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METHOD

BEE-quest of the nest: A novel method for eDNA-based, nonlethal detection of cavity-nesting hymenopterans and other arthropods

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Abstract

Wild bee populations are facing strong declines due to a variety of stressors, such as habitat loss and pesticide use. Research focused on wild bees has increased over the last three decades, yet species identifications are still relying on lethal sampling approaches, followed by time- and labor-intensive pinning and morphological analysis by experts. The use of DNA metabarcoding for species detection offers a time- and cost-effective complement to traditional approaches and can potentially facilitate nonlethal sampling. We developed a DNA metabarcoding workflow to identify cavity-nesting Hymenoptera using environmental DNA collected from vacated nest tubes. We compared different nest sizes and sample types and assessed the effect of these parameters on Hymenoptera species detection rates. We also tested whether our method could detect multiple species in mixed-species nests. The eDNA results were compared to data from morpho-taxonomical species identifications. Despite limited DNA quantity, we detected Hymenoptera at the species level in 57.4% of our samples. Hymenoptera detection was also possible from single brood cells. In mixed-species nests, we correctly detected the presence of multiple species. In nests of solitary wasps, we additionally detected other arthropods, which are probably the remains of larval provisions. Morphological and molecular data agreed in large parts. This eDNA approach offers the opportunity for the implementation of large-scale and nonlethal monitoring of cavity-nesting Hymenoptera that additionally provides valuable information on trophic interactions.

KEYWORDS
biodiversity monitoring, COI, DNA metabarcoding, environmental DNA, nonlethal sampling, wild bees

1 | INTRODUCTION

Wild bees are important pollinators of a variety of flowering plants and crops (Garibaldi et al., 2013; Klatt et al., 2014) and are indispensable for several ecosystem services (Matias et al., 2017) and human well-being (Klein et al., 2018). Wild bee richness, including species and phylogenetic diversity, has been declining globally since the 1990s (LeBuhn et al., 2013; Zattara & Aizen, 2021).

This decline is attributed to climate change, habitat degradation, fragmentation, and loss, as well as the interaction thereof (Goulson et al., 2015; IPBES, 2016; Soroye et al., 2020).

The importance of wild bees and other pollinators has been acknowledged throughout society (Domroese & Johnson, 2017; Drossart & Gerard, 2020; Wilson et al., 2017), and political programs to protect pollinators have been instigated, such as the Nature Restoration Law (European Commission, 2022) and the New Deal for Pollinators (European Commission, 2023). Consequently, the development of EU-wide pollinator monitoring gained political momentum (Potts et al., 2021). Novel approaches could accelerate its successful implementation by (1) overcoming the limitation in taxonomic expertise (Brown & Paxton, 2009; Hausmann et al., 2020), (2) utilizing nonlethal sampling and identification approaches (Tepedino & Portman, 2021), and (3) strengthening the involvement of the public (Domroese & Johnson, 2017; Drossart & Gerard, 2020; European Commission, 2023; Wilson et al., 2017).

The majority of studies on cavity-nesting Hymenoptera rely on trap nests, which is a reliable, straightforward, and cost-effective method (Staab et al., 2018), but frequently involves killing adult specimens for taxonomic identification. As a potential alternative, trap nests made of grooved wooden boards that can be stacked on top of each other (Straffon-Diaz et al., 2021) offer the opportunity for a nonlethal and citizen science-based monitoring approach simultaneously (henceforth referred to using the term nesting aid). Volunteers can take photos of the boards without interfering with the nesting Hymenoptera. Based on such photos and the visual appearance of nest building material and cocoons, a broad identification on the genus level can be achieved (Lindermann et al., submitted). Additionally, environmental DNA (eDNA) analysis (Taberlet et al., 2012) using DNA traces left behind in nest cavities after hatching can deliver species-level identifications. However, eDNA quantity and quality may be too low (Bruce et al., 2021) to achieve reliable species-level identifications. Moreover, confounding DNA sources such as pollen residues and micro-organisms (Keller et al., 2013; Voulgari-Kokota, Ankenbrand, et al., 2019; Voulgari-Kokota, McFrederick, et al., 2019) in the nests of cavity-nesting Hymenoptera may dilute the target DNA, resulting in reduced detection rates. This may be exacerbated due to DNA degradation processes and simultaneous microbial growth, the rate of which is influenced by environmental factors such as temperature and humidity (Sikorowski & Lawrence, 1994).

We developed a method for nonlethal species detection for cavity-nesting Hymenoptera for a potential citizen science-based monitoring approach. In order to determine ideal sampling conditions and processing workflows suitable for the involvement of volunteers, we collected eDNA from vacated nest tubes and compared detection rates under various conditions (Figure 1). Based on DNA metabarcoding of the *cytochrome oxidase I* (*COI*) gene, we compared detection success rates obtained from different nest sizes (based on brood cell counts), different sample types (fecal pellets and other residues

