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

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20 **Abstract**

21 As mammalian diversity declines, effective biodiversity monitoring tools are urgently
22 needed. To address this issue, we assessed the detectability of mammalian environmental
23 DNA (eDNA) using the eDNA metabarcoding method with stemflow. We collected
24 stemflow samples six times from June 2024 to January 2025 on 11 trees in the Oji Zoo,
25 Kobe, Japan. One tree was inside the cage, which could be directly contacted by captive
26 mammals whereas the others were outside the cages, which could not be directly
27 contacted by captive mammals. Then, we amplified eDNA extracted from the stemflow
28 samples with MiMammal primers targeting the mitochondrial 12S rRNA gene and the
29 PCR products were then sequenced. We detected 25 mammal species eDNA, 16 of which
30 were zoo-kept species and 9 of which were not. Environmental DNA of a captive mammal
31 species which could directly contact the target tree was detected all the six sampling times.
32 According to statistical analysis, there was a significant negative relationship between
33 DNA detection and the distance from each mammal cage to each tree, as well as
34 significant differences in the composition of the detected species between the trees inside
35 and outside the cages. Moreover, the results suggested that significantly fewer species
36 were detected in stemflow collected from trees with rough bark. We hereby show that
37 eDNA analysis using stemflow is useful for detecting mammalian eDNA, indicating that
38 this method has the potential to contribute to understanding the mammalian fauna in the
39 field.

40 **Keywords**

41 biodiversity conservation, biomonitoring, environmental DNA, metabarcoding, mammal,
42 stemflow, zoo

43 **Introduction**

44 Mammals are widely recognized as flagship species for biodiversity conservation because
45 of their taxonomic diversity and extensive ecological roles (Ceballos & Ehrlich 2002;
46 Ceballos et al. 2005). However, as approximately one-quarter of mammalian species are
47 currently threatened with extinction (IUCN 2025), urgent measures are required to
48 address this issue. Thus, effective monitoring to assess the presence and distribution of
49 mammals is essential, highlighting the need for the development of comprehensive and
50 accurate monitoring techniques.

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

65 Standardizing eDNA-based mammalian monitoring remains challenging due to
66 variability in detection probability and substrate-specific limitations. The spatial and

67 temporal detectability of eDNA is influenced by the uneven distribution of sources such
68 as feces and the stochastic nature of animal-substrate contact. Each sampling method
69 presents unique challenges: soil eDNA may reflect past rather than current presence
70 (Leepoel et al. 2020; Guthrie et al. 2024), and air eDNA is affected by wind, making
71 habitat attribution difficult (Bohmann & Lynggaard 2023; Lynggaard et al. 2024).
72 Aquatic eDNA survey may miss species observed via conventional camera traps and are
73 limited by water availability (Harper et al. 2019; Sales et al. 2020; Mena et al. 2021).
74 Additionally, fly sampling can be biased by insect preferences (Calvignac-Spencer et al.
75 2013). On trees, alternative approaches such as the roller method, rubbing tree surfaces
76 to collect eDNA, have shown promise for detecting arboreal mammals (Allen et al.
77 2023). Thus, while various methods have successfully detected mammalian eDNA, each
78 has inherent constraints and challenges.

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

88 The aim of this study was to assess the detectability of mammalian eDNA
89 using the eDNA metabarcoding method with stemflow. To test this, the study was
90 conducted in a zoo where the number of mammal species and individuals, and locations

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

97

98 **Materials and Methods**

99 **Sampling**

100 Stemflow sampling was performed at the Oji Zoo, Kobe, Japan where 263 mammal
101 individuals of 51 species are kept (Table S1, Figure S1). A total of six sampling sessions
102 was conducted from June 2024 to January 2025 on 11 trees in the zoo, with additional
103 sampling on two trees because water could not be recovered from some samples (Figure
104 1; Table S2 and S3). As a result, the total number of samples was 62. Of the 11 trees, one
105 tree (T9) was inside the cage where Japanese squirrels (*Sciurus lis*) are kept and can be
106 directly contacted by the captive animals, and the others were outside the cages and
107 cannot be directly contacted by captive animals (Table S2). To examine differences in
108 results due to the shape of the trees, T1 and T2, T3 and T4, and T5 and T6 were chosen
109 to be adjacent trees of different tree species (Table S2).

110

111 **Collecting stemflow**

112 Stemflow samplers were made and installed according to Sakata et al. (2023) with slight
113 modification regarding the height. The height at which the rubber rope and gauze were

114 wrapped was set at 80 cm from the ground. Both ends of the rubber rope were fixed
115 through a funnel made of silicon material, and the gauze was also placed in the funnel.
116 Next, both ends of the rubber rope were inserted into a hose cut to approximately 10 cm
117 in length. The hose was then connected to a backflow prevention unit attached to the
118 water collection bag. After the rainfall, the water collection bag was removed from the
119 sampler, capped, and brought back to the laboratory. One water sampling bag containing
120 1 L distilled water was prepared for each sampling time as a field blank and placed near
121 one of the sampling trees. For both samples and field blanks, 500 μ L of 10%
122 benzalkonium chloride solution (Nippon Pharmaceutical Co., Ltd.) was added beforehand
123 to inhibit DNA degradation (Yamanaka et al. 2017). Field blanks were analyzed as other
124 actual samples from DNA extraction to next-generation sequencings. Rainfall data was
125 obtained from the Japan Meteorological Agency (Table S3) (Ministry of Land,
126 Infrastructure, Transport and Tourism, Japan Meteorological Agency “Historical Weather
127 Data Search” <https://www.data.jma.go.jp/obd/stats/etrn/index.php>, data at the nearest
128 point [Kobe observatory] was checked on February 16, 2025).

129

130 Filtration, DNA extraction, and removal of inhibitors

131 On the same day that the water collection bags were collected, gravity filtration was
132 performed according to the method of Oka et al. (2022). Sterivex (Merck, USA) with a
133 pore size of 0.45 μ m was connected to the water sampling bag, a hose cut to 1.5 m was
134 hung below it. From a height of approximately 2 m, the 1.5 m hose was dropped and left
135 for 1 to 4 nights. After filtration was completed, the Sterivex was collected and unfiltered
136 water was discarded (Table 1). After filtration, 1 mL of Buffer ATL was added to the
137 Sterivex, sealed top and bottom with a cap, and stored at -25°C.

138 DNA extraction from Sterivex was performed using DNeasy Blood and Tissue Kit
139 (Qiagen), according to Wu & Minamoto (2023). Finally, 100 μ L of sample solution was
140 obtained and stored at -25°C .

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

144

145 eDNA metabarcoding

146 To produce the library, a two-round PCR was performed: in the 1st PCR, a mix of
147 MiMammal-U primers, MiMammal-B primers, and MiMammal-E primers were used to
148 amplify the mitochondrial 12S rRNA genes in mammalian DNA (median insert length =
149 $\sim 171\text{bp}$) (Ushio et al. 2017) (Table S4); the MiMammal-U is for mammals in general, and
150 the MiMammal-B and MiMammal-E are for bears and elephants, respectively, which are
151 difficult to amplify with the MiMammal-U.

152 The reaction mixture for the 1st PCR contained 6.0 μ L of $2\times$ KAPA HiFi HotStart
153 ReadyMix (KAPA Biosystems, USA), each primer at a final concentration of 0.3 μM , and
154 2.0 μ L of template DNA with the final volume of 12 μ L. All PCRs were performed in
155 four replications. To check for contamination during the PCR process, 2.0 μ L of pure
156 water was used as the PCR non-template controls and was also performed in four
157 replicates. The PCR no-template controls were analyzed with the next generation
158 sequencing. The reaction conditions for the 1st PCR were as follows: initial step at 95°C
159 for 3 min, followed by 40 cycles of denaturation at 98°C for 20 s, annealing at 65°C for
160 15 s, extension at 72°C for 15 s, and final extension at 72°C for 5 min.

161 After 1st PCR, amplicons were purified using SPRIselect (Beckman Coulter, USA)

162 and the concentration of purified DNA was measured using Qubit 3.0 Fluorometer and
163 dsDNA Quantification Assay Kits (Thermo Fisher Scientific, USA). The purified products
164 were then diluted in TE buffer (pH 8.0) to 0.1 ng/ μ L, respectively. Negative controls,
165 whose concentrations could not be measured due to low concentrations, were diluted
166 using the average dilution factor of the actual samples (The eDNA Society 2019;
167 Minamoto et al. 2021). The diluted samples were then stored at -25°C.

168 The 2nd PCRs were performed to add tag primers to identify each sample (Table S4).
169 The reaction mixture contained 6.0 μ L of 2 \times KAPA HiFi HotStart ReadyMix (KAPA
170 Biosystems), each tag primer at a final concentration of 0.3 μ M, and 1.0 μ L of the diluted
171 1st PCR product with the total volume of 12 μ L. PCR conditions were initial 95°C for 3
172 min, followed by 12 cycles of 98°C for 20 s and 72°C for 20 s, and the final extension of
173 72°C for 5 min.

174 Pooled 2nd PCR amplicons were purified using the E-Gel Agarose Electrophoresis
175 System (Thermo Fisher Scientific, USA) and 2% E-Gel SizeSelect (Thermo Fisher
176 Scientific, USA). The Agilent 2100 BioAnalyzer and Agilent DNA 1000 Kit (Agilent
177 Technologies, USA) were then used to confirm that the quality of the library. The
178 concentration of the library was measured using a Qubit 3.0 Fluorometer and dsDNA
179 Quantification Assay Kits, then diluted to 1 nM with pure water, and the libraries were
180 sequenced using iSeq100 plat form (Illumina, USA) with 2 \times 150 bp paired-end
181 sequencing.

