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ORIGINAL ARTICLE

Comparison of mosquito and fly derived DNA as a tool for sampling vertebrate biodiversity in suburban forests in Berlin, Germany

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Abstract

The use of invertebrate-derived DNA (iDNA) is a promising non-invasive tool to monitor wildlife. While most studies have been carried out in dense tropical and subtropical forests and have focused on the use of a single category of invertebrates, this study compares the use of flies and mosquitoes-derived DNA to assess vertebrate diversity in semi-urban environments. We conducted our sampling in four different forest plots in Berlin, Germany. Pools of flies and non-bloodfed mosquitoes were metabarcoded using 108-bp vertebrate-specific 12S rRNA (12S-V5) and 94-bp mammal-specific 16S rRNA (16Smam) mitochondrial markers, and individual bloodfed mosquitoes were sequenced using the 340-bp vertebrate-specific 12S rRNA fragment (Mam-12S-340). Most sequencing was only successful for mammal species. From the fly pools, we detected 10 mammal species using 16Smam, and six species using 12S-V5. From the non-bloodfed mosquito pools, we only amplified putative contaminant DNA, indicating that mosquito females without visual signs of a blood meal carry no traces of vertebrate DNA. Finally, in the bloodfed mosquitoes, we identified four mammal species. We did not find significant differences in the proportion of mammal species detected regarding the total available number of species between sampling localities. Fly samples were easier to obtain and more abundant over the sampled localities compared to mosquito samples. We conclude that, while there are a few advantages in using mosquito blood meals, the use of flies in the detection of wildlife in a suburban environment is more effective in terms of collection of samples and detection of vertebrates, although this technique is limited to few mammal species in the urban environment.

KEYWORDS
DNA metabarcoding, iDNA, mammals, mosquito bloodmeal, non-invasive sampling, urban ecology

1 | INTRODUCTION

The detection of vertebrates using invertebrates has been used primarily on medically important arthropods to determine host preference, to understand the route of disease transmission, and to identify vectors (Kent, 2009). These arthropods have been limited to hematophagous insects such as mosquitoes, ticks, blackflies, triatomine bugs, and sandflies. In recent years, the extension from vectors to other invertebrates such as leeches (Alfano et al., 2021; Schnell et al., 2012; Wilting et al., 2021) and flies (Calvignac-Spencer et al., 2013) and the development of the metabarcoding protocol have enabled the sampling of vertebrates through invertebrate samples beyond the scope of disease transmission. This resulted in the development of invertebrate-derived DNA (iDNA) within the umbrella of environmental DNA and its use as monitoring tool, also for rare and elusive species (Nguyen et al., 2021).

Thus far, the use of iDNA has shown that vertebrate DNA can be recovered from flies, in particular, from carrion flies consisting of families that use feces or carcasses for oviposition, mating, or feeding sites. This method has been used to monitor wildlife in Africa (Calvignac-Spencer et al., 2013; Schubert et al., 2015), Peninsula Malaysia (Lee et al., 2015), Panama (Rodgers et al., 2017), Germany (Hoffman et al., 2018), and the USA (Owings et al., 2019). Besides, carrion flies, mosquitoes, and sandflies feeding on vertebrate blood have been used in biodiversity studies (Kocher, de Thoisy, Catzefils, et al., 2017; Kocher, de Thoisy, Catzeflis, et al., 2017) in French Guiana.

Although iDNA has been used in dense forested areas in Asia and Africa either as a monitoring tool on its own or in combination with other detection methods (Abrams et al., 2019), only a few studies have been conducted in urbanized areas. Urbanization is a big driver of biodiversity loss (Blair & Johnson, 2008, Planillo et al., 2021), but the impact of suburban areas on biodiversity remains unclear. While urbanization has a negative impact on native and rare species (Mckinney, 2002), suburban areas can inadvertently provide unique environmental conditions and new habitats for other species to thrive (DeStefano & DeGraaf, 2003; Kowarik, 2011; Mckinney, 2002), resulting in an increase of local species richness and diversity compared to more urbanized areas. Suburban parks are often located at the edge of the urban area (Shochat et al., 2006), connecting large cities to rural or wild areas, thus resulting in a transitional habitat for species to adapt and colonize urban environments and sometimes increasing the number of species (Evans et al., 2009; Faeth et al., 2011; McKinney, 2008). However, due to low densities of some species and difficulties to implement classical monitoring schemes such as camera trapping in urban and suburban areas due to privacy protection reasons, getting a picture of the true community remains difficult. Developing a monitoring method for elusive species is needed to support urban planning and urban biodiversity strategies (e.g., Berlin Strategy for Biodiversity, Senate of Berlin, 2012).

Currently, urban or suburban areas worldwide can host many wild species and at least a portion of the regional biodiversity (Beninde et al., 2015; Faeth et al., 2011), but the use of iDNA techniques to identify the urban wild species is challenging. For instance, urban samples are exposed to massive quantities of confounding DNA by humans and domestic animals likely masking wildlife DNA (Hoffman et al., 2018). Building on the results of Hoffman et al. (2018), we aim to investigate and compare the suitability of using two types of iDNA sources based on ubiquitous insects –carrion flies and mosquitoes– for detecting wild mammal species in suburban forests in a large city. Specifically, we focused on four suburban areas in Berlin and applied metabarcoding using next-generation sequencing to identify species in iDNA samples from carrion flies, non-bloodfed mosquitoes, and bloodfed mosquitos. Moreover, a customized and incremental database of molecular markers was built to support the species identification analysis within this and future studies involving vertebrate, mosquito, and fly molecular studies from Berlin.

2 | MATERIALS AND METHODS

2.1 | Study area and sampling locations

Our study area was the city-state of Berlin, Germany (52°31′N, 13°24′E). Berlin is the capital and largest city in the country, with a population of *c*. 3.8 million people, an area of 892 km^2 and an average population density of 4260 people/km^2 (Amt für Statistik Berlin-Brandenburg, 2020). Almost 20% of Berlin's area is covered by forests (Berlin Environmental Atlas, 2015). The main suburban forests are located in Spandau ("Tegeler Forst"), in "Grunewald", and in Mueggelsee ("Koepenicker Forst") (Figure 1). Although these forests are heavily used by humans, for example, the biggest forest, "Grunewald" is visited by *c*. 1 million people annually (Franusch, 2015), they are regulated under the State Forestry Law to make compatible recreational activities by humans, like hiking, dog walking, or hunting, with forest and nature conservation.

