Airborne environmental DNA for terrestrial vertebrate community monitoring

Highlights
- 49 vertebrate species detected through metabarcoding of airborne eDNA from the zoo
- Detections included 30 mammal, 13 bird, 4 fish, 1 amphibian, and 1 reptile species
- 6 to 21 vertebrate species were detected per air filtering sample
- Shorter geographical distance and higher biomass increased probability of detection

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In brief
Lynggaard et al. demonstrate that airborne environmental DNA coupled with metabarcoding and high-throughput sequencing can be used to detect terrestrial vertebrates. The 49 detected species are known to occur in or around the zoo study site. Animals in closer proximity to the sampler and present in larger biomass have higher detection probability.
Airborne environmental DNA for terrestrial vertebrate community monitoring

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SUMMARY

Biodiversity monitoring at the community scale is a critical element of assessing and studying species distributions, ecology, diversity, and movements, and it is key to understanding and tracking environmental and anthropogenic effects on natural ecosystems.1–4 Vertebrates in terrestrial ecosystems are experiencing extinctions and declines in both population numbers and sizes due to increasing threats from human activities and environmental change.5–8 Terrestrial vertebrate monitoring using existing methods is generally costly and laborious, and although environmental DNA (eDNA) is becoming the tool of choice to assess biodiversity, few sample types effectively capture terrestrial vertebrate diversity. We hypothesized that eDNA captured from air could allow straightforward collection and characterization of terrestrial vertebrate communities. We filtered air at three localities in the Copenhagen Zoo: outside between the outdoor enclosures, and in the Rainforest House. Through metabarcoding of airborne eDNA, we detected 49 vertebrate species spanning 26 orders and 37 families: 30 mammal, 13 bird, 4 fish, 1 amphibian, and 1 reptile species. These spanned animals kept at the zoo, species occurring in the zoo surroundings, and species used as feed in the zoo. The detected species comprise a range of taxonomic orders and families, sizes, behaviors, and abundances. We found shorter distance to the air sampling device and higher animal biomass to increase the probability of detection. We hereby show that airborne eDNA can offer a fundamentally new way of studying and monitoring terrestrial communities.

RESULTS

The air is filled with particles, such as fungal spores, bacteria, vira, pollen, dust, sand, droplets, and fibrous material, which can be airborne for days and transported over long distances.9,10 These contain DNA and/or carry DNA attached to them, and DNA sequencing has been used to identify the taxonomic origins of airborne fungal spores, algae, pollen, and microbiota collected on adhesive tape, in air filters, and in dust traps.11–15 Further, two recent studies demonstrated vertebrate detection through DNA filtered from air in small, confined rooms containing tens to hundreds of individuals of the target species,16,17 and one study sequenced DNA from atmospheric dust samples in the Global Dust Belt over the Red Sea and detected eukaryotes, including small quantities of human, cetacean, and bird DNA.18 However, the use of airborne environmental DNA (eDNA) for studying and monitoring local vertebrate communities in a wider context has been unexplored.

We detected vertebrate airborne eDNA in Copenhagen Zoo, Denmark, by filtering air with three air sampling devices. Specifically, we filtered air using a water vacuum and a 24 V and 5 V blower fan. The two latter had class F8 fibrous filters for airborne particulate matter attached. Sampling times were between 30 min and 30 h. We first tested airborne eDNA monitoring in a semi-confined space by collecting 12 samples of airborne particulate matter using the water vacuum (n = 4, September), the 5 V sampler (n = 2, December), and 24 V sampler (n = 6, December) in a stable in the southern section of the zoo holding two okapis (Okapia johnstoni) and two red forest duikers (Cephalophus natalensis) (Figure 1A). Using this approach, we detected both species present in the stable in all samples. Further, we detected 13 birds and mammals that were kept in neighboring outdoor enclosures in the southern section of the zoo, one zoo animal that was located in the northern section of the zoo, two animals known to be pests in the zoo (i.e., brown rat and house mouse) of which one is also kept at the zoo and used as feed (house mouse), two...
wild or domestic non-zoo species known to occur in and around the zoo (i.e., European water vole and dog), and two fish species used as feed in the zoo (i.e., smelt and salmon) (Figure 2). Overall, we detected 22 non-human vertebrate species (Figure 2; Table S1) with 6 to 17 species detected per sample (mean = 11.33, SD = 3.17) (Table S2).

To further explore the potential of airborne eDNA to monitor terrestrial vertebrate communities in natural settings, we deployed air samplers in an open-air location proximal to multiple outdoor mammal and bird enclosures in the southern section of the zoo (Figure 1B). In total, 16 samples of airborne particulate matter were collected, split between the water vacuum (n = 4, September; n = 4, December), the 5 V sampler (n = 2, December), and the 24 V sampler (n = 6, December). Between 8 and 21 non-human vertebrates were detected per sample (mean = 14.5, SD = 4.69) (Table S2), totaling 30 non-human vertebrate species (Figure 2; Table S1). Among these, we detected 21 of the 35 bird and mammal species that had access to an outdoor enclosure in the southern section of the zoo (Figures 1B and 2). We further detected one zoo animal present in the north section of the zoo, three animals known to be pests in the zoo (i.e., brown rat, house mouse, and yellow-necked mouse) of which two are also used as feed (brown rat and house mouse) and also kept at the zoo (house mouse), four wild or domestic non-zoo mammal species known to occur in and around the zoo (e.g., cat and squirrel), and one fish species used as feed (smelt).

