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Discovery of an unrecorded population of Yamato salamander (*Hynobius vandenburghi*) by GIS and eDNA analysis

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Abstract

Background: Biodiversity loss is a serious environmental problem, and human activities might be primarily responsible for the marked decline in animal populations globally. Amphibians, in particular, have significantly decreased in number in recent decades. One example is the endangered Yamato salamander (*Hynobius vandenburghi*), which is distributed in Central Japan and has a very restricted distribution in Gifu Prefecture.

Aims: We aimed to discover new populations of *H. vandenburghi* using a combination of GIS and environmental DNA (eDNA) analysis.

Materials & Methods: Firstly, we designed two primer sets for amplifying Hynobius species targeting mitochondrial 12S rRNA and cytochrome *b* genes. Next, we performed aquarium experiments to detect *H. vandenburghi* DNA in aquarium water. We also conducted sequential eDNA detection surveys in five known habitats in Gifu City, Japan, 17 times from January to August 2016. Following these basic eDNA studies, we used GIS to characterize the vegetation and topography of known habitats of *H. vandenburghi*. We collected water samples in the potential habitats identified by GIS and analyzed eDNA for the presence of *H. vandenburghi* using the designed primers. Finally, we conducted physical collection surveys in these potential habitats.

Results: We successfully developed two Hynobius-specific primer sets and detected *H. vandenburghi* eDNA in aquarium water. The eDNA of the target species was detected in almost all (94%–100%) samples from four habitats, whereas only 29% of samples were positive for one habitat. We identified five potential habitats by GIS analysis. The DNA of *H. vandenburghi* was detected in three of five potential habitats. Finally, we discovered a new population in one of the potential sites.

Discussion: Our approach combining GIS and eDNA enabled the detection of novel population of an endangered amphibian species. This study was conducted by high school students under the supervision of teachers with the help of university researchers, suggesting the applicability of eDNA studies as a tool of citizen science.

Conclusion: The combination of GIS and eDNA will allow to detect cryptic populations on which conservation efforts may be focused and to alert people to the need for conservation action.

KEYWORDS
environmental DNA, geographic information system, *Hynobius vandenburghi*, water

1 | INTRODUCTION

Human survival depends on a variety of ecosystem services, and humans and their cultures have coevolved with nature. In Japan, there is a sustainable practice of creating "*satoyama*", which is a traditional Japanese agricultural landscape found throughout Japan, that circulates resources and energy within it (Nakamura, 2012). Typical *satoyama* is mainly composed of multiple ecosystems such as woodlands, paddy fields, crop fields, grasslands, and settlements (Ichikawa et al., 2006; Figure 1), resulting in high biodiversity. However, loss of biodiversity due to invasion of alien species and abandonment of agricultural fields due to declining residence has recently become a serious problem in the *satoyama* areas. Particularly, the decrease in amphibian abundance is a concern. In Japan, approximately 60% of amphibian species are listed as endangered (Herpetological Society of Japan, 2018; Japanese Ministry of the Environment, 2018). Amphibians generally are dependent on water during the larval period, while adults move to the land following metamorphosis. Therefore, they require terrestrial and aquatic habitats and their connecting ecotones. To conserve amphibian biodiversity, rapid monitoring of endangered species is needed; however, conventional field capture requires considerable time, energy, and money, and might not allow timely monitoring in the event of rapidly declining biodiversity.

In our study, we used environmental DNA (eDNA) sampling and GIS to reveal the presence of a cryptic population of an endangered species. GIS is used to analyze spatial information by overlaying different kinds of spatial data such as altitude, precipitation, temperature, land use, and geographic characters. The ability of GIS to handle large data sets allows for efficient surveys (Mitsuhashi and Kamata, 2006). Environmental DNA is the total DNA in environmental media such as water and sediment that comes from living and dead microbes in the samples, as well as secretions, excretions, feces, gametes, shed skin, hair, and other parts of macro-organisms (Barnes and Turner, 2016). The presence of an organism may be inferred by analyzing water collected from the survey area (Fukumoto et al., 2015). The combination of these technologies should make surveys for rare or hard-to-detect taxa, more effective. We can identify potential habitats of taxa within a larger area using GIS, analyze eDNA, and then conduct field surveys to confirm their presence at sites yielding positive eDNA signals. With this strategy, we can rapidly and effectively identify sites most likely to harbor the taxon in question, thus cutting field survey costs.