2.2 | Sampling design

For the three invertebrate groups (bloodfed and non-bloodfed mosquitoes, carrion flies), we standardized the sample pools to ease statistical analysis and avoid confounding effects when having different numbers of individuals per sample. As non-bloodfed mosquitoes might contain residual bloodmeals that may not be readily visible, we decided to include them in our insect collection to test their efficiency as a potential source of vertebrate DNA, in comparison with the bloodfed mosquitoes and the carrion flies. Mosquitoes were sampled at five localities, while flies were collected at four localities (Figure 1).

Field sampling days were aimed at maximizing captures, selecting the times when the insects were active and avoiding days with rain or strong winds. To maximize the representation of the insect taxa and the mammal detections, as well as to minimize the risk of multiple insect taxa detecting the same vertebrate individual at a

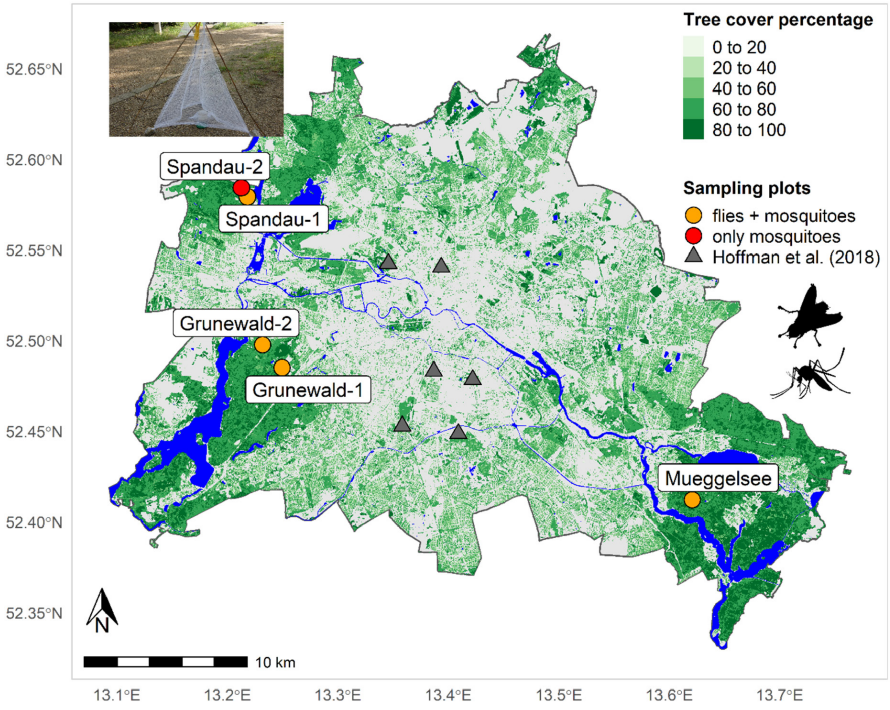

FIGURE 1 Sampling sites in Berlin with one example of fly trap used in the study in the top left corner. Orange circles denote collection sites of both flies and mosquitoes, red circle indicates collection of bloodfed mosquitoes only, and gray triangles indicate fly detection locations used by Hoffman et al. (2018).

TABLE 1 Sampling localities in Berlin.

Localities	Pools of flies (10 each)	Pools of NBF mosquitoes (10 each)	BF mosquitoes (individuals)	Sampling dates (2017)
Grunewald_1	4	6	9	22.06*, 24.07*, 07.08, 23.08
Grunewald_2	5	4	10	24.07*, 07.08, 04.09, 20.09*
Mueggelsee	6	4	15	26.05*, 06.06*, 26.08, 18.09
Spandau_1	6	4	15	08.06*, 31.07*,17.08, 30.08
Spandau_2#	-	-	9	08.06, 31.07, 30.08

Note: BF = bloodfed. NBF = non-bloodfed # Only BF mosquitoes were collected.
*Only mosquitoes were collected in this event.

site (e.g., due to the presence of a corpse), at least three to 4 weeks were allowed between sampling visits to the same locality. Resting adult mosquitoes were sampled four times per locality over a 4-month period between 26th of May and 30th of September 2017, except for Spandau 2 locations that was sampled only three times and exclusively for bloodfed mosquitoes. In all cases, mosquitoes were captured with the use of sweep nets (Snow & Medlock, 2008), for a minimum of 30 min (Figure 1, Table 1). Females were selectively collected from the net using glass vials, stored on ice, and transferred to a −20°C freezer. Samples were sorted as being bloodfed (BF) and non-bloodfed (NBF) and stored in 1.5 ml Eppendorf tubes either individually for BF or in pools of 10 individuals for NBF females.

Flies were sampled twice -between August and September 2017- using pyramidal net traps with decomposing fish bait (Hoffman et al., 2018). Three traps were placed in four of the five localities where mosquito collections were carried out, and concomitantly sampled along with mosquitoes in two occasions per locality (Figure 1, Table 1). As pyramidal nets provided more samples per trapping event than the mosquito nets, no more visits were done, to have a comparable sample size between flies and mosquitoes. Pyramidal net traps were placed 10 m apart from each other and left for 45 min each time. After the sampling time, flies were collected, placed in a falcon tube, transported on ice, and stored in a −20°C freezer.

2.3 | DNA extraction

DNA was extracted using a Stool DNA kit (Roboklon GmBH, Berlin, Germany), according to the manufacturer's instructions with the following adjustments. Single BF mosquitoes (n = 58) were placed into individual bead tubes provided in the kit and the sample was homogenized (Precellys 24, France) 3 times for 20 s each. The entire lysate was used for extraction. For the NBF mosquitoes, the pools of 10 individuals were used for the extraction (pools = 18). In a parallel approach, flies were also pooled in groups of 10 per trap per sampling event (pools = 21). Both the NBF and fly pools were first homogenized in 250 µl PBS buffer and 100 µl of the lysate was used for extraction. The remaining lysate was kept in −80°C. The concentration of extracted DNA was measured using a Qubit 4 Fluorometer (Invitrogen, California, USA).