To assess whether sequencing of airborne particulate matter would allow detection of taxonomic groups other than birds and mammals and in an environment resembling that of a rainforest, we collected 12 air filtering samples using the water vacuum (n = 4, September), the 5 V sampler (n = 2, December), and the 24 V sampler (n = 6, December) inside the Tropical House (Figure 1A). The Tropical House consists of two main parts, the Butterfly House and the Rainforest House. We sampled in the latter, which contains multiple reptile, bird, and mammal species, which, except for the Eurasian hoopoe (Upupa epops), were not present in the outdoor enclosures (Table S3). In the 12 samples collected in the Rainforest House, we detected 7 to 17 non-human vertebrate species per sample (mean = 12.17, SD = 2.98) summing to a total of 29 species, including 16 mammal, eight bird, three fish, one amphibian, and one reptile species (Figure 2; Tables S1 and S2). These 29 species included nine of the 24 vertebrate species kept in the Rainforest House of which one of the detected species is kept within a terrarium, namely the Dumeril’s ground boa (Acrantophis dumerili). In addition, we detected five species kept in other parts of the Tropical House than the Rainforest House, four species used as feed in the zoo, and seven zoo species kept outside the Tropical House. Further, we detected two wild or domestic non-zoo species known to occur in and around the zoo (domestic cat and dog) and two rodents known to occur in the zoo as pests (yellow-necked mouse and house mouse).
Figure 2. Vertebrate species detected through metabarcoding of airborne environmental DNA

Detections made through DNA metabarcoding of 40 samples of airborne particles from three sampling locations in Copenhagen Zoo, Denmark: the okapi and red forest duiker stable (n = 12), outside among the outdoor animal enclosures (n = 16), and inside the Rainforest House within the Tropical House (n = 12). Only taxa that could be determined to species level are included. Taxonomic order and family are listed for each species; common names are in bold. Detected species fall within four categories: detected through air eDNA sampling where they are kept (dark blue), detected in another sampling location than where they are kept (blue), detection of wild or domestic non-zoo species (light blue), and species used as animal feed (orange). Some animals kept at the zoo (domestic rabbit, fowl, and (legend continued on next page)

| Order | Family | Genus | Species | Common Name |
|-------|--------|-------|---------|-------------|
| Anura | Bufonidae | Duttaphrynus melanostictus | (Asian common toad) |
| Squamata | Boidae | Acrantophis dumerili | (Dumeril’s ground boa) |
| Cypriniformes | Cyprinidae | Rutilus rutilus | (Common roach) |
| Cyprinodontiformes | Poeciliidae | Poecilia reticulata | (Guppy) |
| Osmeriformes | Osmeridae | Osmerus eperlanus | (European smelt) |
| Salmoniformes | Salmonidae | Salmo salar | (Atlantic salmon) |

- Detected zoo animals kept in sampling location
- Detected zoo animals kept in a different location within the zoo
- Detected non-zoo animals occurring in or around the zoo
- Detected animals used only for feed

1. Zoo animal also used as feed
2. Animal found in and around the zoo as a pest

(legend continued on next page)
mouse) of which one is also kept at the zoo and used as feed (Figure 2).

We collated data from the 40 samples collected in the three sites in an overall inventory. In this collated dataset, we detected between 6 and 21 non-human vertebrate species per sample (mean = 12.8, SD = 3.9) (Table S1), summing to a total of 49 vertebrate species spanning 26 taxonomic orders and 37 families: 30 mammal, 13 bird, four fish, one amphibian, and one reptile species (Figure 2). Of these 49 species, 38 were exotic animals kept in the zoo, three were fish species routinely used as animal feed in the zoo, three were rodent species known to be pests in the zoo, and the remaining five species were wild or domestic non-zoo species known to occur in or around the zoo (e.g., Eurasian red squirrel and water vole). The presence of all 49 detected species could be accounted for. The robustness of our method is demonstrated by 39 matching species detections between the two sets of sampling replicates, with the remaining 10 taxa only being detected by one of the two sampling replicate sets. Further, these are conservative identifications as only those operational taxonomic units (OTUs) that could be identified to the species level. The OTUs that could only be assigned to higher taxonomic levels were assigned to Passeriformes, a large song-bird order; Columbidae, a bird family within Passeriformes consisting of pigeons and doves; and Corvus sp., corvids. These taxonomic groups include wild or feral birds, such as jackdaws, crows, pigeons, and house sparrows, which are common in and around the zoo.

The detected vertebrates represent species with a large variation in sizes, behaviors, and individual numbers (abundance) present in the zoo. This illustrates that a wide range of species can be detected through airborne eDNA. For example, among the species we detected, the zoo holds two ostriches (Struthio camelus) each weighing ca. 90 kg, five white rhinoceros (Ceratotherium simum) each weighing ca. 1,800 kg, 25 helmeted guineafowls (Numida meleagris) each weighing ca. 1.3 kg, and 47 Javan sparrows (Lonchura oryzivora) each weighing ca. 22 g. Furthermore, although most of the detected vertebrate species were cursorial (e.g., impala, Aepyceros melampus; and Java mouse-deer, Tragulus javanicus), animals with other lifestyles were also detected, including volant birds (e.g., kea, Nestor notabilis), a crawling snake (Dumerili’s ground boa, Acrantophis dumerilii), and arboreal animals (e.g., two-toed sloth, Choloepus didactylus).

