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(Citation)
Landscape and Ecological Engineering,19(1):3-10

(Issue Date)
2023-01

(Resource Type)
journal article

(Version)
Accepted Manuscript

Development of environmental DNA detection assays for snakes in paddy fields in Japan

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Abstract

Paddy ecosystems are among the most biodiversity-rich ecosystems, but they can be degraded by field consolidation or the abandonment of rice cultivation. Snakes are the top predators in paddy ecosystems; therefore, understanding the distribution of snakes is important for the conservation of paddy biodiversity. Traditionally, surveys of snakes have been primarily conducted by sighting and trapping, but such methods require encountering snakes during surveys and require expertise in species identification. In this study, we developed environmental DNA (eDNA) detection assays for snakes as a method for surveying a wide area relatively easily. We designed species-specific assays for three common snake species in Japanese paddy ecosystems: *Elaphe quadrivirgata*, *Rhabdophis tigrinus*, and *Gloydius blomhoffii*. After confirming the detection of target species DNA from the rearing environment, the assays were applied to field samples. Eighty-seven and 51 samples were collected from paddy fields, reservoirs, and ditches in summer and autumn, respectively. We collected eDNA samples from eight traditional paddy areas that had not been consolidated, eight consolidated paddy areas, and two abandoned paddy areas in southeastern Hyogo Prefecture, Japan. We attempted to detect the DNA of the three target species in these samples using our eDNA assays and succeeded in identifying the DNA of *R. tigrinus* and *G. blomhoffii* in three areas and one area, respectively. Statistical analysis suggested that land-use type affected the detection of *R. tigrinus* eDNA. This study is the first attempt to survey snakes in paddy ecosystems using eDNA analysis and will contribute to the development of paddy ecosystem monitoring.

Keywords: environmental DNA; paddy field ecosystems; real-time PCR; snake

Introduction

Paddy ecosystems support rich wetland biodiversity by providing habitats for plants, fish, reptiles, mollusks, crustaceans, and insects (Natuhara 2013; Ramsar Convention 2008). Paddy ecosystems, which are human-made landscape complexes composed of paddy fields, irrigation ponds, ditches, semi-natural grasslands on levees, and adjacent secondary (*Satoyama*) forests, are among the most biodiverse ecosystems in Japan and other monsoon Asian countries (Hidaka 1998; Ramsar Convention 2008; Katoh et al. 2009). However, land-use changes, such as field consolidation and abandonment, have led to the rapid degradation of paddy ecosystems in terms of biodiversity (Uematsu et al. 2010).

Along with the degradation of paddy ecosystems, populations of paddy-associated snakes, which are often top predators in these ecosystems, have declined throughout Japan (Hasegawa 1995). Assessing the habitat availability for snakes after land-use changes is essential for understanding the status of paddy ecosystem health, but detailed distributions of snakes among different types of paddy fields have not yet been described. To date, the main methods of surveying snakes have been based on observation, capture, and the collection of shed skins (Kadowaki 1996). However, quantitative surveys using these physical survey methods are difficult to conduct because encounters with snakes during surveys are accidental and infrequent because of the cryptic nature of most snake species (Durso et al. 2011).

Recently, environmental DNA (eDNA) analysis has been developed as a method for estimating the habitat of macroorganisms (Taberlet et al. 2012). Environmental DNA analysis enables the monitoring of target organisms by detecting and analyzing DNA fragments, which are thought to be derived from excreta, cell fragments, and gametes of