2.4 | DNA amplification

As vertebrate DNA found in both NBF mosquitoes and in carrion flies can be heavily degraded, we opted for using two short mitochondrial markers (mtDNA). To identify blood sources in pools of NBF and flies, a 108-bp fragment of the 12 s rRNA gene (12 S-V5) was used to target vertebrate DNA (Riaz et al., 2011) and a 94-bp fragment of the 16 S rRNA gene (16Smam) was used to target mammal DNA (Boessenkool et al., 2012) (Table 2), following the method described in Hoffman et al. (2018). To reduce the amount of human and pig contaminants that could be present in the fly samples due to the urban environment and outside eating areas, both human and pig blocking primers were used (Hoffman et al., 2018). Additionally, all PCR mixes contained 0.3 U Amperase® uracil *N*-glycosylase (UNG; Invitrogen, Carlsbad, CA) and digestion with UNG was carried out to degrade potential contaminant PCR products prior to the start of the PCR cycle. As such PCR products were generated using 0.2 mM of dN(U)TP. Briefly, two amplicons per sample were generated and samples with both amplicons present were cleaned (Purelink gel extraction, Invitrogen) and pooled. Cycling conditions for 12 S-V5: UNG digest-45°C 7 min, 95°C 10 min; PCR cycle 95°C 5 min, 42 cycles (95°C 30 s, 60°C 30 s, 72°C 1 min), 72°C 10 min and 16Smam: 45°C 7 min, 95°C 10 min, 42 cycles (95°C 30 s, 64°C 30 s, 72°C 1 min), 72°C 10 min. Illumina adapters were added in a second PCR, cleaned (Agencourt® AMPure XP PCR purification, Beckman Coulter, California) and indexes added in a third short amplification round.

For the purpose of building a DNA reference library, insects were identified using the 658-bp COI barcoding fragment (Folmer et al., 1994) following the protocol outlined above, to obtain the species identity of the flies and mosquitoes collected. However, only one amplicon was generated per sample. Cycling conditions: 95°C 5 min, 35 cycles (95°C 30 s, 50°C 30 s, 72°C 1 min), 72°C 10 min. Concentration of clean indexed DNA was measured using a plate reader with the High Sensitivity DNA kit (Bioanalyser, Agilent Technologies, California). Samples were pooled in equimolar volumes and further diluted to 4 nM for sequencing on the Illumina MiSeq.

The same three markers were used on the BF mosquitoes. Instead of using the 108-bp 12 S fragment, we used the primers of Kocher et al. (2017, Mam-12 S_340) to amplify a longer 12 S (340 bp) fragment (Table 2). A single amplicon was generated for each marker. These were cleaned using Agencourt® AMPure XP PCR purification (Beckman Coulter, California, USA). Cleaned products were then sanger sequenced (SMB Services, Berlin, Germany).

2.5 | Customized sequence database

The database was built for all three markers (Database 1–4) with the goal of curating a local database of the 59 terrestrial mammal species occurring in Berlin (Klawitter et al., 2005).

Briefly, an initial database was created by downloading sequences from the NCBI database using a list of known markers and species of interest. As sequences downloaded would be of varying fragment lengths, in silico PCR was conducted using ecoPCR within the OBItools package to obtain the fragment used in this study, forming the initial database. A second search was conducted via Genbank or BOLD, using ecoPCRSequences.sh script (Heeger et al., 2022; url: doi.org/10.5281/ZENODO.7322543), to obtain a comprehensive list of sequences, as sequences in the NCBI database are limited to the date of download. These additional sequences were aligned to

TABLE 2 Summary of the results of DNA barcode primers used for mammal identification in samples of each type of insect, primer used for insect identification (COI-insect).

Locality (total # pools; # of BF mosquitoes)	Primers	Type	Fly pools (#taxa detected)	NBF mosquitoes (#taxa detected)	BF mosquitoes (#taxa detected)
Grunewald_1 (4, 9)	16 S-mam	16 S-rRNA/mammals	6	—	—
	12-V5	12 S-rRNA/vertebrates	3	—	—
	Mam-12_340	12 S-rRNA/vertebrates	—	—	3
Grunewald_2 (5; 10)	16 S-mam	16 S-rRNA/mammals	8	1	—
	12-V5	12 S-rRNA/vertebrates	6	—	—
	Mam-12_340	12 S-rRNA/vertebrates	—	—	3
Mueggelsee (6; 15)	16 S-mam	16 S-rRNA/mammals	9	—	1
	12-V5	12 S-rRNA/vertebrates	1	—	—
	Mam-12_340	12 S-rRNA/vertebrates	—	—	3
Spandau_1 (6; 15)	16 S-mam	16 S-rRNA/mammals	7	1	2
	12-V5	12 S-rRNA/vertebrates	1	2	—
	Mam-12_340	12 S-rRNA/vertebrates	—	—	3
Spandau_2 (0; 15)	16 S-mam	16 S-rRNA/mammals	—	—	—
	12-V5	12 S-rRNA/vertebrates	—	—	—
	Mam-12_340	12 S-rRNA/vertebrates	—	—	2

Note: bp: number of base pairs, NBF = non-bloodfed. BF = non-bloodfed.

the initial database as a reference to retain sequences with the fragment of interest and fragment length at a 99%–100% identity match. Sequences were then dereplicated using obiuniq *–m sample samplename and* maximum likelihood tree was built with IQ-TREE (Nguyen et al., 2015) using ModelFinder to select a model of nucleotide evolution (Kalyaanamoorthy et al., 2017) and *Macropus giganteus* (GenBank accession number: NC027424) as an outgroup for mammals.

A comparison was made between the phylogenetic trees constructed in this study with existing phylogenetic trees from published papers. Sequences that fall into a clade outside of their group were deemed to be mis-annotations and removed from the database. This resulted in a database of sequences for which identity of species was high: 16S ($n = 34$), 12S ($n = 42$), mosquito ($n = 45$), and flies ($n = 62$).