The two employed metabarcoding primer sets both yielded vertebrate species detections (Table S1). Forty-eight non-human OTUs were obtained from the 16S mammal primer set (excluding positive controls) from which 41 could be assigned to species level. Of the remaining seven OTUs, three could be assigned to genus, one to family, two to order, and one to class. The OTUs assigned to species in the 16S dataset (and included in the analyses) each contained 21 to 125,153 sequence copies per detection (mean = 2,547.64, SD = 12,065.26). As the two primer sets are designed to have different taxonomic coverage, their detections complemented each other. Specifically, the 16S mammal primer set had 22 unique species detections spanning one amphibian, two bird, 18 mammal, and one reptile species, while the 12S vertebrate primer set had 13 unique detections spanning four fish, eight bird, and one mammal species. Overall, three bird and 11 mammal species were detected by both primer sets.

Biomass and distance to air sampling device influence detection

In studies of natural systems, airborne eDNA will predominantly be collected in open air. Thus, we explored putative factors influencing the detection of vertebrate DNA in the outdoor sampling site. We used a logistic regression model with air filtering method, sampling time, average distance of animal to the samplers, animal biomass (no. individuals × average weight for individuals, log transformed), and the taxonomic group (mammal and bird) as independent variables. We found no significant effect of the taxonomic class, the choice of sampling device, or sampling time. These variables were therefore removed from the model. In the resulting model (formula: detection ~ distance + log(biomass)), shorter distance between animal and sampler significantly increased detection probability (beta = −8.046, 95% CI [−0.01, −1.68e−03], p = 0.015; SD beta = −0.14, 95% CI [−0.44, 0.15]). The explanatory power of the model was substantial (Tjur’s R² = 0.30). Further, higher animal biomass increased detection probability (beta = 0.47, 95% CI [0.34, 0.61], p < 0.001; SD beta = 1.57, 95% CI [0.78, 2.52]).

When assuming that mammals and birds had the same biomass and therefore not using animal biomass as a potential explanatory variable in the model, the effect of taxonomic group was statistically significant. However, the explanatory power of this model (formula: value ~ distance + class) was weak (Tjur’s R² = 0.10). As in the previous model, within this model the effect of distance was statistically significant and negative (beta = −6.22e−03, 95% CI [−0.1, −1.88e−04], p = 0.046; SD beta = −0.31, 95% CI [−0.62, −9.31e−03]). However, in this model, mammals had a significantly higher probability of detection (beta = 1.45, 95% CI [0.81, 2.12], p < 0.001; SD beta = 1.45, 95% CI [0.81, 2.12]).

Despite not finding significant differences in detections between the three air sampling devices, we did observe practical differences. The water vacuum sampler was larger than the two particle samplers. It measured 535 × 289 × 345 mm while the 5 V and 24 V measured 40 × 40 × 40 mm and 100 × 100 × 90 mm, respectively. In addition to the size, the water vacuum was less flexible due to the need for an external power supply and molecular grade sterilized water. Further, with its 66 dB(A) it was noisier than the 5 V and 24 V samplers, which were 20 and 55.0 dB(A), respectively. Thereby, we found that...
the 5 V and 24 V samplers allowed for more flexible sampling with less disturbance of animals.

DISCUSSION

We demonstrate airborne eDNA as an untapped source of vertebrate distribution data. We do so by filtering eDNA from air and using metabarcoding to detect vertebrate species. The 49 detected species included zoo animals, locally occurring animals, and animals used as feed at the zoo. The ability to detect this range of vertebrates through airborne eDNA is supported by Clare et al.19

We found an increased probability of detecting airborne eDNA from animals present in high biomass and within a shorter geographical distance to an air sampler. A similar observation regarding distance was made in a study carried out at Hamerton Zoo Park, UK, where airborne eDNA was found to be concentrated around areas recently inhabited by zoo animals.19 Accordingly, in the samples that we collected at the outdoor sampling location in the southern part of the zoo, we only detected one species located in the northern section, namely the eastern gray kangaroo (Macropus giganteus). However, in addition to distance, wind direction can have played a role. On sampling days, the wind came from southwest, south, southeast, and east. There was thereby no wind coming from the north (Figure 1). This can have favored the transport of DNA from vertebrates from the southwest, south, southeast, and east parts of the zoo. However, we speculate that the presence of several buildings and a trafficked road between our sampling site in the southern part of the zoo and the northern part also prevented us from detecting vertebrate species present in the northern part of the zoo. Similarly, the failure to detect 14 species present in the southern part of the zoo could not only be due to the biomass, and distance, but also the presence of buildings between the enclosure and the air sampler (Figure 1B).

We found the 16S mammal and 12S vertebrate metabarcoding primer sets to detect many of the same species, but as they were designed to have different taxonomic range, each of them also had unique species detections (Table S1). The complementarity of vertebrate detections between these two metabarcoding primer sets has previously been documented in metabarcoding studies of, e.g., carrion fly (iDNA) and bulk biomass, and distance, but also the presence of buildings between the enclosure and the air sampler (Figure 1B).