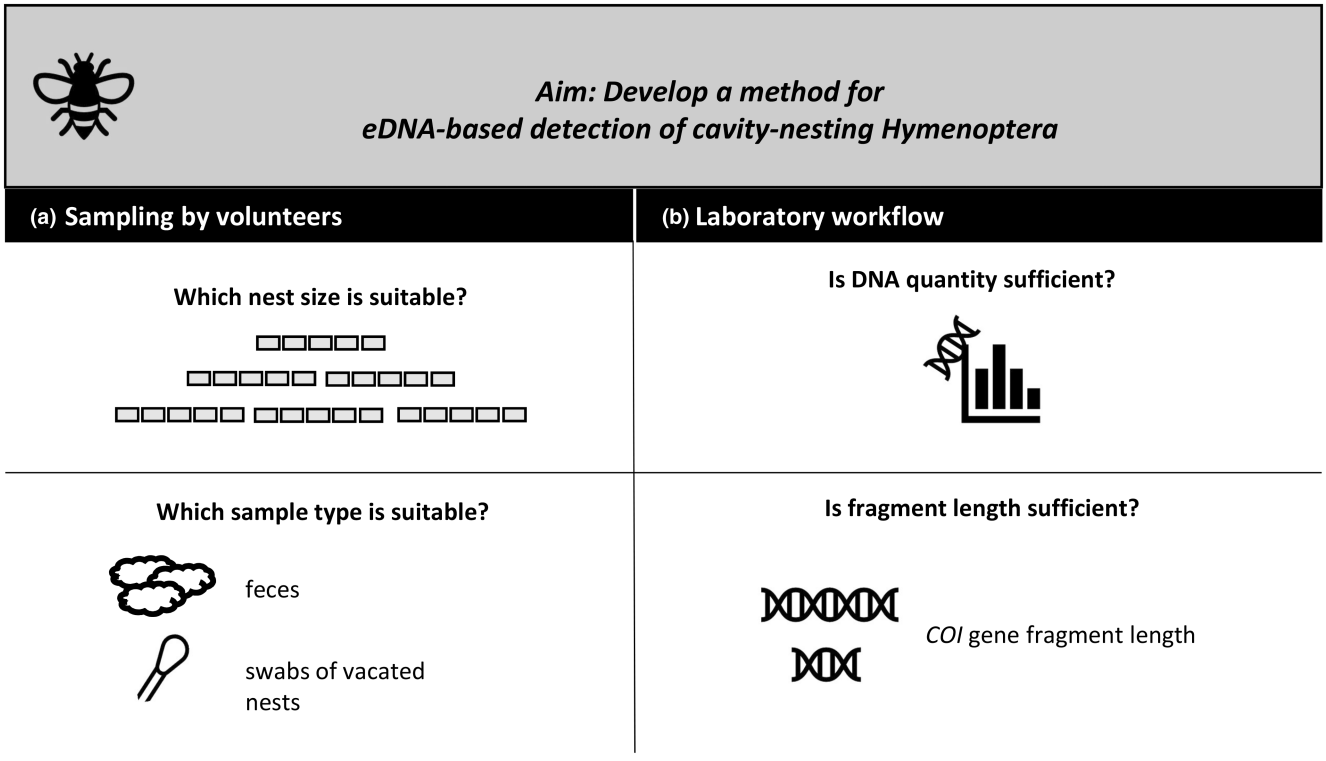

FIGURE 1 Conditions to be assessed for eDNA based Hymenoptera detections. (a) In order to facilitate sampling by volunteers, different nest sizes (top left) and sample types (bottom left) need to be evaluated. Nest sizes are indicated via brood cell counts; possible sample types are fecal pellets or swab samples obtained from the nest tube (bottom left). (b) DNA quantity (top right) and fragment length (bottom right) need to be sufficient for species-level detections.

versus DNA traces collected with cotton swabs), and assessed DNA quantities and overall PCR success. We expected DNA quantities and PCR success to be limited but sufficient for species-level detections. We expected detection rates to be affected by nest size and sample type, with higher detection rates potentially being obtained from larger nests (several brood cells) due to the accumulation of more DNA traces. Regarding sample type, we expected higher detection rates from swab samples, as fecal samples might contain PCR inhibitors as well as confounding DNA traces (Ali et al., 2019; Ando et al., 2020; Cheng & Lin, 2016; Nagarajan et al., 2020). Further, in the case of nests and/or brood cells shared between two different species (henceforth: mixed-species nests), we assessed whether several hymenopteran and/or other arthropod species could be detected simultaneously. Lastly, we compared the eDNA results with species identifications based on morphological characteristics and discussed the results in light of their potential implementation in future monitoring programs of cavity-nesting Hymenopterans.

2 | MATERIALS AND METHODS

2.1 | Experimental setup

We designed two experiments in order to evaluate eDNA-based species detection for cavity-nesting Hymenoptera (Figure 2). In *experiment 1* (Figure 2a), we assessed whether the quantity and fragment lengths of eDNA in vacated nests were sufficient for Hymenoptera detections at species level. We grouped 50 trap nests into three different nest sizes: (1) small nests (1–5 brood cells; $N=20$), (2) medium nests (6–10 brood cells; $N=20$), and (3) large nests (>10 brood cells; $N=10$). Across all nest sizes, we tested two different sample types: feces collected from nests of cavity-nesting hymenopterans and swabs of the same nests after adults hatched (($N=100$); see Section 2.2). During PCR, we amplified two different *COI* gene fragment lengths (205 and 313 base pairs (bp)). In *experiment 2* (Figure 2b), we assessed whether we could also analyze mixed-species nests. We included 55 nests that harbored more than one species. This included nests that were likely parasitized ($N=37$), for example, by *Cacoxenus indagator*, as well as nests that were colonized by two Hymenoptera species without apparent parasitation ($N=18$). Thus, this included nests colonized by cavity-nesting Hymenopterans, that is, wild bees as well as wasps (including potential parasitoids such as Chrysididae) as well as other arthropods, for example, Dipterans. Here, only swab samples and the 205-bp gene fragment were tested. Feces were excluded from this experiment based on preliminary test results, which indicated a lower amplification success from feces overall.

2.2 | Nest processing and eDNA sampling

We used trap nests that were set up by Beyer et al. (2023) to attract cavity-nesting hymenopterans in various agricultural landscapes across Germany in 2018. After the activity period of cavity-nesting Hymenopterans, the trap nests were collected in October 2018 and stored as single tubes (henceforth: nest tubes) at 4°C in a climate chamber for 3 months. Then, nest tubes were carefully opened with a scalpel and visually inspected to collect feces samples as well as obtain preliminary taxa identifications as follows: Fecal pellets and other residues (henceforth: feces), such as residual pollen or remains of larval food of solitary wasp species, were removed using sterilized forceps and stored at −20°C. Preliminary taxa identifications were obtained based on nest building material and other characteristics. Nest building material and the visual appearance of wild bee nests are rather characteristic and thus allow preliminary taxa identifications at the genus and, in some cases, even species level (Lindermann et al. (2023); see Figure S1 for example photos). Additionally, in many cases, individual brood cells can be distinguished visually (see Figure S1 for example photos) and thus serve as a means to determine nest sizes via simple brood cell counting. After feces collection, preliminary identification, and nest size determination, nest tubes were resealed and further stored at room temperature to allow the hatching of adult specimens. After hatching, specimens were collected and morphologically identified to the lowest taxonomic level possible (data available from the GitHub repository; see data availability statement). In June 2020, nests were reopened using a scalpel, and remaining DNA was collected with DNA-free cotton swabs (Sarstedt AG & Co. KG, Nümbrecht, Germany, Supplemental Material S1), which were stored at −20°C until further processing.

2.3 | DNA extraction, PCR and sequencing

DNA was extracted using the ZymoBIOMICS® DNA Microprep Kit (Zymo Research Europe GmbH), eluting in 20 μL as specified by the manufacturer. Samples were then split into duplicates, to serve as technical replicates, and distributed over 96-well plates following the layout of Elbrecht and Steinke (2019).