Out of 63 vertebrate species known in Berlin, 5 species did not have 12S sequences in Genbank and were replaced with similar species found in Europe: *Crocidura leudocon* and *C. suaveolens* was replaced with *C. gueldenstaedti*, *Eptesicus nilssoni* with *E. serotinus*, *Myotis dasycneme*, and *M. nattereri* with *M. brandtii*. However, none of these were detected in our samples. For the 16S sequences, 14 out of the 63 vertebrates had no sequences available at the time and as with the 12S, were replaced with similar species existing in Europe and none were detected in our samples.

2.6 | Sequence analysis

Primers were trimmed from the sequences of both 12S-V5 and 16Smam metabarcodes using Geneious 8.1 (Biomatters, New Zealand). The trimmed paired-end reads were merged using OBItools (Boyer et al., 2016), chimeras removed using Vsearch (Rognes et al., 2016), and the filtered sequences dereplicated to identical sequences using the OBItools pipeline. For COI data, following the removal of primers, low-quality sequences were removed and paired-end reads were concatenated with the gap between reads filled with 10 N bases. Concatenated sequences were dereplicated using OBItools to identical sequences. Unique sequences from samples were concatenated with the sequences from the database and pairwise distances were calculated using Vsearch. Based on these distances, clusters with an identity threshold of 99%–100% were created using clusterFromPairs.py (Heeger et al., 2022). An analysis of the clusters, using clusterAnalysis.py (Heeger et al., 2022), was carried out to ensure that clusters were formed between sequences from samples and those from the database. Clusters formed from sequences from samples only were not assigned to a species. To determine the effectiveness of the 12S and 16S primers, in silico PCR was run using Primersearch (Rice et al., 2000) on sequences obtained from Genbank.

2.7 | Statistical analysis

Based on the identifications obtained from processing the iDNA samples, we focused our statistical analyses on mammal diversity. This decision was taken as only the primer 12S-V5 used in the fly pools was able to identify non-mammals, although at very low rates with a total of 4 bird genera and 2 reptiles/amphibians genera. These detections were considered anecdotic and did not constitute enough data for statistical analyses.

To assess the ability of the iDNA samples to detect mammal species in the suburban environment, we compared the mammals identified in our iDNA samples with the complete potential mammal list for Berlin (Klawitter et al., 2005), focusing on terrestrial mammals. We therefore excluded bat species ($n = 17$), as they were neither detected in fly nor mosquito samples, hence the list was narrowed from 59 to 42 mammal species (Table S1).

We analyzed the differences in the proportion of mammals detected (number detected over total potential number of 42 mammals) by a generalized linear model (GLM) with binomial error distribution and logit link function. For the fly pools, we used as explanatory variables the marker type (16Smam vs 12S-V5) and the sampling locality. For BF mosquitoes, we used only the sampling locality, as most of the mammals were detected only by the 12S_340 primer, thus including marker type in the models would have been uninformative. Due to the low number of samples, we used each fly pool as an independent sample for the fly analysis. In the BF mosquito analysis, we pooled together the results from all mosquitoes captured in the same date and locality. We assessed the significance of the explanatory variables through likelihood ratio tests (LRT).

We further investigated differences in the detected mammal community composition by analyzing changes in species identity through beta-diversity metrics. We ran a Principal Coordinate Analyses (PCoA) based on Jaccard dissimilarities, and analyzed the influence of the marker type and the locality in the mammal community composition detected by fly pools using permutational multivariate analysis of variance (permanova). We ran a similar PCoA for BF mosquitoes and analyzed the effect of locality and mosquito species in the mammal community composition detected by permanova.

In the comparison of the diversity detected by each method, we discarded the use of accumulation curves for diversity estimates or rarefaction approaches, as the samples sizes were not large enough to provide meaningful results (Figure S1).

Statistical analyses were performed in R v4.0.5 (R Core Team, 2021). Beta diversity analyses were done using vegan package and functions *cmdscale* to compute the PCoA and *adonis* to run the permanova (Oksanen et al., 2020).

3 | RESULTS

3.1 | Insect identification COI

In the case of mosquitoes, we identified five taxa of mosquitoes among 58 individual females detected with a bloodmeal: *Aedes cinereus* ($n = 4$), *Ae. vexans* ($n = 23$), *Culex pipiens s.l.* ($n = 1$),

Ochlerotatus sticticus ($n = 9$) and from the Annulipes Group ($n = 14$). As the species with the Annulipes complex cannot be distinguished using the COI marker alone (Heym et al., 2019), we have identified them as the Annulipes Group. A total of 51 mosquitoes were identified taxonomically using COI, while the others ($n = 7$) were unidentified.

A total of five mosquito taxa belonging to four genera were detected from 11 pools of NBF adults: *Aedes vexans* ($n = 8$), *Ae. cinereus* ($n = 1$), *Culex pipiens* ($n = 1$), *Ochlerotatus sticticus* ($n = 4$) and Annulipes Group ($n = 2$). *Aedes vexans* was detected in all localities, while both *Cx. pipiens* and *Ae. cinereus* were found only in Spandau_1 (note that only BF mosquitoes were sampled in Spandau_2).

For flies, pooling was done by the random addition of 10 flies per trap to a sample. We identified 18 fly species belonging to 13 genera in all pools using the 658 bp COI barcoding primer (Folmer et al., 1994). We obtained a total of 76,650 reads (R1 and R2) from 19 out of 21 pools (Figure 2). Flies belonging to the genus *Sarcophaga* were the most common, followed by those belonging to genera *Thricops* and *Lucilia*. *Thricops simplex* was detected in all localities and *Lucilia caesar* and *Sarcophaga albiceps* were detected in both sites in Grunewald and in Muegglesee, while *S. carnaria* was detected in Grunewald_2, Muegglesee and Spandau_1. In some instances, species were not easy to differentiate genetically namely *S. carnaria/S. pyrenaica*, *S. carnaria/S subvicina*, *S. lehmanni/S. variegata*, and *L. caesar/L. illustris*.