182

183 Bioinformatics

184 Bioinformatics processing was performed using USEARCH v10.0.240_i86osx32 (Edgar,
185 2010). The “fastq_mergepairs” command was used to merge paired-end reads. Sequences

186 with the 16 bases or less of paired-end sequences and those with more than 5 different
187 bases in the aligned regions were removed. The “fastx_truncate” command was used to
188 remove primer sequences. The “fastq_filter” command was used to check the quality
189 score and expected error rate (Edgar, 2010). Sequences with an expected error count
190 greater than 1 and reads shorter than 140 bp were considered low quality reads and
191 removed. The “fastx_uniques” command was used to summarize the exact matching
192 sequences and, following Edgar's (2010) recommendation, all singletons, doubletons, and
193 tripletons were excluded from subsequent analyses. The “unoise3” command was used to
194 remove noise and generate amplicon sequence variants (ASVs). Next, data from the
195 National Center for Biotechnology Information (NCBI: <https://www.ncbi.nlm.nih.gov/>)
196 were used as reference sequences and taxonomic assignments to species were made using
197 the Basic Local Alignment Search Tool (BLAST). Sequences with >99% query coverage
198 and >98.5% concordance with the reference sequence were selected. Non-mammalian
199 species were then removed. Human (*Homo sapiens*) and house mouse (*Mus musculus*)
200 were also removed. The number of ASV reads detected in field blanks and PCR negative
201 controls was subtracted from the number of reads in the corresponding samples (Allen et
202 al. 2023), and set to zero if the number of reads was less than zero.

203

204 Statistical analysis

205 Of the mammals detected by eDNA metabarcoding, species not kept in the zoo were
206 excluded from the following analyses. To examine factors affecting mammal eDNA
207 detection, a generalized linear mixed model (GLMM, family=binomial) was used. The
208 objective variable was the detection/non-detection (0/1) in each sample of mammals kept
209 in the zoo that had been detected at least once in our eDNA survey, and the explanatory

210 variables were the distance from each tree to the cage where mammals were kept and
211 filtration volume, with sampling times, mammal species and tree ID as random effects.
212 The function “glmer()” in the package lme4 of the statistical software R version 4.4.1 (R
213 Core Team, 2024) was used for the analysis. The distances from cages to trees were
214 obtained using QGIS 3.38 based on a map created by the Kobe Oji Zoo (Table S5). Only
215 for capybaras (*Hydrochoerus hydrochaeris*), since there are two cages (*a* and *am*) where
216 they are kept, analyses were conducted separately for *a* and *am*, but the results were not
217 affected, and then we show the case of *a*. The function “predict” in R was used to compute
218 the predictions of the model, and the regression curve was shown.

219 To examine the factors affecting the number of mammalian species detected in each
220 tree, a GLMM (family = poisson) was used. The objective variable was the number of
221 species detected from each tree, and the explanatory variables were bark type and
222 filtration volume, with sampling times as a random effect. To compare the number of
223 species detected by different bark types, we used data from three pairs of adjacent trees
224 of different species, T1 and T2, T3 and T4, and T5 and T6. Bark types were determined
225 visually and classified into three categories: smooth, slightly rough, and rough (Table S2).
226 The function “glm” from the R package stats was used for the analysis.

227 To compare the species composition detected in each tree, a pairwise permutational
228 multivariate analysis of variance (PERMANOVA) was performed. First, the total number
229 of reads of the detected rearing mammal species was set to 1, and the relative number of
230 reads of each rearing mammal species was calculated. Next, the relative number of reads
231 was used to calculate the beta diversity of each sample by the Bray-Curtis index. A
232 pairwise PERMANOVA was then conducted with the β -diversity obtained by the Bray-
233 Curtis index as the objective variable and each tree as the explanatory variable. Due to

234 the small number of permutations in the dataset used alone, a Monte Carlo random
235 sampling procedure was used (permutation: 999). For the analysis, the function “vegdist”
236 from the R package *vegan* and the function “pairwise.adonis” from the package
237 *pairwiseAdonis* were used. Then, to evaluate whether the assumption of homogeneity of
238 multivariate dispersion was met, we assessed the within-group variation using the
239 function "betadisper" from the R package *vegan* (permutations: 999).

240 To visually show the differences in population structure among trees, we performed
241 non metric multidimensional scaling (NMDS) using the function “metaMDS” in the
242 package *vegan* in R.

243

244 Results

245 eDNA metabarcoding for stemflow samples

246 The analysis yielded 6,590,492 raw reads in total, with a final count of 1,176,633 reads
247 after filtering (Table S6). A total of 25 mammal species eDNA were detected. In T1,
248 eDNA of red kangaroo, Chapman's zebra, and hippopotamus was detected; in T5, that of
249 rabbits (*Oryctolagus cuniculus*), horses (*Equus caballus*), capybaras, sheep (*Ovis aries*),
250 llamas (*Lama glama*), and goats (*Capra hircus*); in T7, that of crested porcupine; in T9,
251 that of Japanese squirrel all species kept in the enclosures located within 50 m from the
252 each tree where they were detected (Table S9). The maximum number of reads of species
253 detected was 203,020 for the Japanese squirrel detected from T9 (Table S7). In terms of
254 the number of reads of mammalian species other than Japanese squirrel detected from T9,
255 the minimum was 4 and the maximum 23,518 reads for the Llama (*Lama glama*) (Table
256 S7). eDNA of Japanese squirrel was detected in all samples of T9 (Table S7, Table S8).

257 Japanese squirrel DNA was detected in some field blanks and PCR negative controls; the
258 minimum and maximum numbers of reads were 26 and 85, respectively. Llamas DNA
259 was also detected in one PCR negative control with a read number of 6.

260 The maximum distance from the cage to the tree where a captive mammal species
261 was detected was 314 m for Japanese squirrels (Table S9). Thirteen of the 16 captive
262 mammal species detected had detections in the tree closest to the cage (Table S5, Table
263 S9). There were no detections of species kept in enclosed spaces separated by walls or
264 glass.

265

266 Factors affecting mammalian eDNA detection and composition

267 The GLMMs showed a significant negative relationship between DNA detection and the
268 distance from each mammal cage to each tree ($p < 0.05$) (Table S10, Figure 2). The
269 GLMMs conducted to examine factors affecting the number of mammalian species
270 detected from each tree showed significantly fewer species detected from trees with rough
271 bark ($p < 0.05$) (Table S11, Figure S2). The species composition detected in each tree was
272 compared using pairwise PERMANOVA, and significant differences were found in all
273 pairs including T9, as well as in the pairs T1 and T5 and T7 and T8 (all $p < 0.05$) (Table
274 S12). PERMDISP analysis showed significant differences in within-group dispersions
275 among trees ($p < 0.05$) (Table S13). Differences in community structure per sample were
276 visualized by NMDS (stress value =0.125) (Figure 3).

277

278 Discussion

279 Detectability of mammalian eDNA via stemflow

280 In this study, we showed that mammalian eDNA is detectable using stemflow. This
281 indicates that this method has the potential to contribute to understanding the mammalian
282 fauna in the field. Indeed, the eDNA of mammalian species was detected in all 11 target
283 trees (Figure 1). This method is simpler than the methods with felling branches or
284 scrubbing the tree surface with a roller (Allen et al. 2023; van Beeck Calkoen et al. 2019)
285 because sampling can be done simply by leaving a water sampler attached to the tree.

286

287 Spatial patterns of eDNA detection

288 Not only eDNA of species in direct contact with trees (Japanese squirrels), but also eDNA
289 of species that do not have physical contact with trees (all other rearing species) was
290 detected in the stemflow (Table S7, Figure 1). Japanese squirrel eDNA was detected on
291 all six sampling occasions from T9, a tree that can be contacted by the specie. Thus, it is
292 likely that mammalian contact with trees facilitates the attachment of animal residues,
293 from which eDNA is derived, to the trees, making eDNA more likely to be detected. These
294 results indicate, first, that this method is particularly effective for monitoring species that
295 can directly contact trees, such as arboreal mammals. On the other hand, mammalian
296 eDNA was also detected in trees located outside the cages where the mammals were kept
297 (Table S7, Figure 1). Previous studies have shown that mammalian eDNA can also be
298 detected in the air (Clare et al. 2022; Lynggaard et al. 2022), and it is likely that eDNA,
299 or the residue from which it is derived, reaches the trees via air. The lack of detection of
300 eDNA in species kept in closed enclosures also supports that eDNA reaches the trees via
301 the air. In addition, eDNA of mammalian species not kept in zoos were also detected
302 (Table S7). These are all wild animal species that have been confirmed to inhabit Kobe
303 City (Kobe City, 2020), are kept as pets, or are processed and distributed as livestock

304 products. eDNA from these species may have reached the trees via the air or humans, or
305 wild individuals may have entered the zoo. The eDNA of those species should reach the
306 trees through the air, but there is also the possibility of transmission through people who
307 touch their pets or through wildlife entrance.

308 Thirteen of the 16 rearing mammal eDNA were detected at least once at the tree
309 closest to each cage, suggesting that the method is likely to detect eDNA of species
310 particularly close to trees (Table S5, Table S9). This was statistically supported by the
311 significant negative relationship between distance and detection results (Table S10,
312 Figure 2). In two previous studies that detected mammalian eDNA using air samples in
313 zoos, the conclusion of the animal-sampler location relationship is controversial. Clare et
314 al. (2022) found that between the number of mammal reads detected and the distance
315 showed no significant relationship, and Lynggaard et al. (2022) showed that the closer
316 the animal and sampler, the higher the detection rate. In this study, the detection rate was
317 also shown to increase with trees closer to the animal, and it may be possible to detect
318 mammals in proximity from the tree, but further validation is needed to draw more
319 generalized conclusions.