A concern with demonstration of a novel eDNA substrate is authenticity of results. Metabarcoding is a technique that uses universal primers to PCR amplify short DNA markers.22 When this is carried out on low DNA quantities of target taxa, such as in eDNA extracts, there is a risk of amplifying contaminant templates.23 Further, library preparation, PCR, and sequencing artifacts can lead to inflated diversity and false positives.24–26 To ensure authenticity of results for the demonstration of such novel eDNA substrate with which to monitor terrestrial vertebrates, we therefore followed strict sampling, laboratory, and bioinformatic workflows. We created a dedicated eDNA pre-PCR laboratory for DNA extractions of the air filtering samples in which we followed guidelines commonly used for ancient DNA analyses.27

To enable identification of potential contamination, we included negative controls in all steps of the workflow, including samples from the air in the DNA extraction lab. To reduce risk of PCR-introduced artifacts, we used the so-called tagged PCR approach28 and only carried out one PCR amplification step prior to sequencing. A crucial step to ensure authenticity was the inclusion of four differently tagged PCR replicates per sample, which allowed stringent filtering of sequences across each sample’s PCR replicates.24,28,29 Our stringent measures resulted in a dataset in which we could account for the presence of all detected species. We are aware that we detected species known to be contaminants in laboratory reagents, such as pig, cow, chicken, and to a lesser extent mouse, rabbit, goat, and guinea pig.30 For example, we used bovine serum albumin (BSA) in PCR amplifications, which is synthesized from cow’s blood.31 We did not, however, detect any of these taxa in the negative controls.

It is important to note that the confinement and density of animals in the zoo’s enclosures may have artificially increased their probability of detection in air samples compared to sampling in a natural environment. In addition, the movement of people within the zoo could have impacted the detection of vertebrate airborne eDNA. In the Rainforest House, we detected species that zoo visitors can come in close proximity to, and maybe even in contact with (e.g., okapi, pygmy goat, Eastern gray kangaroo, and domestic rabbit), but that were not found within the Rainforest House (Figure 2). This may have been caused by movement of people, as has also been shown for plant airborne eDNA.32 Still, we also detected six animals not kept at the zoo but present in the surrounding area. Detection of locally occurring animals has also been documented in other studies on airborne eDNA.19,33 This further highlights the potential of airborne eDNA for detection of wildlife. In addition, we detected fish (i.e., roach, smelt, and salmon) that are used as feed to various zoo animals on a daily basis. As part of this practice, fish are thawed in the zoo commissary and transported through the zoo in buckets before being presented to the zoo animals (M. Bertelsen, personal communication). While roach could originate from nearby lakes and salmon from zoo restaurants, smelt does not occur in the region where the air samples were collected.34 Therefore, the most likely origin of these detections is from the use of it as feed in the zoo.

Collection and sequencing of airborne eDNA has the potential to transform the way natural ecosystems are studied and surveyed. It can act as a cost-effective and efficient tool to inform conservation efforts and track progress in achieving biodiversity targets, something of great global importance given the ongoing climate and biodiversity crisis.1,34 As with any novel methodology, including the first demonstrations of eDNA in aquatic environments,35 the use of airborne eDNA for vertebrate community surveys will require optimizations and developments to match natural habitats and the aim of specific research applications. Pending this, and with time, we envision terrestrial airborne eDNA vertebrate surveys can parallel the field of aquatic eDNA monitoring with the potential to revolutionize and form the cornerstone in future ecosystem studies, including global next generation biomonitoring frameworks.36,37
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### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE                      | SOURCE                             | IDENTIFIER |
|------------------------------------------|------------------------------------|------------|
| **Chemicals, peptides, and recombinant proteins** |                                    |            |
| AmpliTaq Gold                            | Applied Biosystems                 | N8080241   |
| 1 × Gold PCR Buffer                      | Applied Biosystems                 | N8080241   |
| MgCl₂                                    | Applied Biosystems                 | N8080241   |
| dNTP mix                                 | Invitrogen                         | R1121      |
| Bovine serum albumin (BSA)               | New England BioLabs                | B9000S     |
| SYBR Green I nucleic acid gel stain      | Invitrogen                         | S7563      |
| ROX Reference Dye                        | Invitrogen                         | Cat#12223-012 |
| DMSO                                     | LabLife                            | D2650-5X5ML |
| EB buffer                                | QIAGEN                             | Cat#19086  |
| PBS pH 7.4 (1X)                          | GIBCO, Thermo Fisher               | Cat#10010023 |
| T4 Polynucleotide Kinase, 10 U/µl        | New England BioLabs                | M0201S     |
| Klenow Fragment (3′-5′ exo-), 5 U/µl     | New England BioLabs                | M0212S     |
| T4 DNA Ligase, 400 U/µl                  | New England BioLabs                | M0202S     |
| T4 DNA Ligase Buffer, 10x                | New England BioLabs                | M0202S     |
| dATP                                     | Thermo-Fisher                      | Cat#18252015 |
| Tween-20                                 | Sigma-Aldrich                      | P5927-100ML |
| PEG-8000                                 | Sigma-Aldrich                      | Cat#89510-250G-F |
| EDTA, 0.5 M, pH8.0                       | Thermo-Fisher                      | Cat#15570520 |
| Tris-HCl, 1M, pH8.0                      | Thermo-Fisher                      | Cat#15568025 |
| MagBio HiPrep beads                      | LabLife                            | Cat# AC-60050 |
| **Critical commercial assays**           |                                    |            |
| DNeasy Blood & Tissue Kit                | QIAGEN                             | Cat#69506   |
| NEBNext Library Quant Kit for Illumina   | New England BioLabs                | E7603S     |
| Qubit dsDNA HS Assay Kit                 | Invitrogen                         | Q32854     |
| **Deposited data**                       |                                    |            |
| Raw and analyzed data                    | This paper                         | https://doi.org/10.17894/ucph.82e86c81-77ce-4172-8508-4bca32d82892 |
| **Oligonucleotides**                     |                                    |            |
| primers 16S mam1 (forward 5′-CGGTTGGGGTGACCTCGGA-3) and 16S mam2 (reverse 5′-GCTGTATCCCTAGGGTAACT-3) | 39 | N/A |
| primers 12SV05 (forward 5′-TTAGATAACCCCCACTATGC-3) and 12SV05 reverse (5′-TAGAA CAGGCTCTCTCTAG-3′) | 40 | N/A |
| human blocker (5′-3′ GCGACCTCGGAGCA GAACCC spacerC3) | 41 | N/A |
| human blocker (5′-3′ TACCCACTATGCT TAGCCCTAAACCTCAAGTAAA TC spacerC3) | 42 | N/A |
| TagSteady P5/P7 adapters                 | 43 | N/A |
| **Software and algorithms**              |                                    |            |
| AdapterRemoval v2.2.2                    | https://adapterremoval.readthedocs.io/en/stable/installation.html; RRID:SCR_011834 |
| Begum                                    | https://github.com/shyamsg/Begum/wiki |            |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Kristine Bohmann (kbohmann@sund.ku.dk).