3.2 | Mammal species identification

3.2.1 | Mosquitoes

Vertebrate hosts were identified from 54 out of 58 individual mosquitoes using Mam-12S-340. Four out of the five vertebrates identified were mammals: roe deer (*Capreolus capreolus*, $n = 21$), fallow deer (*Dama dama*, $n = 11$), wild boar (*Sus scrofa*, $n = 19$), and red fox (*Vulpes vulpes*, $n = 1$).

The red fox was identified from an *Ae. vexans* female collected in Grunewald_1. Fallow deer was found in almost all localities except Grunewald_1, and roe deer was found in both sites in Spandau and in Grunewald_2. The species of mosquitoes from which these two species of deer were found included *Ae. cinereus*, *Ae. vexans*, the Annulipes Group and *Och. sticticus*. A total of 21 out of 58 samples worked for the 16Smam RNA fragment, but only sequences from 17 samples gave results at the species level. Nine samples were identified as wild boar and eight were roe deer. All samples amplified with both markers showed almost identical species identification ($n = 15$, Figure 3).

DNA amplification was successful for only six out of the 18 NBF mosquito pools examined. Four pools from Grunewald_1 ($n = 1$), Grunewald_2 ($n = 2$), and Spandau_1 ($n = 1$) were sequenced using 16Smam. Two pools from Spandau_1 ($n = 1$) and Grunewald_2 ($n = 1$) were sequenced using 12S-V5. All three Grunewald_2 and Spandau_1 mosquito pools detected human DNA, while the pool from Grunewald_1 detected wild boar. We therefore did not include the NBF samples in further statistical analyses.

3.2.2 | Flies

Together, 12S-V5 and 16Smam identified 11 mammal taxa, and 12S-V5 identified four genera of birds and five of amphibians. For mammal taxa, nine out of 21 pools were successful for 12S-V5, identifying 6 mammal genera and generating 223,470 reads in both directions (Figure 2); there were no mismatches found in between the reference sequences and primer pair used. 16 pools were successfully sequenced for 16Smam and these identified 10 genera of mammals (Figure 2, Table S2). Both markers identified European badger (*Meles meles*), dog (*Canis lupus familiaris*), and red squirrel (*Sciurus vulgaris*) (Figure 3). We obtained a total of 164,620 reads for 17 out of the 21 pools sampled (Figure 2), analysis of the primers showed to have a single mismatch on the forward primer and no mismatches on the reverse. The 16Smam marker detected raccoon (*Procyon lotor*, Spandau_1, 1 pool only), roe deer (*C. capreolus*, 2 pools), and European rabbit (*Oryctolagus cuniculus*, Mueggelsee, 1 pool), while the 12S-V5 marker picked up wood mouse (*Apodemus sylvaticus*) in both sites in Grunewald (Grunewald_1: 1 pool, Grunewald_2: 2 pools). European badger was detected in all localities with both 12S-V5 and 16Smam, while red fox was detected in three out of four localities using 16Smam and only in one locality with 12S-V5 (Figure 3). Generally, vertebrates could not be assigned to a specific fly species. One potential exception included a pool in Grunewald_1 for which *Thricops simplex* was the only fly species identified. The mammals detected in the latter included European badger and wild boar.

3.3 | Mammal detection probability by flies and mosquitoes

Fly pools were able to identify a mean of 2.65 species (range 0–5 species), with a total of 11 different mammal species identified across all fly pools, which represent about 26% of the potential mammal species. BF mosquitoes, on the other hand, gave information about one mammal species per mosquito and only identified four mammal species, one of them being the common red fox, which was detected only once. For the fly pools, the GLMs showed no statistical difference in the proportion of the mammal community detected in relation to either locality (LRT: $\chi^2 = 2.5069$, df = 3, $p = 0.474$) or genetic marker (LRT: $\chi^2 = 1.0595$, df = 1, $p = 0.303$) (Figure 4). Similar results were found for the BF mosquitoes, with no significant effect of the locality (LRT: $\chi^2 = 0.9305$, df = 4, $p = 0.920$). The detection proportions in BF mosquitoes were around 4% (Grunewald_1 mean 3%, range 2%–7%; Grunewald_2: mean 4%, range 2%–8%, Mueggelsee mean 3%, range 1%–7%, Spandau_1 mean 5%, range 2%–9%, Spandau_2 mean 4%, range 2%–9%).

