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Research Article

Stemflow for detecting mammalian environmental DNA: a case study in a zoo

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Abstract

As mammalian diversity declines, effective biodiversity monitoring tools are urgently needed. To address this issue, we assessed the detectability of mammalian environmental DNA (eDNA) by applying eDNA metabarcoding to stemflow samples. We collected stemflow samples six times from June 2024 to January 2025 from 11 trees in the Oji Zoo, Kobe, Japan. One tree was inside the cage and thus in direct contact with the captive mammals, whereas the others were outside the cages, *i.e.*, could not be directly contacted by captive mammals. We amplified eDNA extracted from the stemflow samples with MiMammal primers targeting the mitochondrial 12S rRNA gene, and the PCR products were then sequenced. We detected eDNA of 22 mammal species, 14 of which were zoo species and eight of which were not. Environmental DNA of Japanese squirrels (*Sciurus lis*), which could directly contact the target tree, was detected at all six sampling time points. In addition, we found a significant negative relationship between eDNA-based detection and the distance from each mammal cage to each tree, as well as significant differences in the composition of the detected species between the trees inside and outside the cages. In conclusion, our pioneering analysis shows that stemflow eDNA metabarcoding can be useful for detecting and monitoring mammals. The potential for its application in natural settings will need to be explored in subsequent studies.

Key words: Biodiversity conservation, biomonitoring, metabarcoding, MiMammal

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Introduction

Mammals are widely recognized as flagship species for biodiversity conservation because of their taxonomic diversity and extensive ecological roles (Ceballos and Ehrlich 2002; Ceballos et al. 2005). However, as approximately one-quarter of mammalian species are currently threatened with extinction (IUCN 2025), urgent measures are required to address this issue.

Thus, effective monitoring to assess the presence and distribution of mammals is essential, highlighting the need for the development of comprehensive and accurate monitoring techniques.

Environmental DNA (eDNA) analysis has recently emerged as a promising method for biological monitoring. It involves detecting genetic material present in environmental media such as water, soil, or air to identify the species in each habitat (Minamoto et al. 2012; Newton et al. 2025). This technique has advantages in that it does not require specialized taxonomic expertise for species identification and allows for non-invasive and cost-effective monitoring. Compared to traditional mammalian monitoring methods such as visual surveys, camera traps, and pitfall traps, eDNA analysis has been shown to be more cost-efficient (Burton et al. 2015; Thomsen and Willerslev 2015; Yonezawa et al. 2020; Lyet et al. 2021). Various approaches have been reported for mammalian eDNA surveys, including the use of non-biological substrates such as water, soil, and air (Ishige et al. 2017; Ushio et al. 2017; Leempoel et al. 2020; Clare et al. 2022), as well as biological substrates such as invertebrates and plant tissues (Schnell et al. 2012; Calvignac-Spencer et al. 2013; Allen et al. 2023). However, no standardized and widely accepted eDNA monitoring method for mammals has yet been established.

Standardizing eDNA-based mammalian monitoring remains challenging due to variability in detection probability and substrate-specific limitations. The spatial and temporal detectability of eDNA is influenced by the uneven distribution of sources such as feces and the stochastic nature of animal substrate contact. Each sampling method presents unique challenges: soil eDNA may reflect past rather than current presence (Leempoel et al. 2020; Guthrie et al. 2024), and air eDNA is affected by wind, making habitat attribution difficult (Bohmann and Lynggaard 2023; Lynggaard et al. 2024). Aquatic eDNA surveys may miss species observed via conventional camera traps and are limited by water availability (Harper et al. 2019; Sales et al. 2020; Mena et al. 2021). Additionally, fly sampling can be biased by insect preferences (Calvignac-Spencer et al. 2013). On trees, alternative approaches such as the roller method (rubbing tree surfaces to collect eDNA) have shown promise for detecting arboreal mammals (Allen et al. 2023). In addition, rainwash eDNA sampling, which collects eDNA washed from the canopy by rainfall, has recently been demonstrated as a minimally invasive method to characterize canopy-associated biodiversity (Macher et al. 2023). Thus, while various methods have successfully detected mammalian eDNA, each has inherent constraints and challenges.

A comparable method for detecting eDNA on trees, the stemflow method, entails collecting rainwater that flows over the tree surface (stemflow) to detect eDNA on the tree (Sakata et al. 2023). This method is advantageous due to its simplicity and cost-effectiveness, as sampling is feasible in any environment where trees are present and rainfall occurs (Sakata et al. 2023). Consequently, mammalian eDNA attached to trees can be readily and cost-effectively recovered using the stemflow method, potentially exhibiting high sensitivity, making it a promising tool for mammal monitoring. However, there have been no reports of mammalian detection achieved using the stemflow method.

