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Development of Species-Specific Primers for Detecting Raccoon (*Procyon lotor*) eDNA from Field Water

Hiroyoshi KOYAMA[1], Chinatsu KOZAKAI[2]*, Hiroki MATSUMURA[3] and Hiroyuki SHIBAIKE[2]

[1] Saitama Agricultural Technology Research Center, Kumagaya, Japan
[2] National Agriculture and Food Research Organization, Tsukuba, Japan
[3] Chiba Prefectural Agriculture and Forestry Research Center, Chiba, Japan

Abstract

In Japan, invasive alien raccoons (*Procyon lotor*) cause severe agricultural damage. To address this issue, it is essential to understand their distribution and ecology. Using environmental DNA (eDNA) is an efficient strategy for quickly detecting their presence and determining their distributions; however, species-specific eDNA detection methods are limited for terrestrial mammals. In this study, we developed a specific qPCR-based detection method for raccoons and tested its effectiveness through captive and field samples. Species-specific primers and probes were designed for the cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cyt*b*) regions of mitochondrial DNA. Water samples were collected from cage water and from field ponds and rivers in a raccoon habitat. For a comparison of detection sensitivity, a metabarcoding analysis targeted to mammals was performed. In captivity, raccoon eDNA was detected in cage water that had been in contact with raccoons for a short time. In field surveys, eDNA was detected at a pond where footprints were found, as well as at other sites. Additionally, the developed qPCR assay detected raccoon eDNA at a higher frequency than the metabarcoding analysis. These results indicate that the newly developed raccoon-specific detection assay is a valuable tool for eDNA monitoring.

Discipline: Agricultural Environment
Additional key words: agricultural damage by wildlife, invasive alien species, monitoring

Introduction

The raccoon (*Procyon lotor*) is native to North America (Gehrt 2003) and has become established as an introduced species outside of its native range. In Japan, this first occurred when raccoons escaped from captivity in Aichi Prefecture in 1962 (Agetsuma-Yanagihara et al. 2004). The distribution expanded from 35 prefectures in a 2007 survey to 44 prefectures in a 2015-2017 survey (Ministry of the Environment 2018). Raccoons are omnivores, and a variety of food sources, including insects, fish, amphibians, reptiles, birds, mammals, and plants, have been detected in their feces (Osaki et al. 2019). Agricultural damage by raccoons in Japan is a serious issue, with damage reported to be around JPY 414 million nationwide in FY2021 (https://www.maff.go.jp/j/seisan/tyozyu/higai/hogai_zyoukyou/index.html, Accessed on 26 June 2023). In addition to causing agricultural damage, raccoons adversely affect ecosystems (Hayama et al. 2006) and carry zoonotic diseases (Matoba et al. 2006, Hinenoya et al. 2020). For these reasons, this species is designated as a Specified Invasive Alien Species, and its appropriate management is considered crucial in resolving these issues (Act on the Prevention of Adverse Ecological Impacts Caused by Designated Invasive Alien Species: Act No. 78 of 2004).

To prevent damage and habitat expansion, methods for quickly assessing their distribution must be developed and appropriate measures, such as trapping, must be implemented. Mazzamuto et al. (2020) improved raccoon capture rates and reduced capture effort by adjusting capture locations. Several monitoring methods have been employed to confirm the distribution of raccoons. However, sighting by laypeople is not reliable due to the

*Corresponding author: kozakai.chinatsu891@naro.go.jp
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potential for misidentification. Camera trapping is an effective method, but requires an enormous amount of effort in data collection and animal species identification from images. Therefore, it is essential to develop monitoring methods that have low rates of misidentification and are highly efficient.