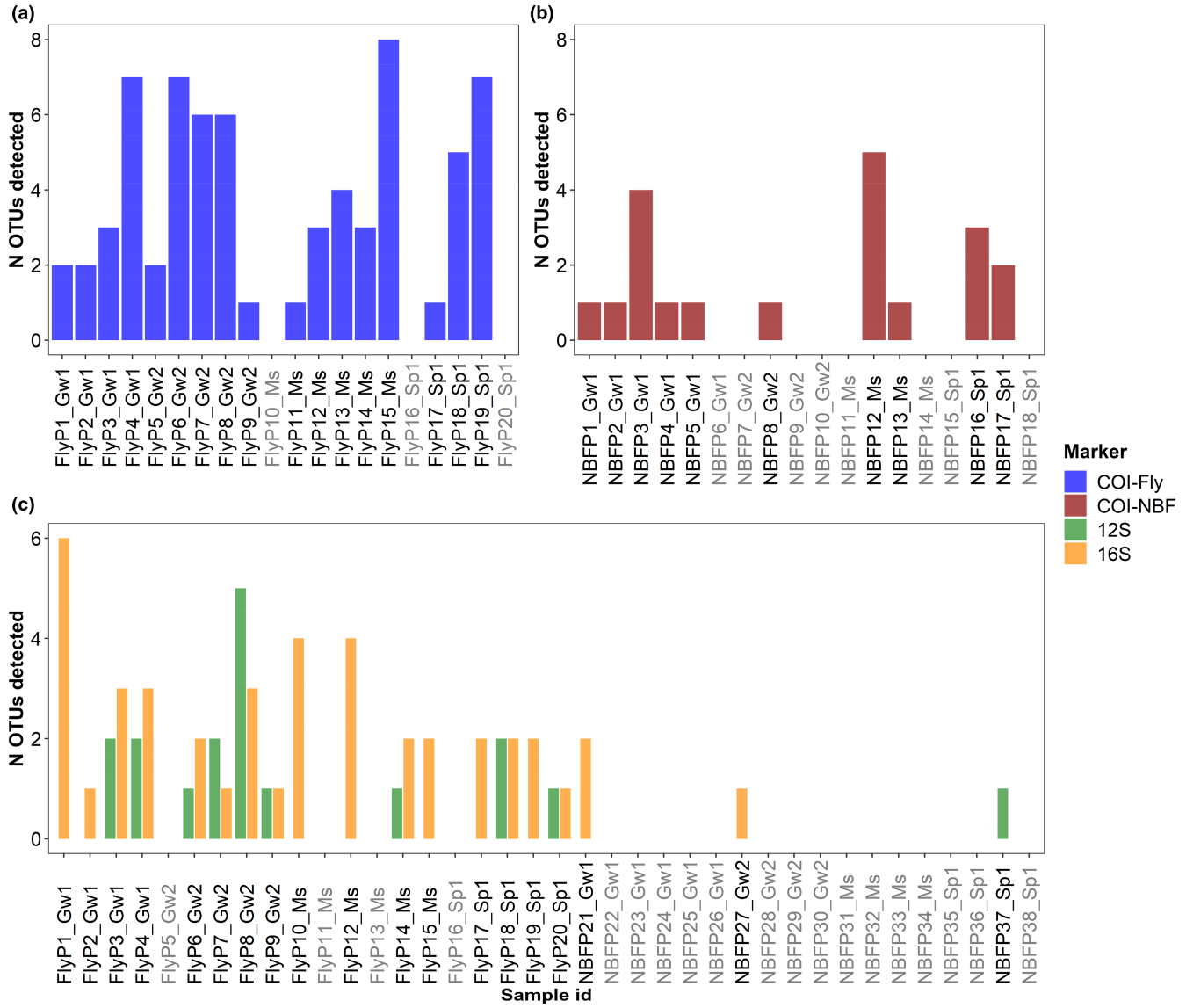

FIGURE 2 Number of different OTUs detected from COI, 12S-V5, and 16SMam markers. COI markers were used to identify insect taxa in A. fly pools ("FlyP") and B. non-bloodfed mosquito pools ("NBFP"), while 12S-V5 ("12S") and 16Smam ("16S") markers were used to identify C. mammal species. Samples are coded by sequential id and locality (Gw1: Grunewald_1, Gw2: Grunewald_2, Ms: Mueggelsee, Sp1: Spandau_1). Samples from which no taxa was identified are colored in gray.

3.4 | Differences in mammal community composition

In the beta diversity analysis, the first two axes of the PCoA based on fly pool data explained more than 80% of the variance in the detected mammal community composition (Figure 5). Within the fly pools, the highest dissimilarities between samples stemmed from the detections of four main species: European badger, wild boar, wood mouse and dog. Permanova analyses showed no significant effect of locality ($F = 0.318$, $df = 3$, $p = 0.415$), but a significant effect of the mtDNA marker on the mammal community detected ($F = 5.181$, $df = 1$, $p = 0.001$), with 16Smam being able to detect a wider variety of species (Figure S2).

The PCoA based on samples from BF mosquitoes accounted for more than 90% variance in the community composition (variance explained: axis 1 = 58%, axis 2 = 38.2%). As the BF mosquitoes were analyzed individually and each one only detected one mammal species, the PCoA showed three clear groups of points, based on the identification of the three main mammal species. Permanova analysis showed a significant effect of locality ($F = 2.689$, $df = 4$, $p = 0.011$), mainly due to the detection of red fox and fallow deer in only one and three out of five localities, respectively (Figure 3).

Comparing the detections from flies and BF mosquitoes, the mammal species presented low overlap. While flies detected a wider range of mammal species, BF mosquitoes detected species usually of larger size, such as ungulates (Figure S2). Additionally, samples based on fly pools only failed to detect one species present on BF mosquito samples, the fallow deer.

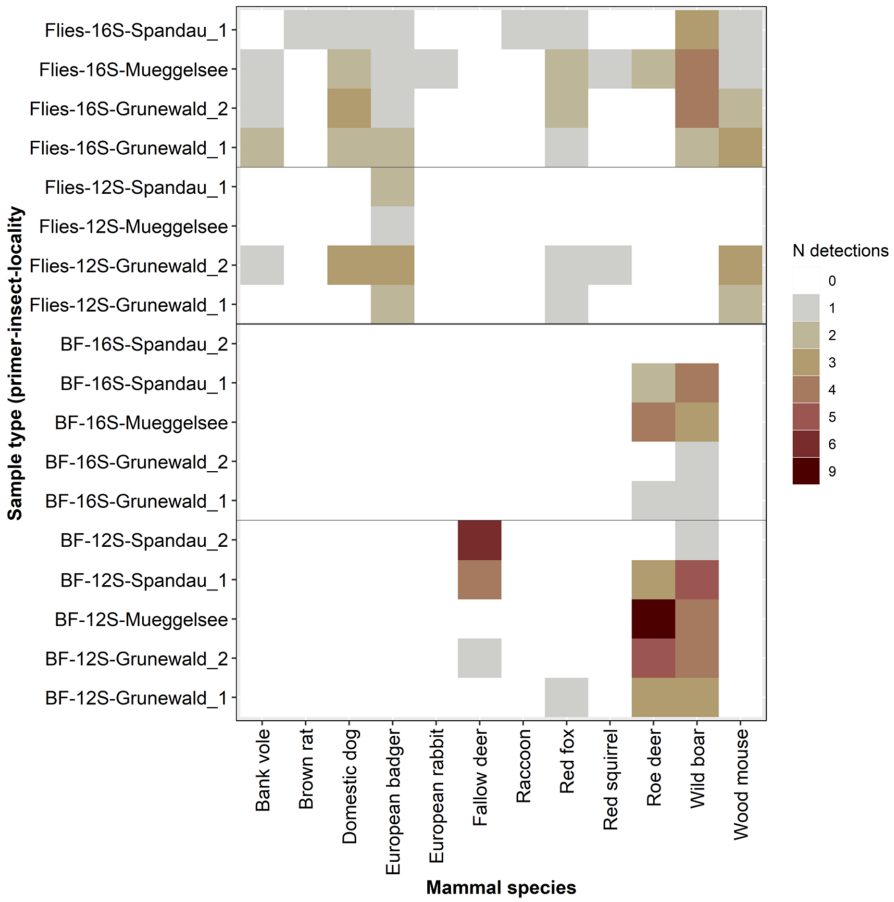

FIGURE 3 Heatmap showing the mammal species detected using flies and bloodfed mosquitoes, divided by locality and primer. The row names follow the structure "insect"_"primer"_"locality," for example, "Flies_16s_Spandau_1" refers to mammals identified from samples taken from flies, using 16s primer, from the samples collected in Spandau 1.