320 As for the results of the large difference in species composition between T9 and the
321 other trees, the possibility of direct contact may have influenced (Table S12, Figure 3);
322 T9 is an only tree with target animal have direct contact. On the other hand, the species
323 composition of eDNA detected was also different in the combinations of T1 and T5 and
324 T7 and T8 (Table S8, Table S12, Figure 3). eDNA of species kept within 50 m of trees
325 such as red kangaroo (*Osphranter rufus*), Chapman's zebra (*Equus quagga chapmani*),
326 and hippopotamus (*Hippopotamus amphibius*) were detected in T1 (Table 10). In T5,
327 eDNA of species kept in enclosures adjacent to trees, including rabbits (*Oryctolagus*

328 *cuniculus*), horses (*Equus caballus*), capybaras, sheep (*Ovis aries*), llamas, and goats
329 (*Capra hircus*), were detected. The results of each tree probably reflected the surrounding
330 species. T8 had no cages where mammals were kept within 100 m of its perimeter, and
331 the number of species detected was especially low, therefore its species composition may
332 have differed from that of T7, which had a higher number of species detected. These
333 results also suggest that eDNA of species close to trees is more likely to be detected.
334 However, taking the results showed by PERMDISP analysis into account, the observed
335 PERMANOVA differences might be partly attributable to heterogeneity in group
336 dispersions rather than solely to shifts in species composition.

337

338 Effect of bark roughness on eDNA recovery

339 The number of species detected differed significantly depending on the bark type of the
340 trees used, suggesting that the eDNA detectability by stemflow was affected by bark types.
341 Of the three bark type categories, the number of species detected was significantly lower
342 in trees with rough bark (*Cinnamomum camphora* and *Quercus serrata*), suggesting that
343 the roughness of the bark may have inhibited eDNA collection. The fact that the number
344 of species detected was lower than in the paired trees even when a sufficient amount of
345 stemflow could be filtered suggests that this rough bark may have caused eDNA to be
346 trapped in the bark. Therefore, it may be advisable to avoid trees with rough bark when
347 selecting target trees for this method. However, the number of tree species used in this
348 study was limited, and a detailed examination of whether the detection pattern differs
349 among tree species is needed in the future study.

350

351 Effect of filtration volume on detection sensitivity

352 The mammalian eDNA was more likely to be detected as filtration volume increases with
353 this method (Table S10, Table S11). With respect to filtration volume, a study of eDNA
354 metabarcoding in riverine fish showed that the number of species detected increased
355 significantly as filtration volume increased (Sakata et al. 2021). This may be because as
356 the volume of water handled increases, the expectation of containing eDNA of the target
357 species increases. To improve the detection rate in this method, it is important to ensure
358 sufficient filtration volume, and it is necessary to take measures such as sampling during
359 the rainy season.

360

361 Comparison with other eDNA sampling methods

362 Comparisons will be made regarding this method with reported eDNA methods for
363 mammals. First, regarding which species eDNA is likely to be detected by the substrates
364 used in each method: aquatic and semi-aquatic mammals from water sources (Ushio et al.
365 2017), semi-fossil small mammals from soil sources (Tetzlaff et al. 2024), arboreal
366 mammals from trees (Allen et al. 2023), and various mammalian species from air eDNA
367 (Lynggaard et al. 2024). This study showed that stemflow method had a high detectability
368 for arboreal mammals which directly contact with trees. Compared to other tree-based
369 methods, this method has the advantage of being low labor-intensive. Furthermore,
370 compared to methods using water from rivers or ponds, which depend on the water source
371 of the sampling site, this method can sample in any environment where trees and
372 precipitation are present, which may make this method more effective in many cases.
373 However, these eDNA methods for mammals have their own advantages and
374 disadvantages, as their efficiency and detection rates tend to vary depending on the target
375 species and environmental factors. Therefore, it is expected that by complementing each

376 other's weaknesses with the strengths of each method, they will be established as tools
377 that enable comprehensive and accurate understanding of the mammalian fauna.

378

379 Limitations and future perspectives

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

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

390 Third, this study used trees located at a maximum distance of 347 m from each animal's
391 cage, and the detectable distance limit is not certain. The detection of a Japanese squirrel
392 from a tree 314 m from the cage and a red kangaroo (*Osphranter rufus*) from a tree 309
393 m from the cage, and the overall similarity of species composition detected from trees
394 other than T9, are examples of the need to clarify the detectable distance and the species
395 range that the detection results reflect. It is necessary to validate this method in a wider
396 range of survey environments. For example, the use of trees located outside the zoo would
397 provide an indication of the maximum reachable distance.

398

399 Conclusion

400 In this study, we showed that eDNA analysis using stemflow is useful for detecting
401 mammalian eDNA. This method is highly effective, especially for mammals in contact
402 with trees, and therefore has the potential to contribute to the monitoring of arboreal
403 mammals. Furthermore, even for mammals that do not directly contact trees, a
404 relationship of increased detection rate was observed when the distance between their
405 location and the tree was close, suggesting that the method is likely to reflect the species
406 composition around the tree being surveyed. This study is a limited example of how the
407 distance between mammals and eDNA sampling points affects mammal detection, which
408 will be an important finding in the development of mammal monitoring techniques via
409 eDNA.

410

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424 Author contributions

425 Conceptualization: YI, KA, MKS and TM; Methodology: YI, AS, MM and TM; Formal

426 analysis: YI, MKS, and QW; Investigation: YI, KA, KH; Writing - Original Draft; YI and

427 TM. Writing - Review & Editing: all authors; Visualization: YI; Supervision: TM; Project

428 administration: YI and TM; Funding acquisition: TM and MKS.

429

430 Data availability

431 The raw data from the metabarcoding analysis are provided in the Supplementary

432 Information.

433

434 Declarations

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

436 Reference

437 Allen MC, Kwait R, Vastano A, Kisurin A, Zoccolo I, Jaffe BD, Angle JC, Maslo B,
438 Lockwood JL (2023) Sampling environmental DNA from trees and soil to detect cryptic
439 arboreal mammals. *Scientific Reports*, 13: 180. [https://doi.org/10.1038/s41598-023-](https://doi.org/10.1038/s41598-023-27512-8)
440 [27512-8](https://doi.org/10.1038/s41598-023-27512-8)

441

442 Bohmann K, Lynggaard C (2023) Transforming terrestrial biodiversity surveys using
443 airborne eDNA. *Trends in Ecology & Evolution*, 38: 119–121.
444 <https://doi.org/10.1016/j.tree.2022.11.006>

445

446 Burton AC, Neilson E, Moreira D, Ladle A, Steenweg R, Fisher JT, Bayne E, Boutin S
447 (2015) Wildlife camera trapping: A review and recommendations for linking surveys to
448 ecological processes. *Journal of Applied Ecology*, 52: 675–685.
449 <https://doi.org/10.1111/1365-2664.12432>

450

451 Calvignac-Spencer S, Merkel K, Kutzner N, Kühl H, Boesch C, Kappeler PM, Metzger
452 S, Schubert G, Leendertz FH (2013) Carrion fly-derived DNA as a tool for
453 comprehensive and cost-effective assessment of mammalian biodiversity. *Molecular*
454 *Ecology*, 22: 915–924. <https://doi.org/10.1111/mec.12183>

455

456 Ceballos G, Ehrlich PR (2002) Mammal population losses and the extinction crisis.
457 *Science*, 296: 904–907. <https://doi.org/10.1126/science.1069349>

458

- 459 Ceballos G, Ehrlich PR, Soberón J, Salazar I, Fay JP (2005) Global mammal
460 conservation: What must we manage? *Science*, 309: 603–607.
461 <https://doi.org/10.1126/science.1114015>
462
- 463 Clare EL, Economou CK, Bennett FJ, Dyer CE, Adams K, McRobie B, Drinkwater R,
464 Littlefair JE (2022) Measuring biodiversity from DNA in the air. *Current Biology*, 32:
465 693–700. <https://doi.org/10.1016/j.cub.2021.11.064>
466
- 467 Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST.
468 *Bioinformatics*, 26: 2460–2461. <https://doi.org/10.1093/bioinformatics/btq461>
469
470
- 471 Harper LR, Lawson Handley L, Carpenter AI, Ghazali M, Di Muri C, Macgregor CJ,
472 Logan TW, Law A, Breithaupt T, Read DS, McDevitt AD, Hänfling B (2019)
473 Environmental DNA (eDNA) metabarcoding of pond water as a tool to survey
474 conservation and management priority mammals. *Biological Conservation*, 238:
475 108225. <https://doi.org/10.1016/j.biocon.2019.108225>
476
- 477 Ishige T, Miya M, Ushio M, Sado T, Ushioda M, Maebashi K, Yonechi R, Lagan P,
478 Matsubayashi H (2017) Tropical-forest mammals as detected by environmental DNA at
479 natural saltlicks in Borneo. *Biological Conservation*, 210: 281–285.
480 <https://doi.org/10.1016/j.biocon.2017.04.023>
481
- 482 IUCN (2025). The IUCN Red List of Threatened Species Version 2024-2

483 Retrieved 3 February 2025, from <https://www.iucnredlist.org/en>

484

485 Kobe City (2020) List of flora and fauna observed in Kobe City (FY2020). Retrieved

486 March 13, 2025, from

487 https://www.city.kobe.lg.jp/documents/41456/reddata2020kobever_alllist.pdf

488

489 Leempoel K, Hebert T, Hadly EA (2020) A comparison of eDNA to camera trapping for

490 assessment of terrestrial mammal diversity. Proceedings of the Royal Society B:

491 Biological Sciences, 287: 20192353. <https://doi.org/10.1098/rspb.2019.2353>

492

493 Lyet A, Pellissier L, Valentini A, Dejean T, Hehmeyer A, Naidoo R (2021) eDNA

494 sampled from stream networks correlates with camera trap detection rates of terrestrial

495 mammals. Scientific Reports, 11: 90598. <https://doi.org/10.1038/s41598-021-90598-5>

496

497 Lynggaard C, Bertelsen MF, Jensen CV, Johnson MS, Frøslev TG, Olsen MT, Bohmann

498 K (2022) Airborne environmental DNA for terrestrial vertebrate community monitoring.

499 Current Biology, 32: 701-707.e5. <https://doi.org/10.1016/j.cub.2021.12.014>

500

501 Lynggaard C, Frøslev TG, Johnson MS, Olsen MT, Bohmann K (2024) Airborne

502 environmental DNA captures terrestrial vertebrate diversity in nature. Molecular

503 Ecology Resources, 24: e13840. <https://doi.org/10.1111/1755-0998.13840>

504

505 Mena JL, Yagui H, Tejada V, Bonifaz E, Bellemain E, Valentini A, Tobler MW,

506 Sánchez-Vendizú P, Lyet A (2021) Environmental DNA metabarcoding as a useful tool

507 for evaluating terrestrial mammal diversity in tropical forests. *Ecological Applications*,
508 31: e2335. <https://doi.org/10.1002/eap.2335>

509

510 Minamoto T, Miya M, Sado T, Seino S, Doi H, Kondoh M, Nakamura K, Takahara T,
511 Yamamoto S, Yamanaka H, Araki H, Iwasaki W, Kasai A, Masuda R, Uchii K. (2021)
512 An illustrated manual for environmental DNA research: Water sampling guidelines and
513 experimental protocols. *Environmental DNA*, 3: 8–13

514 <http://dx.doi.org/10.1002/edn3.121>

515

516 Minamoto T, Yamanaka H, Takahara T, Honjo MN, Kawabata Z (2012) Surveillance of
517 fish species composition using environmental DNA. *Limnology*, 13: 193–197.