Environmental DNA (eDNA) has been utilized as a tool for monitoring the distributions of aquatic and semi-aquatic species in ponds, rivers, and seawater. eDNA metabarcoding analyses of mitochondrial DNA regions are also used for monitoring terrestrial animals. This approach has been employed to detect raccoons by Cannon et al. (2016) and Ushio et al. (2017). However, there are few examples of species-specific detection systems for terrestrial mammals (e.g., the North American river otter *Lontra canadensis*: Padgett-Stewart et al. 2016), and a system for raccoons has not been developed. The advantages of eDNA monitoring include the ability to efficiently survey a wide range of locations through simple water sampling and the low risk of misidentification because DNA information is used. The specific detection of raccoons using eDNA would contribute to the quick identification of their distribution. In this study, we developed a species-specific qPCR-based detection system for raccoon eDNA and tested its usefulness through captive and field studies.

Materials and methods

1. Design of species-specific primers and probes

Species-specific primers and probes were designed to amplify the cytochrome *c* oxidase subunit I (COI) and cytochrome *b* (cyt*b*) regions of mitochondrial DNA. Reference sequences for Procyonidae (COI: 7 species, cyt*b*: 13 species) were obtained from the DNA Data Bank of Japan (DDBJ) (Table 1). These sequences were aligned using ClustalW (Thompson et al., 1994) in MEGA11 (Tamura et al. 2021), and unique regions were identified through comparisons between the raccoon and other species. Species-specific primers were designed for these unique regions. The specificity of the designed primer sets was checked using Primer-BLAST, and two highly specific sets were selected (Table 2). Probes were designed for each amplified region using Edesign (Kimura et al. 2016).

2. PCR amplification test

DNAs of raccoon and 17 non-target mammals (*Martes melampus*, *Meles anakuma*, *Mustela itatsi*, *Ursus thibetanus*, *Canis lupus*, *Nyctereutes procyonoides*, *Vulpes vulpes*, *Felis catus*, *Paguma larvata*, *Sus scrofa*, *Cervus nippon*, *Capricornis crispus*, *Ondatra zibethicus*, *Mus musculus*, *Lepus brachyurus*, *Macaca fuscata*, *Homo sapiens*) were extracted from tissue fragments using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). PCR was conducted in a 20 μL reaction volume containing 10 μL of EmeraldAmp PCR Master Mix (2×Premix) (TaKaRa Bio Inc., Kusatsu, Japan), 2 μL of 2 μM Forward primer, 2 μL of 2 μM Reverse primer, 4 μL of nuclease-free water, and 2 μL of 5 ng/μL template DNA. PCR amplification was performed in Mastercycler nexus GSX1 (Eppendorf) with a thermal profile consisting of an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 40 s, with a final extension at 72°C for 7 min. PCR products were electrophoresed on a 2% agarose gel. The gels were then stained with Atlas ClearSight DNA Stain (BioAtlas).

3. Standard curve test

Plasmids containing COI and cyt*b* were generated as standard controls by gene synthesis (Eurofins Genomics, Tokyo, Japan). They were diluted to 10^1–10^6 copies/μL and used as templates for quantitative PCR (qPCR). Two qPCR methods, intercalating dye detection (IDD) and hydrolysis-based probe detection (HPD), were used with the primer set Pl_COI_F4/R3 or Pl_cyt*b*_F3/R4. In this study, the limit of detection (LOD) was defined as the minimum DNA concentration that produces a positive detection in one or more replicates (following Tréguier et al. 2014). The limit of quantification (LOQ) was defined as the minimum DNA concentration at which positive detections were recorded in all replicates (following Tréguier et al. 2014).

4. Experiment in captivity

A 2 L water bowl was placed in a raccoon cage, and the raccoon behavior was observed using a video camera. These video images were analyzed to measure the time that the raccoon's limbs and rostrum touched the water. This experiment involved three animals. A water sample was collected from each bowl using a φ0.45 μm Sterivex filter (Merck Millipore, Darmstadt, Germany), following the protocol described in Ushio et al. (2018) (Table 3). As a negative control, water samples were similarly collected before being placed in a cage. eDNA was extracted from the filters using a DNeasy Blood and Tissue Kit, following the protocol described in Miya et al. (2016). For the IDD method, the primer set Pl_COI_F4/R3 was used. For the HPD method, the primer set Pl_COI_F4/R3 or Pl_cyt*b*_F3/R4 was used.

