	137.8
Full model	*Trifolium pratense*	Min	Flower_abundance_plot + Flowers_pooled_Y_N + Num_pooled_flowers	0.49	132.9
Final model	*Trifolium pratense*	Min	Flowers_pooled_Y_N + Num_pooled_flowers	0.46	138.0
Full model	*Trifolium pratense*	Max	Flower_abundance_plot + Flowers_pooled_Y_N + Num_pooled_flowers	0.49	132.9
Final model	*Trifolium pratense*	Max	Flowers_pooled_Y_N + Num_pooled_flowers	0.46	137.9

indication of potential species richness between plant species. We remain speculative about the reasons underlying the high rates of species turnover among flower visitors across our plots. It is possible that with a relatively large community of flower visitors with high evenness, there will be large differences between subsamples of species detected through flower eDNA just because of stochastic processes. It is also possible that these flower visitor communities are structured by factors that we failed to measure. Alternatively, the environment may be so hostile or lacking in resources that searching for food in these areas is not a viable strategy, which would also explain our observations with zero flower visitors. Similarly, low arthropod detection from flower eDNA has been reported in simplified ecosystems such as apple orchards, where resources may also be limited (Gamonal Gomez et al. 2023). A methodological explanation would be that eDNA does not accumulate on flower heads, suggesting a short residence time of genetic material. The small overlap in flower-associated species between *F. vesca* and *T. pratense* samples could be due to the differences in flower morphology, as well as by the turnover of potential pollinators captured by the different sampling times between these species.

4.3 | Effects of Microclimatic Temperature and Forest Structure on Flower-Visitor Richness

Although temperature can shape pollinator communities in some contexts (Geppert et al. 2023), our forest setup showed that microclimatic temperature had no impact on flower-visitor diversity. However, since we only used the average, minimum, or maximum temperature values across the experimental period, increasing the resolution of microclimatic data and matching it with fine-scale flower-visitor data may provide different results. In our setup, it seems that forest structure is more important than temperature when it comes to determining flower-associated arthropod richness. Forest density and the proportion of broadleaves negatively and positively affected the number of flower visitors on *F. vesca,* respectively, but not those of *T. pratense*. Plots with open canopies and a dominance of broadleaves hosted more flower-associated arthropods than conifer-dominated closed plots, possibly due to the increased light availability increasing diurnal pollinator activity (Bartholomée et al. 2023; Eckerter et al. 2019). In fact, the expected diversity of flower visitors in the most open plots where *F. vesca* was collected was higher than in the densest plots, and very similar to the total expected diversity across all plots (24.5 ± 10.30 out of 24 ± 13.71). *Trifolium pratense* showed the opposite patterns, with the densest plots expected to host more flower-associated diversity than the most open plots but we did not detect an effect of forest density or the proportion of broadleaves on their richness. One advantage of eDNA is that it can detect species interactions that can otherwise be missed when using traditional methods, as for example in the detection of nocturnal pollinators (Avalos et al. 2024). Moths are important nocturnal pollinators and contribute to *T. pratense* seed set (Alison et al. 2022), and were more frequently recorded on flowers of *T. pratense* than on flowers of

F. vesca (see Table S3). Vertical forest structure plays a role in shaping moth communities in forests (De Smedt et al. 2019), and this could explain why a higher flower visitor richness was expected in denser plots for *T. pratense*. In fact, it could be that the balance between diurnal (potentially more abundant in open plots) and nocturnal (potentially more abundant in denser plots) flower visitors confounded the effect of forest structure on the observed diversity of flower visitors for *T. pratense*.

4.4 | Field Control Contamination

Meshing plants to avoid pollinator visits to flowers is common in pollination research, as it is a method to quantify pollinator contribution to fruit and seed yield (Tetreault and Aho 2021). In our study, it is possible that the taxa that we detected in our field controls (i.e., meshed plants) did not originate from airborne eDNA contamination, but rather from insects that were trapped in the mesh bags and were transported from the greenhouse, or even from insects that managed to enter the mesh bags. We suggest this due to their consistently small size. These taxa were an aphid parasitoid (*Aphidius tarsalis*) that could have been introduced from some of the potentially aphid-infested plants in the greenhouse where our plants were grown; a fungus gnat (*Bradysia* sp.) whose larvae would have been in the potted soil when meshing the plants; as well as a barklouse (*Peripsocus subfasciatus*) and a fungivorous mite (Eupodidae). We do not think that watering was a source of contamination, as plants were watered with a hose and not by misting or overhead spraying.