Materials availability
This study did not generate new unique reagents.

Data and code availability
Sequence data that support the findings of this study have been deposited in the Electronic Research Archive (ERDA) from the University of Copenhagen with https://doi.org/10.17894/ucph.82e86c81-77ce-4172-8508-4bca32d82892 (https://erda.ku.dk/archives/83c87a1dc0f120f6256143ec71cc493/published-archive.html)

Published methods and software were used, and these are fully referenced.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Vertebrate DNA was collected from air at the Copenhagen Zoo, Denmark, and extracted from filters at the University of Copenhagen. See Method details section for more information.

METHOD DETAILS

Study site
Forty air samples were collected at the Copenhagen Zoo, Denmark, during September and December 2020. Air samples were collected: 1) inside a 155 m² stable inhabited by two okapis (Okapi johnstoni) and two red duikers (Cephalophus natalensis), which had the option to use an adjoining outdoor enclosure during the day. 2) in open air at a fixed location in the part of the zoo containing multiple outdoor enclosures with a mixed variety of mammals and other terrestrial vertebrates. 3) inside the Rainforest House, a 442 m²/2200 m³ confined enclosure in which smaller vertebrates and other animals from the tropics move freely around both day and night, located within the Tropical House (Figure 1; Table S3). Inside the stable and the Rainforest House, the temperature was kept constant during September and December, ranging from 18.6 to 20.5 °C, and from 22 to 27° C, respectively. None of the locations were directly exposed to the wind, but the stable did have openings to the outdoors and the Rainforest House had an internal ventilation system. During the outdoor sample collection, on the 11th of September the temperature ranged from 17.1 to 17.3 °C, wind speed from 4.4 to 5.2 m/s with wind coming from the SW; on the 22th of September the temperature ranged from 21 to 21.2 °C, wind speed of 2.6 to

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| SUMACLUST           | 46     | https://git.metabarcoding.org/obitools/sumaclust/wikis/home |
| LULU                | 46     | https://github.com/tobiasgf/lulu |
| MEGAN Community Edition v6.12.5 | 46 | https://software-ab.informatik.uni-tuebingen.de/download/megan6/welcome.html |
| R version 4.0.3     | 47     | https://www.R-project.org/ |
| Geneious Prime 2020.1.2 | Geneious | https://www.geneious.com/download/; RRID:SCR_010519 |

Other

- Kärcher DS5800 Water Vacuum, Alfred Kärcher GmbH & Co. KG, Germany; DKM A/S, 1195.210.DS0
- Brushless radial blower fan, 97.2 mm x 33 mm 24 V, 0.550 A DC, Delta Electronics, BFB1024HH
- Class F8 pleated fibrous particulate filter, Dongguan Wonen Environmental Protection Technology, WE180423-350-W10
- 3D-printed filter housing, Airlabs, Copenhagen, Denmark, NA
- Brushless radial blower fan, 40 mm x 40 mm x 10 mm, 5 V, 0.10 A DC, Hawkung/Long Sheng Xin, HK-337
- Sterivex filters (pore size 0.22 μm), Merck Millipore, SVGPL10RC
4.5 m/s and with wind coming from S; on the 10th of December, the temperature was 3 °C, wind speed was 5.9 to 5 m/s and the wind came from the East; and on the 11th of December, the temperature was 2.4 °C, wind speed 5.6 to 5.4 m/s and the wind came from SE/E direction. Although there was some precipitation before and after the outdoor sampling in September (0.4 to 0.7 mm), there was none during the time of sample collection (https://www.dmi.dk/).

**Airborne eDNA samplers**

Environmental DNA was collected from air using three different samplers; a water-based commercial vacuum and two air particle filters with different power sources and airflows. The water-based commercial vacuum was the Kärcher DSS800 Water Vacuum (VV) (Alfred Kärcher GmbH & Co. KG, Germany), which consists of a high-flow-rate impinger with an outer part that creates suction and an inner vortex chamber where the particles flow into. This VV was connected to the electrical circuit and provided an average airflow of 8.8 m³/min. The second sampler was a custom made air particle filter sampler consisting of a Delta Electronics 97.2 mm x 33 mm 24 V, 0.550 A DC brushless radial blower fan, a class F8 pleated fibrous particulate filter (Dongguan Wonen Environmental Protection Technology), and a 3D-printed filter housing (Airlabs, Copenhagen, Denmark; 3D-printing blueprints available in Figure S1). The filter was placed approximately 40 mm from the intake of the blower fan and was connected to the electrical circuit, providing an airflow of 0.8 m³/min. We call this the 24 V sampler (Figure S1). The third sampler was overall similar to the 24 V sampler, except that the filter was placed on a 3D-printed filter housing approximately 20 mm from the intake of the blower fan, which is a battery-driven Hawkung/Long Sheng Xin 40 mm x 40 mm x 10 mm, 5 V, 0.10 A DC brushless radial blower fan, providing an airflow of 0.03 m³/min (Figure S1). We call this the 5 V sampler. For both 24 V and 5 V samplers, we used class F8 pleated fibrous particulate filters (Dongguan Wonen Environmental Protection Technology). This type of filter is usually implemented in A/C units and is designed to capture airborne particulate matter and micro- and nanofibers with high efficiency and low pressure drop. As the filter is cut and stretched out to a single layer around the size of the filter housing, the airflow and retention efficiency is expected to decrease slightly from the official F8 rating (https://www.emw.de/en/filter-campus/filter-classes.html).