The aim of this study was to assess the detectability of mammalian eDNA using the eDNA metabarcoding method with stemflow. To test this, the study

was conducted in a zoo where the number of mammal species and individuals, and their locations, were known. Stemflow was collected from trees in the zoo, and the extracted eDNA was used for eDNA metabarcoding analysis, which allows simultaneous detection of multiple mammal species. Based on the data obtained, we evaluated the number of mammal species detected, the distance from mammal enclosures to sampled trees, and the influence of bark roughness on eDNA detectability to determine the effectiveness and limitations of the stemflow method for monitoring mammals.

Materials and methods

Sampling

Stemflow sampling was performed at the Oji Zoo, Kobe, Japan, where 263 mammal individuals of 51 species are kept (Suppl. material 1: table S1, fig. S1). A total of six sampling sessions were conducted from June 2024 to January 2025 from 11 trees in the zoo, with additional sampling on two trees because water could not be recovered from some samples (Fig. 1, Suppl. material 1: tables S2, S3). As a result, the total number of samples was 62. Of the 11 trees, one tree (T9) was inside the cage where Japanese squirrels (*Sciurus lis*) are kept and could be directly contacted by the captive animals, whereas the others were outside the cages and could not be directly contacted by captive animals (Suppl. material 1: table S2). To examine differences in the results due to the shape of the trees, T1 and T2, T3 and T4, and T5 and T6 were chosen to be adjacent trees of different species (Suppl. material 1: table S2).

Collecting stemflow

Stemflow samplers were made and installed according to Sakata et al. (2023), with a slight modification regarding the height. The height at which the rubber rope and gauze were wrapped was set at 80 cm from the ground. Both ends of the rubber rope were fixed through a funnel made of silicone material, and the gauze was also placed in the funnel. Next, both ends of the rubber rope were inserted into a hose cut to approximately 10 cm in length. The hose was then connected to a backflow prevention unit attached to the water collection bag. After the rainfall, the water collection bag was removed from the sampler, capped, and brought back to the laboratory. One water sampling bag containing 1 L distilled water was prepared for each sampling time as a field blank and placed near one of the sampling trees. For both samples and field blanks, 500 µL of 10% benzalkonium chloride solution (Nippon Pharmaceutical Co., Ltd.) was added beforehand to inhibit DNA degradation (Yamanaka et al. 2017). Field blanks were analyzed as regular samples from DNA extraction to high-throughput sequencing. Rainfall data were obtained from the Japan Meteorological Agency (Suppl. material 1: table S3) (Ministry of Land, Infrastructure, Transport and Tourism, Japan Meteorological Agency "Historical Weather Data Search" https://www.data.jma.go.jp/obd/stats/etrn/index.php; data at the nearest point [Kobe observatory] were checked on 16 February 2025).

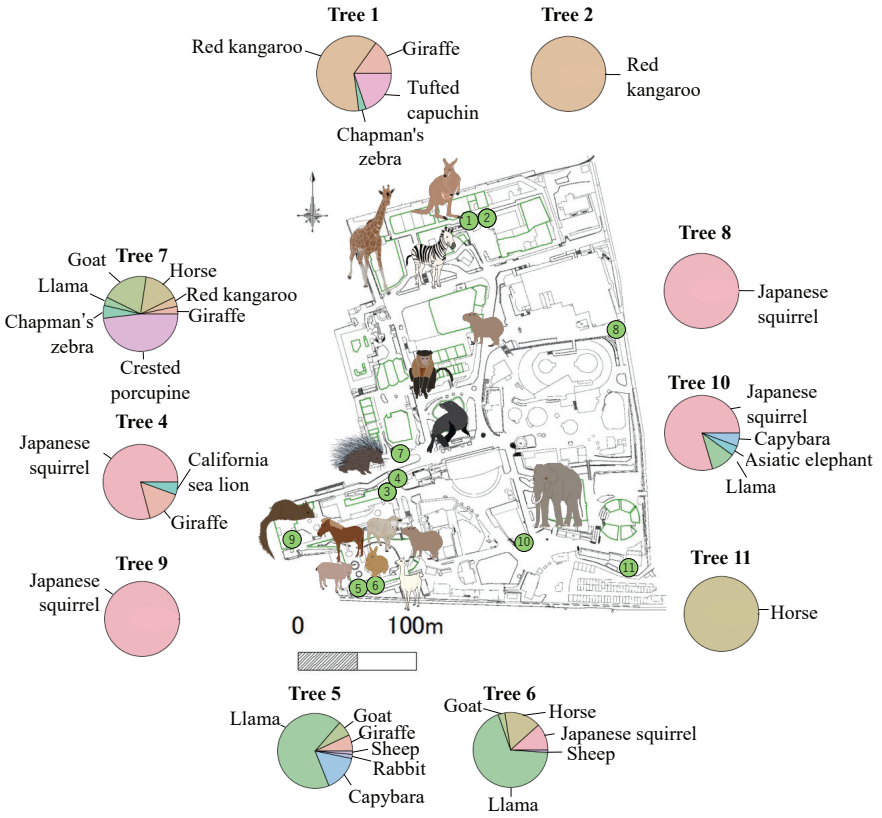

Figure 1. Location of trees and detected species of captive mammals. Approximate locations of the 14 captive mammal species detected by eDNA are presented. The circled numbers on the map indicate the locations of the sampled trees. The pie charts show the proportion of reads for each animal species detected from each sampled tree.