518 <https://doi.org/10.1007/s10201-011-0362-4>

519

520 Newton JP, Allentoft ME, Bateman PW, van der Heyde M, Nevill P (2025) Targeting
521 terrestrial vertebrates with eDNA: Trends, perspectives, and considerations for
522 sampling. *Environmental DNA*, 7: e70056. <https://doi.org/10.1002/edn3.70056>

523

524 Oka S, Miya M, Sado T (2022) Gravity filtration of environmental DNA: A simple, fast,
525 and power-free method. *MethodsX*, 9: 101838.

526 <https://doi.org/10.1016/j.mex.2022.101838>

527

528 R Core Team. (2024) The R project for statistical computing, from <https://www.r->
529 [project.org](https://www.r-project.org)

530

531 Sakata A, Sado T, Oka S, Ushio M, Miya M (2023) Collection of environmental DNA
532 from stemflow for monitoring arboreal biodiversity: Preliminary validation using
533 lichens. *MethodsX*, 11: 102448. <https://doi.org/10.1016/j.mex.2023.102448>
534

535 Sakata MK, Watanabe T, Maki N, Ikeda K, Kosuge T, Okada H, Yamanaka H, Sado T,
536 Miya M, Minamoto T (2021) Determining an effective sampling method for eDNA
537 metabarcoding: A case study for fish biodiversity monitoring in a small, natural river.
538 *Limnology*, 22: 221–235. <https://doi.org/10.1007/s10201-020-00645-9>
539

540 Sales NG, Kaizer MC, Coscia I, Perkins JC, Highlands A, Boubli JP, Magnusson WE,
541 Da Silva MNF, Benvenuto C, Mcdevitt AD (2020) Assessing the potential of
542 environmental DNA metabarcoding for monitoring Neotropical mammals: A case study
543 in the Amazon and Atlantic Forest, Brazil. *Mammal Review*, 50: 221–225.
544 <https://doi.org/10.1111/mam.12183>
545

546 Schnell IB, Thomsen PF, Wilkinson N, Rasmussen M, Jensen LRD, Willerslev E,
547 Bertelsen MF, Gilbert MTP (2012) Screening mammal biodiversity using DNA from
548 leeches. *Current Biology*, 22: R262–R263. <https://doi.org/10.1016/j.cub.2012.02.058>
549

550 Tetzlaff SJ, Katz AD, Wolff PJ, Kleitch, M. E (2024) Comparison of soil eDNA to
551 camera traps for assessing mammal and bird community composition and site use.
552 *Ecology and Evolution*, 14: e70022. <https://doi.org/10.1002/ece3.70022>
553

554 The eDNA Society (2019) Environmental DNA Sampling and Experiment Manual (ver
555 2.1). [https://ednasociety.org/wp-](https://ednasociety.org/wp-content/uploads/2022/03/eDNA_manual_Eng_v2_1_3b.pdf)
556 [content/uploads/2022/03/eDNA_manual_Eng_v2_1_3b.pdf](https://ednasociety.org/wp-content/uploads/2022/03/eDNA_manual_Eng_v2_1_3b.pdf)

557

558 Thomsen PF, Willerslev E (2015) Environmental DNA – An emerging tool in
559 conservation for monitoring past and present biodiversity. *Biological Conservation*, 183:
560 4–18. <https://doi.org/10.1016/j.biocon.2014.11.019>

561

562 Ushio M, Fukuda H, Inoue T, Makoto K, Kishida O, Sato K, Murata K, Nikaido M,
563 Sado T, Sato Y, Takeshita M, Iwasaki W, Yamanaka H, Kondoh M, Miya M (2017)
564 Environmental DNA enables detection of terrestrial mammals from forest pond water.
565 *Molecular Ecology Resources*, 17: e63–e75. <https://doi.org/10.1111/1755-0998.12690>

566

567 van Beeck Calkoen STS, Leigh-Moy K, Cromsigt JPGM, Spong G, Lebeau LC,
568 Heurich M (2019) The blame game: Using eDNA to identify species-specific tree
569 browsing by red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) in a
570 temperate forest. *Forest Ecology and Management*, 451: 117483.

571 <https://doi.org/10.1016/j.foreco.2019.117483>

572

573 Wu Q, Minamoto T (2023) Improvement of recovery yield of macro-organismal
574 environmental DNA from seawater samples. *Analytical Sciences*, 39: 713–720
575 <https://doi.org/10.1007/s44211-023-00280-1>

576

577 Yamanaka H, Minamoto T, Matsuura J, Sakurai S, Tsuji S, Motozawa H, Hongo M,
578 Sogo Y, Kakimi N, Teramura I, Sugita M, Baba M, Kondo A (2017) A simple method
579 for preserving environmental DNA in water samples at ambient temperature by addition
580 of cationic surfactant. *Limnology*, 18: 233–241. [https://doi.org/10.1007/s10201-016-](https://doi.org/10.1007/s10201-016-0508-5)
581 [0508-5](https://doi.org/10.1007/s10201-016-0508-5)
582
583 Yonezawa S, Ushio M, Yamanaka H, Miya M, Takayanagi A, Isagi Y (2020)
584 Environmental DNA metabarcoding reveals the presence of a small, quick-moving,
585 nocturnal water shrew in a forest stream. *Conservation Genetics*, 21: 1079–1084.
586 <https://doi.org/10.1007/s10592-020-01310-5>
587

588 Table 1. Filtration volume of each sample

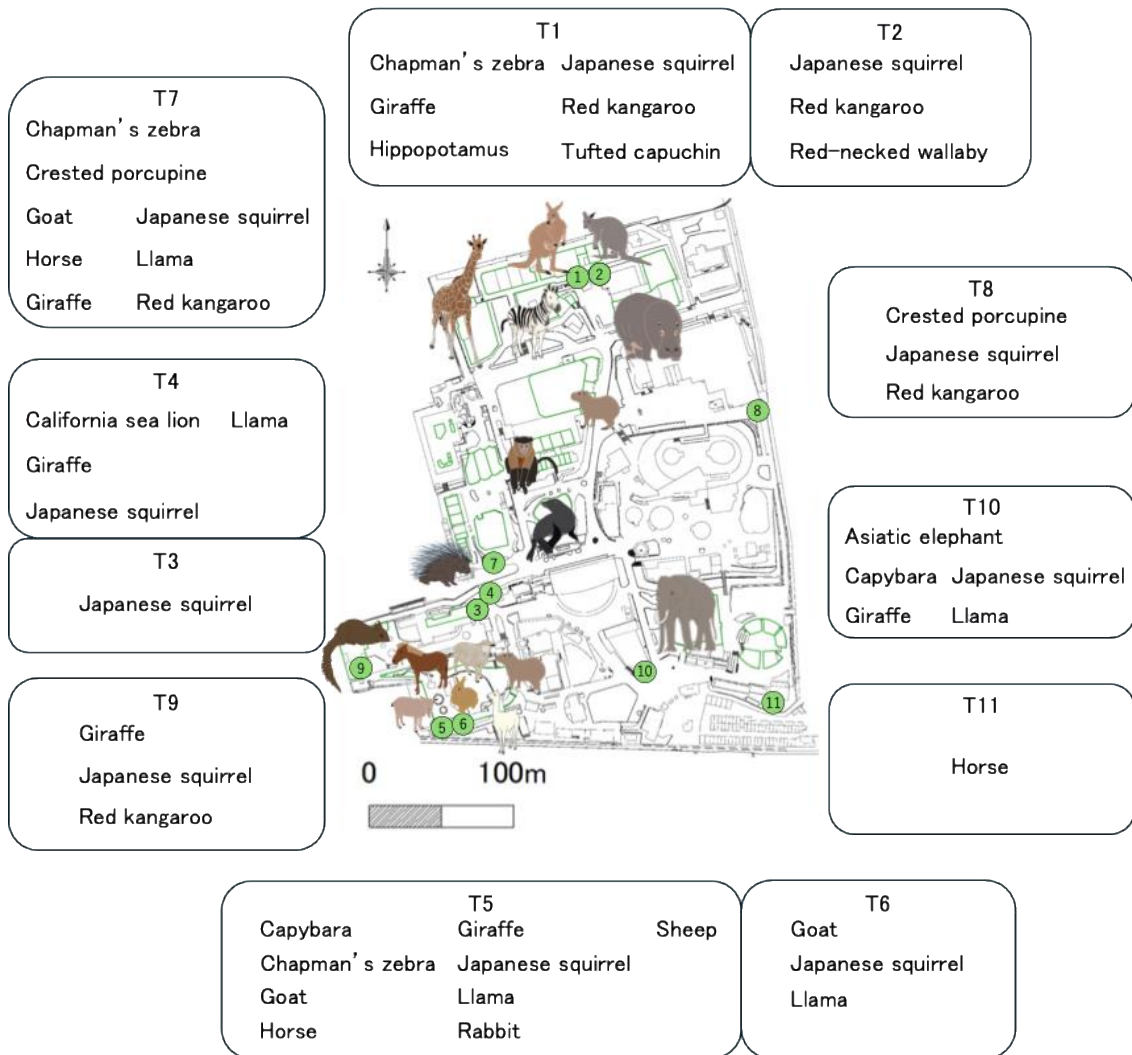
Samplin	T1	T2	T3	T4	T5	T6	T7	T8	T9	T1	T1	FN
g times										0	1	C
1st	315	NA*	NA*	50	250	19	50	250	50	25	30	100
		1	1			5				0		0
2nd	530	350	450	31	800	50	450	400	75	46	60	100
				0		0			0	0	0	0
3rd	700	550	400	25	800	80	550	NA*	80	80	30	100
				0		0		1	0	0	0	0
4th	200	500	100	20	750	50	800	800	40	40	40	100
				0		0			0	0	0	0
5th	800	600	400	50	100	90	100	900	80	90	60	100
				0	0	0	0		0	0	0	0
6th	NA* ¹	NA*	NA*	50	600	50	100	400	20	80	50	100
		1	1			0			0	0		0
7th		600*	200*									100
		2	2									0

589 *¹NA: Filtration was not available due to broken water sampling equipment or insufficient
 590 water sampling volume.