Table 1. Procyonidae reference sequences used for primer and probe design

Family	Genus	Species	COI	Cyt*b*
Procyonidae	*Bassaricyon*	*alleni*	-	EF107710, DQ660299
		beddardi	JF459105	KX756273
		gabbii	DQ533937	EF107704, DQ660300
		neblina	-	EF107709, EF107708
		medius	-	MK144297, EF107706
	Bassariscus	*astutus*	DQ533935	AF498159
		sumichrasti	-	DQ660301
	Nasua	*narica*	DQ533934, JF446036	DQ533940, DQ660302
		nasua	KF771221, HM106331	GQ214530, KU253485
	Nasuella	*olivacea*	-	GQ169038, GQ169039
	Potos	*flavus*	DQ533936, MK990569, JF459232, JF459231, MW257234	KX756246, MW257234
	Procyon	*cancrivorus*	-	AB564099, KT626625, DQ660305
		lotor	AB462046, AB462049, AB462048, AB462047, AB462045, AB297804, MT410951, AB291073, AM711899, AB462066, AB462180, AB462142, AB462218, AB462104, JF443368, JF443366, JF443367, JF443369, AH014079	AB462123, AB462161, AB462237, AB462199, AB462085, GU175439, KX357307, KX357310, KX357313, KX357316, KX357319, KX357322, KX357325, KX357328, KX357331, KX357334, KX357337, KX357340, KX357308, KX357311, KX357314, KX357317, KX357320, KX357323, KX357326, KX357329, KX357332, KX357335, KX357338, KX357341, KX357306, KX357309, KX357312, KX357315, KX357318, KX357321, KX357324, KX357327, KX357330, KX357333, KX357336, KX357339, KX357342, DQ660306

Table 2. Primers and probes used in this study

Name	Property	Sequence	PCR product size
Pl_COI_F4	Forward primer	5′-GTGCCATCAACTTCATCACC-3′	
Pl_COI_R3	Reverse primer	5′-GCTAGTACTGGCAGCGATAATAAG-3′	130 bp
Pl_COI_F4/R3	Probe	5′-FAM-CCCCGCTATATCACAATACCAAATCCCA-TAMRA-3′	
Pl_cyt*b*_F3	Forward primer	5′-ACACACCCGCTAACCCCT-3′	
Pl_cyt*b*_R4	Reverse primer	5′-GGTATGTAGGAGTGGAATGATGATT-3′	161 bp
Pl_cyt*b*_F3/R4	Probe	5′-FAM-AGTTTGTTGGGAATGGAACGTAGAATTGC-TAMRA-3′	

Table 3. Breeding cage water sampling volume and sample details

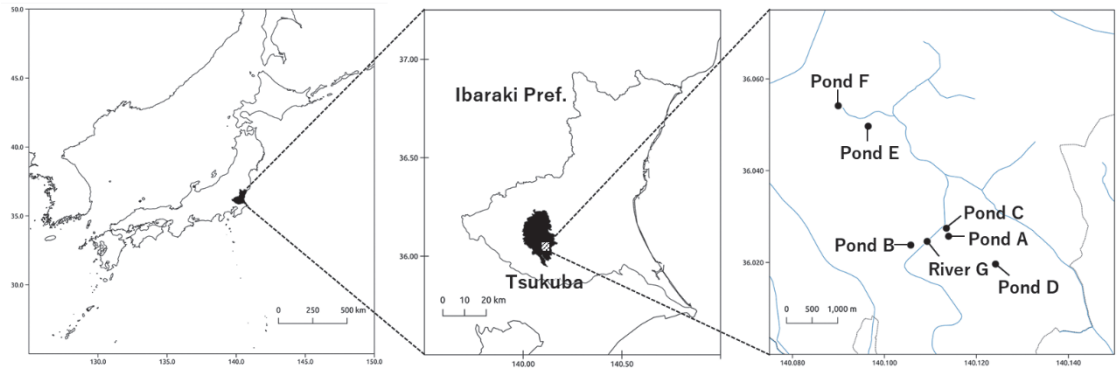
Sample name	Sample volume (mL)	Total time in contact with water (hh:mm:ss)	Elapsed time after placement (hh:mm)	Notes
R002F_1	500	00:00:55	2:30	Rostrum contact.
R002F_2	500	00:00:55*	18:00	Same water as R002F_1 The water contained bait fragments.
R004M_1	15	00:01:19*	18:00	Rostrum, buttock, and forelimb contact. The water contained feces.
R005M_1	500	00:00:20*	18:00	Rostrum contact. The water contained body hairs.