4.5 | Caveats and Future Recommendations

Trifolium pratense had a higher proportion of flowers that yielded DNA than *F. vesca*. Although we did not explore the reasons behind this, a possible explanation could be that *T. pratense* is more attractive to flower visitors than *F. vesca*, as shown by both our observed and estimated richness. However, it may also be possible that insects leave more DNA on *T. pratense* because its flower shape forces a more intimate contact with the corolla when reaching for nectar compared to the open flowers of *F. vesca* and/or due to longer flower visiting times. Similarly, flower morphology may also affect eDNA persistence, as closed flowers host microclimates that have the potential to protect eDNA from environmental factors that degrade it, such as UV radiation (Pilliod et al. 2014) or temperature (Yu et al. 2022). In addition, differences in floral longevity may contribute to the observed patterns. *T. pratense* inflorescences consist of multiple flowers that open sequentially over several days, effectively prolonging the functional lifespan of the floral unit. In contrast, *F. vesca* produces single flowers with relatively short lifespans. From the perspective of flower-associated arthropods, the extended availability of a *T. pratense* inflorescence may provide a more consistent foraging resource over time, increasing the likelihood of arthropod visits and cumulative DNA deposition. These hypotheses will need to be tested to ensure better evidentiary foundations for the conclusions derived from these molecular techniques.

It is not unexpected that extracting and sequencing DNA from single flowers separately increases species detectability, as there are more PCRs involved than when pooling all samples into a single DNA extraction and PCR (e.g., Macher et al. 2021). Moreover, the chances of amplifying different species through stochastic primer binding are higher when sequencing biological sample replicates, as opposed to when samples are pooled together and a single species with a high DNA yield can dominate the amplification. Additionally, pooling can also have a dilution effect on DNA from individual samples, potentially affecting the detectability of low-abundance taxa (Sato et al. 2017). However, besides the extra cost of increasing the number of replicates, this also presents its own challenges. eDNA is typically found at low concentrations and is usually concentrated before extraction. For instance, in aquatic ecology different volumes of water are filtered to obtain eDNA (Altermatt et al. 2023), and the same can be done for airborne (Roger et al. 2022) and soil eDNA (Saccò et al. 2022). Filtering higher volumes of water, air, or soil typically yields more genetic material, overcoming the issue of low eDNA concentrations (Hunter et al. 2019; Bessey et al. 2020). In situations where sampling is limited and hence the concentration of eDNA is challenging, such as with the number of flowers of a plant, field replication comes with its own risks. In our study, only 20% of the individually sequenced flowers of *F. vesca* and 60% of *T. pratense* flowers amplified DNA, presumably due to low initial eDNA concentrations. Hence, for flower eDNA, it is critical to optimize the sampling approach (e.g., soaking and filtration of the samples), DNA extraction (particularly the lysis step), and PCR conditions to enhance the likelihood of successful amplification. This could involve increasing the number of biological replicates per sample, as indicated by the fact that the number of flowers pooled before DNA extraction positively influenced the number of arthropod species detected in our study. Similarly, more PCR replicates or the inclusion of more molecular markers can increase detected diversity in eDNA studies (Shirazi et al. 2021). In our study, we only used the COI region to detect flower visitors, as this region has a better taxonomic coverage in sequence databases. However, other markers, such as 16S, may have increased the number of species detected, as well as potentially the overlap in flower visitors between our plant species. In fact, it seems that some pollinator groups such as Hymenoptera do not amplify optimally with COI primers or additional COI markers compared to other markers such as 16S (Marquina et al. 2019). Although some studies have successfully amplified Hymenoptera from flower eDNA using COI (e.g., Harper et al. 2023), this inefficiency has been reported by others (Avalos et al. 2024; Gamonal Gomez et al. 2023; Johnson et al. 2023; Newton et al. 2023) and may introduce bias to PCR amplification (Clarke et al. 2014). Nevertheless, when using both these primers to amplify flower eDNA, Thomsen and Sigsgaard (2019) detected more families and more species with the COI primers than with the 16S. Other flower eDNA studies find that primer choice substantially influences arthropod detection (Avalos et al. 2024). We also found that the COI primers we used (see Zeale et al. 2011), which were designed to be specific for degraded arthropod DNA, also amplified chloroplast sequences from *F. vesca*. Interestingly, this was not the case for the samples from *T. pratense*, nor for any of the plant species examined in studies using the same COI primer, including *Centaurea jacea*, *Tanacetum vulgare*, *Eupatorium cannabinum*, *Solidago canadensis*, *Daucus carota*, *Angelica archangelica*, *Echium vulgare* (Thomsen and Sigsgaard 2019), and *Malus* sp. (Gamonal Gomez et al. 2023). Thus, it seems that such co-amplification