Filtration, DNA extraction, and removal of inhibitors

On the same day that the water collection bags were collected, gravity filtration was performed according to the method of Oka et al. (2022). Sterivex filters (Merck, USA) with a pore size of 0.45 µm were connected to the water sampling bag, and a hose cut to 1.5 m was hung below it. From a height of approximately 2 m, the 1.5 m hose was dropped and left for one to four nights for gravity-based filtration. After filtration was completed, Sterivex was collected, and unfiltered water was discarded (Table 1). After filtration, 1 mL of Buffer ATL was added to the Sterivex, sealed at the top and bottom with a cap, and stored at −25 °C.

DNA extraction from Sterivex was performed using the DNeasy Blood and Tissue Kit (Qiagen), according to Wu and Minamoto (2023). Finally, 100 µL of sample solution was obtained and stored at −25 °C.

To remove PCR inhibitors in the extracted DNA solution, further purification with the DNeasy Power Clean Pro Cleanup Kit (Qiagen, Germany) was performed as per the manufacturer's protocol. Finally, 100 µL of sample was obtained and stored at −25 °C.

eDNA metabarcoding

To produce the sequencing library, untagged two-step PCR (Bohmann et al. 2022) was performed: in the first PCR, a mix of MiMammal-U primers, MiMammal-B

Table 1. Filtration volume of each sample.

Sampling times	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	FB
1st	315	NA*	NA*	50	250	195	50	250	50	250	30	1000
2nd	530	350	450	310	800	500	450	400	750	460	600	1000
3rd	700	550	400	250	800	800	550	NA*	800	800	300	1000
4th	200	500	100	200	750	500	800	800	400	400	400	1000
5th	800	600	400	500	1000	900	1000	900	800	900	600	1000
6th	NA*	NA*	NA*	50	600	500	100	400	200	800	50	1000
7th		600**	200**									1000

*NA: Filtration was not available due to broken water sampling equipment or insufficient water sampling volume.
** Because enough water was not available for the T2 and T3 samples in the sixth sampling, an additional seventh sampling was performed.

primers, and MiMammal-E primers (column-purified) were used to amplify the mitochondrial 12S rRNA genes in mammalian DNA (median insert length = ~171 bp) (Ushio et al. 2017) (Suppl. material 1: table S4); the MiMammal-U is for mammals in general, and the MiMammal-B and MiMammal-E are for bears and elephants, respectively, which are difficult to amplify with the MiMammal-U.

The reaction mixture for the first PCR contained 6.0 µL of 2× KAPA HiFi HotStart ReadyMix (KAPA Biosystems, USA), each untagged primer at a final concentration of 0.3 µM, and 2.0 µL of template DNA, with the final volume of 12 µL. All PCRs were performed in four replications for each actual sample and field blank. To check for contamination during the PCR process, 2.0 µL of pure water was used as the PCR no-template control and was also performed in four replicates for each PCR plate. The PCR no-template controls were also sequenced. The reaction conditions for the first PCR were as follows: an initial step at 95 °C for 3 min, followed by 40 cycles of denaturation at 98 °C for 20 s, annealing at 65 °C for 15 s, extension at 72 °C for 15 s, and a final extension at 72 °C for 5 min.

After the first PCR, replicate amplicons were pooled and then purified using SPRIselect (Beckman Coulter, USA), and the concentration of purified DNA was measured using a Qubit 3.0 Fluorometer and dsDNA Quantification Assay Kits (Thermo Fisher Scientific, USA). The purified products were then diluted in TE buffer (pH 8.0) to 0.1 ng/µL, respectively. Negative controls, whose concentrations could not be measured due to low concentrations, were diluted using the average dilution factor of the actual samples (The eDNA Society 2019; Minamoto et al. 2021). The diluted samples were then stored at –25 °C.

The second PCRs were performed to add flow-cell binding sequences and library indices (Bohmann et al. 2022) (Suppl. material 1: table S4, Suppl. material 2: Appendix S1). The reaction mixture contained 6.0 µL of 2× KAPA HiFi HotStart ReadyMix (KAPA Biosystems), each tag primer at a final concentration of 0.3 µM, and 1.0 µL of the diluted first PCR product, with the total volume of 12 µL. PCR conditions were an initial 95 °C for 3 min, followed by 12 cycles of 98 °C for 20 s and 72 °C for 20 s, and a final extension at 72 °C for 5 min.