*: Recording was stopped 13-15 h after installation. The time of confirmation in the movie was noted.

5. Field survey

Water samples were collected from field ponds (A-F) and a river (G) in a raccoon habitat using a φ0.45 µm Sterivex filter (Merck Millipore, Darmstadt, Germany), following the protocol described in Ushio et al. (2018) (Figs. 1, 2; Table 4). During sampling, the condition of the revetment around the sampling site and the presence or absence of mammal tracks were visually checked. eDNA was extracted from the filters using a DNeasy Blood and Tissue Kit, following the protocol described in Miya et al. (2015). Two qPCR analyses, IDD and HPD, were performed using the primer sets Pl_COI_F4/R3 or Pl_cyt*b*_F3/R4. If at least one of the three technical replicates showed an exponential amplification curve, the sample was considered positive (following Rees et al. 2014). To confirm the specificity of each primer set in the field sample, positive PCR products from the IDD method were subjected to Sanger sequencing and compared with the reference sequence of the raccoon.

a)



b)

Fig. 1. Environmental DNA (eDNA) sampling sites in Tsukuba, Japan

6. Quantitative PCR

qPCR using the IDD method was conducted in a 20 µL reaction volume containing 10 µL of TB Green Premix Ex Taq (Tli RNaseH Plus) (2×) (TaKaRa Bio Inc.), 3µL of 2 µM Forward primer, 3 µL of 2 µM Reverse primer, 0.4 µL of ROX Reference Dye (50×), 1.6 µL of nuclease-free water, and 2 µL of DNA template. qPCR analyses were performed using Quant Studio 3 (Applied Biosystems, Foster City, CA) with a thermal profile consisting of an initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, and annealing & extension at 60°C for 30 s. Melting curve analysis was performed after qPCR. A sample was considered positive if a sample melting peak was included within ± 0.5°C of the standard plasmid single melting peak.

qPCR using the HPD method was conducted in a 20 µL reaction volume containing 10 µL of TaqMan Environmental Master Mix 2.0 (2×), 3 µL of 2 µM Forward primer, 3 µL of 2 µM Reverse primer, 0.5 µL of 10 µM Probe, 0.08 µL of ROX Reference Dye (50×), 1.42 µL of nuclease-free water, and 2 µL of DNA template. qPCR analyses were performed using a Quant Studio3 (Applied Biosystems) or Stratagene Mx3000P with a thermal profile consisting of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing & extension at 60°C for 1 min.

Fig. 2. Raccoon footprints at an eDNA sampling site (Pond D)

7. Metabarcoding analysis

eDNA samples that were positive by qPCR were subjected to a metabarcoding analysis using MiMammal-U/E/B mixed primers (Ushio et al. 2017). The metabarcoding analysis was contracted to the Bioengineering Lab. Co., Ltd. (Kanagawa Pref., Japan) and was performed as follows. Preparation of the metabarcoding library was performed according to a two-step PCR protocol, with the first PCR amplification of the target fragments using MiMammal-U/E/B mixed primers and second PCR addition of the index and sequencing adapters. Briefly, paired-end sequencing was performed using Miseq (Illumina) for 2 × 300 bp. The obtained sequences with Phred scores < 20 and lengths < 40 bases were removed using Sickle (ver. 1.33). Reads were joined with a minimum overlap of 10 bases using FLASH (ver. 1.2.11). Chimera and noise sequences were removed using the dada2 plugin from Qiime2 (ver. 2021.11). The processing sequences were assembled into amplicon sequence variants. Representative sequences from each amplicon sequence variant were subjected to