problems may be plant species-specific. A possible solution to overcome this issue would be to design species-specific oligonucleotides (e.g., Deagle et al. 2013) tailored to the study plants in which co-amplification occurs. This would reduce the loss of sequencing depth that has the potential to affect metabarcoding results. Moreover, although PCR bias is not caused directly by the DNA extraction method (e.g., whether eDNA is extracted directly from flowers or by soaking and filtering), the extraction process strongly influences the composition, quality, and purity of the DNA template. If the extraction method preferentially recovers DNA from certain taxa or co-extracts inhibitors, those biases can amplify PCR bias. Therefore, careful selection and optimization of both extraction and amplification protocols is essential to minimize taxonomic bias in eDNA metabarcoding studies.

In conclusion, our study revealed a relatively high diversity of flower-associated arthropods, which consisted of a highly variable community composition, exhibiting a great deal of spatial variation in the understory of a Swedish forest. We show that forest structure has a positive effect on the species richness of flower-associated arthropods, particularly in response to the increased light availability found in broadleaf-dominated open plots, and that microclimate seems to play only a small role, if any, in species richness. Moreover, these effects may be plant species-specific. Future studies should explore the drivers of eDNA persistence on flowers, including the effects of flower shape and foraging time on eDNA yield. Future similar studies should also include baseline sampling of model plant species, sequencing controls separately, and optimizing the filtration procedure, including soaking time, water volume, and filter type, as well as performing in silico analyses for taxa expected in the study region. Additionally, eDNA sampling designs must be carefully planned to concentrate enough eDNA before extraction. Whenever possible, biological replicates should be prioritized. Moreover, primers should be assessed for potential co-amplification of plant chloroplast sequences, and blocking primers (e.g., Vestheim and Jarman 2008) should be designed to reduce the loss of sequencing depth. By improving our understanding of flower eDNA and refining current sampling techniques, we can increase species detectability and help to ensure that the collected data more accurately represents the biodiversity of the sampled environment.

Author Contributions

J.D.-C., P.D.F., A.F., E.Ö., S.A.O.C., and P.-O.H. conceptualized and designed the study. J.D.C., F.R., and P.O.H. conducted data collection, statistical analyses, and interpretation. All authors contributed to the writing of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Number of flowers (open pollination) and control flowers (bagged flowers with no insect interactions) collected per plant species, plot, and date for the arthropod eDNA extraction. **Table S2:** COI sequence list for all identified species, obtained with the primers developed in Zeale et al. (2011). **Table S3:** List of arthropod flower visitors identified through flower eDNA. The columns *Trifolium* and *Fragaria* show whether a flower visitor was present (1) or absent (0) from the samples of each respective plant species. **Figure S1:** Rarefaction curves of samples. The X-axis represents the number of sequences per sample, and the Y-axis is the number of ASVs found based on the number of sequences, which is used to reflect the sequencing depth. Different samples are represented by different curves. Note that all curves plateau, meaning that sequencing depth captured all sequence diversity.