66 macroorganisms, in environmental medium, such as water and soil (Ficetola et al. 2008;
67 Barnes & Turner 2016). This method has been mainly used in aquatic environments such
68 as oceans (Thomsen et al. 2012a), rivers (Minamoto et al. 2012), lakes (Takahara et al.
69 2013), and marshes (Ficetola et al. 2008), to target a variety of aquatic organisms, such
70 as fish (Jerde et al. 2011; Minamoto et al. 2012), amphibians (Ficetola et al. 2008;
71 Goldberg et al. 2011), insects (Bista et al. 2017; Thomsen et al. 2012b), and aquatic plants
72 (Fujiwara et al. 2016; Scriver et al. 2015). Environmental DNA analysis has also been
73 reported for turtles in lentic and lotic systems (Kakuda et al. 2019; Kessler et al. 2020).
74 The earliest studies on snake eDNA were performed on large snakes, such as the Burmese
75 python (Piaggio et al. 2014; Hunter et al. 2015). More recently, similar studies of smaller
76 snakes and very low-density species have been reported (Baker et al. 2018; Ratsch et al.
77 2020; Katz et al. 2021). However, no studies have been reported on snakes in paddy field
78 ecosystems.
79 The purpose of this study was to investigate whether the eDNA analysis method is
80 effective for surveying snake populations in paddy ecosystems. First, we established
81 species-specific eDNA detection assays for *Elaphe quadrivirgata*, *Rhabdophis tigrinus*,
82 and *Gloydius blomhoffii*, which are widely distributed in Japanese paddy fields. Next, we
83 conducted eDNA surveys for these three snake species in traditional and altered paddy
84 ecosystems, including water-filled paddy fields, adjacent ponds, and ditches in Hyogo
85 Prefecture, Japan. Finally, the effects of environmental factors on eDNA detection were
86 examined.
87
88 **Materials and Methods**
89 Development of specific assays for three snake species

DNA sequences of mitochondrial cytochrome b (*Cytb*) genes of three target snake species (*E. quadrivirgata*, *R. tigrinus*, and *G. blomhoffii*) and three non-target snake species (*Elaphe climacophora*, *Hebius vibakari*, and *Euprepiophis conspicillata*) that coexist in the survey area were downloaded from the National Center for Biotechnology Information (NCBI) (Table 1). For each species, the sequences of the target and non-target species were aligned, and primer regions containing bases specific to the target species within five bases of the 3'-ends were searched by eye. Then, a probe containing nucleotides specific to the sequence of each target species was designed using Primer Express software (version 3.0; Applied Biosystems) (Table 2).

We validated the assays in vitro using DNA from the target species and from two sympatric species to assess the specificity of the designed primers and probes. DNA was extracted from the shed skins of the target species (*E. quadrivirgata*, *R. tigrinus*, and *G. blomhoffii*) and from the tissues of two coexisting species (*E. climacophora* and *H. vibakari)*. The shed skins were purchased from the Japan Snake Institute, and tissue samples of *E. climacophora* and *H. vibakari* were provided by Dr. Yasuoki Takami (Graduate School of Human Development and Environment, Kobe University). We extracted DNA from these tissues and shed skins using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Each sample was diluted in TE buffer (10 mM Tris HCl [pH 8], 1 mM EDTA) to obtain a 50 pg/μL shed skin or tissue-derived DNA sample. Real-time polymerase chain reaction (PCR) was performed to test the specificity of the three primer sets using shed skin or tissue-derived DNA as templates using a CFX96 real-time PCR analysis system (Bio-Rad, Hercules, CA, USA). We also conducted experiments to determine the limit of quantification (LOQ) and limit of detection (LOD) for each assay using 100 pg/reaction to 1 fg/reaction of the

target species DNA as templates. The reagent composition of the PCR reaction solution (20 μL) was 1× TaqMan Environmental Master Mix 2.0, 900 nM of each primer, 125 nM of each probe, 0.1 μL of AmpErace Uracil N-glycosylase, 2 μL of DNA sample, and ultrapure water. The real-time PCR conditions were as follows: 2 min at 50°C, followed by 10 min at 95°C, and 55 cycles of 15 s at 95°C and 1 min at 60°C.

eDNA detection in the rearing environment

To test whether snake eDNA can be detected in environmental samples, we conducted in-house testing of water from the rearing environments of *E. quadrivirgata* and *G. blomhoffii*. Because the three target species share similar ecological traits (Kadowaki 1996), testing for two species was considered sufficient, and so we did not conduct this analysis for *R. tigrinus*. Water samples were collected from the Japan Snake Institute in Ota City, Gunma Prefecture, Japan, on August 19, 2019. Water samples were collected from three puddles (Fig. S1-A) in an *E. quadrivirgata* release area and three plastic cases in the rearing cages of *E. quadrivirgata* (Fig. S1-B). Water was also collected from three plastic cases in the rearing cages of *G. blomhoffii*. One liter of tap water was also sampled as a field blank. One milliliter of 10% benzalkonium chloride solution was added to each sample to prevent eDNA degradation (Yamanaka et al. 2017), and the samples were sent to Kobe University under refrigeration (~5 °C) on the same day.