Pooled second PCR amplicons were purified using the E-Gel Agarose Electrophoresis System (Thermo Fisher Scientific, USA) and 2% E-Gel SizeSelect (Thermo Fisher Scientific, USA). The Agilent 2100 BioAnalyzer and Agilent DNA

1000 Kit (Agilent Technologies, USA) were then used to confirm the quality of the library. The concentration of the library was measured using a Qubit 3.0 Fluorometer and dsDNA Quantification Assay Kits, then diluted to 1 nM with molecular-grade water, and the libraries were sequenced using the iSeq100 platform (Illumina, USA) with the 2 × 150 bp paired-end sequencing kit.

Bioinformatics

Bioinformatics processing was performed using USEARCH v10.0.240_i86osx32 (Edgar 2010). The "fastq_mergepairs" command was used to merge paired-end reads. Sequences with 16 bases or fewer of paired-end sequences and those with more than five different bases in the aligned regions were removed. The "fastx_truncate" command was used to remove primer sequences. The "fastq_filter" command was used to check the quality score and expected error rate (Edgar 2010). Sequences with an expected error count greater than one and reads shorter than 140 bp were considered low quality and removed. The "fastx_uniques" command was used to summarize the exact matching sequences, and, following Edgar's (2010) recommendation, all singletons, doubletons, and tripletons were excluded from subsequent analyses. The "unoise3" command was used to remove noise and generate amplicon sequence variants (ASVs). Next, data from the National Center for Biotechnology Information (NCBI: https://www.ncbi.nlm.nih.gov/) were used as reference sequences, and taxonomic assignments to species were made using the Basic Local Alignment Search Tool (BLAST). Sequences with > 99% query coverage and > 98.5% identity to the reference sequence were selected. Sequences with identity ≤ 98.5% were not used for the downstream analysis. Among those lower-identity reads, most occurred at very low counts. One exception was a relatively abundant cluster matching the genus *Capra*, detected in a single sample, but it could not be assigned to a species and was therefore excluded. Non-mammalian species and humans (*Homo sapiens*) were then removed because they are not targets of the study. Because house mouse (*Mus musculus*) reads were consistently detected in most of the samples and negative controls, as well as in multiple unrelated samples processed during the same period, these detections were considered unlikely to represent true biological signals. Therefore, *M. musculus* was excluded from all analyses. To control for contamination, we applied a conservative threshold filter based on negative controls. Specifically, 0.02% was defined as the proportion of total reads observed across all negative controls relative to the total read count of the dataset, and for each sample, any taxon-specific read count at or below the read number implied by this proportion was treated as non-detection.

Statistical analysis

To examine factors affecting mammal eDNA detection, a generalized linear mixed model (GLMM, family = binomial) was used. We fitted two versions of the model: (i) using as the objective variable the detection/non-detection (0/1) in each sample restricted to mammals kept in the zoo that had been detected at least once in our eDNA survey, and (ii) using the same objective variable including all captive mammal species (i.e., detected and undetected species, with undetected treated

as 0). In both versions, the explanatory variables were the distance from each tree to the cage where mammals were kept and filtration volume, with sampling times, mammal species, and tree ID as random effects. Preliminary analyses showed that precipitation was significantly correlated with the filtration volume, and therefore we did not include precipitation as an explanatory variable. To assess robustness to contamination-control settings, we repeated the GLMMs under alternative read-threshold filters (0.001%, 0.01%, 0.02%, and 0.1%) for the model (i). The function "glmer()" in the package lme4 of the statistical software R version 4.4.1 (R Core Team 2024) was used for the analysis. The distances from cages to trees were obtained using QGIS 3.38 based on a map created by the Kobe Oji Zoo (Suppl. material 1: table S5). Only for capybaras (*Hydrochoerus hydrochaeris*), since there are two cages (*a* and *am*) where they are kept, analyses were conducted separately for *a* and *am*, but the results were not affected, and then we show the case of *a*. The function "predict" in R was used to compute the predictions of the model, and the regression curve was shown.

To examine the factors affecting the number of mammalian species detected in each tree, a GLMM (family = Poisson) was used. The objective variable was the number of species detected from each tree, and the explanatory variables were bark type and filtration volume, with sampling times as a random effect. To compare the number of species detected by different bark types, we used data from three pairs of adjacent trees of different species, T1 and T2, T3 and T4, and T5 and T6. Bark types were determined visually and classified into three categories, *sensu* Wojtech (2013): smooth, shallowly fissured, and fissured (Suppl. material 1: table S2). The function "glm" from the R package stats was used for the analysis.