We followed a two-step PCR protocol with a dual-tagging strategy. We specifically aimed to detect a wide range of arthropod taxa, such as the dipteran *Cacoxenus indagator*, a common parasitoid of cavity-nesting wild bees (Steffan-Dewenter, 2002). Thus, we used primers that amplify both hymenopteran and non-hymenopteran DNA. We applied two sets of primers that amplify *COI* gene fragments of different lengths: The first set (henceforth: short fragment) amplifies ~205 bp (Elbrecht et al., 2019) and was used on all samples. The second set (henceforth: long fragment) amplifies ~313 bp (Folmer et al., 1994; Leray et al., 2013) and was used on feces only, as this fragment could not be amplified from swab samples, as determined during preliminary testing (data not shown). Both fragment sizes do not cover the complete *COI* gene but have successfully been used in DNA metabarcoding of terrestrial arthropods (Elbrecht et al., 2019). Generally, longer fragments are preferred for species-level identifications, but preliminary testing revealed that we were unable to amplify the desired 313 bp fragment from swab samples (data not shown). All oligos were purchased from Eurofins

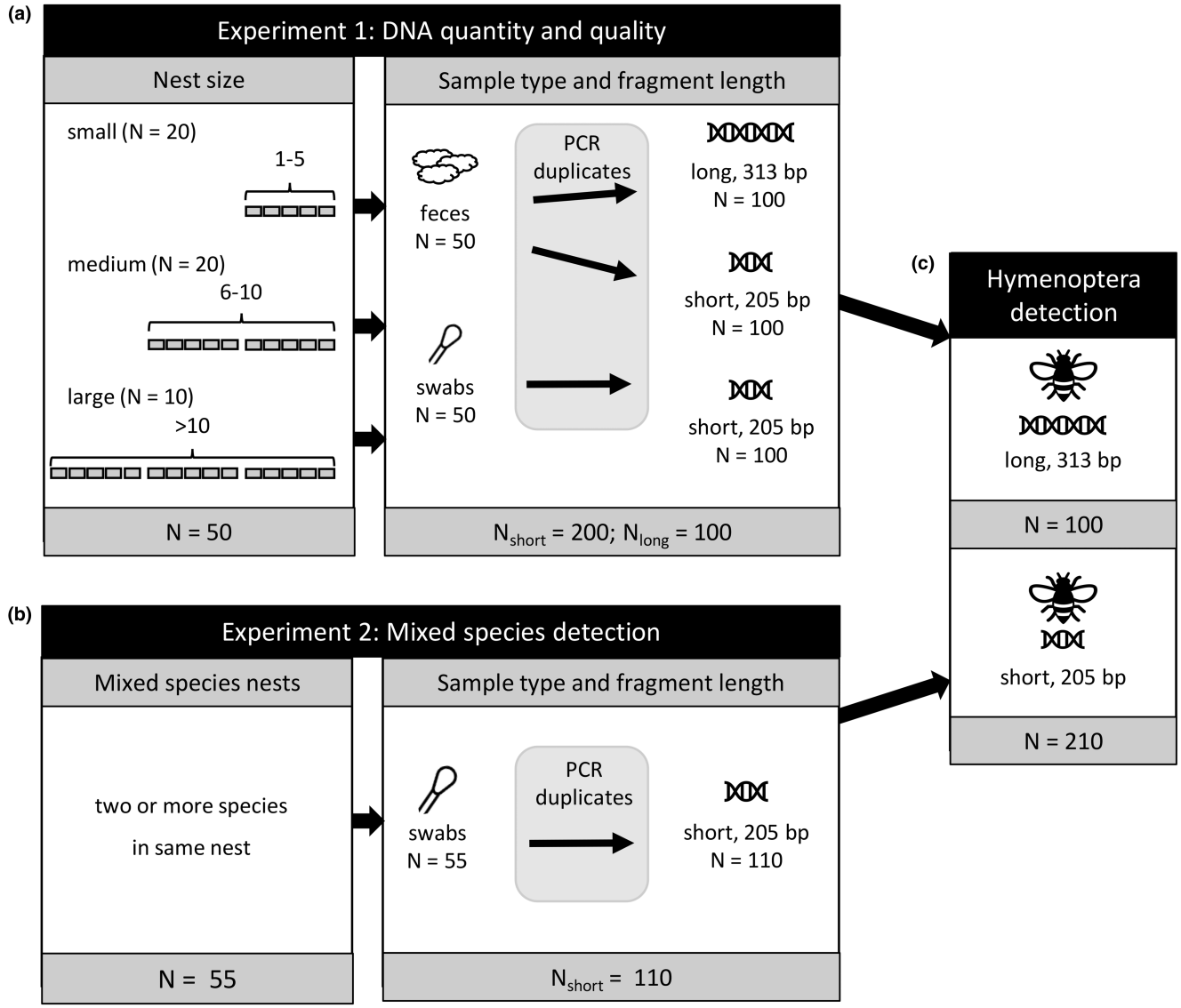

FIGURE 2 Experimental design. (a) Experiment 1, where different nest sizes and sample types were compared and DNA quantity and PCR success were assessed based on *COI* gene fragments of different lengths. Nest sizes were small: 1–5 brood cells, medium: 6–10 brood cells, and large: >10 brood cells; sample types were feces and swabs; feces were collected during visual inspection of the nests; swabs were collected approximately 1 year after adult hatching; the long *COI* fragment was assessed for feces samples only and the short *COI* fragment was assessed for both feces and swab samples; PCRs were performed in duplicate for all samples; (b) experiment 2, where mixed-species nests were assessed based on swab samples and the short *COI* gene fragment; PCRs were performed in duplicate for all samples; (c) for both experiments, the rate of Hymenoptera species detection was assessed.

Genomics (Ebersberg, Germany) at NGS-grade (HPLC-cleaned), and complete sequences, including tags and their allocation to samples, are available from GitHub (see Data Availability Statement).

PCRs were performed in 10 μL (first PCR) and 50 μL (second PCR) reactions, both containing 0.25 U HotStarTaq polymerase, 1× PCR buffer, 200 μM each of dNTPs (all Qiagen), sterile filtered PCR grade water (*quantum sadis*), 0.3 μM oligos, and 1 μL DNA template. In the first PCR, oligos were forward and reverse primers with HEAD-/TAIL-sequences (Table S1); in the second PCR, oligos were sample-specific combinations of forward and reverse tags containing HEAD-/TAIL-sequences as well (Table S1). PCR conditions were: initial denaturation for 15 min at 95°C; 40 (first PCR) or 20 (second PCR) cycles of denaturation for 45 s at 94°C; annealing for 45 s at different temperatures (Table S1); elongation for 1 min at 72°C; and final elongation for 10 min at 72°C (performed on a MasterCycler ×50s, Eppendorf).