591 *²Because enough water was not available for the T2 and T3 samples in the 6th sampling,
 592 an additional 7th sampling was performed.

593

594



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596

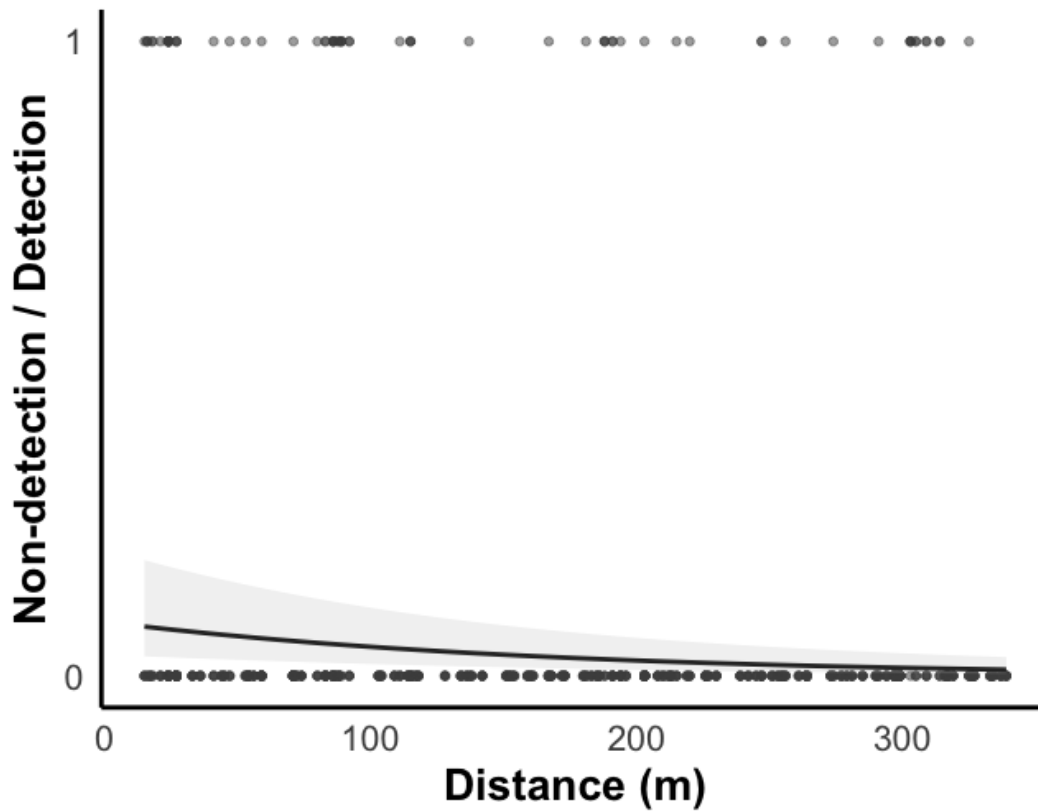
597 Figure 1. Location of trees and detected species of captive mammals. Approximate

598 locations of the 16 captive mammal species detected by eDNA are presented.

599 NumberS121 correspond to each tree number.

600

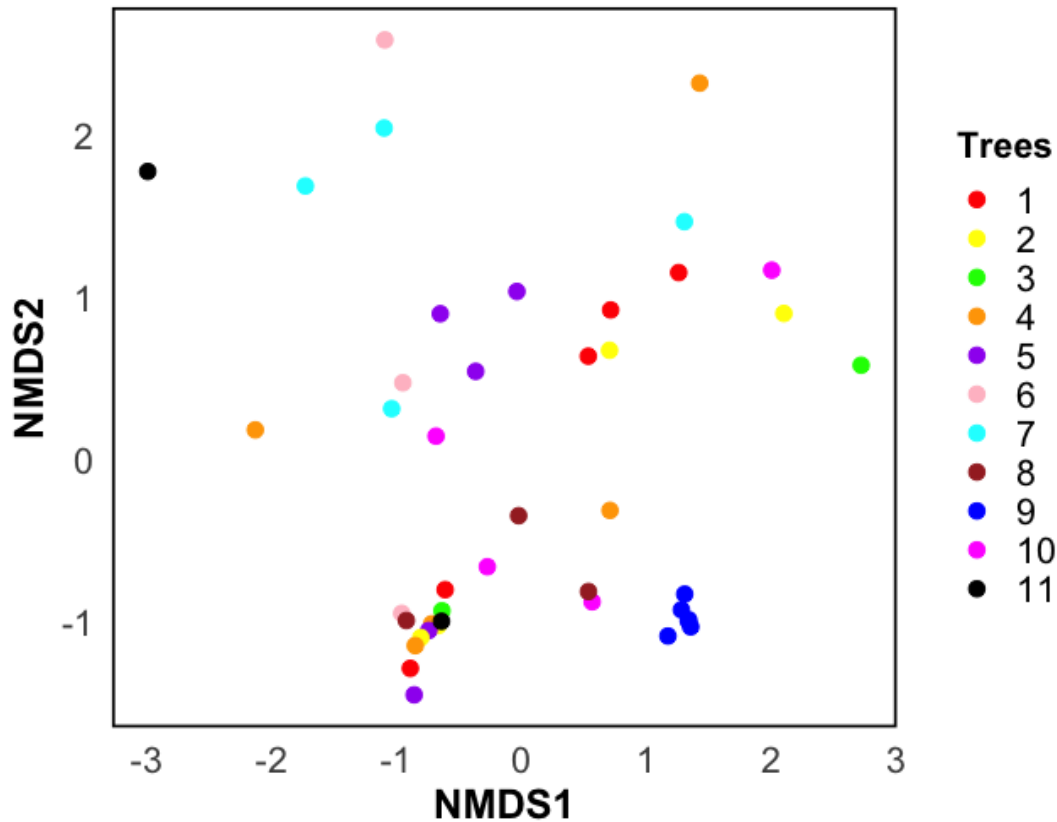
601



602

603

604 Figure 2. Relationship between eDNA detection or non-detection of captive mammals
 605 with the distance to trees. Regression curves were drawn by plotting the detection or
 606 non-detection (1/0) from 11 trees for 16 captive mammal species that had been detected
 607 at least once. The curve was drawn using the standard function “predict()” in R. Results
 608 from T9 where Japanese squirrels directly contact the tree were excluded.



609

610

611 Figure 3. Species composition similarity for each tree shown by NMDS. Using the
612 Bray-Curtis index, species composition dissimilarity based on relative read counts was
613 calculated and plotted among samples in which a captive mammal species was detected.
614 Samples from Tree 9, shown in blue, form a distinct cluster, indicating a high degree of
615 similarity in species composition.

616

Table S1. Mammalian species kept in Oji Zoo, Kobe (as of April 5, 2024)

Species name	Scientific name	Order	Family	Number of individuals (as of May 2024)	Number of individuals (as of Dec 2024)
Koala	<i>Phascolarctos cinereus</i>	Diprotodontia	Phascolarctidae	6	7
Red-necked wallaby	<i>Notamacropus rufogriseus</i>	Diprotodontia	Macropodidae	3	3
Red kangaroo	<i>Osphranter rufus</i>	Diprotodontia	Macropodidae	21	21
Giant anteater	<i>Myrmecophaga tridactyla</i>	Pilosa	Myrmecophagidae	2	2
Southern two-toed sloth	<i>Choloepus didactylus</i>	Pilosa	Megalonychidae	4	4
Egyptian rousette	<i>Rousettus aegyptiacus</i>	Chiroptera	Pteropodidae	55	54
Senegal galago	<i>Galago senegalensis</i>	Primates	Galagidae	1	1
Ring-tailed lemur	<i>Lemur catta</i>	Primates	Lemuridae	4	4
Douroucouli	<i>Aotus trivirgatus</i>	Primates	Aotidae	3	3
Tufted capuchin	<i>Sapajus apella</i>	Primates	Cebidae	13	12
Bolivian squirrel monkey	<i>Saimiri boliviensis</i>	Primates	Cebidae	4	4
Common squirrel monkey	<i>Saimiri sciureus</i>	Primates	Cebidae	5	5
White-tufted-ear marmoset	<i>Callithrix jacchus</i>	Primates	Cebidae	2	1
De Brazza's monkey	<i>Cercopithecus neglectus</i>	Primates	Cercopithecidae	3	3
Mantled guereza	<i>Colobus guereza</i>	Primates	Cercopithecidae	4	4
Common gibbon	<i>Hylobates lar</i>	Primates	Hylobatidae	3	3
Siamang	<i>Symphalangus syndactylus</i>	Primates	Hylobatidae	2	2
Chimpanzee	<i>Pan troglodytes</i>	Primates	Hominidae	2	2
Bornean orangutan	<i>Pongo pygmaeus</i>	Primates	Hominidae	1	1
Tibetan brown bear	<i>Ursus arctos pruinosus</i>	Carnivora	Ursidae	1	1
Hokkaido brown bear	<i>Ursus arctos yesoensis</i>	Carnivora	Ursidae	2	2
Polar bear	<i>Ursus maritimus</i>	Carnivora	Ursidae	1	1
Japanese black bear	<i>Ursus thibetanus japonicus</i>	Carnivora	Ursidae	2	2