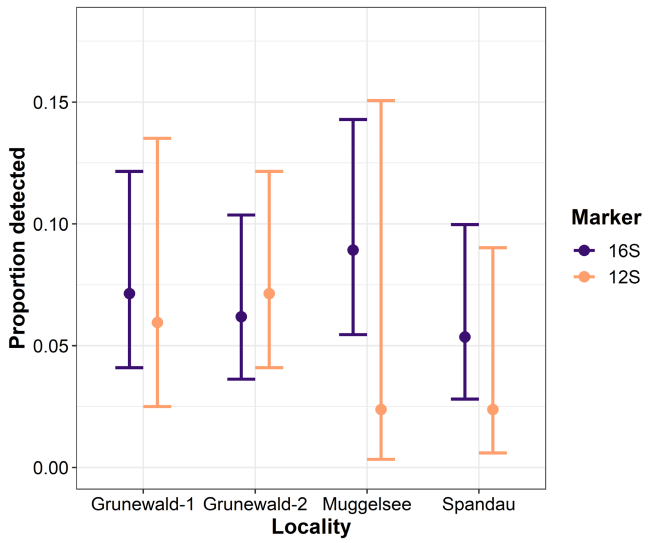

FIGURE 4 Effects of the GLM model analyzing the proportion of the mammal community detected by fly pools based on locality and genetic marker. Vertical bars represent the 95% confidence intervals.

4 | DISCUSSION

We tested the use of flies and mosquitoes as iDNA sources for the detection of mammal species in four suburban forested areas in the city-state of Berlin. We further assessed the efficacy of using metabarcoding of two mitochondrial markers - 12SrRNA and 16SrRNA - as monitoring tools for terrestrial mammals. Our results highlight that, in general, iDNA samples obtained from fly species pools detected more mammal species than samples from mosquitoes. In addition, the lack of visible bloodmeal in NBF resulted in the amplification of humans and pigs, both a common source of lab contamination.

Out of the total 11 mammal species that we detected using the fly pools, five of them were also found in the inner-city parks studied previously by Hoffman et al. (2018): *Procyon lotor*, *Rattus norvegicus*, *Oryctolagus cuniculus*, *Sciurus vulgaris*, and *Vulpes vulpes*. All of them are considered common synurbic species that can be categorized as urban dwellers (Santini et al., 2019). In the study by Hoffman et al. (2018), the sampling took place within the inner city of Berlin, while the four plots in this study were forested suburban areas. The different location of the sampling sites could explain the findings of moles and hedgehogs in Hoffman et al. (2018), which were not detected in our study. In contrast, we detected species that were lacking in the city parks, such as *Myodes glareolus*, *Apodemus sylvaticus*, *Meles meles*, and *Capreolus capreolus*, which typically avoid highly urbanized areas. Additionally, the degree of urbanization has an effect of species composition of flies (Kavazos & Wallman, 2012). We detected more Sarcophagidae and Muscidae compared to Hoffman et al. (2018), who detected more Calliphoridae, especially genus *Lucilia*. This is congruent with overall findings of genus *Lucilia* typically characterized as urban exploiters (Kavazos & Wallman, 2012;

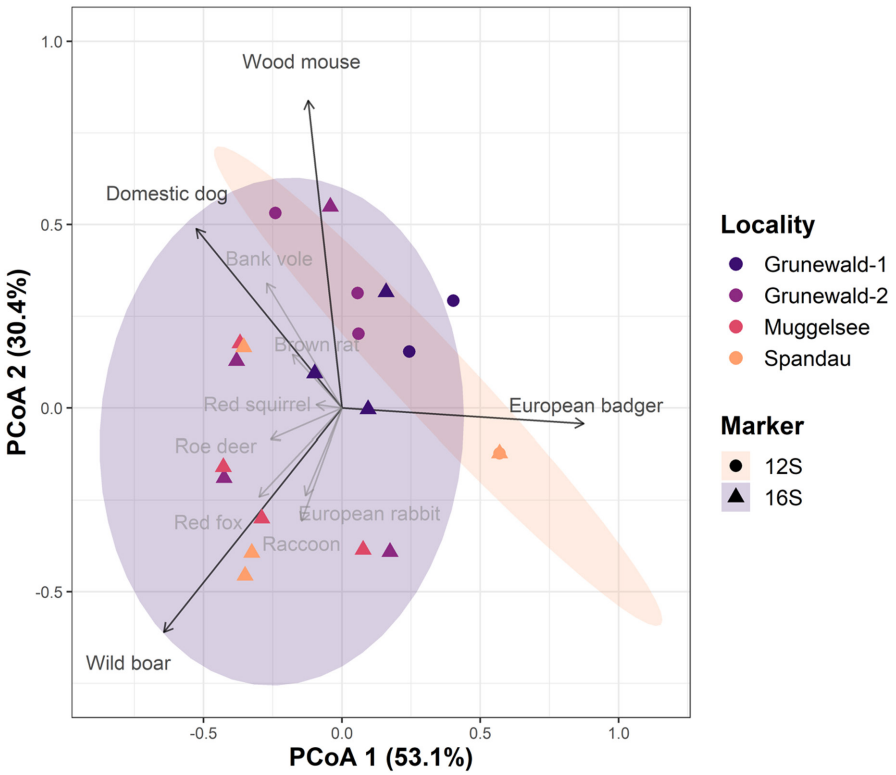

FIGURE 5 PCoA based on fly pool data. Point shapes represent the different genetic markers and point colors represent the sampling localities. Shaded areas represent the elliptical space of each marker type. Mammal species with a significant effect on the axes are shown in black, while mammal species with weak effects are shown in gray.