To compare the species composition detected in each tree, two permutational multivariate analyses of variance (PERMANOVA) based on Bray–Curtis index and Jaccard dissimilarities were performed. For the Bray–Curtis index, the total number of reads of the detected captive mammal species was set to 1, and the relative number of reads of each captive mammal species was used to calculate the beta diversity of each sample by the Bray–Curtis index. The presence/absence data were used to calculate Jaccard dissimilarities.

To visually show the differences in population structure among trees, we performed non-metric multidimensional scaling (NMDS) using the function "metaMDS" in the package vegan in R.

Results

eDNA metabarcoding for stemflow samples

The analysis yielded 6,674,861 raw reads in total, with a final count of 1,175,322 reads after filtering (Suppl. material 1: table S6). The raw read data have been deposited to the DDBJ BioProject database under the accession number PRJDB40379 (https://ddbj.nig.ac.jp/search/entry/bioproject/PRJDB40379). A total of 22 mammal species eDNA were detected. In T1, eDNA of red kangaroos (*Osphranter rufus*) and Chapman's zebras (*Equus quagga chapmani*) was detected; in T5, that of rabbits (*Oryctolagus cuniculus*), capybaras, sheep (*Ovis aries*), llamas (*Lama glama*), and goats (*Capra hircus*); in T7, that of crested porcupines (*Hystrix cristata*) was detected; in T9, that of Japanese squirrels

was detected. All species described above kept in the cages located within 50 m of each tree were detected at least once (Suppl. material 1: table S9). The maximum number of reads of species detected was 203,049 for the Japanese squirrels detected from T9 (Suppl. material 1: table S7). In terms of the number of reads of mammalian species other than Japanese squirrels detected from T9, the minimum was 265 reads for horses, and the maximum was 23,518 reads for llamas (Suppl. material 1: table S7). eDNA of Japanese squirrel was detected in all samples of T9 (Suppl. material 1: tables S7, S8). Japanese squirrel DNA was detected in some field blanks and PCR negative controls; the minimum and maximum numbers of reads were 26 and 85, respectively. Llama DNA was also detected in one PCR negative control with a read number of six.

The maximum distance from the cage to the tree where a captive mammal species was detected was 314 m for Japanese squirrels (Suppl. material 1: table S9). Twelve of the 14 captive mammal species detected had detections in the tree closest to the cage (Suppl. material 1: tables S5, S9). There were no detections of species kept in enclosed spaces separated by walls or glass. The minimum filtration volume was 30 mL among the samples in which mammalian eDNA was detected (Table 1).

Factors affecting mammalian eDNA detection and composition

The GLMMs showed a significant negative relationship between DNA detection and the distance from each mammal cage to each tree ($p < 0.05$) based on the analysis using the 0.02% contamination-control threshold (Suppl. material 1: table S10, Fig. 2). The same pattern was observed both when restricting the response to species detected at least once and when including all captive species. Sensitivity analyses, conducted only for the analysis restricted to species detected at least once, examined alternative contamination-control thresholds (0.001%, 0.01%, 0.02%, and 0.1%) and similarly revealed significant negative relationships (all $p < 0.05$; Suppl. material 1: table S11). The GLMM conducted to examine factors affecting the number of mammalian species detected from each tree showed no significant difference among bark categories ($p > 0.05$) (Suppl. material 1: table S12, fig. S2). PERMDISP analysis based on both Bray–Curtis index and Jaccard dissimilarities showed significant differences in within-group dispersions among trees ($p < 0.05$ for both) (Suppl. material 1: table S13). Differences in community structure among samples were visualized using NMDS (Fig. 3: stress values = 0.02 for Jaccard index and 0.02 for Bray–Curtis index).

Discussion

Detectability of mammalian eDNA via stemflow

In this study, we showed that mammalian eDNA is detectable from stemflow water. This indicates that this method has the potential to contribute to understanding the mammalian fauna in the field. Indeed, the eDNA of mammalian species was detected in 10 target trees (Fig. 1). This method is simpler than methods involving felling branches or scrubbing the tree surface with a roller (Allen et al. 2023; van Beeck Calkoen et al. 2019), and it offers a level

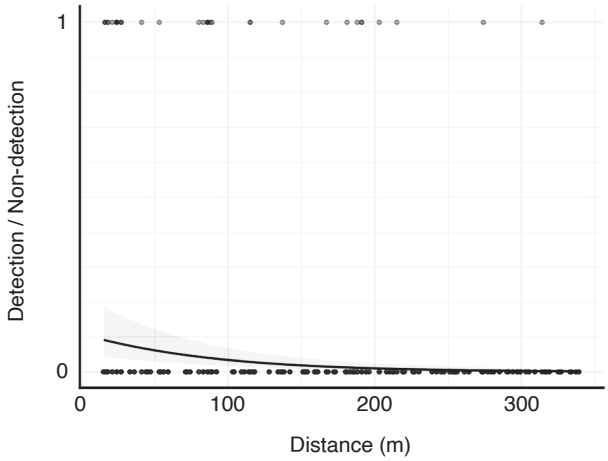

Figure 2. Relationship between eDNA detection or non-detection of captive mammals and the distance to trees. Regression curves were drawn by plotting the detection or non-detection (1/0) from 11 trees for 14 captive mammal species that had been detected at least once. The curve was drawn using the standard function "predict()" in R. Results from T9, where Japanese squirrels directly contact the tree, were excluded.