Table 4. Field water sampling volume and sample details

Water sampling location	East longitude	North latitude	Sample volume (mL)		Revetment	Notes
			1st	2nd		
Pond A	36.025705	140.113937	500	500	Concrete revetment	
Pond B	36.023796	140.105724	500	500	Concrete revetment	
Pond C	36.027466	140.113465	500	500	No revetment (Soil)	
Pond D	36.019661	140.124151	150	150	No revetment (Soil)	Raccoon footprints were found near the sampling point.
Pond E	36.049680	140.096465	265	320	Crushed stone revetment	Animal feces were found near the sampling point.
Pond F	36.054134	140.089953	500	500	Concrete revetment	
River G	36.024594	140.109292	500	500	Concrete revetment	

BLASTN searches against the NCBI nt database. The top BLAST hit with at least 97% sequence identity was used for species assignment of each representative sequence.

Results

1. Confirmation of amplified products generated using each primer set

For each primer set, a single DNA fragment of approximately 100-200 bp was amplified from the raccoon template DNA only (Fig. 3). No DNA fragments were observed in the DNA of the 17 non-target mammalian species or the blank control template.

2. Comparison of each primer set and detection methods

DNA amplification was confirmed in all replicates of 10^2–10^6 copies/µL samples with each primer set and detection method (Table 5). For 10^1 copies/µL, DNA amplification was confirmed in 2-5 of 8 replicates with each primer set and detection method. Following Tréguier et al. (2014), the LOD and LOQ in these assays were 10^1 and 10^2 copies/µL, respectively.

3. Detection from cage water in captivity

In captivity, each animal touched water in the bowl (Table 3). The contact time ranged from 20 s at the shortest to 1 min 19 s at the longest. We detected the eDNA of raccoons in all water samples, except the negative control, using each primer set and detection method (Table 6). Using Pl_COI_F4/R3 and the HPD method, 1 of the 3 replicates of R002-F1 was excluded owing to a lack of exponential amplification.

4. eDNA detection from field ponds or rivers in a raccoon habitat

Raccoon footprints were observed at Pond D but not at the other six sites (Table 4). In Pond D-1, Pond D-2, and Pond F-1, raccoon eDNA was detected using each primer set and detection method (Table 7). In River G-1 and G-2,