FIGURE 1 Typical landscape of *satoyama* in Japan. Paddy fields, woodland, and settlements are closely distributed

It has been reported that there are approximately 50 *Hynobius* (Amphibia: Caudata: Hynobiidae) species globally, and 32 of which are endemic to Japan (Frost, 2018; Matsui et al., 2019). Yamato salamander, *Hynobius vandenburghi* Dunn, 1923, is a small, lentic salamander, endemic to and distributed in central to western Japan; it was formerly classified as *H. nebulosus* and was very recently re-classified as *Hynobius vandenburghi* (Matsui et al., 2019). The former *H. nebulosus* was designated as Vulnerable in the Red List of Japan (Japanese Ministry of the Environment, 2018). *Hynobius vandenburghi* breeds in water around December to April, and the most frequent breeding season in Gifu is from late February to early March. It lays eggs that attach to fallen branches or leaves, and prefers *satoyama* environments, inhabiting rice fields adjoining houses (Miyoshi and Natuhara, 2003). Usually, field surveys for small salamanders include fumbling under fallen leaves or stones, capturing adults with hand nets and visual searches for eggs, requiring more than an hour. Thus, considerable time and energy are required to locate *H. vandenburghi* with such approaches because the animal is small (approximately 30–60 mm long), nocturnal, and lives under fallen leaves, rubble, and in leaf mold during day.

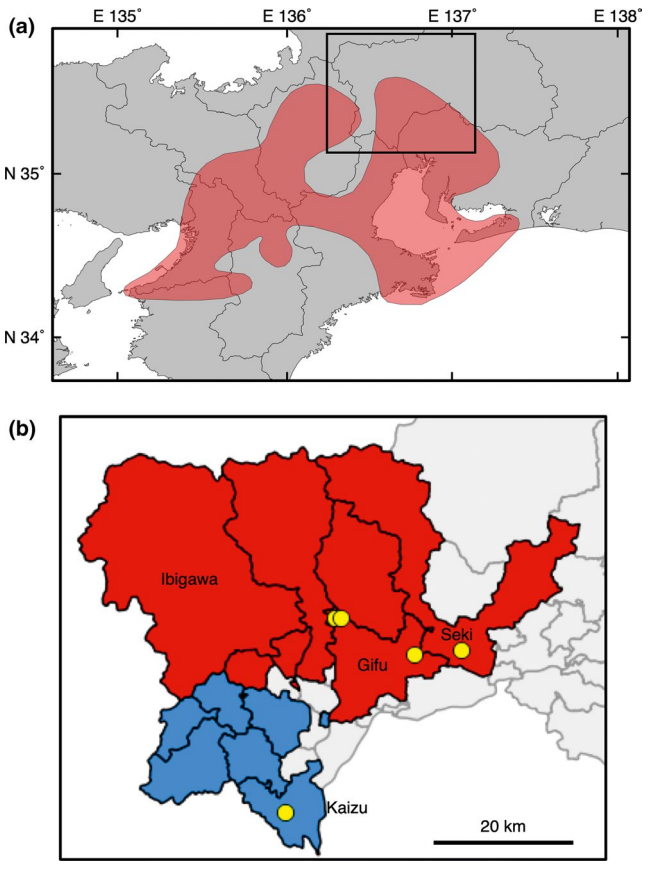

FIGURE 2 (a) Rough distribution map of *Hynobius vandenburghi* (red shaded area) in central Japan based on Matsui et al. (2019) and our experiences. (b) Map of the study area. The red (western Gifu) and blue (southwestern Gifu) areas are the targets in this study. Yellow circles indicate potential habitats identified with GIS analysis. Note that the size of the circles is enlarged and the points are roughly indicated for conservation purpose