Patitucci et al., 2010), while flies belonging to families Muscidae and Sarcophagidae are generally associated to areas with a lower degree of urbanization (Beltran et al., 2012; Patitucci et al., 2010). This differentiation in habitat preference of the different fly families could also have an impact on mammal species that can detected using iDNA samples, as the different fly families might have different preferences for some mammal species. Therefore, comparison between studies should be done with caution and the captured fly taxa should always be reported in the studies.

Despite the use of blocking primers, we frequently detected sequences that matched perfectly both domestic pig and wild boar. As BF mosquitoes carry substantial amounts of host DNA and were sampled in forested areas with known high density of wild boars, we consider that these detection events are more likely to reflect wild boar detection. However, carrion flies visit carcasses and stool, but also human food leftovers, and therefore the assignment of *S. scrofa* sequences to either pig or wild boar remains ambiguous.

Analysis of bloodmeals from BF mosquitoes, on the other hand, not only gave a clear indication of the vertebrates identified but also of its location relative to where the mosquito was collected; fallow deer (*Dama dama*), a species not native to Germany and which is not supposed to range freely in the urban forests, was identified from 10 BF mosquitoes collected in two sites in Spandau and one in Grunewald, where petting zoos are housing this species. Interestingly, fallow deer was not identified in any of the fly pools. A comparison of the 12S showed only a single base pair insertion in the alignment of both the roe and fallow deer sequences. However, the 16S human and pig blocking primers showed a 65% and 92% similarity to the roe and fallow deer sequence, respectively. Thus, they likely blocked the detection of fallow deer when using the 16S primer, which could explain the lack of detection of this species from fly samples. These blocking primers were not used with the BF mosquitoes.

Mosquitoes detected very low diversity of mammals compared to fly pools. The mammal diversity detected by the mosquitoes seemed to be limited to medium or large body size species, while fly pools detected a wider range of species. As the DNA found on flies are in trace amounts and subject to contamination compared to that of the BF mosquitoes, blocking primers were only used with the fly pools. This allowed us to reduce the signal of common contaminants such as the domestic pigs and humans. The use of these primers would not have influenced the number of species detected in either fly pools or BF mosquitoes. Thus, further supporting the ability of fly pools to detect more mammal species. The low diversity detected by mosquitoes could be attributed to either the host preference of the mosquito or a site-specific bias. Insect species with high host preference might not represent the real diversity. For example, the mosquito *Culex pipiens* is a known ornithophilic species (Rizzoli et al., 2015) that will select birds over mammals if available, thus providing a reduced and bias representation of the mammal species in the surroundings. Additionally, different environmental preferences of the insect species might affect the mammal community that is available at each site, thus creating a site-specific bias (Kocher, de Thoisy, Catzefils, et al., 2017) in the detections. A further exploration of the environmental preferences of flies and mosquitoes would be recommended to better understand the differences in detections.

As we focused our study on the presence of mammalian wildlife in Berlin, we found that the 16S marker was more effective in

detecting mammal across localities compared to 12S, which had detected seven of the 11 mammal species picked up by 16S. However, it also detected 4 taxa of birds (*Sitta*, *Strix*, *Sturnidae*, and *Turdidae*) and 2 species of amphibians (*Bufo bufo*; *Lissotriton vulgaris*). This was similar to the findings of Schnell et al. (2018) who suggested that use of multiple markers would help in the detection of vertebrates. Given that there was little to no mismatches on both the primer pairs and that our restricted sampling of flies was limited to 2 months of the year in a particular type of locality could have accounted for the low diversity of animals detected. Frequent sampling of different localities using two primers could increase the number of species detected. Further to this, the use of two primers helped to resolve ambiguities. In 3 samples, the 16S sequences were assigned to wood mouse based on the 12S results of the same sample; should only the 16S have been used, those samples would have been assigned to order Murinae.

The use of flies as iDNA sources could be a cost-effective tool to obtain a first impression of wildlife in a city. A 45-min sampling effort with a minimum of three sampling events in 4 localities results in the detection of 11 non-domestic mammal species. As a comparison, a large citizen science project that consisted of camera traps in more than 150 sites in Berlin during 5 repeats each lasting for 4 weeks (Louvrier et al., 2022) detected only a total of 14 mammal species. Interestingly, while camera traps failed to detect some of the rare non-native species (racoon dog and fallow deer), the iDNA samples failed to detect species such as European hare (*Lepus europaeus*), European hedgehog (*Erinaceus europaeus*), or Eurasian beaver (*Castor fiber*). While these differences may partly be explained by differences in the sampling locations for both methods, the detection of non-native species by iDNA samples deserves to be highlighted. These results make the fly iDNA approach very promising, as a complementary method to other monitoring efforts. However, the cost per sample should also be taken into account. The average cost for the fly collections and laboratory work per pool amounted to approximately €15. Additionally, iDNA samples integrate information over a small temporal window of 45 min and an unknown spatial scale, as it is uncertain where the invertebrates took up the mammal DNA. The cost of a camera trap is around €200 and can provide hundreds of images across a long time span. The use of iDNA alone would not result in a comprehensive understanding of the wildlife present in a given locality, such as activity plots from exact time stamps of the images or wildlife densities via marked individuals can provide (Niedballa et al., 2016). Therefore, a combined approach setting up camera traps in places where access is restricted and collection of flies in semi-natural and suburban areas could offer a complete picture of the diversity of wildlife present (Rodgers et al., 2017; Gogarten et al., 2020).

AUTHOR CONTRIBUTIONS

CM, SCS, and SKS designed and supervised the work. SD collected the samples. SB and RD performed the labwork. CT curated the customized sequence database. GP and AP ran the statistical analysis. RD and AP wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest in the preparation of this manuscript.

DATA AVAILABILITY STATEMENT

All raw reads have been deposited to DRYAD and is available with the following DOI: https://doi.org/10.5061/dryad.xsj3tx9j8. R code to run the analyses is available on Github: https://github.com/EcoDynIZW/Danabalan_Planillo_2023_eDNA.

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SUPPORTING INFORMATION

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