**Sample collection**

Sampling with the WV sampler followed Šantl-Temkiv et al., i.e., the inner vortex chamber was filled with 1.7 L sterile Milli-Q H₂O. After running the impinger, the water from the vortex chamber was filtered using Sterivex filters (pore size 0.22 μm). In between samplings, the vortex chamber and the suction hole were cleaned with 5% sodium hypochlorite (bleach) and 70% ethanol. Using this sampler, air was collected for 30 min and 60 min at each site during September. Samples collected outside with the WV were also collected during December. At every location (inside the stable, outdoors, and inside the Rainforest House during September and outdoors during December), a sampling negative control consisting of 200 mL of sterile Milli-Q H₂O was added to the vortex chamber and thereafter filtered with Sterivex filters. Prior to sampling with the 24 V and 5 V air samplers, the F8 filters were cut into a smaller size to fit the housing, autoclaved, placed under UV light for 20 min and thereafter stored individually in sterile plastic bags. In between sampling events, the housing of the 24 V and 5 V samplers was cleaned with 5% bleach and 70% ethanol. Sterilized filters were handled using sterile tweezers and medical gloves. To test the effect of sampling time, the 24 V sampler was left running for 30 min, 60 min and 5 h. For the 5 V sampler, to test the effect of long sampling time, this sampler was left running for 30 h at each location. Both samplers were run during December. For all three samplers, samples were taken at 1 m above the ground and in duplicates. Interference with the samplers by physical contact with birds and people was not observed but contact with animals cannot be ruled out for the 5 V sampler running overnight. Upon sampling, filters were stored individually in sterile 50 mL Falcon tubes in a cooling box for up to 5 h and thereafter at −20 °C until DNA extraction.

**DNA extraction**

Due to their large size, the filters used with the 24 V sampler were cut in half with sterile blades. Both halves of filters used with the 24 V and the entire filter from the 5 V samplers were transferred independently to 5 mL Eppendorf tubes and 3 mL of autoclaved PBS pH 7.4 (1X) (GIBCO, Thermo Fisher) was added. After an incubation of 45 min, the filters were transferred to a new Eppendorf tube, and the PBS was centrifuged at 6000 xg for 10 min to create a pellet, and the supernatant removed. PBS was added directly to the water vacuum Sterivex filters, which were sealed with luer lock caps and parafilm, incubated and centrifuged as described above to obtain a pellet. The DNeasy Blood & Tissue Kit (QIAGEN, USA) was used for DNA extraction of the PBS pellets, the Sterivex filters, and the 5 V, and 24 V filters. To test for contamination during handling of filters, DNA extractions were performed directly on autoclaved (clean and non-used) filters and PBS. In addition, to test for contamination in the DNA extraction room, two falcon tubes containing 50 mL sterile Milli-Q H₂O were left open for 48 h and subjected to DNA extraction.

The DNA extraction followed manufacturer’s instructions (DNeasy Blood & Tissue Kit by QIAGEN, USA; Purification of Total DNA from Animal Tissues protocol), with slight modifications: the ratio 9:1 of ATL buffer to Proteinase K was kept but the volume was increased to 720 μL ATL and 80 μL Proteinase K and an incubation step of 37 °C for 15 min was added after the addition of 50 μL EBT (EB buffer with 0.05% Tween-20 (WVR)). This elution step was carried out twice to increase DNA yield.

In order to ultimately yield one DNA extract per sample, the digest of the filters and from the PBS pellets belonging to the same sample were passed through the same spin column. However, for three samples collected inside the okapi stable using the 24 V sampler, the digests of each half of the filter presented many particles clogging the spin column and therefore the digests could not be combined into one spin column. This resulted in a total of 49 DNA extracts, representing 40 samples. Negative extraction controls were added for every 16 samples. Eluted DNA was stored in Eppendorf LoBind tubes at −20 °C.
To minimize contamination risk during DNA extraction, we set up a specialized environmental DNA pre-PCR laboratory, which was thoroughly cleaned prior to its use and in which many of the guidelines follow those used in ancient DNA laboratories, such as unidirectional workflow and the use of hair net, sleeves, facemask, two layers of medical gloves, dedicated footwear, and decontamination with ≥3% bleach. All steps of the workflow were carried out in laminar flowhoods and using filter tips.