of practicality similar to recently developed rainwash eDNA methods in which rainwater is collected beneath the canopy (Macher et al. 2023), because sampling can be done simply by leaving a water sampler attached to the tree.

Spatial patterns of eDNA detection

Not only eDNA of species in direct contact with trees (Japanese squirrels), but also eDNA of species that do not have physical contact with trees (all other captive species) was detected in the stemflow (Suppl. material 1: table S7, Fig. 1). Japanese squirrel eDNA was detected on all six sampling occasions from T9, a tree that can be physically contacted by the species. Thus, it is likely that mammalian contact with trees facilitates the attachment of animal residues, from which eDNA is derived, to the trees, making eDNA more likely to be detected. These results indicate, first, that this method is particularly effective for monitoring species that can directly contact trees, such as arboreal mammals. On the other hand, mammalian eDNA was also detected in trees located outside the cages where the mammals were kept (Suppl. material 1: table S7, Fig. 1). Previous studies have shown that mammalian eDNA can also be detected in the air (Clare et al. 2022; Lynggaard et al. 2022), and it is likely that eDNA, or the residue from which it is derived, reaches the trees via air. The lack of detection of eDNA in species kept in closed enclosures also supports that eDNA reaches the trees via the air. In addition, eDNA of mammalian species not kept in zoos were also detected (Suppl. material 1: table S7). As discussed below, the eDNA of those species may reach the trees through the air, but there is also the possibility of transmission through people who touch their pets or through the entry of wild animals.

Among the captive mammal species from which eDNA was detected (14 species in total; Suppl. material 1: table S1), 12 species were detected at the tree closest to each cage at least once, suggesting that the method is likely to detect eDNA of species particularly close to trees (Suppl. material 1:

tables S5, S9). This was statistically supported by the significant negative relationship between distance and detection results (Suppl. material 1: table S10; Fig. 2). In two previous studies that detected mammalian eDNA using air samples in zoos, the conclusion regarding the animal–sampler location relationship is controversial. Clare et al. (2022) found that the relationship between the number of mammal reads detected and distance showed no significant association, whereas Lynggaard et al. (2022) showed that the closer the animal and sampler, the higher the detection rate. In this study, the detection rate was also shown to increase with trees closer to the animal, and it may be possible to detect mammals in proximity to the tree, but further validation is needed to draw more generalized conclusions.

As for the results of the large difference in species composition between T9 and the other trees, the possibility of direct contact may have influenced this pattern (Suppl. material 1: table S13; Fig. 3); T9 is the only tree with target animals having direct contact. On the other hand, the species composition of eDNA detected was also different in the combinations of T1 and T5, and T1 and T7 (Suppl. material 1: tables S8, S12; Fig. 3). eDNA of species kept within 50 m of trees, such as red kangaroo and Chapman's zebra (*Equus quagga chapmani*), was detected in T1 (Suppl. material 1: table S10). In T5, eDNA of species kept in enclosures adjacent to trees, including rabbits, capybaras, sheep, llamas, and goats, was detected. The results of each tree probably reflected the surrounding species. T8 had no cages where mammals were kept within 100 m of its perimeter, and the number of species detected was especially low; therefore, its species composition may have differed from that of T7, which had a higher number of species detected. However, these comparisons should be interpreted with caution, given the limited sample size, and future studies with a larger sampling effort will be necessary to evaluate these patterns more rigorously. In addition, taking the results shown by PERMDISP analysis into account, the observed PERMANOVA differences might be partly attributable to heterogeneity in group dispersions rather than solely to shifts in species composition.