The next day, the collected water samples and field blank samples were filtered using a glass fiber filter (GF/F, Cytiva, Tokyo, Japan). Pure water was filtered under the same conditions and used as a filtration blank. The water sampling and filtration volumes are presented in Table 3. After filtration, the filter paper of each filtered sample was wrapped with aluminum foil and stored at −20 °C until DNA extraction. DNA extraction from the

filters was performed using a centrifugal elution device (Salivette, Sarstedt, Nümbrecht, Germany) and an eDNA extraction method using the DNeasy Blood & Tissue Kit (Minamoto et al. 2019; The eDNA Society, 2019). Each filter was placed in a Salivette tube (Sarstedt) and a solution of 400 µL Buffer AL (Qiagen) and 40 µL Proteinase K (Qiagen) were added to the filter before incubation at 56 °C for 30 min. DNA extraction was performed according to the manufacturer's instructions. The extracted DNA was eluted with 100 µL of Buffer AE (Qiagen) and stored at −20 °C until qPCR analysis. The extraction solution was stored at −20 °C.

Real-time PCR was performed on the eDNA extracted from the rearing environments of *E. quadrivirgata* and *G. blomhoffii* using assays specific for each species (Table 2). The field and filtration blanks were treated in the same manner as the samples. A positive control was prepared by adding 0.5 pg/µL of the target species DNA instead of the DNA sample, and three replicates of PCR negative controls were prepared by replacing the DNA sample with the same amount of ultrapure water.

Field surveys

We conducted field surveys in traditional, consolidated, and abandoned paddy areas. Traditional paddy areas have been managed using traditional methods and generally consist of small, irregularly shaped, and poorly drained paddy fields (Uematsu et al. 2010; Uchida & Ushimaru 2014). Extensive traditional field management is known to support higher biodiversity levels than modern intensive field management (Tscharntke et al. 2005; Uchida & Ushimaru 2014). Consolidated paddy areas are composed of large, quadrangular, well-drained fields to improve productivity and allow mechanized farming (Fukamachi et al. 2005; Yamaguchi et al. 1998; Uematsu et al. 2010). Abandoned paddy

areas are old fields that have been abandoned due to aging or depopulation of farmers (Fukamachi et al. 2005). The management of paddy levees varies among the three paddy area types, and both intensive and no (or less) levee management in consolidated and abandoned paddy areas, respectively, have caused lower plant and insect diversity than in traditional paddy areas (Fukamachi et al. 2005; Uchida & Ushimaru 2014).

In June and July 2018 (summer survey) and November 2018 (autumn survey), we conducted field surveys in paddy ecosystems in southeastern Hyogo Prefecture. A total of 18 areas (Areas 1–18) were defined for further study: eight traditional paddy areas, eight consolidated paddy field areas, and two abandoned paddy field areas (Fig. 1, Table S1). Two paddy field samples, two pond samples, and a single ditch water sample were collected from each study area for each survey. Pond samples were collected from two different ponds in Areas 1, 7, and 9, and at two distant points in a single pond in the other 15 areas. For paddy samples, two paddy fields in each area were arbitrarily selected, and irrigation water was collected from the levees. The water was collected from either vegetated soil or concrete ditches that connected the ponds and fields. Paddy field samples could not be collected during the autumn survey, which took place after rice harvesting or when waterlogging was not observed owing to abandonment.