Gifu Prefecture is thought to be the north-eastern limit of *H. vandenburghi's* distribution (Matsui et al., 2001; Matsui et al., 2019) and is designated as Critically Endangered in the Red List of Gifu (as *H. nebulosus*; Gifu Prefecture, 2010). Although there had been known only two populations in Gifu Prefecture for several decades (one each in Gifu City and Ibigawa Town; Figure 2), a third population was found in Kaizu City in 2016 by the 10th author. This suggests that there may be other undiscovered populations in Gifu Prefecture. Another two *Hynobius* species, *H. kimurae* and *H. stejnegeri*, are potentially distributed around fields; however, these species breed in lotic habitats, while *H. vandenburghi* breeds in lentic habitats. We attempted to locate an undiscovered population by (a) developing primer sets to amplify eDNA of *Hynobius* species and applying them to eDNA from aquarium water and known wild populations, (b) using GIS to specify the habitat characteristics of three known *H. vandenburghi* habitats, (c) using a model based on these data to locate additional potential habitats, (d) testing for the presence of *H. vandenburghi* in these locations using eDNA analysis, and (e) conducting field surveys to confirm the presence of *H. vandenburghi*. This study was conducted by high school students under the supervision of high school teachers with the help of professional researchers. The molecular tools were developed by professional researchers, and GIS analysis, field surveys, and eDNA experiments were conducted by high school students. Our study shows that eDNA analysis is a useful tool that can be used by citizen scientists for conservation purposes.

2 | MATERIALS AND METHODS

2.1 | Designing primers for eDNA analysis

We designed two primer sets specific to 12S rRNA and Cytb of the genus *Hynobius* for the detection of *H. vandenburghi* eDNA. There are more than 30 endemic *Hynobius* species in Japan, and most of these species show habitat segregation, that is we rarely see two species sharing their breeding habitat. Therefore, genus-specific primers are useful for eDNA studies of *Hynobius* species in Japan. Although the use of COI is recommended for Barcode of Life, we used these genes for the following reasons. The Cytb gene is the most frequently sequenced in *Hynobius* species, especially among close relatives of *H. vandenburghi*, and 12S is often used, providing positive outcomes in eDNA metabarcoding studies (e.g., Miya et al., 2015; Ushio et al., 2018).

We obtained sequence data from the NCBI database for the mitochondrial 12S rRNA for all available sequences (16 species) in the genus *Hynobius* (Table S1) and aligned them using CLUSTAL W algorithm (Thompson et al., 1994). We then chose the locus conserved across all the 16 species by eye. The designed primer sets are as follows:

*Hynobius*_12S_F1(5′-TTAATAAAAACGGCCTAAAGCGTG-3′)and *Hynobius*_12S_R1 (5′-TCAATTATAGAACAGGCTCCTCTAGGG-3′). The expected amplification length was 290 bp.

We obtained sequence data for the Cytb of six *Hynobius* species (Table S2) and aligned them using CLUSTAL W algorithm (Thompson et al., 1994). We then located the site that has a high commonality in sequences of these six species. The designed primer sets are as follows:

CBL181: (5′-ACACTATACAGCCGACACAT-3′) and CBH759: (5′-GGGGTAAAATTATCTGGGTC-3′). The expected amplicon size was 615 bp.

The specificity of the primer sets was checked in silico using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The applicability of the primers for other *Hynobius* species is under evaluation (Tomita et al., in preparation).

The limit of detection of each primer set was determined as follows. Extracted tissue DNA of *H. vandenburghi* was diluted, and 1, 0.1, 0.01, and 0.001 pg of DNA was used as template. The PCR conditions for each primer set were the same as those for eDNA analysis as described below. Five replicate reactions were performed for each template volume.

2.2 | Contamination control measures

To reduce the risk of contamination, the pre- and post-PCR experiments were performed in independent rooms, and a unidirectional workflow was adopted. Laboratory bench was cleaned with DNA Away (Cosmobio) before experiments. During sampling and experiments, surveyors wore nitrile groves and used filter tips. All equipment used in the eDNA experiments were disposable or bleached before experiments.

2.3 | Extracting eDNA from aquarium water

We maintained 18 *H. vandenburghi* individuals in an aquarium (86 cm long, 86 cm wide, and 54 cm high). The aquarium contained 45 L of water, gravel, and sphagnum. The water temperature was controlled at 7°C (in winter) and 17°C (in summer), and each animal was fed one or two *Gryllus bimaculatus* or *Galleria mellonella* twice weekly.