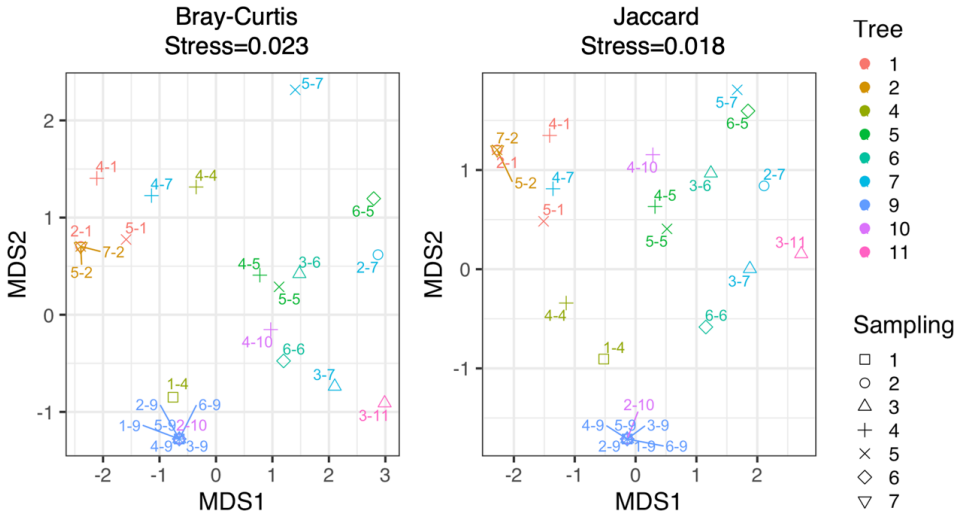

Figure 3. Species composition similarity for each tree shown by NMDS. Using the Bray–Curtis index (left) and Jaccard dissimilarity (right) among samples in which a captive mammal species was detected. X–Y on each plot denotes sampling time–tree number. Samples from Tree 9, shown in blue, form a distinct cluster, indicating a high degree of similarity in species composition.

In addition to spatial variation, we also examined whether temporal replicability could be assessed within individual trees. However, temporal comparisons were difficult because many stemflow samples yielded no mammal eDNA, providing insufficient information for evaluating within-tree replicability. Consequently, no clear temporal pattern was identified, and temporal consistency within trees should be interpreted with caution.

Detection of non-zoo species

DNA of eight non-zoo species (Siberian weasel [*Mustela sibirica*], raccoon [*Procyon lotor*], masked palm civet [*Paguma larvata*], Japanese marten [*Martes melampus*], sika deer [*Cervus nippon*], dog [*Canis lupus familiaris*], domestic cat [*Felis catus*], and domestic cattle [*Bos taurus*]) were also detected (Suppl. material 1: table S7). Among these, the Siberian weasel, raccoon, masked palm civet, Japanese marten, and domestic cat inhabit the environment surrounding the zoo and are likely to enter the zoo grounds (Kobe City 2020). The eDNA of domestic cattle may originate from food items brought in by zoo visitors. In addition, because many people keep dogs, dog DNA may have been introduced into the zoo via their owners. The interpretation of the detection of sika deer is difficult. Although the species is unlikely to be common around the zoo, a small number of individuals may occur in the surrounding area, and the zoo also kept this species until 2018. Therefore, either of these factors may explain the presence of its eDNA. Overall, these observations indicate that the detection of non-zoo species can be plausibly explained by the surrounding fauna, human-mediated transport, and past husbandry records.

Effect of tree morphological traits on eDNA recovery

In our analysis, the number of detected species did not differ among bark categories. Although trees with fissured bark (*Cinnamomum camphora* and *Quercus serrata*) tended to yield fewer detections, this trend was not statistically supported and should be interpreted with caution. In addition, canopy structure may also influence stemflow because the density and arrangement of branches and foliage determine how much rainfall is intercepted before reaching the stem. As a result, variation in canopy coverage could affect the stemflow volume available for eDNA collection, and incorporating canopy metrics into future studies will be important for a better understanding of tree-level effects.

Effect of filtration volume on detection sensitivity

Mammalian eDNA was more likely to be detected as filtration volume increased with this method (Suppl. material 1: tables S10, S11). With respect to filtration volume, a study of eDNA metabarcoding in riverine fish showed that the number of species detected increased significantly as filtration volume increased (Sakata et al. 2021). This may be because, as the volume of water handled increases, the likelihood of containing eDNA of the target species increases. To improve the detection rate in this method, it is important to ensure sufficient filtration volume, and it is necessary to take measures such as sampling during the rainy season.

Considering the minimum filtration volume at which mammalian eDNA was detected, mammals may be detectable with as little as 30 mL of stemflow. However, even within the same tree, there were instances where mammalian eDNA was detected at lower filtration volumes but not at higher ones. This inconsistency indicates that detection probability is not determined by filtration volume alone. Therefore, although approximately 30 mL appears to be a practical lower bound, it remains unclear how much volume is sufficient to ensure reliable detection. Identifying an appropriate and robust filtration volume is thus an important topic for future research.