PCR success was assessed after the first PCR via agarose gel electrophoresis, but all samples were processed further, irrespective of whether a PCR band was visible. After the second PCR, we quantified all products using the Quantus™ and Quantifluour ONE assays (Promega) and created two pools, one for each of the different fragment lengths. Each pool was cleaned up using magnetic beads (NucleoMag Clean-up & Size Select, Macherey Nagel), eluting in 30 μL. The two pools were then combined into a common

library (ratio short: long = 2:1), with a final concentration of 14 ng/μL. This library was sequenced at LGC Genomics (Berlin, Germany) for 2 × 300 bp using Illumina MiSeq v3 chemistry, with 5%–10% PhiX added (information provided by LGC Genomics). A detailed description of the methods can be found in Supplemental Material S1. DNA extraction and PCR setup were performed under a clean bench that was UV-treated and cleaned with DNA-away® (Thermo Fisher Scientific Inc.) before and after each extraction batch and PCR setup, respectively. Only sterile pipette tips with filters were used.

2.4 | Bioinformatic processing of sequencing data

Bioinformatic processing of sequencing data mainly followed the *VSEARCH* pipeline (Rognes et al., 2016). Here, we report a summary of the main steps, whereas details, including filtering parameters, are given in Supplemental Material S2. Raw data were preprocessed, demultiplexed, and trimmed using *Cutadapt 3.7* (Martin, 2011) with *Python 3.9.6* and *seqkit 2.2.0* (Shen et al., 2016). Short and long fragments were processed separately, following the same pipeline. The remaining steps were performed using *VSEARCH* 2.17.1 (Rognes et al., 2016), which included read joining, quality filtering, dereplication, denoising, chimera checking, and community table generation based on amplicon sequence variants (ASVs). For taxonomic classification, we followed a step-wise approach similar to Wilson et al. (2021), but based on *COI* reference data. Our reference databases were a combination of GenBank (Sayers et al., 2021) and BOLD v3 (Ratnasingham & Hebert, 2007), which included arthropods known to occur in Germany. We also used the MIDORI-UNIQ database GB241 (Machida et al., 2017) to classify the remaining taxa (see Supplemental Material S2 for a detailed description). Bioinformatic scripts as well as reference databases are publicly available (see Data Availability Statement).

2.5 | Data analysis

2.5.1 | Hymenoptera detection and mixed-species nests (experiments 1 and 2)

The short fragment data set, including all samples from both experiments (Figure 2), was subsetted to only include metazoa. ASVs with more than 75 reads in laboratory controls (extraction blanks, PCR negative controls) were removed from the data set. This threshold was determined after assessing raw read counts of ASVs in extraction and PCR controls (Figure S2) and removing the most abundant contaminant ASVs.

Positive controls were checked for the occurrence of *Apis mellifera* and subsequently removed from the data set. We then agglomerated ASVs on the species level (i.e., ASVs with the same taxonomic classification in the species column of the taxonomy table) and subsetted them to non-*Apis* Hymenoptera and Diptera. Hymenoptera detection was counted as successful only when a species-level detection was achieved. Comparisons between sample types and nest sizes were assessed using the Fisher's exact test.

We further assessed the effect of nest size and sample type on Hymenoptera detection rates and mixed-species detections using generalized linear models (GLMs, categorical variables converted to numeric, binomial distribution). For Hymenoptera detections, we included two explanatory variables: nest size, sample type and their interaction. The response variable was successful Hymenoptera detection, counted as true only when both duplicates detected Hymenoptera at species level (coded as 0/1). In this model, we only included samples from experiment 1 (Figure 2a).

Using the swab samples collected from mixed species (Figure 2b), we assessed the effect of nest size on detection rates of more than one taxon per nest tube (only including Hymenoptera and Diptera). Nest size was used as the explanatory variable, and mixed-species detection was the response variable, counted as true only if at least two taxa were detected in both duplicates. Additional GLMs also included the effect of DNA yield and PCR success on Hymenoptera and mixed-species detections (see Tables S2 and S3, shortly discussed in Supplemental Material S4).

The models were assessed and evaluated via chi-square goodness of fit, McFadden's pseudo R-squared and likelihood ratio tests. The statistical significance of individual terms was determined using Wald's chi-square test. Additionally, we analyzed the confusion matrices, accuracy, precision, recall, and F1-score of the predicted values of the different models.

2.5.2 | DNA quantity and PCR success (experiment 1)

Using both feces and swab samples from different nest sizes (Figure 2a), DNA quantity was assessed as total yield (DNA concentration [ng/μL] * elution volume [20 μL]). PCR success was assessed as a "visible band after first PCR" for both the short and long fragments. DNA yields between sample types were assessed via the Kruskal-Wallis test, as DNA yields were not normally distributed (Shapiro-Wilk-Test; data not shown). The effect of nest size was then assessed for both sample types combined as well as separately, again with the Kruskal-Wallis test. Significant differences between sample types were assessed with Kruskal-Nemenyi's All-Pairs Rank Comparison Test. For PCR results, we assessed differences between sample types and/or nest sizes using the Fisher's exact test.

2.5.3 | Validation of eDNA data—Hymenoptera and other arthropods

For both experiments, we validated the eDNA-based species detections obtained with the short fragment. The reference data set was based on morphological species identifications by a trained expert (Christian Schmid-Egger), complemented by indirect identifications

made during visual inspection of the nest tubes during sample processing (the dataset can be obtained from GitHub; see the Data Availability Statement). Some taxa could only be identified as belonging to the groups Ichneumonoidea, Chalicidoidea, or Spheciformes, respectively. In such cases, we used a list of taxa within those groups known to inhabit trap nests in Germany (Supplemental Material S3) to validate the eDNA data set.

2.5.4 | Analysis of the long COI fragment reads

Analogous to the short fragment data set (see above), the long fragment data set was subsetted to only include metazoa. We removed ASVs with more than 75 reads in laboratory controls. Positive controls were checked for the occurrence of *Apis mellifera* and subsequently removed from the data set. As this data set contained too few reads to analyze comprehensively (see Section 3.4), we only checked for the occurrence of Hymenoptera and Diptera across all samples combined.