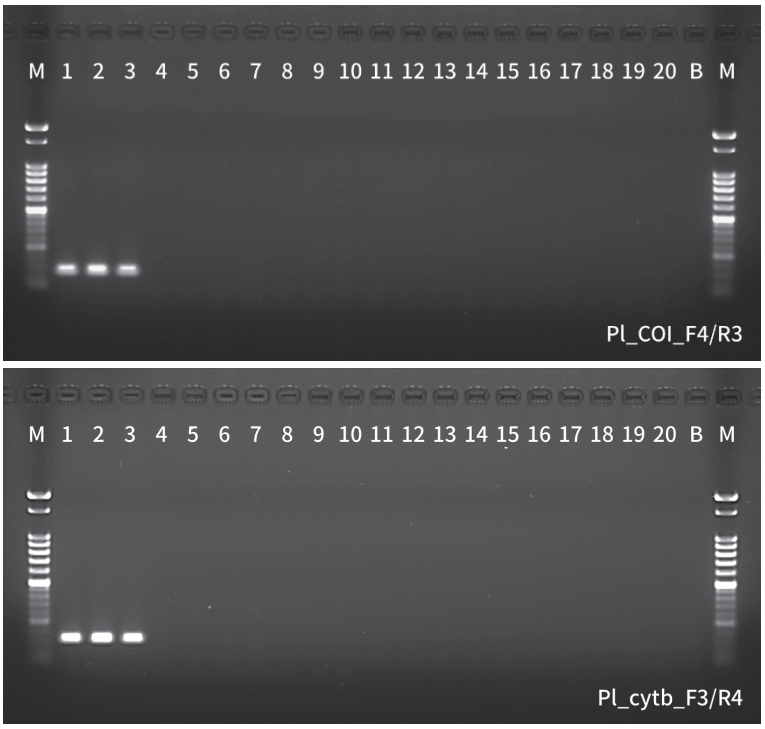


Fig. 3. PCR amplification using raccoon (*Procyon lotor*)-specific primer sets

M: 50bp DNA Ladder, 1-20: DNA sample of mammals (1-3: *Procyon lotor*, 4: *Martes melampus*, 5: *Meles anakuma*, 6: *Mustela itatsi*, 7: *Ursus thibetanus*, 8: *Canis lupus*, 9: *Nyctereutes procyonoides*, 10: *Vulpes vulpes*, 11: *Felis catus*, 12: *Paguma larvata*, 13: *Sus scrofa*, 14: *Cervus nippon*, 15: *Capricornis crispus*, 16: *Ondatra zibethicus*, 17: *Mus musculus*, 18: *Lepus brachyurus*, 19: *Macaca fuscata*, 20: *Homo sapiens*), B: Blank (Nuclease-Free Water)

eDNA was detected only using the HPD method. The DNA sequences of the amplification products detected by the IDD method were identical to the raccoon reference sequence.

5. Metabarcoding analysis

In total, 284,526 raw sequence reads were generated from five samples: Pond D-1, Pond D-2, Pond F-1, River G-1, and River G-2 (Table 8). The first PCR attempt for Pond F-2 was unsuccessful, and no reads were obtained. In total, 237,264 sequence reads remained following trimming, merging, and length filtering (Table 8). After a bioinformatic analysis, mammalian sequences were confirmed in Pond D-1, Pond F-1, and River G-2 (Table 9). Raccoon sequences were detected only in Pond F-1 and accounted for approximately 3% of the total reads. In addition to sequences for mammals, those from fish, birds, and bacteria were detected at most sites.

Table 5. Standard curve analysis using artificially synthesized plasmids

Primer set	Pl_COI_F4/R3											
Detection methods	IDD						HPD					
Template concentration (copies/μL)	10^1	10^2	10^3	10^4	10^5	10^6	10^1	10^2	10^3	10^4	10^5	10^6
qPCR results	3/8	8/8	8/8	4/4	4/4	4/4	5/8	8/8	8/8	4/4	4/4	4/4
Cq mean	36.3	35.4	31.2	27.2	24	20.3	38	35.3	32.2	28.9	25.3	21.8
SD	0.356	1.185	0.401	0.309	0.226	0.293	0.686	0.64	0.536	0.468	0.444	0.522
Standard curve	$y = -3.52\log_{10}(x) + 41.6$						$y = -3.27\log_{10}(x) + 41.7$					
Coefficient of determination	$R^2 = 0.970$						$R^2 = 0.988$					
Amplification efficiency	0.943						1.017					
Primer set	Pl_cyt*b*_F3/R4											
Detection methods	IDD						HPD					
Template concentration (copies/μL)	10^1	10^2	10^3	10^4	10^5	10^6	10^1	10^2	10^3	10^4	10^5	10^6
qPCR results	2/8	8/8	8/8	4/4	4/4	4/4	3/8	8/8	4/4	4/4	4/4	4/4
Cq mean	35	33.4	29.4	25.7	22.2	18.6	35.8	33.9	30.4	26.6	23.9	19.7
SD	0.769	0.853	0.065	0.13	0.126	0.056	1.122	0.983	0.735	1.049	0.435	0.67
Standard curve	$y = -3.54\log_{10}(x) + 40.0$						$y = -3.33\log_{10}(x) + 40.2$					
Coefficient of determination	$R^2 = 0.986$						$R^2x = 0.973$					
Amplification efficiency	0.938						0.997					

SD, standard deviation; IDD, intercalating dye detection method; HPD, hydrolysis-based probe detection method
The denominator for "qPCR results" represents the number of PCR replicates, and the numerator represents the number of detections.
QuantStudio 3 was used for the IDD method, and the Stratagene Mx3000P was used for HPD.