Comparison with other eDNA sampling methods

Comparisons will be made regarding this method with reported eDNA methods for mammals. First, regarding which species eDNA is likely to be detected by the substrates used in each method: aquatic and semi-aquatic mammals from water sources (Ushio et al. 2017), semi-fossil small mammals from soil sources (Tetzlaff et al. 2024), arboreal mammals from trees (Allen et al. 2023), and various mammalian species from air eDNA (Lynggaard et al. 2024). This study showed that the stemflow method had a high detectability for arboreal mammals that directly contact trees. Compared to other tree-based methods, such as tree rolling and spray aggregation methods (Allen et al. 2023; van Beeck Calkoen et al. 2019; Valentin et al. 2020), this method, as well as the rainwash method (Macher et al. 2023), has the advantage of being low labor-intensive. Furthermore, compared to methods using water from rivers or ponds, which depend on the water source of the sampling site, this method can sample in any environment where trees and precipitation are present, which may make this method more effective in many cases. However, these eDNA methods for mammals have their own advantages and disadvantages, as their efficiency and detection rates tend to vary depending on the target species and environmental factors. Therefore, it is expected that, by complementing each other's weaknesses with the strengths of each method, they will be established as tools that enable a comprehensive and accurate understanding of the mammalian fauna.

Limitations and future perspectives

Finally, some limitations regarding eDNA studies of mammals using stemflow should be discussed. First, because this method incorporates the uncertainty of rainfall, it is important to note that the timing of water sampling, the amount of water sampled, and the amount of filtration will vary depending on rainfall. For example, in areas or seasons where rainfall is infrequent, a pseudo-rainfall survey may be possible if it is possible to spray water from above trees.

Second, the detection of mammals in this method is determined by whether mammals come into contact with trees and whether mammal-derived eDNA eventually reaches the trees via the air, in which there can be stochastic fluctuations. Increasing the number of trees sampled, for example, would improve the stability of the results.

Third, this study used trees located at a maximum distance of 347 m from each animal's cage, and the detectable distance limit is uncertain. The detection of a Japanese squirrel from a tree 314 m from the cage and the overall

similarity of species composition detected from trees other than T9, are examples of the need to clarify the detectable distance and the species range that the detection results reflect. This is a pioneering study, and it is necessary to validate this method in a wider range of survey environments. For example, the use of trees located outside the zoo would provide an indication of the maximum reachable distance.

Conclusion

We showed that eDNA metabarcoding of stemflow water is useful for detecting mammalian eDNA. This method is highly effective, especially for mammals in contact with trees; however, even for mammals that do not directly contact trees, an increased detection rate was observed when the distance between their location and the tree was close. Thus, our pioneering data suggest that the method can reflect the species composition around the tree surveyed and contribute to improved arboreal monitoring of mammals. This study is a limited example of how the distance between mammals and eDNA sampling points affects mammal detection, which will be an important finding in the development of mammal monitoring techniques via eDNA.

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Additional information

Conflict of interest

TM is an inventor of the patent for the use of BAC for eDNA preservation.

Ethical statement

No ethical statement was reported.

Use of AI

The following AI tools were used in the preparation of this manuscript:
Description: The authors utilized Microsoft Copilot for the translation of the manuscript from Japanese to English and for proofreading the English version. All content was reviewed and verified by the authors to ensure accuracy and appropriateness.

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Author contributions

Conceptualization: YI, KA, MKS, and TM; Methodology: YI, AS, MM, and TM; Formal analysis: YI, MKS, and QW; Investigation: YI, KA, KH; Writing—original draft: YI and TM; Writing—review and editing: all authors; Visualization: YI; Supervision: TM; Project administration: YI and TM; Funding acquisition: TM and MKS.

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Data availability

The raw data from the metabarcoding analysis have been deposited in the BioProject database of the DNA Data Bank of Japan under accession number PRJDB40379 (https://ddbj.nig.ac.jp/search/entry/bioproject/PRJDB40379). The metadata, documented according to the FAIRe checklist, are provided as Suppl. material 2: Appendix S1.

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Supplementary material 1

Additional figures and tables

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Data type: pdf

Explanation note: **table S1.** Mammalian species kept in Oji Zoo, Kobe (as of 5 April 2024). **table S2.** Sampled trees. **table S3.** Rainfall information. **table S4.** Primers used in this study. **table S5.** Distance in meters from the cage where each mammal is kept to each tree. **table S6.** Numbers of reads in each processing stage. **table S7.** Results of metabarcoding. **table S8.** Count of detections per tree and species. **table S9.** Detected captive mammal species and distance to trees. **table S10.** Results of GLMM analysis between sampling conditions and detection/non-detection of captive mammals. **table S11.** Results of sensitivity analysis on GLMMs between sampling conditions and detection/non-detection of captive mammals. **table S12.** Results of GLMM analysis between number of species detected and sampling conditions for captive mammals. **table S13.** Results of permutation test for homogeneity of multivariate dispersions. **fig. S1.** Map of Oji Zoo, Kobe City (as of 5 April 2024). **fig. S2.** Number of captive mammal species detected from each tree in adjacent sets of trees.

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Supplementary material 2

Appendix S1

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