At each site, 1 L of water was collected in plastic bottles, and 1 mL of 10% benzalkonium chloride solution was added to the water samples on site to avoid DNA degradation (Yamanaka et al. 2017). The water samples were transferred to the laboratory using a cooler box and filtration was performed within 6 h of water collection. We used 1 L of purified water as a field blank for each study area, added 1 mL of 10% benzalkonium chloride solution in the field, and stored it in the same box as the other samples. At the same time, water temperature, pH, dissolved oxygen (DO),

electrical conductivity (EC), and turbidity were measured at each site using a multi-item water quality meter (WQC-24, DKK-TOA, Tokyo, Japan). Filtration, DNA extraction, and real-time PCR of eDNA samples from *E. quadrivirgata*, *R. tigrinus*, and *G. blomhoffii* were performed using the methods described above in the section "eDNA detection in the rearing environment." Samples amplified by real-time PCR were subjected to direct sequencing to confirm whether the amplified DNA sequence was of the target species.

Statistical Analysis

We used a generalized linear mixed model (GLMM) using the glmer function with binomial distribution in the lme4 package to assess the relationships between snake eDNA detection and environmental variables and to explore the factors that influence the distribution of *R. tigrinus* in rice paddy ecosystems. However, statistical analysis could not be applied to the *E. quadrivirgata* and *G. blomhoffii* data because of the small number of detections. The response variable was the detection/non-detection (1/0) of snake eDNA at each sampling site, and the explanatory variables were land use, type of paddy field (traditional, consolidated, and abandoned), habitat type (paddy field, pond, and ditch), water temperature (water temperature at each water sampling point [°C]), and elevation (elevation of a representative point in each area (m)), and the random effects were the water sampling point and season (summer/autumn). The Akaike information criterion (AIC) was used to select the model with the best explanation and the lowest AIC using the R dredge function in the MuMIn package. We also conducted the Wilcoxon rank-sum test to compare the elevation of representative points in two groups: study areas in which eDNA of *R. tigrinus* was detected and not detected. The

area where eDNA was detected in one or more replicates as a result of real-time PCR was defined as "area where eDNA was detected." All statistical analyses were performed using R version 3.5.1 (R Core Team 2018).

Results

Development of eDNA assays and detection in the rearing environment

Specificity tests using shed skins of the target species confirmed the amplification of DNA of the target species in all three assays. In addition, no amplification was observed for DNA of non-target species. The LOQ and LOD were determined for each assay. The lowest concentration of template for which all replicates were positive was defined as the LOQ, and the lowest concentration of template for which any of the replicates was positive was defined as the LOD. The LOQ and LOD of assays for *E. quadrivirgata*, *R. tigrinus*, and *G. blomhoffii* were 100 and 10 fg/reaction, 10 and 10 fg/reaction, and 10 and 10 fg/reaction, respectively. The PCR efficiency for each assay was between 99.2% and 106.0%, and the R^2 values of the regression curves were >0.99 for all assays (Table S2).

Amplification of target species eDNA was confirmed in all samples from the rearing environments of *E. quadrivirgata* and *G. blomhoffii* (Table 3). No amplification was observed in the field, filtration blanks, or PCR negative controls. The sequences of the PCR products matched those of each target species. Real-time PCR using eDNA from an *E. quadrivirgata* release area as a template resulted in the following mean Cq values: 36.44 (Puddle 1), 36.83 (Puddle 2), and 31.66 (Puddle 3). The average Cq value of the three puddle samples, 34.98, corresponds to 0.97 pg/reaction according to the regression curve. Considering that the release area of 207 m^2, houses approximately 30