**DNA metabarcoding**

Metabarcoding was conducted using two different primer sets. To target mammals, a ca. 95 bp 16S rRNA mitochondrial marker was PCR-amplified with the primers 16Smam1 (forward 5'-CGGTTGGGTGACCTCGGA-3') and 16Smam2 (reverse 5'-GCTTATCCCTAGGGTAACT-3'). To target vertebrates, a ca. 97 bp fragment of the 12S gene was PCR-amplified with the primer set 12SV05 forward 5'-TAGATACCCACTATGC-3' and 12SV05 reverse 5'-TAGAACAGGCTCCTTAG-3'. The two metabarcoding primer sets are from here on referred to as 16S mammal and 12S vertebrate primers, respectively. Nucleotide tags were added to the 5' ends of forward and reverse primers of both primer sets to allow parallel sequencing. Nucleotide tags were six nucleotide tags in length and had min. three nucleotide differences between them. One to two nucleotides were added to the 5' end to increase complexity on the flowcell. DNA extracts from fin whale (*Balaenoptera physalus*) and bowhead whale (*Balaena mysticetus*) were used as positive controls, as none of the species are found close to the sampling site in Copenhagen Zoo.

Prior to metabarcoding PCR amplification, dilution series of a subset of the DNA extracts were screened using SYBR Green quantitative PCR (qPCR). This was done to determine the optimal cycle number and DNA template volume to ensure optimal amplification in the following metabarcoding PCR amplifications. Further, all negative controls were included in the qPCR to screen for contamination.

For the 16S mammal primer set, the 20 μL reactions consisted of 2 or 4 μL DNA template, 0.75 U AmpliTaq Gold, 1 × Gold PCR Buffer, and 2.5 mM MgCl₂ (all from Applied Biosystems); 0.6 μM each of 5' nucleotide tagged forward and reverse primer; 0.2 mM dNTP mix (Invitrogen); 0.5 mg/mL bovine serum albumin (BSA, Bio Labs); 3 μM human blocker (5'-3' GGCAGCTCGAGCA-GAACCCC-spaceCr3); and 1 μL of SYBR Green/ROX solution [one part SYBR Green I nucleic acid gel stain (S7563) (Invitrogen), four parts ROX Reference Dye (12223-012) (Invitrogen) and 2000 parts high-grade DMSO]. The thermal cycling profile was 95°C for 10 min, followed by 40 cycles of 95°C for 12 s, 59°C for 30 s, and 70°C for 25 s, followed by a dissociation curve. For the 12S vertebrate primer, the 20 μL reaction was the same except for the human blocker (5'-3' TACCCTACTATGCTTACCCATACCTCAACGTTAAATC-spaceCr3) and the thermal cycling profile of 95°C for 10 min, followed by 40 cycles of 94°C for 30 s, 59°C for 45 s, and 72°C for 60 s, followed by a dissociation curve. The amplification plots from the qPCR indicated that 2 μL DNA template, and 35 and 38 cycles were optimal for the 16S mammal and 12S vertebrate primers, respectively. The negative extraction controls incorporated during the laboratory work showed no contamination.

For the metabarcoding PCR, the 20 μL reactions were set up as described for the qPCR above but omitting SYBR Green/ROX and replacing the dissociation curve with a final extension time of 72°C for 7 min. Four tagged PCR replicates were carried out for each of the 49 DNA extracts, negative and positive controls, and for both primer sets. The PCR replicates from each sample were differently tagged. Negative controls were included every seven PCR reactions.

Amplified PCR products were visualized on 2% agarose gels with GelRed against a 50 bp ladder. All negative controls incorporated during laboratory analyses appeared negative and all positive controls showed successful amplification. Some PCR replicates of the negative sampling controls collected with the water vacuum showed faint bands. All PCR products were pooled. This included both samples, negative, and positive controls and even if they did not show successful amplification. The pooling resulted in four amplicon pools: one pool for each of the four PCR replicates.

Amplicon pools were purified with MagBio HiPrep beads (LabLife) using a 1.6x bead to amplicon pool ratio and eluted in 35 μL EB buffer (QIAGEN). Purified amplicon pools were built into sequence libraries with the TagSteady protocol to avoid tag-jumping. Libraries were purified with a 1.6x bead to library ratio and eluted in 30 μL EB buffer and qPCR quantified using the NEBNext Library Quant Kit for Illumina (New England BioLabs). Purified libraries were pooled equimolarly according to the qPCR results and sequenced at the GeoGenetics Sequencing Core, University of Copenhagen, Denmark. Libraries were sequenced using 150 bp paired-end reads on an Illumina MiSeq sequencing platform using v3 chemistry aiming at 30,000 reads for each of the four PCR replicates, equaling an estimated 120,000 reads per sample.

**Data analyses**

Sequence data for each primer set was processed separately. Illumina adapters and low quality reads were removed and paired ends merged using AdapterRemoval v2.2.26. Within each amplicon library, sequences were sorted based on primers and tag sequences using Bgum, allowing two primer-to-sequence mismatches. Further, for each sample Bgum was used to filter sequences across the PCR replicates. This was guided by the positive and negative controls, and retaining sequences found in three out of the four PCR replicates and with a minimum copy number of 10 and 6 for the 16S mammal and 12S vertebrate primer sets, respectively. As the aim of the present study was to detect and identify species, and not intraspecific variation, we decided to create clusters of sequences (OTUs), instead of denoising and creating amplicon sequence variants (ASV). Although clustering relies on the combination of groups of sequences with a certain percentage of similarity and denoising aims to detect and remove erroneous sequences while keeping the correct ones, they have proven to be complementary, instead of alternatives. However, when working with eukaryotes, clustering should be the standard as long as the correct parameter settings are used during data analysis. The filtered sequences with a similarity score of 97% were therefore clustered into operational taxonomic units (OTU) using SUMACLUST. Curation of the OTUs was carried out with the LULU algorithm, using default settings to remove erroneous OTUs.
Taxonomic identification of the OTU sequences was carried out using BLASTn against the NCBI GenBank database. The output was imported into MEGAN Community Edition v6.12.28 using a weighted LCA algorithm with 80% coverage, top percent of 10, and a minimum score of 150. The taxonomic identification of all OTU sequences was manually validated and species-level identification was assigned if the OTU sequence had a 100% identity match to a NCBI reference sequence. We assigned those that matched 100% to more than one species to the species found in the Copenhagen Zoo. In a few cases where multiple OTUs were assigned to the same species, the corresponding DNA sequences were checked visually in Geneious Prime 2020.1.2 to assess whether the OTUs resulted from genuine haplotype variation or biases caused by minor variations in sequence length. OTUs that could not be identified to species level were discarded before further analysis. In addition, OTUs identified as human were removed, as well as one matching chimpanzee (Pan troglodytes) due to its close similarity to human sequences.