All statistical analyses were conducted in the R environment v.4.1.2 (R Core Team, 2020) with a set of different packages (Supplemental Material S2).

3 | RESULTS

3.1 | Experiment 1 and 2: Hymenoptera detection and mixed-species nests

Hymenoptera detection rates were assessed for the short *COI* fragment only, due to larger sample sizes and sequencing output. Across both experiments, we detected 274 metazoan ASVs, 17 of which were removed by contamination filtering.

Agglomerating the remaining 257 ASVs on species level (see Section 2.5) reduced this further to 55 taxa in 49 genera and 31 families (including ASVs that were not taxonomically classified). Reduction to non-*Apis* Hymenoptera and Diptera left 17 taxa, of which 13 were Hymenoptera (one unclassified taxon). Overall, we detected on average 5.4 metazoan taxa per sample (SD: 3.2). When only including Hymenoptera and Diptera, we detected on average 1.5 taxa per sample (SD: 1.4).

Hymenoptera could be detected in 198 of 310 samples (63.9%), and 178 (57.4%) included a taxonomic classification at the species level. Swab samples performed significantly better than feces, with 61.4% and 49% of samples having Hymenoptera species-level detection, respectively (Figure 3, Fisher's exact test, $p=0.049$). There was no significant difference in nest size (small nests: 58.1%, medium nests: 50.9%, large nests: 69.2%; Fisher's exact test, $p=0.085$). This was also true when analyzing the two sample types separately (feces: $p=0.57$, swabs: $p=0.136$). In small nests, it was possible to detect Hymenopterans from as little as one singular brood cell, although this was the case only for swab samples (Figure S3). Regarding mixed-species nests, we detected more than one taxon in

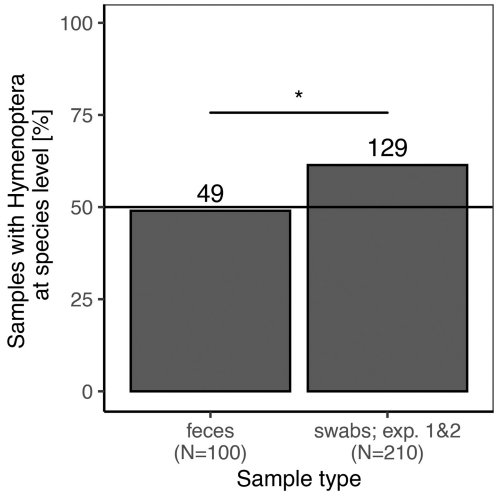

FIGURE 3 Hymenoptera detections in both experiments. Feces versus swab samples of both experiments; asterisk indicates a statistically significant difference (Fisher's exact test); numbers on top of columns indicate absolute counts of samples with positive Hymenoptera detections; the horizontal line indicates a 50% threshold.

64.5% of the samples (71/110), with 47.3% (26/55) of the duplicated samples showing concordant results.

We used GLMs to assess whether sample type and/or nest size could be used to infer successful Hymenoptera or mixed-species detections (Table 1). The models' performance was variable but generally not satisfactory. For example, chi-square goodness of fit estimates were small (<0.2), and Wald's test did not show any statistical significance for individual terms. Additionally, pseudo-R^2 was low (0.01). Confusion matrices, accuracy, precision, recall, and the F1-score varied across the two different models (Table 1). Among these metrics, the F1-score (likelihood of predictions being correct) is particularly informative for evaluating model performance. The F1-score was undefined due to zero true and false positives and F1 = 0.57, respectively, which shows that neither model performed well (Table 1).

3.2 | Experiment 1: Assessing DNA quantity and PCR success

DNA quantities ranged from 0 to 1860 ng (mean: 539.89 ng; standard deviation (SD): 524.38 ng, Figure 4) and were not statistically different between the two sample types, feces and swabs (mean feces samples: 570.0 ng; SD: 520.1 ng; mean swab samples: 509.8 ng; SD: 532.2 ng; Kruskal-Wallis test; chi-squared (χ^2) = 0.495, df = 1, $p=0.482$). Nest size had a significant effect on DNA yields across swabs and feces combined ($\chi^2=23.3$, df = 2, $p=8.58\times10^{-6}$), as well as for each sample type separately (feces: $\chi^2=13.6$, df = 2, p-value = 0.00111; swabs: $\chi^2=10.1$, df = 2, $p=0.00637$). Small nests (1–5 brood cells) were statistically different from the other two nest sizes ($p<0.05$).

PCR success was assessed as a "visible band after first PCR." For the short fragment, this was the case for 57% of the samples

TABLE 1 GLM analysis for the detection of Hymenoptera at the species level and mixed-species detection.

X^2-goodness of fit	McFadden's pseudo-R^2	Likelihood ratio test	Wald's X^2-test[1]	Confusion matrix (TP|FN FP|TN)	Accuracy	Precision	Recall	F1-score
Hymenoptera detection ~ sample type * nest size								
0.11	0.01	0.73	*Sample type*: 0.83 *Nest size*: 0.50 *type*size*: 0.02	0|26 0|74	0.74	NA[a]	0	NA[a]
Mixed-species detection ~ nest size								
0.02	0.01	0.49	*Nest size*: 0.48	17|9 17|12	0.53	0.50	0.65	0.57

Note: All values were rounded to two decimal places.

Abbreviations: FN, false negative; FP, false positive; TN, true negative; TP, true positive.

[1]All p-values > 0.05.

[a]Undefined as TP and FP are both zero.

FIGURE 4 DNA yields in experiment 1. (a) Feces and swab samples combined; (b) swab samples only; (c) feces only; nest size abbreviations: S = small (1–5 brood cells), M = medium (6–10 brood cells), L = large (>10 brood cells), each dot represents an individual sample, dots are jittered to avoid overlaps, and asterisks indicate significant differences between nest sizes (Nemenyi's All-Pairs Rank Comparison Test).

(114/200 samples; processed in duplicate during PCR). In 78% (78/100) of the samples, the duplicates showed consistent PCR results (i.e., both showed a band or not); the other 22% (22/100) had discordant PCR results, that is, one duplicate showed a band, the other did not. In both sample types, 57% (57/100 samples each) of the samples were PCR positive, with 78% of the duplicates in agreement (39/50 samples each). Regarding nest size, all sizes performed similarly well, with 55%–59% of samples being PCR positive and concordance between replicates ranging from 50% to 92.5% (Table S2). For swab samples, medium-sized nests performed slightly better than the other two nest sizes, while for fecal samples the reverse was true (Table S2). These differences were, however, not

TABLE 2 Comparison of taxon detections between morphological identification and eDNA data.