234 *E. quadrivirgata* individuals and the LOD of the assay (10 fg/reaction), the detection
235 limit in the field can be estimated to be 1,494 individuals/km^2. The validity of the three
236 assays generally met level 4 of the index proposed by Thalinger et al. (2021).
237
238 Field surveys
239 The water quality parameters during the summer and autumn surveys are shown in
240 Tables S3 and S4, respectively. During the summer surveys, positive eDNA signals for
241 *R. tigrinus* were detected in two paddy fields and in ditches in Areas 1, 3, and 5, all of
242 which were traditionally managed paddy field areas (Fig. 1, Table S5). We also detected
243 eDNA from *G. blomhoffii* at four sampling points: a paddy field, two ponds, and a ditch
244 point in Area 15, a traditionally managed paddy field area (Fig. 1, Table S5). During the
245 autumn survey, we successfully detected *R. tigrinus* eDNA in ponds in two traditionally
246 managed paddy field areas (Areas 3 and 11, Table S5). Most areas where snake eDNA
247 was detected were at relatively high elevations (Tables S1 and S5), except for one area
248 (Area 11: elevation: 54 m) where *R. tigrinus* eDNA was detected (Fig. S2). The best-
249 performing model included only land-use as the explanatory variable (Table 4).
250
251 **Discussion**
252 In this study, we developed species-specific eDNA detection assays for *R. tigrinus*, *G.*
253 *blomhoffii*, and *E. quadrivirgata*, which are common snake species in Japanese paddy
254 ecosystems. Our results showed that the developed assays are useful for detecting the
255 eDNA of *R. tigrinus* and *G. blomhoffii* in paddy ecosystems. In this study, water
256 samples were collected from 18 areas of paddy fields, ponds, and ditches under different
257 land-use conditions. We were able to detect the eDNA of *R. tigrinus* and *G. blomhoffii*

in only traditionally managed paddy field areas. The GLMM and model selection analyses revealed that paddy consolidation and abandonment decreased the detection of eDNA from *R. tigrinus,* implying that the population of *R. tigrinus* was reduced following consolidation or abandonment. This is the first report on snake population decline in paddy ecosystems affected by land-use and is consistent with reported patterns for other plant and animal taxa (Uchida & Ushimaru 2014; Katayama et al. 2015).

Snake species in paddy ecosystems are known to predominantly prey on paddy-associated frogs (Hirai 2004), some of which have declined in recent years and are listed as endangered species in national and local red lists (Ministry of the Environment of Japan 2020). Land-use-induced changes in paddy ecosystems have reduced the populations of these frogs and toads (Hasegawa 2000; Tsuji et al. 2011). The detection pattern of eDNA in this case could reflect the fact that land consolidation in paddy fields has reduced the number of paddy-associated frogs and toads and, consequently, the number of *R. tigrinus*. Paddy consolidation and abandonment might have strong negative impacts not only on plants and insects (Uchida & Ushimaru 2014) but also on top predators, such as snakes and birds (Katayama et al. 2015).

Although *E. quadrivirgata* is the most common paddy-associated anuran specialist snake species with a large population in mainland Japan (Kadowaki 1996; Hirai 2004), no eDNA of this species was detected in any study area. The frequency of *E. quadrivirgata* entering the aquatic environment is known to be lower than that of *R. tigrinus* (Mori 2008); therefore, the eDNA concentration of *E. quadrivirgata* in environmental water could be low. Alternatively, different species may release different amounts of eDNA, but this is unlikely, because we successfully detected *E.*

282 *quadrivirgata* eDNA from the environmental water of rearing areas. The study period
283 coincided with the active period of the target species, but it is possible that conducting
284 the study during April, which is the breeding season for *E. quadrivirgata*, might
285 improve the detection rate (Kadowaki et al. 1992).
286 Among the 18 study areas, Area 15, where *G. blomhoffii* eDNA was detected, was
287 surrounded by the largest forest area (within a 300 m radius). *G. blomhoffii* is known to
288 be abundant on the floors of forests and under bushes (Uchiyama et al. 2002), and the
289 results of eDNA detection might reflect this habitat preference. Our results suggest that
290 the detection of eDNA in paddy ecosystems reflects the currently known ecology and
291 habitat preferences of snakes. Because eDNA in environmental water was examined in
292 this study, interspecific differences in aquatic and forest environment use might affect
293 eDNA detection.
294 This study is the first to apply eDNA analysis to detect snakes inhabiting paddy
295 ecosystems. We successfully detected eDNA from two species of snakes in paddy
296 fields, but the detection rate was very low. The LODs of the three assays were
297 comparable to those of previous studies (e.g., Jo et al. 2020; Mauvisseau et al. 2019),
298 suggesting that the sensitivity of the assays is not likely to be the cause of the low
299 detection rates. Given the results of the in-house experiment, it is likely that eDNA can
300 only be detected when the target species is present at a relatively high density and that
301 our target snakes may release relatively low amounts of DNA into the water. There have
302 been few previous reports on eDNA detection in semi-aquatic reptiles (Baker et al.
303 2018; Hunter et al. 2019), and detection rates varied depending on the target species and
304 study environment (Ratsch et al. 2020; Katz et al. 2021). To better understand the
305 habitat and distribution of snakes using eDNA surveys, it may be necessary to consider