Lesser panda	<i>Ailurus fulgens</i>	Carnivora	Ailuridae	3	3
Ring-tailed coati	<i>Nasua nasua</i>	Carnivora	Procyonidae	2	2
Kinkajou	<i>Potos flavus</i>	Carnivora	Procyonidae	1	1
California sea lion	<i>Zalophus californianus</i>	Carnivora	Otariidae	6	5
Binturong	<i>Arctictis binturong</i>	Carnivora	Viverridae	1	1
Pallas's cat	<i>Otocolobus manul</i>	Carnivora	Felidae	1	1
Bobcat	<i>Lynx rufus</i>	Carnivora	Felidae	1	1
Eurasian lynx	<i>Lynx lynx</i>	Carnivora	Felidae	2	2
Jaguar	<i>Panthera onca</i>	Carnivora	Felidae	1	1
Amur leopard	<i>Panthera pardus orientalis</i>	Carnivora	Felidae	2	2
Amur tiger	<i>Panthera tigris altaica</i>	Carnivora	Felidae	2	2
Snow leopard	<i>Panthera uncia</i>	Carnivora	Felidae	1	1
Asiatic elephant	<i>Elephas maximus</i>	Proboscidea	Elephantidae	2	2
Ass	<i>Equus asinus</i>	Perissodactyla	Equidae	2	2
Chapman's zebra	<i>Equus quagga chapmani</i>	Perissodactyla	Equidae	2	2
Horse	<i>Equus caballus</i>	Perissodactyla	Equidae	1	1
Hippopotamus	<i>Hippopotamus amphibius</i>	Artiodactyla	Hippopotamidae	2	2
Llama	<i>Lama glama</i>	Artiodactyla	Camelidae	3	3
Giraffe	<i>Giraffa camelopardalis</i>	Artiodactyla	Giraffidae	4	4
Goat	<i>Capra hircus</i>	Artiodactyla	Bovidae	4	4
Sheep	<i>Ovis aries</i>	Artiodactyla	Bovidae	3	3
Sitatunga	<i>Tragelaphus spekii</i>	Artiodactyla	Bovidae	4	4
Japanese squirrel	<i>Sciurus lis</i>	Rodentia	Sciuridae	28	28
Crested porcupine	<i>Hystrix cristata</i>	Rodentia	Hystriidae	1	1
Indian crested porcupine	<i>Hystrix indica</i>	Rodentia	Hystriidae	2	2
Domestic guinea pig	<i>Cavia porcellus</i>	Rodentia	Caviidae	17	16
Capybara	<i>Hydrochoerus hydrochaeris</i>	Rodentia	Caviidae	5	4
Rabbit	<i>Oryctolagus cuniculus</i>	Lagomorpha	Leporidae	16	16

Table S2. Sampled trees

No.	Scientific name	Circumference (cm) ^{*1}	Latitude	Longitude	Remark	Bark shape
T1	<i>Celtis sinensis</i>	166, 90 ^{*2}	34.7119751	135.2140604	Adjacent different	slightly rough
T2	<i>Cinnamomum camphora</i>	124	34.7119762	135.2140735	tree species	Rough
T3	<i>Cinnamomum camphora</i>	228	34.7099108	135.2137345	Adjacent different	Rough
T4	<i>Ilex integra</i>	223	34.7098173	135.2137124	tree species	slightly rough
T5	<i>Quercus glauca</i>	64.5	34.7090941	135.2137757	Adjacent different	smooth
T6	<i>Quercus serrata</i>	117	34.7090423	135.2137674	tree species	rough
T7	<i>Ilex integra</i>	103	34.7100706	135.2137892		
T8	<i>Prunus species</i>	41.5	34.7111204	135.2156493		
T9	<i>Cryptomeria japonica</i>	32	34.7093345	135.2129593		
T10	<i>Ilex rotunda</i>	198	34.7096203	135.2149750		
T11	<i>Prunus sp.</i>	146.5	34.7095494	135.2159577		

^{*1}Circumference was measured at breast height, 1.2 m from the ground.

^{*2} Since T1 was divided into two trunks below breast height, the circumference of each was measured.

Table S3. Rainfall information (cited from the Japan Meteorological Agency, Ministry of Land, Infrastructure, Transport and Tourism, “Historical Weather Data Search” (<https://www.data.jma.go.jp/obd/stats/etrn/index.php>, data from Kobe Observatory, confirmed on February 16, 2025).

Sampling times	Date (2024–2025)	Precipitation duration (h) ^{*1}	Precipitation (mm) ^{*2}	Maximum precipitation per hour (mm)	Number of days since last precipitation
1st	June 9–10	27	11.5	3	7
2nd	June 27–July 1	36	93	16.5	3
3rd	July 1–3	10	39.5	30	0.5
4th	September 22–23	8	45	19	7
5th	October 3–4	19	58.5	15.5	11
6th	November 28–29	5	8.5	4	1
7th	November 28 –January 7	9	20	4	39

^{*1}Actual time it rained between the installation of the water sampler and the collection of the sample.

^{*2}The total precipitation during the precipitation duration.

Table S4. Primers used in this study (Cited from Ushio et al. 2018)

	Primer	Sequence (5' > 3')
1stPCR	MiMammal-U (Forward)	ACACTCTTCCCTACACGACGCTCTCCGATCTNNNNNNGGGTTGGTAAATTCGTGCCAGC
	MiMammal-U (Reverse)	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNCATAGTGGGGTATCTAATCCCAGTTTG
	MiMammal-E (Forward)	ACACTCTTCCCTACACGACGCTCTCCGATCTNNNNNNGGACTGGTCAATTCGTGCCAGC
	MiMammal-E (Reverse)	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNCATAGTGAGGTATCTAATCTCAGTTTG
	MiMammal-B (Forward)	ACACTCTTCCCTACACGACGCTCTCCGATCTNNNNNNGGGTTGGTTAATTCGTGCCAGC
	MiMammal-B (Reverse)	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNCATAGTGGGGTATCTAATCCCAGTTTG
2ndPCR	Forward	AATGATACGGCGACCACCGAGATCTACAXXXXXXXXXXACACTCTTCCCTACACGACGCTCTCCCATCT *
	Reverse	CAAGCAGAAGACGGCATAACGAGATXXXXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT *

*Eight consecutive X's indicate the index sequence.

Table S5. Distance from the cage where each mammal is kept to each tree

Species	Distance (m)										
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11
Japanese squirrel	303	305	83	88	89	92	111	314	11	188	279
Goat	297	299	54	59	24	27	86	264	71	115	203
Horse	297	299	54	59	24	27	86	264	71	115	203
Llama	297	299	54	59	24	27	86	264	71	115	203
Sheep	297	299	54	59	24	27	86	264	71	115	203
Capybara	297	299	54	59	24	27	86	264	71	115	203
Capybara	88	89	164	159	238	240	131	130	238	184	241
Red-necked wallaby	17	15	261	255	337	339	227	183	326	285	333
Red kangaroo	16	18	247	242	325	327	215	194	309	281	334
Tufted capuchin	137	138	109	103	185	186	74	161	180	152	226
California sea lion	167	167	88	83	159	160	56	153	168	117	194
Asiatic elephant	254	253	154	151	173	172	142	128	230	53	72
Chapman's zebra	41	44	213	208	291	294	181	191	273	255	316
Hippopotamus	47	45	245	239	317	319	210	136	317	251	290
Giraffe	80	83	196	191	274	277	167	219	247	256	326
Crested porcupine	212	213	36	33	114	118	21	220	104	134	226
Rabbit	297	299	54	59	24	27	86	264	71	115	203

Table S6. Numbers of reads in each processing stage

Raw reads	After merging	After denoising	Final reads
6,590,492	6,508,761	3,825,770	1,176,633

Table S7. Results of metabarcoding

Sampling	Species name	Scientific name	Status	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11
1st	Japanese squirrel	<i>Sciurus lis</i>	Zoo-kept	8	NA*	NA*	7919	5	0	0	0	66382	0	0
	Giraffe	<i>Giraffa reticulata</i>	Zoo-kept	0	NA*	NA*	482	0	0	0	0	0	0	0
	Dog	<i>Canis lupus familiaris</i>	Non-zoo	0	NA*	NA*	0	0	0	0	0	0	1831	0
	Domestic cattle	<i>Bos taurus</i>	Non-zoo	0	NA*	NA*	0	0	0	0	0	127	0	0
	Wild boar	<i>Sus scrofa</i>	Non-zoo	0	NA*	NA*	0	0	0	0	0	5	0	0
	Siberian weasel	<i>Mustela sibirica</i>	Non-zoo	0	NA*	NA*	0	0	0	0	0	0	0	1204
2nd	Japanese squirrel	<i>Sciurus lis</i>	Zoo-kept	12	0	0	0	0	0	0	0	203020	7187	0
	Goat	<i>Capra hircus</i>	Zoo-kept	0	0	0	0	0	0	4303	0	0	0	0
	Red kangaroo	<i>Osphranter rufus</i>	Zoo-kept	4744	0	0	0	0	0	0	0	0	0	0
	Horse	<i>Equus caballus</i>	Zoo-kept	0	0	0	0	0	0	1638	0	0	0	0
	Dog	<i>Canis lupus familiaris</i>	Non-zoo	0	0	0	0	0	0	693	0	0	0	0
	Raccoon	<i>Procyon lotor</i>	Non-zoo	0	0	0	0	0	0	0	4071	0	0	0
3rd	Japanese squirrel	<i>Sciurus lis</i>	Zoo-kept	57	31	39	26	23	6	14	NA*	151320	0	0
	Goat	<i>Capra hircus</i>	Zoo-kept	0	0	0	0	0	775	0	NA*	0	0	0
	Horse	<i>Equus caballus</i>	Zoo-kept	0	0	0	0	0	0	2801	NA*	0	0	265
	Dog	<i>Canis lupus familiaris</i>	Non-zoo	0	0	0	0	0	0	0	NA*	0	628	0