We filtered 1 L of aquarium water using a 47-mm glass fiber filter of pore size 0.7 μm (GF/F, GE Healthcare) using an aspirator (GAS-1N: AS ONE). Environmental DNA was extracted from the filter using the Qiagen DNeasy Blood and Tissue Kit (Qiagen) according to a previous study (Uchii et al., 2016), with slight modifications. Each filter was treated with 20 μl of Proteinase K and 200 μl of Buffer AL at 56°C for 30 min, and then centrifuged at 5,000 *g* for 3 min. Then, 200 μl of Buffer AL and 200 μl of EtOH were added to the recovered solution. The mixture (approximately 650 μl) was added to a DNeasy column, and then centrifuged at 6,000 *g* for 1 min. This step was repeated until the mixture was completely processed. Finally, DNA was eluted with 110 μl of Buffer AE instead of 200 μl, which is the manufacturer's recommendation, to increase eDNA concentration. The DNA solution was stored at −20°C and used as the positive controls for the eDNA analysis of field samples.

2.4 | Conventional PCR

The PCR for 12S rRNA and Cytb was performed under the same conditions. The PCR solution consisted of 1.0 μl each of primer (10 μM), 12.5 μl of 2× KAPA HiFi Hot Start Ready Mix (Kapa Biosystems), 2 μl of DNA solution, and 8.5 μl of distilled water; the total amount was 25 μl. The PCR (GeneAtlas 482; Astec) was run under the following conditions: 95°C for 3 min, and then, 55 cycles at 98°C for 20 s, 62°C for 15 s, and 72°C for 15 s. Environmental DNA from the aquarium water was used as the positive control. One liter of distilled water was filtered and processed in the same way as eDNA samples and used as the negative control to confirm that the sample was uncontaminated. A single PCR-positive control and a single negative control (filtration blank) were performed with each PCR run. For these samples, all PCRs were duplicated. The PCR products were visualized using 2% agarose gel.

The 12S rRNA and Cytb PCR products derived from the eDNA of aquarium water and known habitats in Gifu City were sequenced to confirm that the DNA was from *H. vandenburghi*. The PCR products were purified using the Exo SAP-IT Kit (GE Healthcare). The purified DNA products were directly sequenced with the same primers used for amplification using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit ver. 3.1 in an ABI PRISM 3100 Genetic Analyzer (Thermo Fisher Scientific).

2.5 | Analysis of eDNA from environmental samples

We collected a single 1 L of water sample in each of five known population sites in Gifu City 17 times (January 12, 23, 28; February 4, 24; March 4, 11, 18; April 5; June 11, 17, 24; July 1, 14, 21, 28; and August 9 of 2016) to determine optimal survey times. We collected 82 water samples (three samples on August 9 were not collected due to water shortage) (Table 2). The detection of 12S-eDNA was performed with these samples. For these surveys, PCR was duplicated.

2.6 | GIS analysis

GIS analysis was conducted using QGIS (version 2.14). We created approximately 6,000 circles of radius 500 m onto a map of 12 cities on the Nobi Plain (i.e., Gifu City, Yamagata City, Seki City, Motosu City, Ikeda Town, Ohno Town, Ibigawa Town, Ohgaki City, Kaizu City, Tarui Town, Yoro Town, and Sekigahara Town) (colored area in Figure 2b). We analyzed environments by calculating the proportion of each parameter of known habitats (vegetation coverage, elevation, slope inclination, and slope direction) on GIS. Firstly, we produced five vegetation maps (i.e., paddy field, weed community in paddy field, fallow paddy field, weed community in fallow paddy field, and bamboo forest) extracted from a vegetation map for Gifu Prefecture (vg_21a) from the Biodiversity Center of the Japanese Ministry of the Environment (http://gis.biodic.go.jp/webgis/sc-023.html [Accessed August 13, 2018]) using GIS. To analyze topography around their populations, we downloaded fifth mesh data version 2.2 published by the National Land Numerical Information of Japan (http://nlftp.mlit.go.jp/ksj-e/gml/datalist/KsjTmplt-G04-d.html [Accessed October 12, 2018]), and 6,000 circles of

TABLE 1 Results of analysis of three known populations in Gifu Prefecture. The GIS search criteria for search in Gifu Prefecture were based on these results

	Vegetation cover	Elevation (m)	Inclination (°)	Slope direction
Gifu City	27.27	55.88	9.79	East to south
Ibigawa Town	27	98.96	11	East to south
Kaizu City	11.9	41.68	9.3	East to south

radius 500 m were generated. Average elevation, average grade, and average slope direction in each circle were calculated. We set search conditions based on the result, and then searched the 6,000 circles for those meeting the specified conditions.

From the 6,000 circles, we identified 51 circles in which vegetation coverage was similar to that in which known populations occurred (see Results). By stratifying further on the basis of average elevation, average grade and average slope direction of the known habitats (Table 1), we identified five areas in which the overall environmental conditions were similar to those of known habitats.