306 the effect of seasonality in eDNA detection or to increase the frequency of water
307 sampling, as well as the number of samples collected. With improvements in sampling,
308 eDNA analysis as described in this paper could be useful for the biological monitoring
309 of paddy ecosystems in the future.
310
311 **Acknowledgements**
312 We thank Atsushi Sakai (The Japan Snake Institute) for his help with experiments in the
313 rearing environment and for his advice on snake biology. We would like to thank Dr.
314 Yasuoki Takami (Graduate School of Human Development and Environment, Kobe
315 University) for providing us with tissue samples and for his advice on this research.
316 This study was financially supported by the Organization of Advanced and Integrated
317 Research, Kobe University.
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524 Table 1. Accession numbers of the DNA sequences of target species and coexisting

525 species used for designing primers and probes.

526

Species	Accession numbers
Elaphe quadrivirgata	LC327648, HQ122366, HQ122365, HQ122365, HQ122363
Rhabdophis tigrinus	AB979245, AB979243, AB979241, AB979240, AB979239
Gloydius blomhoffii	AF039271, AY352751, JF357942
Elaphe climacophora	LC327646, LC327645, LC327644, LC327643, LC327642
Hebius vibakari	AB989302, KJ685677, JQ798798, JQ798799
Elaphe conspicillata	LC369627, DQ902106, KF669242, KF669241

527

528 Table 2. Sequences of primers and probes designed for this study.

529 Equ, *Elaphe quadrivirgata*; Gbl, *Gloydius blomhoffii*; Rti, *Rhabdophis tigrinus*

scientific name	Amplification length	Primer /probe name	Sequence (5′ > 3′)
Elaphe quadrivirgata	112bp	Equ-Cytb-F	CGGCACAACACTCACCTC
		Equ-Cytb-R	GGATATTGAGAGGATAGTGAATGGAA
		Equ-Cytb-P	FAM-TCAATTAATGACCCTACCTTAA-MGB
Gloydius blomhoffii	122bp	Gbl-Cytb-F2	GGGGAATTTATTACGGCTCATATCT
		Gbl-Cytb-R	GAATGATATTTGACCCCATGGTAAG
		Gbl-Cytb-P	FAM-AAGAAGTCTGAGTATCAGGCAC-MGB
Rhabdophis tigrinus	104bp	Rti-Cytb-F	CTCCATGCAATTGGAGCCT
		Rti-Cytb-R	CCAGATAGTCAGACTTCTTTGTTCA
		Rti-Cytb-P	FAM-CATTTACATTCACATCGCACGA-MGB

530

531

532 Table 3. Filtration volume and detection results by real-time PCR in rearing environment.

533 Real-time PCR results show the number of replications where amplification was observed

534 in three PCR replicates. The abbreviations are the same as those listed in Table 1.

535

	Filtration volume (ml)	real-time PCR for Equ	real-time PCR for Gbl
E. quadrivirgata	1000	3/3	-
E. quadrivirgata	800	3/3	-
E. quadrivirgata	250	3/3	-
E. quadrivirgata	250	3/3	-
E. quadrivirgata	410	3/3	-
E. quadrivirgata	450	3/3	-
G. blomhoffii	340	-	3/3
G. blomhoffii	390	-	3/3
G. blomhoffii	330	-	3/3
Field blank	1000	0/3	0/3
Filtration blank	1000	0/3	0/3

536

537

538 Table 4. Results of model selection using Akaike information criterion (AIC) of
539 generalized linear mixed models.
540 The response variables were the detection/non-detection of eDNA of *Rhabdophis*
541 *tigrinus* in each water sample, and the explanatory variables were the management type
542 of the paddy field (traditional area, consolidated area, abandoned area), habitat type
543 (paddy field, pond, and a single ditch), water temperature, elevation, and the random
544 effects (the water sampling point and season). In this section, the weight is shown as
545 0.05 or higher.