Order/Family	Species	Both	Morphological identification	DNA
Hymenoptera		X		
Crabronidae		X		
	Trypoxylon figulus	X		
	Trypoxylon clavicerum	X		
Megachilidae		X		
	Osmia bicornis	X		
	Heriades truncorum	X		
	Hoplitis leucomelana[a]		X	
	Stelis breviuscula			X
Colletidae		X		
	Hylaeus communis	X		
Sapygidae		X		
	Sapygina decemguttata	X		
Chrysididae		X		
	Chrysis solida			X
	Trichrysis cyanea		X	
Vespidae				X
	Ancistrocerus nigricornis			X
	Euodynerus quadrifasciatus			X
	Symmorphus bifasciatus			X
	Allodynerus rossii			X
Eulophidae			X	
	Melittobia acasta		X	
Torymidae			X	
	Monodontomerus obscurus		X	
Diptera		X		
Drosophilidae				
	Cacoxenus indagator	X		
Agromyzidae				X
	Chromatomyia primulae			X
Syrphidae				X
	Paragus haemorrhous			X
	Brachypalpoides lentus			X
Coleoptera		X		
Coccinellidae			X	

Note: Forty-six additional taxa detected and identified at the species level with eDNA only are excluded, but can be found in Supplemental File S2; for wild bee taxonomy, we followed Scheuchl et al. (2023).

[a]Previously: *Osmia leucomelana*.

significant. Large nests had significantly less samples in concordance than medium sized nests (Fisher's exact test, both sample types combined: $p = 0.00114$, swab samples only: $p = 0.001061$, feces only: $p > 0.05$). Feces were also used for the long *COI* fragment, and 56% (56/100) were PCR positive, with 64% (32/50) of the duplicates in concordance. When comparing nest sizes, the large nest size performed better than the other two (Table S2). Regarding the numbers of PCR-positive samples, no significant differences between sample types and/or nest sizes were found (Fisher's exact test, $p > 0.05$). Supplemental GLM analyses further showed that PCR success was only partially informative to infer sequencing success (Tables S3 and S4, Supplemental Material S4).

3.3 | Validation of eDNA-data

Validation of eDNA results via morphological analysis could not be performed for all samples since adult specimens for morphological

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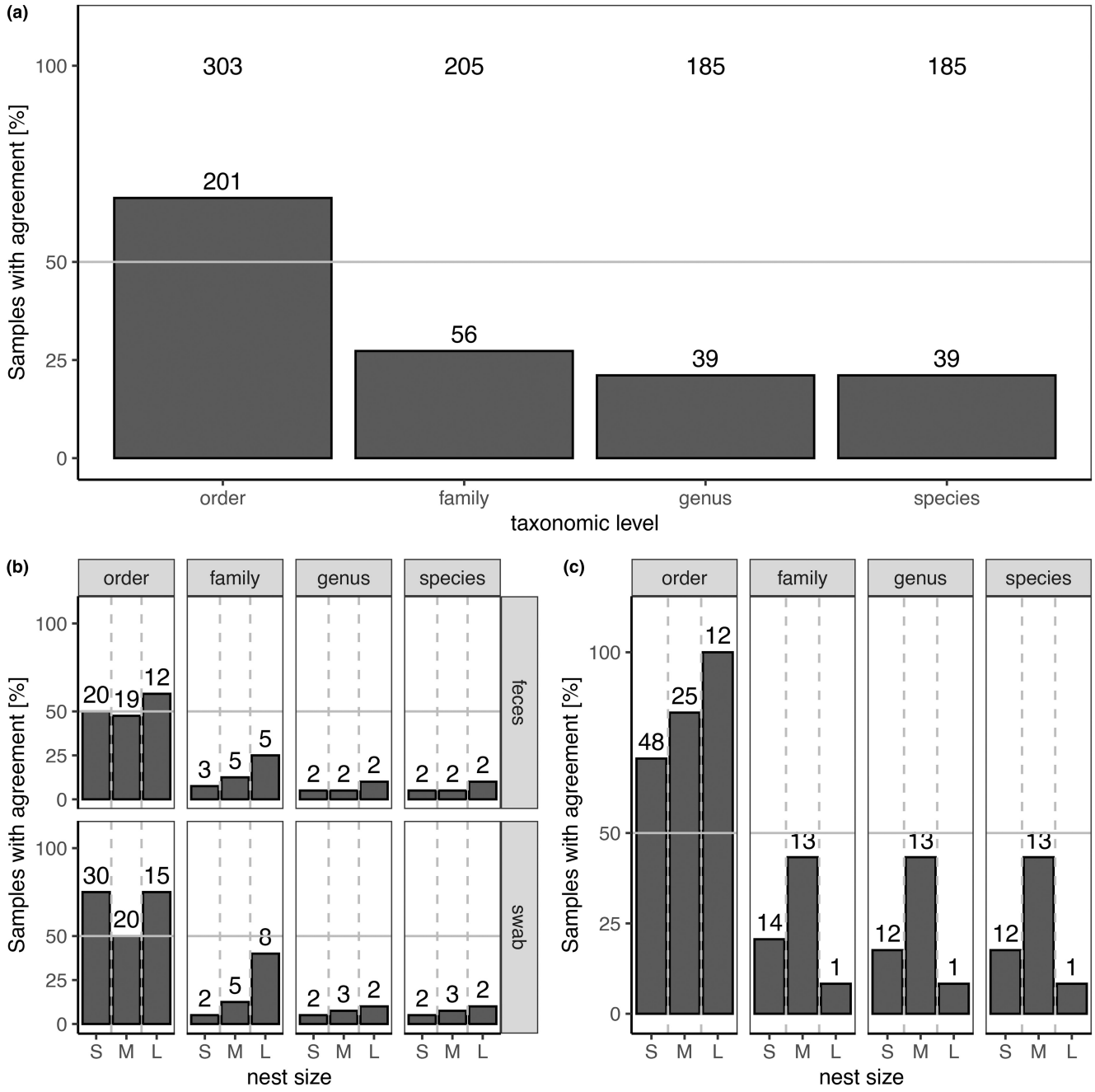

FIGURE 5 Validation of eDNA detections for Hymenoptera and other Arthropods. (a) validation across all samples combined; (b) validation for experiment 1; (c) validation for experiment 2; nest size abbreviations: S = small (1–5 brood cells); M = medium (6–10 brood cells); L = large (>10 brood cells); numbers at the upper margin of the plotting area indicate total sample size per column; numbers on top of columns indicate absolute counts of samples with positive validation results; a horizontal line indicates a 50% threshold.

analysis were available from only 57 nests or because identifications could only be made at the genus or family level. Seven samples did not have an Arthropod species detected via eDNA. Thus, validation of eDNA detections was possible for only a subset of the samples, and this varied depending on the taxonomic level. For the order level, 303 eDNA samples could be validated, 205 at the family level and 185 at the genus and species level, respectively.