One of the few undetected mammals in the outdoor sampling site in the southern part of the zoo was wallaby (Macropus rufogriseus). However, the Eastern gray kangaroo was detected (M. giganteus) even though it is found on the North part of the zoo. Both animals belong to the same genus, Macropus, but the detected 12S and 16S OTUs both show a 100% match to Eastern gray kangaroo DNA, therefore showing that it is a correct detection. Finally, we identified Canis lupus OTUs with all samplers, in all three locations, and in both September and December. Both the 12S and 16S OTUs identified as Canis lupus had 100% identity to dog (Canis lupus familiaris) and wolf (Canis lupus). Three gray wolves were present in the zoo during the sampling in September, but they were absent during the sampling in December. This, the difficulty discerning wolf and dog with the two utilized metabarcoding markers, and the fact that we detected Canis lupus 12S and 16S OTUs with all samplers and in all three locations made us conclude that the detected OTUs were from dogs in the area.

No OTUs were detected in the negative controls implemented during the laboratory work. However, DNA was detected in two of the three sampling negative controls of the water vacuum vortex chamber. These spanned all three locations. Within the okapi and red forest duiker stable, an okapi OTU was detected in the sampling negative control, but as that stable is inhabited by the okapi the detection was retained in the samples. From the sampling in the outdoor sampling site in December, an OTU from the Sclater’s crowned pigeon was detected in the water vacuum sampler negative control (19,997 sequence copies with the 12S primer set). From that sampling day at the outdoor location, an OTU from the Sclater’s crowned pigeon was also detected in the four samples collected with the water vacuum (2741 to 17,464 sequence copies with the 12S primer set). Prior to this outdoor sampling, the water vacuum had been used in the Rainforest House where the Sclater’s crowned pigeon is kept. No other animals from the Rainforest House were detected in the outdoor samples with any of the three air samplers. Therefore, the Sclater’s crowned pigeon was considered cross-contamination from the previous sampling within the Rainforest House and deleted from the data from the water vacuum from the outdoor sampling site. The sampling negative control from water vacuum collection in the Rainforest House also yielded a species detection, namely okapi (OTU detected with the 16S primer set in 669 sequence copies). Further, we detected okapi in the four samples collected with the water vacuum in the Rainforest House (103 to 494 sequence copies with the 16S primer set). In addition, okapi DNA was detected in four out of eight of the samples collected with the 5 V and 24 V samplers in the Rainforest House. The Rainforest House is placed at the northern part of the zoo, while the okapis are only kept at the southern part of the zoo in a stable and an adjoining outdoor enclosure. Zoo visitors have access to their stable. Due to the consistent okapi detections within the Rainforest House, we hypothesize that they can be caused by movement of people within the zoo. Further supporting this is the fact that we detected other species in the Rainforest House that are not kept at the Rainforest House, but which people can come in close proximity to, and maybe even in direct contact with (e.g., pygmy goat, Eastern gray kangaroo, and domestic rabbit) (Figure 2). To summarize, the detection of the two OTUs in the negative controls of the water vacuum indicates that the water vacuum is a very sensitive sampler of airborne eDNA - especially when it has previously been used in a confined space. Further, it shows that the vortex chamber of the water vacuum is difficult to clean sufficiently for eDNA sampling. The design of the two filter-based samplers did not allow for sampling negative controls. However, the fact that none of the negative controls incorporated during the laboratory work yielded detections shows that all animal detections in this study arose from the zoo.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

We collated data across replicates and the two primer sets in an overall inventory. For the statistical analysis, only data from species present at the southern part of the zoo was used (Table S4). Detected non-zoo animals and those also used as feed were removed from the dataset, as it was not possible to assess location and biomass. The distance of the animals to the samplers was measured using a satellite view of the Copenhagen Zoo using Google Earth (https://earth.google.com/) and using an average point of reference for animals with a large enclosure. Average body weight data was obtained from Species360 Zoological Information Management System (ZIMS) (2021) (Table S4). We fitted a logistic model (estimated using ML) using R 4.0.347 to predict detection with distance, biomass (log transformed), taxonomic group (class: bird or mammal), sampler type (WV, 5 V, 24 V), and sampling time as potential explanatory variables. Effect of sampler type, taxonomic group, and sampling time were insignificant, and they were removed one at the time from the model. Therefore, the final formula for the model was: detection ~ distance + log(biomass). The model’s intercept, corresponding to distance = 0 and biomass = 0, is at −1.50 (95% CI [-2.29, −0.76], p < 0.001). Finally, animal biomass was removed as a potential explanatory variable in the model, to further explore the probability of detection between birds and mammals. The formula for that model was: value ~ Distance + Class. The model’s intercept, corresponding to distance = 0 and Class = Aves, is at −0.67 (95% CI [-1.33, −0.04], p = 0.040).