Models	AIC	ΔAIC	weight
Land use type	45.1	0	0.334
Land use type, Elevation	46.5	1.37	0.168
Land use type, Water temperature	47.0	1.95	0.126
Land use type, Elevation, Water temperature	48.4	3.35	0.063
Land use type, Habitat type	48.5	3.41	0.061
Elevation	48.6	3.48	0.059

546

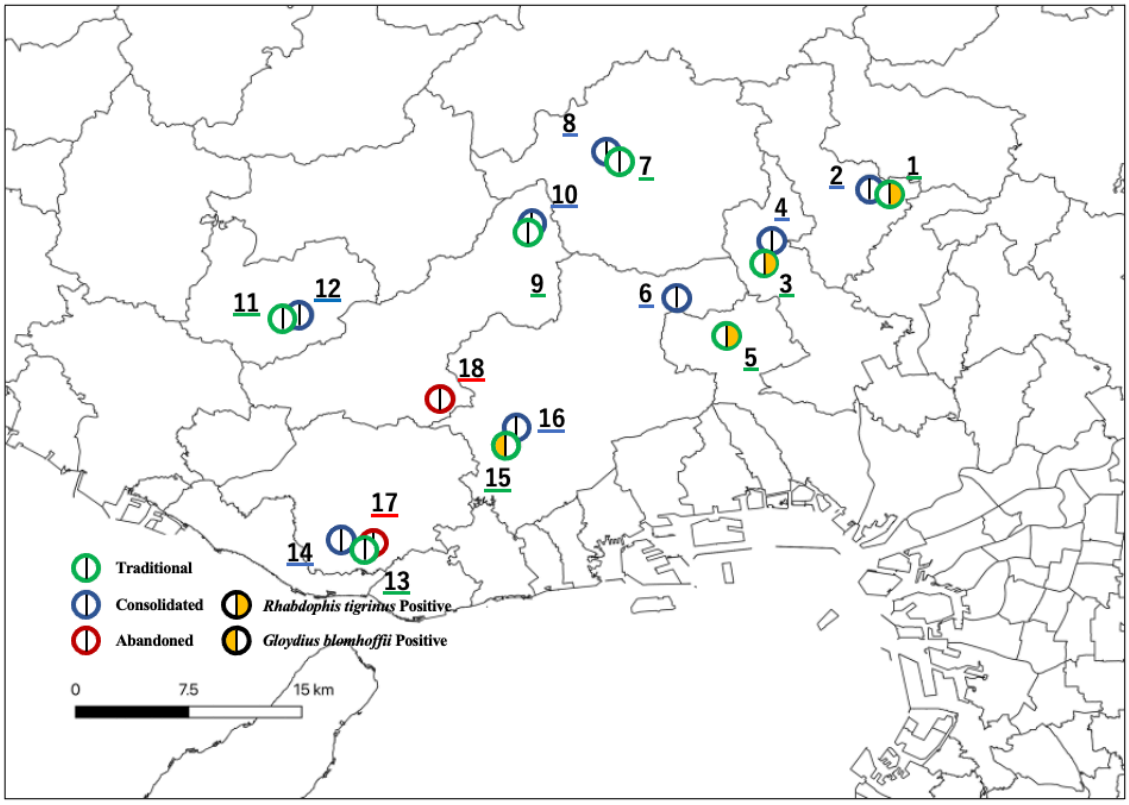

Fig. 1. Water sampling areas and results of eDNA detection in summer and autumn surveys in 2018. Detection in either of the two surveys was considered as positive for the area. Numbers in the map indicate area numbers.