Table 6. Environmental DNA detection from breeding cage water

	Number of detections (n = 3) [eDNA concentration (copies/L)]					
	Pl_COI_F4/R3				Pl_cyt*b*_F3/R4	
	IDD		HPD		HPD	
R002F-1	3	[6.9×10^6]	2*	[8.1×10^6]	3	[2.1×10^6]
R002F-2	3	[4.2×10^6]	3	[2.8×10^6]	3	[1.9×10^6]
R004M-1	3	[6.9×10^7]	3	[9.1×10^7]	3	[4.2×10^7]
R005M-1	3	[4.2×10^6]	3	[8.7×10^5]	3	[1.6×10^6]

*: One PCR replicate was excluded because an exponential amplification curve was not obtained.
No eDNA was detected in the negative control.

Discussion

The LOD and LOQ for each detection system were verified through a standard curve analysis using standard control plasmids. For each detection method, the LOD and LOQ were 10^1 copies/μL and 10^2 copies/μL (Table 5), respectively. Williams et al. (2018) demonstrated that eDNA can be detected in artificial wallow water after only 15 min of exposure by a single pig. Similarly, we demonstrated that eDNA can be detected in cage water that was in contact with the rostrum, buttocks, and limbs for a short time (Table 7). Raccoons use waterside areas for feeding and drinking. Therefore, hair, feces, and saliva released into water during feeding and drinking are sources of eDNA, providing a basis for detecting raccoons in field samples.

Raccoon eDNA was detected not only in Pond D, where footprints were found, but also in Pond F and River G through the qPCR analysis. Therefore, the presence of raccoons can be estimated by eDNA detection using the developed qPCR method. In contrast, raccoon eDNA was not detected in Ponds A, B, C, or E. In Ponds A and B, where raccoon DNA was not detected, the surface of the water is far below the bank (Fig. 1b). That is, even if raccoons were present in the surrounding area, eDNA would not be detected because raccoons could not touch the water. In ponds C, D, E, and F, the water surface is

Table 7. Environmental DNA detection from field water by qPCR

	Number of detections (n = 3) [eDNA concentration (copies/L)]							
	Pl_COI_F4/R3				Pl_cyt*b*_F3/R4			
	IDD		HPD		IDD		HPD	
Pond A-1	0	[ND]	0	[ND]	0	[ND]	0	[ND]
-2	0	[ND]	0	[ND]	0	[ND]	0	[ND]
Pond B-1	0	[ND]	0	[ND]	0	[ND]	0	[ND]
-2	0	[ND]	0	[ND]	0	[ND]	0	[ND]
Pond C-1	0	[ND]	0	[ND]	0	[ND]	0	[ND]
-2	0	[ND]	0	[ND]	0	[ND]	0	[ND]
Pond D-1	3	[1.0×10^4]	1	[5.4×10^3]	3	[4.4×10^4]	3	[2.9×10^4]
-2	3	[3.8×10^3]	1	[5.2×10^3]	2	[1.4×10^4]	2	[7.9×10^3]
Pond E-1	0	[ND]	0	[ND]	0	[ND]	0	[ND]
-2	0	[ND]	0	[ND]	0	[ND]	0	[ND]
Pond F-1	2	[3.4×10^3]	1	[1.5×10^3]	2	[7.1×10^3]	1	[3.3×10^3]
-2	0	[ND]	0	[ND]	0	[ND]	0	[ND]
River G-1	0	[ND]	1	[1.7×10^3]	0	[ND]	1	[1.5×10^4]
-2	0	[ND]	1	[8.5×10^2]	0	[ND]	0	[ND]