2.7 | eDNA surveys in potential habitats

We analyzed eDNA in water samples from the five potential sites identified with GIS. We collected 1 L of water sample in each site. Sampling was performed at one potential site in Kaizu City in February 2017, and at the other four sites (three in Gifu City and one in Seki City) in August 2017 (Figure 2b). Aqueous eDNA for these samples was extracted and the eDNA analysis for *Hynobius* species was conducted as mentioned above. As filtration blanks, 1 L of distilled water was filtered, extracted, and PCR-amplified in the same manner as samples. The PCRs with both primer sets were performed. For these samples, all PCRs were performed in five replicates. We detected eDNA of *Hynobius* in potential sites and performed species identification in each sample, by direct sequencing using a commercial sequencing service (Fasmac).

2.8 | Field surveys for physical collection

In the GIS-identified potential habitat in Kaizu City, we searched for eggs or adults on March 12, 2017. Seven persons searched the area on foot. Because we finished searching when we found eggs, this search lasted for only 5 min. At three potential habitats in Gifu City, the same field surveys were conducted on February 17 and April 4, 2018, by eight persons for 30 (February 17) and 60 (April 4) min. In a potential habitat of Seki City, the field survey was conducted on February 17 by eight persons for 60 min.

3 | RESULTS

3.1 | Designing the primers for 12S rRNA and Cytb

The specificity of the designed primer sets was checked in silico using Primer-Blast. The 12S primers potentially amplify only the sequence of *Hynobius* species among species distributed in mainland Japan. The results of Primer-Blast for Cytb primers suggested the potential amplification of *Hyla japonica*, which is distributed in mainland Japan; however, there was a substitution in the second nucleotide from the 3′ end of the forward primer. The 3′ end of the primer is important for specificity (Wilcox, Carim, McKelvey, Young, & Schwartz, 2015), and the sequences obtained in our study were from the target species as described below. Therefore, we considered that the specificity of the primers was sufficient.

The results of limit of detection test for 12S rRNA primer set revealed that five, four, two, and zero out of five replicates were positive when 1, 0.1, 0.01, and 0.001 pg of genomic DNA per reaction were used as template, respectively. For Cytb primer set, five, four, one, and zero out of five replicates were positive when the template was 1, 0.1, 0.01, and 0.001 pg, respectively. Thus, the limit of detection for both primer sets was 0.01 pg per reaction.

The PCR with 12S rRNA primer sets using DNA from the aquarium water of *H. vandenburghi* resulted in amplification of the 300 bp band (Figure 3). The same size of positive bands was obtained from water samples in the Gifu City population, and no band was detected from the negative control. We sequenced the amplified fragments of 12S rRNA gene and determined 218 bp. The sequences from aquarium (LC485184) and DNA from the tissue sample (LC485183) matched perfectly. These sequences showed 91.0%–91.4% and 91.0%–93.2% identities with those of *H. kimurae* and *H. stejnegeri*, respectively, which are potentially sympatric *Hynobius* species, suggesting sufficient resolution of the gene marker.

The PCR with Cytb primer set using DNA from *H. vandenburghi* resulted in the amplification of an approximately 600 bp band. The sequence (615 bp) of the amplified DNA (LC485190) perfectly matched that of *H. vandenburghi* collected in Gifu City (AB972628). These sequences showed 83.6% and 84.8%–87.1% identities with those of *H. kimurae* and *H. stejnegeri*, respectively, also suggesting sufficient resolution.

3.2 | Optimal period for eDNA survey

The results from the 17 sampling occasions in Gifu City are shown in Table 2, which shows the presence or absence of bands in the electrophoretic analysis of 12S rRNA in eDNA samples. Environmental DNA was detected irrespective of season, that is breeding and nonbreeding seasons, for all populations except for Gifu City population 4.

3.3 | Identifying potential habitats with GIS

GIS analysis of the three known habitats in Gifu Prefecture (Gifu City, Ibigawa Town, and Kaizu City) showed a vegetation cover of approximately 27% for the Gifu City and Ibigawa Town habitats and 12% for the Kaizu City habitat. The elevation was 40–100 m, slope inclination 9–11°, and slope direction was east to south (Table 1).