Across all samples, morphological species identification and eDNA-based identification shared seven Arthropod species. Within wild bees, five species were detected in total, and three of them were detected with both approaches, one with morphology only and one with eDNA only (Table 2). For solitary wasps, eleven species were detected in total, and three of them were detected with both approaches, with five species being detected by eDNA only and three species detected by morphology only (Table 2). For other taxa, five dipteran and coleopteran taxa were detected. Of these, only *Cacoxenus indagator* was detected with both methods (Table 2). Moreover, 46 additional taxa were detected and identified at the species level using eDNA, including taxa belonging to Lepidoptera, Coleoptera, Aranae, Psocoptera (or Psocodea), Trombidiformes, and

Thysanoptera (Supplemental File S2). Sample-wise comparisons between morphological and eDNA data (Figure 5) revealed differences depending on taxonomic level. Across all samples (Figure 5a), eDNA detections were validated at 66.3% (201/303) at the order level, at the family level at 27.3% (56/205), and at the genus and species level at 21.1% (39/185). Analyzing these comparisons at the scale of experimental groups, sample types, or numbers of brood cells showed similar numbers and trends (Figure 5b,c).

3.4 | Analysis of the long COI fragment

The long *COI* fragment yielded very few remaining sequences overall (466,109 read pairs compared to 4,665,429 read pairs for the short fragment), which accounted for only 8.8% of total raw read pairs, and after processing (only using forward reads), 195,091 reads remained (41.9% of input reads; controls included; samples processed in duplicate). The mean output per sample was 629.3 reads (SD: 1953.1 reads). This data set included 108 taxa, of which only nine belonged to metazoa and only five were classified at the species level. After removing contaminant taxa and control samples, four metazoan taxa remained. Remaining read counts were very low (mean: 4.5 reads, SD: 16.5 reads, maximum: 132 reads), and only one sample had read counts above 100 reads. These numbers showed that sequencing output was insufficient for comprehensive analyses, and we thus re-combined all remaining reads into an aggregated data set, which contained 444 reads and contained two Hymenopteran taxa (*Heriades truncorum* and *Sapygina decemguttata*) and one unclassified Dipteran taxon.

4 | DISCUSSION

We developed a method for nonlethal detection of cavity-nesting Hymenoptera based on eDNA collected from vacated nest tubes. We compared nests of three different sizes, two different sample types and different fragment lengths by means of DNA yield, PCR success as well as the possibility to detect and identify Hymenoptera at the species level and the possibility to detect mixed-species nests based on *COI* metabarcoding.

4.1 | Hymenoptera species and species mixtures successfully detected

We successfully detected Hymenoptera at the species level in 49% of the feces and 61.4% of the swab samples. This is particularly noteworthy for the swab samples, as these were collected after vacated nest tubes had been stored at room temperature for approximately 1 year. We found little differences in success rates between the three different nest sizes, even though many small nest samples yielded undetectable levels of DNA due to small amounts of residue deposited (see above). Interestingly, swab samples collected from one single brood cell enabled Hymenoptera species detection (Figure S3). This indicates that nests of all sizes can be used to detect Hymenoptera via eDNA, which is especially relevant for small nests (1–5 brood cells in our study) with often undetectable DNA yields after DNA extraction. Additionally, although the fragment lengths that we could amplify were comparably short, they were sufficient for species-level identifications. In a second experiment, we sampled mixed-species nests in order to assess whether we could detect multiple species per nest. Indeed, we were able to detect more than one species (Hymenoptera and Diptera included) in 64.5% of the cases, regardless of nest size (i.e., number of brood cells). For example, we detected the fruit fly *Cacoxenus indagator*, a common parasitoid of cavity-nesting wild bees (Steffan-Dewenter, 2002).

4.2 | DNA quantity and PCR success overall low, but sufficient even after prolonged storage at room temperature

Concerning DNA quantity and PCR success, we found no difference between the two sample types (feces and swabs), but small nests (<= 5 brood cells) yielded significantly less DNA than medium-sized nests (6–10 brood cells) and large nests (>10 brood cells). This difference was mainly driven by samples with undetectable DNA yields from small nests, which is in line with our expectations. An explanation could be that smaller nests contain less residual matter for DNA collection and extraction. Interestingly, this pattern did not hold true for the comparison between medium and large nests.

We were unable to amplify the 313-bp *COI* fragment from swab samples, but we could amplify this from feces. This can be attributed to the difference in sample time and storage conditions. Feces were collected before hatching and directly stored at −20°C. In contrast to this, swab samples were collected after prolonged storage of vacated nest tubes at room temperature. Thus, DNA left in the vacated nest tubes was subjected to biotic and abiotic processes of DNA degradation. However, we also observed no amplification from feces for either of the *COI* fragments. Failure to amplify from feces is commonly reported, and reasons include low concentrations of target DNA compared to nontarget DNA, suboptimal purity, the presence of PCR inhibitors, and DNA degradation even in freshly collected feces (Ali et al., 2019; Ando et al., 2020; Cheng & Lin, 2016; Nagarajan et al., 2020). Thus, PCR success is an important issue in both sample types, but for different reasons.