	Llama	<i>Lama glama</i>	Zoo-kept	0	0	0	0	0	2011	1058	NA*	0	0	0
4th	Japanese squirrel	<i>Sciurus lis</i>	Zoo-kept	25	18	0	0	4	14	0	4747	176209	48	0
	Red kangaroo	<i>Osphranter rufus</i>	Zoo-kept	11909	0	7	0	71	0	1161	0	5	0	0
	Chapman's zebra	<i>Equus quagga chapmani</i>	Zoo-kept	2067	0	0	0	5	0	1599	0	0	0	0
	Giraffe	<i>Giraffa reticulata</i>	Zoo-kept	0	0	0	1050	3900	0	959	0	6	0	0
	California sea lion	<i>Zalophus californianus</i>	Zoo-kept	0	0	0	555	0	0	0	0	0	0	0
	Asiatic elephant	<i>Elephas maximus</i>	Zoo-kept	0	0	0	0	0	0	0	0	0	390	0
	Goat	<i>Capra hircus</i>	Zoo-kept	0	0	0	0	271	0	0	0	0	0	0
	Llama	<i>Lama glama</i>	Zoo-kept	0	0	0	0	14089	0	0	0	0	937	0
	Capybara	<i>Hydrochoerus hydrochaeris</i>	Zoo-kept	0	0	0	0	2129	0	0	0	0	518	0
	Rabbit	<i>Oryctolagus cuniculus</i>	Zoo-kept	0	0	0	0	967	0	0	0	0	0	0
	Raccoon	<i>Procyon lotor</i>	Non-zoo	10945	0	0	0	7	0	0	0	4	0	0
	Domestic cattle	<i>Bos taurus</i>	Non-zoo	0	0	0	1341	81	0	0	0	0	782	0
	Wild boar	<i>Sus scrofa</i>	Non-zoo	0	0	0	0	6	0	51	0	0	0	0
	Dog	<i>Canis lupus familiaris</i>	Non-zoo	0	0	0	0	1163	0	0	4702	0	0	0
	Domestic cat	<i>Felis catus</i>	Non-zoo	0	0	0	700	0	0	0	0	0	0	0
5th	Japanese squirrel	<i>Sciurus lis</i>	Zoo-kept	0	0	0	0	0	0	0	0	152538	123	0

	Red kangaroo	<i>Osphranter rufus</i>	Zoo-kept	23512	10433	0	0	0	0	0	0	14	0	0
	Giraffe	<i>Giraffa reticulata</i>	Zoo-kept	9747	0	0	0	0	0	0	0	0	0	0
	Hippopotamus	<i>Hippopotamus amphibius</i>	Zoo-kept	142	0	0	0	0	0	0	0	0	0	0
	Goat	<i>Capra hircus</i>	Zoo-kept	0	0	0	0	3050	0	1526	0	0	0	0
	Llama	<i>Lama glama</i>	Zoo-kept	0	0	0	0	23518	0	0	0	5	4	0
	Capybara	<i>Hydrochoerus hydrochaeris</i>	Zoo-kept	0	0	0	0	6621	0	0	0	0	0	0
	Sheep	<i>Ovis aries</i>	Zoo-kept	0	0	0	0	838	0	0	0	0	0	0
	Crested porcupine	<i>Hystrix cristata</i>	Zoo-kept	0	0	0	0	0	0	13961	7	0	0	0
	Tufted capuchin	<i>Sapajus apella</i>	Zoo-kept	12770	0	0	0	0	0	0	0	0	0	0
	Raccoon	<i>Procyon lotor</i>	Non-zoo	0	0	0	0	0	0	0	0	6	0	0
	Siberian weasel	<i>Mustela sibirica</i>	Non-zoo	2258	0	0	0	0	0	0	0	0	0	0
	Masked palm civet	<i>Paguma larvata</i>	Non-zoo	0	0	0	0	0	0	0	0	0	0	701
	Dog	<i>Canis lupus familiaris</i>	Non-zoo	0	0	0	0	4084	0	6195	0	0	0	0
	Domestic cat	<i>Felis catus</i>	Non-zoo	0	0	0	0	0	0	3767	0	0	0	0
	Wild boar	<i>Sus scrofa</i>	Non-zoo	0	0	0	0	0	0	0	215	0	0	0
6th/7th	Japanese squirrel	<i>Sciurus lis</i>	Zoo-kept	NA*	0	0	0	2967	0	0	149	161990	0	0
	Red kangaroo	<i>Osphranter rufus</i>	Zoo-kept	NA*	284	0	0	0	0	0	34	0	0	0

Red-necked wallaby	<i>Non-zootamacropus rufogriseus</i>	Zoo-kept	NA*	12	0	0	0	0	0	0	0	0	0
Chapman's zebra	<i>Equus quagga chapmani</i>	Zoo-kept	NA*	4	0	0	0	0	0	0	0	0	0
Giraffe	<i>Giraffa reticulata</i>	Zoo-kept	NA*	0	0	0	0	0	0	0	0	67	0
Llama	<i>Lama glama</i>	Zoo-kept	NA*	0	0	21	16019	0	0	0	0	0	0
Goat	<i>Capra hircus</i>	Zoo-kept	NA*	0	0	0	99	363	0	0	0	0	0
Horse	<i>Equus caballus</i>	Zoo-kept	NA*	0	0	0	4163	0	0	0	0	0	0
Sheep	<i>Ovis aries</i>	Zoo-kept	NA*	0	0	0	284	0	0	0	0	0	0
Domestic cat	<i>Felis catus</i>	Non-zoo	NA*	0	429	0	0	0	0	0	0	0	0
Wild boar	<i>Sus scrofa</i>	Non-zoo	NA*	0	0	0	40	0	0	0	0	12	0
Domestic cattle	<i>Bos taurus</i>	Non-zoo	NA*	0	0	0	0	56	0	0	0	0	0
Dog	<i>Canis lupus familiaris</i>	Non-zoo	NA*	0	0	0	4515	1646	5	0	0	0	0
Raccoon	<i>Procyon lotor</i>	Non-zoo	NA*	0	0	0	0	0	0	5	0	0	0
Japanese marten	<i>Martes melampus</i>	Non-zoo	NA*	0	0	0	0	0	0	0	0	659	0
Sika deer	<i>Cervus nippon</i>	Non-zoo	NA*	0	0	0	0	0	0	555	0	0	0

*1 NA: Filtration was not available due to broken water sampling equipment or insufficient water sampling volume.

Table S8. Count of detections per tree and species

Species	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11
Japanese squirrel	4	2	1	2	4	2	1	2	6	3	0
Giraffe	1	0	0	2	1	0	1	0	1	1	0
Goat	0	0	0	0	3	2	2	0	0	0	0
Red kangaroo	3	2	1	0	1	0	1	1	2	0	0
Horse	0	0	0	0	1	0	2	0	0	0	1
Llama	0	0	0	1	3	1	1	0	1	2	0
Chapman's zebra	1	1	0	0	1	0	1	0	0	0	0
California sea lion	0	0	0	1	0	0	0	0	0	0	0
Asiatic elephant	0	0	0	0	0	0	0	0	0	1	0
Capybara	0	0	0	0	2	0	0	0	0	1	0
Rabbit	0	0	0	0	1	0	0	0	0	0	0
Hippopotamus	1	0	0	0	0	0	0	0	0	0	0
Sheep	0	0	0	0	2	0	0	0	0	0	0
Crested porcupine	0	0	0	0	0	0	1	1	0	0	0
Tufted capuchin	1	0	0	0	0	0	0	0	0	0	0
Red-necked wallaby	0	1	0	0	0	0	0	0	0	0	0
Dog	0	0	0	0	3	1	3	1	0	2	0
Domestic cattle	0	0	0	1	1	1	0	0	1	1	0
Wild boar	0	0	0	0	2	0	1	1	1	1	0
Siberian weasel	1	0	0	0	0	0	0	0	0	0	1
Raccoon	1	0	0	0	1	0	0	2	2	0	0
Domestic cat	0	0	1	1	0	0	1	0	0	0	0
Masked palm civet	0	0	0	0	0	0	0	0	0	0	1
Japanese marten	0	0	0	0	0	0	0	0	0	1	0
Sika deer	0	0	0	0	0	0	0	1	0	0	0

Table S9. Detected captive mammal species and distance to trees

Species	Trees No.	Distance (m)
Capybara	T5	*1 24 / 238
Capybara	T10	*1 115 / 184
Horse	T5	27
Horse	T7	86
Horse	T11	203
Goat	T5	24
Goat	T6	27
Goat	T7	86
Llama	T4	59
Llama	T5	24
Llama	T6	27
Llama	T7	86
Llama	T10	115
Rabbit	T5	24
Sheep	T5	24
Asiatic elephant	T10	53
Japanese squirrel	T1	303
Japanese squirrel	T2	305
Japanese squirrel	T3	83
Japanese squirrel	T4	88
Japanese squirrel	T5	89
Japanese squirrel	T6	92
Japanese squirrel	T7	111
Japanese squirrel	T8	314
Japanese squirrel	T9	11
Japanese squirrel	T10	188
Crested porcupine	T7	21
Crested porcupine	T8	220
Tufted capuchin	T1	137
Giraffe	T1	80
Giraffe	T4	191

Giraffe	T5	274
Giraffe	T7	167
Giraffe	T9	247
Giraffe	T10	256
Chapman's zebra	T1	41
Chapman's zebra	T5	291
Chapman's zebra	T7	181
California sea lion	T4	83
Hippopotamus	T1	47
Red kangaroo	T1	16
Red kangaroo	T2	18
Red kangaroo	T7	215
Red kangaroo	T8	194
Red kangaroo	T9	309
Red-necked wallaby	T2	15

*1Since the capybaras have two cages, the distance to the trees from both was noted.