TABLE 2 Results of eDNA sampling from January to August. The populations 1, 2, and 3 are natural populations and the others are artificially introduced populations

	Jan 14	Jan 23	Jan 28	Feb 4	Feb 24	Mar 4	Mar 11	Mar 18	Apr 5	Jun 11	Jun 17	Jun 24	Jul 1	Jul 14	Jul 21	Jul 28	Aug 9
Gifu City population 1	+	+	+	+[a]	+	+	+[a]	+[a]	+	+	+[a]	+[a]	+	+[a]	+	+	/
Gifu City population 2	–	+	+	+	+	+	+[a]	+[a]	+	+	+[a]	+[a]	+	+	+	+	/
Gifu City population 3	+	+	+	+	+	+	+[a]	+[a]	+	+	+	+	+[a]	+[a]	+	+	+
Gifu City population 4	–	–	–	–	–	–	–	–	+[a]	+	+	+	+	–	–	–	–
Gifu City population 5	–	+	+	+	+	+	+	+[a]	+[a]	+	+	+	+	+	+	+	/

Note: "+" and "–" indicate that "both duplicates were positive" and "both duplicates were negative", respectively.
[a]Indicates that any of the following were found in the survey: adults, eggs, and larvae.

Because it differs between areas, we set search conditions for vegetation as follows: 27.1 ± 2% in western Gifu area (including Gifu City and Ibigawa Town) and 11.9 ± 2% for southwestern Gifu area (including Kaizu City). For geographic conditions, elevation under 100 m, slope inclinations between 7 and 13°, and slope directions from east to south were set. The slope degree was slightly enlarged because we could not find any candidate site when searched between 9 and 11°. As a result, we identified 51 sites in which vegetation coverage was similar to that in which known populations occurred. By stratifying further on the basis of average elevation, average grade and average slope direction of the known habitats (Table 1), we identified five potential habitats (one, three, and one in Kaizu, Gifu, and Seki Cities, respectively; Figure 2b) in which overall environmental conditions were similar to those for known habitats.

3.4 | eDNA analysis in the potential habitats

We attempted to detect eDNA in water samples from five potential sites identified with GIS. For 12S and Cytb markers, we detected positive bands from water (5/5 and 5/5 were positive for 12S and Cytb, respectively) samples collected at a potential site in Kaizu City (Figure 4). We also detected positive bands from water samples collected at one of Gifu sites (3/5 and 2/5 were positive) and a Seki City site (5/5 and 3/5 were positive). Water samples collected in the other two Gifu sites were negative (Table 3). Positive amplicons derived from eDNA samples collected in Gifu and Seki City sites were sequenced. The Cytb sequences of eDNA from a potential site in Gifu and Seki Cities (LC485191 and LC485192, respectively) were completely matched with *H. vandenburghi* sequences (AB972628 and AB972625, respectively; note that these sequences are registered as those of *H. nebulosus*, according to former classification). The 12S sequence of eDNA from a potential site in Gifu and Seki Cities (LC485185 and LC485186, respectively) was completely matched with that from *H. vandenburghi* of known population in Kaizu City (LC485182).

3.5 | Field surveys for specimen collection

We conducted field surveys in the five potential sites identified with GIS. At the potential habitat in Kaizu City, we found a single pair of egg sacks on Mar 12, 2017 (Figure 4). We did not find individuals or eggs at the Gifu and Seki City sites. Thus, we found eDNA evidence in three of five potential sites, and physical

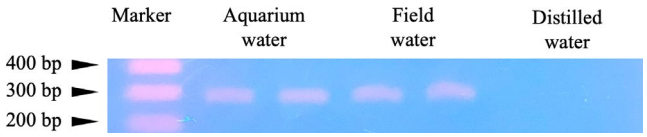

FIGURE 3 Electrophoresis gel image for 12S rRNA primer set. Environmental water samples were collected from the known habitat in Gifu City. We confirmed that the primer sets for 12S rRNA work appropriately. Aquarium water and distilled water are the positive and negative controls for PCR, respectively

FIGURE 4 Site of a newly found population in Kaizu City (left). The single pair of egg sacks found at the site (right)

TABLE 3 Results of eDNA detection at five potential habitats identified by GIS analysis

	12S	Cytb
Kaizu City potential habitat	+++++	+++++
Gifu City potential habitat 1	-----	-----
Gifu City potential habitat 2	-----	-----
Gifu City potential habitat 3	++-+-	-+-+-
Seki City potential habitat	+++++	--+++

Note: "+" and "–" indicate the detection and nondetection of eDNA bands, respectively. The results of five replicates are presented.

evidence at one of those. The found eggs were collected for conservation purpose, and the grown-up adults were released to the original place. In this process, we confirmed that the species identity was *H. vandenburghi*.