IDD, intercalating dye detection method; HPD, hydrolysis-based probe detection method; ND, not detected

Table 8. Metabarcoding analysis using MiMammal from eDNA in field water

	Sequencing results			Filtering results		
	Number of raw reads	Q20(%)	Q30(%)	Number of reads	Number of ASVs	Number of mammalian species
Pond D-1	77,250	98.2	95.2	64,950	15	2
-2	6,053	97.1	92.9	3,736	7	0
Pond F-1	73,721	97.1	92.4	62,396	27	3
-2	-	-	-	-	-	-
River G-1	58,874	95.4	89.1	48,299	34	0
-2	68,628	96.4	91.2	57,883	30	1

ASV, amplicon sequence variant

closer to the bank and the conditions are similar to those of their respective revetments, although eDNA was detected in only two of these ponds. Even if raccoons were present at these sites, eDNA at ponds C and E may have been degraded, and concentrations may have been below the LOD. Thomsen et al. (2012) reported that eDNA could no longer be detected 1-2 weeks after the animals were removed from artificial water. Williams et al. (2018) reported that eDNA concentrations in samples decreased after pigs were removed from an artificial wallow. The relationship between the time since use by animals and the eDNA concentration needs to be verified in the future.

Using the IDD method, the observation of a single peak in the melting curve analysis indicated valid amplification. The River G sample was considered an invalid detection because it also identified a peak different from the single melting peak of the standard plasmid sample. River G samples likely contained DNA responsible for nonspecific amplification in addition to raccoon-derived DNA. The HPD method is more effective for raccoon detection from eDNA containing diverse DNAs because it has higher specificity for detecting the amplification of the target product than the IDD method.

MiMammal was designed as an improvement of MiFish (Miya et al. 2015), targeting the 12S rRNA region, and has been used to detect fish and birds (Yonezawa et al. 2020). Similarly, fish and bird sequences were detected in this study, in addition to mammals (Table 9). The detection sensitivity of qPCR is comparable to or higher than that of metabarcoding (Schneider et al. 2016, Harper et al. 2018). Similarly, in this study, eDNA was detected at some sites by qPCR but not by metabarcoding. For example, in Pond D-1, which had a high estimated template concentration in the qPCR analysis, the majority of the reads were from bovine sources, and no raccoon reads were obtained (Table 9). This suggests that when a sample contains large amounts of DNA from non-target organisms that MiMammal amplifies, it becomes difficult to compare the estimated template concentration in qPCR analysis with the number of reads in metabarcoding analysis.

Conclusion

For this study, we developed raccoon-specific primers and probes for detecting eDNA. Using the qPCR method, raccoon eDNA was detected at a pond where footprints were found, as well as at other sites. The developed qPCR assay detected raccoon eDNA at a higher frequency than the metabarcoding analysis targeting mammals. These results suggest that the proposed raccoon-specific detection assay is a valuable tool for eDNA monitoring. In addition, eDNA was detected using both IDD and HPD methods, with the HPD method proving particularly effective in avoiding the effects of nonspecific amplification in field surveys. These results suggest that the HPD method is effective for field surveys.

Table 9. Number of sequence reads detected from eDNA of field water

	Pond D-1	Pond D-2	Pond F-1	River G-1	River G-2
Total reads	64,950	3,736	62,396	48,299	57,883
Mammals	64,450	0	45,433	0	33,617
Procyon lotor	**0**	**0**	**2,043**	**0**	**0**
Bos taurus	60,769	0	0	0	0
Homo sapiens	3,681	0	41,774	0	33,617
Mus musculus	0	0	1,616	0	0
Birds	316	3,551	190	232	4,546
Fishes	0	0	2,578	16,221	7,992
Microorganisms*	184	114	14,034	31,164	5,359
Others**	0	71	161	682	6,369

*: Includes uncultured bacterium, prokaryote, stramenopile, and *Pavlova* sp.
**: Amplicon sequence variant reads showing < 97% homology.

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