4.3 | Morphological identifications and eDNA matched well

The obtained species-level detections matched well with morphological data, with just one bee species not detected via eDNA but detecting four additional wasp species and one cuckoo bee

species that were not detected with the classical approach. This is in line with previous works comparing DNA metabarcoding and morphological approaches (Fediajevaite et al., 2021; van der Heyde et al., 2020). However, in many cases, direct comparisons were discordant at taxonomic levels below the order level. Many of these cases were samples, where *Osmia bicornis* was identified morphologically but eDNA identified *Heriades truncorum* instead (Figure S4, Supplemental File S3). These two species can be clearly distinguished both morphologically and based on nest building material. *Osmia bicornis* is larger and more pilose than *H. truncorum*, amongst others. Nest building material is clay for *O. bicornis* and resin for *H. truncorum* (Lindermann et al. (2023); see also Figure S1). The misidentification via eDNA was probably caused by the short DNA fragment amplified. Similarly, the identification of *Chrysis solida* in the eDNA data set may be a misclassification, considering that *Trichrysis cyanea* was identified morphologically. In some cases, eDNA failed to detect Hymenoptera at all. This might again be applicable to fragment length issues, and highlights the requirement to work with fresher samples if possible. Thus, the combination of different identification approaches might be a powerful solution to achieving robust species lists. In cases where the eDNA approach fails to unambiguously identify Hymenoptera at the species level, a reference data set containing information on nest building material is useful to achieve a final identification. Such data can be obtained via photographs from boards of nesting aids and is an attractive approach to involving volunteers without taxonomic expertise (Lindermann et al., 2023).

We emphasize that the eDNA approach additionally detected taxa belonging to Coleoptera, Lepidoptera, and Arachnida, among others (Supplemental File S2), which may have been DNA traces of larval provisions of solitary wasps (Evans, 1966; Westerfelt et al., 2015). On top of these, fungal taxa were also detected (see ASV-table available from GitHub), even though the *COI* gene is not a typical genetic marker to analyze fungal communities (Banchi et al., 2020; Schoch et al., 2012). The detection of these additional nontarget taxa indicates that eDNA analysis of the nests of cavity-nesting Hymenoptera can also facilitate the analysis of ecological networks, including parasitoids and, in the case of cavity-nesting wasps, larval provisions (Fornoff et al., 2023).

4.4 | Output of the long COI fragment not sufficient

The long *COI* fragment yielded too few sequences to analyze comprehensively. The low sequencing output may be explained by suboptimal pooling of short and long fragments in combination with the preferred clustering of short fragments on the Illumina platform (Gohl et al., 2019). This may have intensified because long fragments were largely underrepresented in our pooling strategy (132 vs. 384 samples (including controls and duplicates), pooling in favor of short fragments (2:1)). Thus, for the analysis of cavity-nesting Hymenoptera, it seems advisable to keep long and short fragments separate, although successful sequencing of fragments of different lengths has been performed previously (de Kerdrel et al., 2020; Wilson et al., 2021).

4.5 | Recommendations for the workflow

Combining all of the above results holds important implications for applying this approach to other projects in general and to large sample sizes in particular. First, species detection of cavity-nesting Hymenoptera via eDNA is possible using vacated nest tubes and may be successful even after prolonged storage in suboptimal conditions. It is common practice to collect adult specimens via lethal sampling, as many wild bee species cannot be identified in the field (Gibbs et al., 2017). This also applies to previous studies applying DNA-based species identifications (Darby et al., 2020; Gueuning et al., 2019; Tang et al., 2015). In contrast to this, our method applies nonlethal sampling while retaining species-level resolution. Through broad taxa identifications determined during the larval or pupal stage, for example, via photos, we obtain a reference dataset that can be used to validate eDNA detections. This facilitates the analysis of false positives and negatives, which is more difficult in approaches that sample surfaces exposed to the environment or air (Harper et al., 2021; Roger et al., 2022; Thomsen & Sigsgaard, 2019; van der Heyde et al., 2020). Second, fecal pellets and cotton swabs are both suitable sample types. Swabs performed better for Hymenoptera detections, but we were unable to amplify *COI* fragments longer than 205 bp, which was possible from feces. Although the DNA fragment length was sufficient for Hymenoptera detections in 57.4% of the samples, even with the short *COI* fragment, we do recommend collecting samples as quickly as possible after hatching. Third, DNA yields and PCR success as judged by agarose gel electrophoresis were suboptimal predictors to infer the success of Hymenoptera and mixed-species detection (Tables S3 and S4, Supplemental Material S4). Thus, we argue, that it may be sufficient to perform these quality control steps only on positive and negative controls and a random subset of samples, to improve the time- and cost-efficiency of the workflow. Last but not least, certain species were not detected via eDNA or may have been misclassified. Thus, we recommend collecting relevant metadata that may aid in detecting such misclassifications, such as nest tube diameter and nest building material.

Modifications to the workflow may include an alternative quantification method, such as a qPCR assay with Hymenoptera-specific primers, which we expect would improve the suitability of DNA yields (in qPCR measured as DNA copy number) as a predictor for sequencing outcome. Similarly, the application of more specific primers for the endpoint PCR is also an option (Bleidorn & Henze, 2021) and may be advisable for certain research questions that strictly target Hymenoptera. This was not desired in our case, because we specifically aimed at targeting other taxonomic lineages as well (e.g., Diptera, *Cacoxenus indagator*), which would be missed when using Hymenoptera-specific primers.

5 | CONCLUSIONS AND OUTLOOK

We have demonstrated that this method achieves identifications of cavity-nesting Hymenoptera and other insects down to species level, even though the nest tubes were stored at room temperature for a prolonged time interval. The combination with other approaches, such as photographic documentation, presents a powerful strategy for nonlethal and citizen science-based monitoring. Volunteers can be involved in the collection of photographic reference data (Lindermann et al., submitted) that enables broad identifications at the genus, sometimes even at the species level. Moreover, data on brood cell counts can be obtained from the photos and used to infer species counts. In addition to this, the application of eDNA analysis can deliver species-level identifications from nonlethally collected samples. In perspective, this method may be suitable to analyze (phylo-)genetic diversity (Faith, 1992). In conclusion, this strategy successfully combines the advantages of both classic and innovative identification methods and thus opens up novel avenues for pioneering large-scale monitoring programs of cavity-nesting Hymenoptera.

AUTHOR CONTRIBUTIONS

Conception and design of the study: WS and PD; acquisition, analysis, and interpretation of the data: WS, JK, LK; writing of the manuscript: led by WS, all authors contributed critically to the drafts and gave final approval for publication.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Raw sequencing data and reference databases have been uploaded to Zenodo and are publicly accessible via the DOI: 10.5281/zenodo.10125780 (raw sequencing data) and 10.5281/zenodo.7377252 (reference databases). Bioinformatic and data analysis scripts, ASV-table, taxonomy-table, sample data, and species list obtained from morphological analysis are publicly accessible via https://github.com/BWobbii/BeeeDNA.

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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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