4 | DISCUSSION

We developed molecular markers that can amplify DNA of *Hynobius* species. We analyzed eDNA in water samples collected from the potential habitats of *H. vandenburghi* identified with GIS, and conducted field surveys in the eDNA-positive sites. As a result, we found a new population of the endangered *H. vandenburghi*, which had previously been recorded from only three sites in Gifu Prefecture.

In this study, we identified five potential habitats in Gifu Prefecture using GIS to stratify the search area on the basis of vegetation and geographic parameters. In concert with eDNA sampling, this allowed us to isolate one actual and two highly probable habitats, suggesting the appropriateness of the chosen parameters and the usefulness of the approach combining GIS and eDNA analysis. Our approach enabled the detection of novel population of an endangered amphibian species. Furthermore, adding information from past distribution may be useful for this kind of analysis. According to the materials in the Gifu Prefectural Museum, *H. vandenburghi* was more widely distributed in the past. Future research should focus on sampling efforts by considering historical distribution.

Concerning the examination of optimal periods to conduct eDNA surveys, eDNA was detected from January to August except that of one population. For a population (Gifu City population 4 in Table 2), eDNA was detected only from April 5 to July 1, suggesting a smaller population size in this habitat. Thus, the optimal survey season for this species may be April to early July. Environmental DNA of *H. vandenburghi* was always detected when egg sacks or individuals were found by traditional survey (Table 2). These results suggest that the detection sensitivity of the eDNA analysis would be higher than the traditional surveys as reported in previous reports (Takahara, Minamoto, & Doi, 2013; Jerde, Mahon, Chadderton, & Lodge, 2011).

We could not find eggs or individuals in the two eDNA-positive sites. This could be due to the relatively smaller population size. On the contrary, the PCR inhibition and lower sensitivity of the assays potentially cause false-negative results. We did not confirm the absence of inhibition in this study because we mainly aimed to discover a new population rather than confirm the absence of the population. However, to reduce the possibility of false negatives, inhibition checking and the use of inhibitor-resistant reagents will be useful for future studies. The limit of detection of our assays was 0.01 pg of genomic DNA per reaction, and this sensitivity is comparable to that of the real time-PCR or droplet digital PCR (Mauvisseau et al., 2019). However, our assays need Sanger sequencing after PCR, and the use of species-specific TaqMan probes would give results in a timely manner.

Although we found a new population of the endangered species, *H. vandenburghi*, the detection of only a single pair of egg sacks in the new habitat from a single female indicates the critical condition of the population, because generally more than 100 pairs of egg sacks of other known population were found, and the necessity for rapid action. In addition, when we re-visited this habitat in Kaizu City in June 2017, the waterway near the mountain had disappeared because of clean-up activities by citizens who may not have known of the presence of an endangered species. Thus, it is important to enlighten City government and citizens to promote conservation of the population. As there might be other endangered populations in the area, further surveys must be carried out to locate and conserve them.

The approach of combining GIS and eDNA analysis can be applied to organisms requiring specific, known environmental conditions. In the case of rare or threatened organisms, the use of GIS to narrow search areas may prove important in saving time and containing costs. In addition, the use of eDNA analysis allows efficient physical surveys by focusing on the highly potential habitats. Our results offer encouragement to those working to conserve rare,

hard-to-locate, cryptic species, while eDNA analysis offers the additional advantage of simultaneously detecting nonaquatic organisms (Piaggio et al., 2014) which may also require conservation attention. Our method will allow for the location of remnant populations on which conservation efforts may be focused, alerting people to the need for conservation action.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

Conception and study design: YS, KT, TMu, MTak, YY, and TMi. Sample collection and experiments: YS, AK, YT, SO, TK, ST, MTag, MTak, and YY. Writing and editing: YS, TMu, MTak, YY, and TMi.

ETHICAL APPROVAL

All experiments were performed according to the current law of Japan.

DATA AVAILABILITY STATEMENT

All data generated during this study are included in this submitting manuscript (and its Tables S1 and S2).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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