Table S10. Results of GLMM analysis between sampling conditions and detection / non-detection of captive mammals

Capybara (a)				
	Estimate	Std. Error	z value	Pr(> z)
(Intercept)	-3.2062041	0.0012466	-2571.913	< 2e-16
Distance	-0.0066054	0.0010062	-6.565	5.21E-11
Filtration volume	0.0017186	0.0005622	3.057	0.00224
Capybara (an)				
	Estimate	Std. Error	z value	Pr(> z)
(Intercept)	-3.2613788	0.001242	-2626.003	< 2e-16
Distance	-0.0060095	0.0009992	-6.015	1.80E-09
Filtration volume	0.0017155	0.0005582	3.073	0.00212

Since it was impossible to determine from which of the two captive cages the capybara eDNA migrated, statistical analysis was performed for cases (a) and (an), respectively.

Table S11. Results of GLMM analysis between number of species detected and sampling conditions for captive mammals

Term	Estimate	Std. Error	z value	Pr(> z)
(Intercept)	-0.0418725	0.7740684	-0.054	9.57E-01
Filtration volume	0.0015177	0.0009456	1.605	0.10848
Rough bark	-1.1578392	0.4455523	-2.599	0.00936
Slightly rough bark	-0.1835143	0.4910705	-0.374	0.70863

Table S12. Results of pairwise PERMANOVA comparison of mammalian species composition detected in each tree

pairs	SumsOfSqs	F.Model	R2	p.value	p.adjusted sig	monte_carlo_p
1 vs 2	0.179424	0.447675	0.06010937	0.797	1	0.774774775
1 vs 3	0.4031991	0.9759931	0.16331898	0.572	1	0.537537538
1 vs 4	0.496351	1.1862257	0.12913091	0.266	1	0.277277277
1 vs 5	0.7114163	1.9248413	0.19394177	0.112	1	0.042042042
1 vs 6	0.5650399	1.39798	0.1889678	0.261	1	0.232232232
1 vs 7	0.6749053	1.6516393	0.19090478	0.075	1	0.074074074
1 vs 8	0.4336777	1.0716057	0.15153641	0.4	1	0.437437437
1 vs 9	1.7944545	9.306685	0.50837631	0.002	0.11	<10^-9
1 vs 10	0.5904028	1.3712996	0.16380965	0.14	1	0.148148148
1 vs 11	0.4011122	0.9709416	0.16261113	0.57	1	0.594594595
2 vs 3	0.2622131	0.6028058	0.13096485	0.6	1	0.523523524
2 vs 4	0.3193369	0.7397444	0.09557737	0.661	1	0.635635636
2 vs 5	0.5631135	1.4981281	0.17628919	0.192	1	0.158158158
2 vs 6	0.436072	1.0385308	0.17198402	0.312	1	0.283283283
2 vs 7	0.6577998	1.5570699	0.20604149	0.151	1	0.152152152
2 vs 8	0.255244	0.6069835	0.10825491	0.897	1	0.85985986
2 vs 9	1.5869949	9.0062129	0.52958369	0.006	0.33	<10^-9
2 vs 10	0.461406	1.029861	0.14649806	0.35	1	0.316316316
2 vs 11	0.2688482	0.6180592	0.13383527	0.5333333	1	0.566566567
3 vs 4	0.3154154	0.6911406	0.12144149	0.621	1	0.631631632
3 vs 5	0.4814549	1.2728852	0.20291862	0.387	1	0.346346346
3 vs 6	0.406272	0.8965081	0.23007987	0.5	1	0.445445445
3 vs 7	0.5647696	1.2586734	0.23935188	0.2666667	1	0.25025025
3 vs 8	0.2343269	0.5159075	0.14673523	1	1	1
3 vs 9	1.0816515	9.6902235	0.61759627	0.032	1	0.029029029
3 vs 10	0.3675082	0.7545542	0.15870136	0.8666667	1	0.865865866
3 vs 11	0.2511728	0.5023457	0.20074991	1	1	1
4 vs 5	0.5263495	1.3270535	0.14228003	0.204	1	0.222222222
4 vs 6	0.3864367	0.8778124	0.12762959	0.537	1	0.555555556

4 vs 7	0.6223812	1.4160404	0.16825494	0.147	1	0.139139139
4 vs 8	0.2147136	0.487163	0.07509646	0.893	1	0.866866867
4 vs 9	1.5647907	7.2162474	0.44500107	0.002	1	<10 ⁻⁹
4 vs 10	0.3788091	0.8209331	0.10496614	0.636	1	0.600600601
4 vs 11	0.3053804	0.6691519	0.11803387	0.612	1	0.611611612
5 vs 6	0.4876437	1.2999729	0.17807914	0.331	1	0.292292292
5 vs 7	0.7028243	1.8316315	0.20739447	0.078	1	0.059059059
5 vs 8	0.4978577	1.3253792	0.18092978	0.262	1	0.236236236
5 vs 9	1.8514864	10.6752675	0.54257293	0.004	0.22	<10 ⁻⁹
5 vs 10	0.627962	1.5481158	0.18110609	0.127	1	0.117117117
5 vs 11	0.4649536	1.2292586	0.19733626	0.398	1	0.393393393
6 vs 7	0.4757172	1.1040985	0.18087822	0.356	1	0.354354354
6 vs 8	0.427038	0.9918863	0.19869969	0.5	1	0.485485485
6 vs 9	1.3699654	9.317204	0.57100493	0.019	1	0.009009009
6 vs 10	0.4518522	0.9789976	0.16373943	0.538	1	0.532532533
6 vs 11	0.3971028	0.8762746	0.22606103	0.5	1	0.495495495
7 vs 8	0.6562429	1.520898	0.23323445	0.048	1	0.016016016
7 vs 9	1.5548918	8.4935038	0.51496055	0.005	0.275	0.001001001
7 vs 10	0.5396987	1.1805197	0.16440588	0.282	1	0.274274274
7 vs 11	0.4988055	1.1116626	0.21747573	0.4	1	0.38038038
8 vs 9	1.2784941	8.6690347	0.55325902	0.012	0.66	0.001001001
8 vs 10	0.1841355	0.3984196	0.073803	0.862	1	0.823823824
8 vs 11	0.2728156	0.6006464	0.16681626	0.9	1	0.844844845
9 vs 10	1.3453259	6.6519961	0.45399931	0.005	0.275	<10 ⁻⁹
9 vs 11	1.081801	9.6915626	0.6176289	0.038	1	0.025025025
10 vs 11	0.4096496	0.8410773	0.17373763	0.8	1	0.805805806

Table S13. Results of permutation test for homogeneity of multivariate dispersions

Df	Sum Sq	Mean Sq	F	N. perm	Pr (>F)
10	1.0373	0.103727	3.1964	999	0.004



Figure S1. Map of Oji Zoo, Kobe City (as of April 5, 2024). Each number indicates a sampled tree. Alphabets indicate the cages where the animals are kept. *Eurasian lynx* kept in p was moved to g on September 4, 2024, and Bobcat kept in q was moved to g on August 21, 2024. The t–y area has been closed since November 8, 2024. The cages of f–j, o–r, t–ab, and ah–ak are designed with walls covering each cage, creating an environment with less air passage than other species.

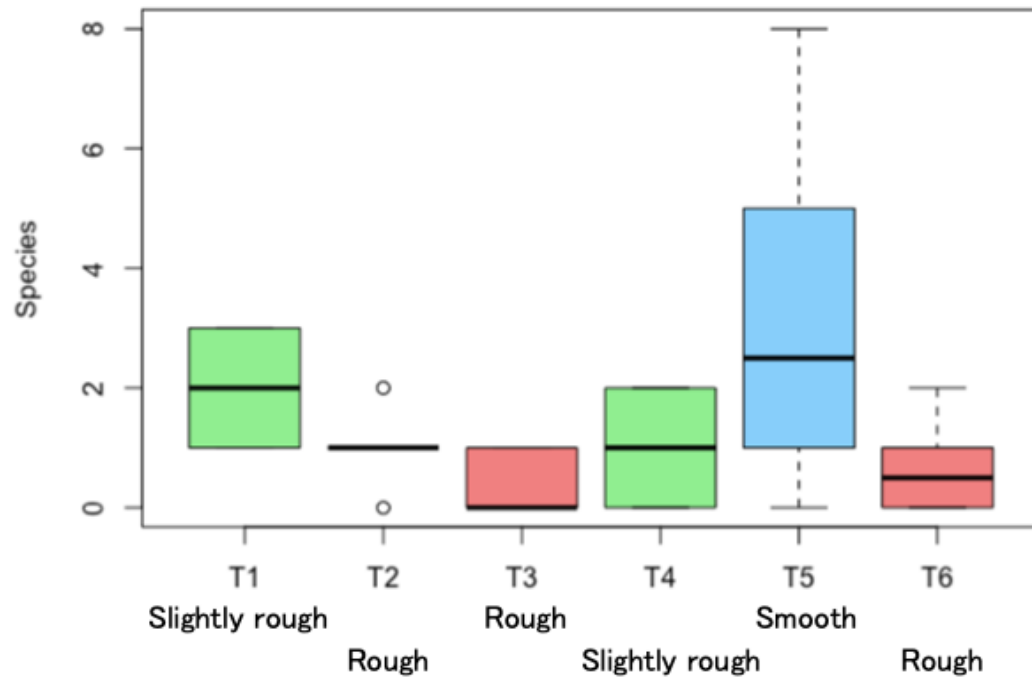


Figure S2. Number of captive mammal species detected from each tree in adjacent sets of trees. The number of captive mammal species detected in each tree for each sampling session are shown. Blue indicates smooth, green indicates slightly rough, and red indicates trees with rough bark. Box ranges first and third quartiles, and the line inside the box represents the median. The whiskers are the maximum and minimum values within 1.5 times of the box.