Comparison of the detection of 3 endangered frog species by eDNA and acoustic surveys across 3 seasons

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Abstract: For conservation of endangered species, basic ecological information is indispensable. Environmental DNA (eDNA) is increasingly used to estimate the presence/absence of aquatic organisms such as frog species. However, the efficiency of detecting eDNA may depend on the ecological characteristics of the target frog species. Here, we adopted an eDNA approach for detecting 3 endangered Japanese species (Babina subaspera, Odorrana splendida, and O. amamiensis). We compared eDNA detection with a traditional acoustic survey method in 3 different seasons. Detection of species was not always consistent between the 2 methods, probably because acoustic surveys target only calling adult males but eDNA detects individuals of both sexes and at any life-history stage in water. Moreover, the differences in detection patterns between the 2 methods among species may be correlated with different characteristics of mating behavior (e.g., calling volume) and life history of tadpoles (e.g., migration ability). The optimal season for detection by eDNA method also differed between the 3 species, probably because of differences in breeding season and larval characteristics. We show that results obtained from eDNA detection methods and traditional field observation techniques, including acoustic surveys, are not always consistent with each other but rather depend on the ecological characteristics of the respective target species. It is therefore necessary to consider the ecological traits of frog species, such as breeding season and seasonal shifts in tadpole abundance, when applying eDNA methods for distribution surveys.

Key words: Amami-Oshima Island, Babina subaspera, Odorrana amamiensis, Odorrana splendida, recorder, tadpole

The loss of species diversity in freshwater environments is of great concern, and numerous species are currently endangered (Dudgeon et al. 2006). Amphibians are among the most threatened animal taxa (Hof et al. 2011). The decline of many amphibian species is the result of habitat degradation, climate change, the spread of disease, or a combination of these factors (Alford and Richards 1999). Their decline shows the urgent need for appropriate conservation measures (Semlitsch 2000). It is vital to gather comprehensive ecological information about endangered amphibian species, including their distribution and abundance, to develop conservation strategies to aid them.

Methods such as acoustic recording of mating calls have typically been used to assess the distribution of frogs (Heyer et al. 1994, Acevedo and Villanueva-Rivera 2006). Mating calls are produced by male frogs during the breeding season, and species-specific sounds facilitate the identification of species at the respective breeding site (Rand and Drewry 1994). Mating calls are often recorded by automated stationary recorders installed at breeding sites for the duration of the breeding season. These recorders can be left at the site for a long time, and the obtained data can be analyzed any time after collection. This approach is useful because observer presence is unnecessary (Heyer et al. 1994).

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However, the detection of male frog mating calls does not necessarily indicate successful reproduction and oviposition at the site (Reseratits and Wilbur 1991). Furthermore, obtaining reliable results from the call data requires the time-intensive step of listening to and scoring the recording prior to analysis (Iwai et al. 2018). Therefore, the development of other survey methods with high detection efficiency and less effort is desirable.

In recent years, bio-monitoring methods that use DNA fragments from the environment, such as those derived from excrement and shed tissues of animals, have progressed considerably and are known as environmental DNA (eDNA) sampling (Thomsen and Willerslev 2015). The eDNA approach was first applied to track the American bullfrog (Lithobates catesbeiana) in France, where it is an invasive species (Ficetola et al. 2008). In the optimal case, 1-time water sampling followed by DNA analysis gives a strong indication of the presence or absence of a target species (Dejean et al. 2012, Takahara et al. 2013). Moreover, the eDNA detection sensitivity of quantitative real-time polymerase chain reactions (qPCR) with target-specific primer-probes may be even higher than that of conventional PCRs (Nathan et al. 2014). Since around 2011, numerous studies employing real-time PCR methods have been conducted successfully on various taxonomic groups such as fishes (Thomsen et al. 2012, Yamanaka and Minamoto 2016), reptiles (Davy et al. 2015, Lacoursière-Roussel et al. 2016), mammals (Foote et al. 2012), crustaceans (Tréguier et al. 2014, Ikeda et al. 2016), aquatic plants (Fujiwara et al. 2016, Matsushashi et al. 2016), and amphibians (Pilliod et al. 2013, Torresdal et al. 2017). Thus, the eDNA approach seems to be a promising tool for detecting the presence of target species and may supersede traditional acoustic survey techniques.

A key challenge of eDNA sampling is that the amount of DNA shed into the environment depends on the life-history stage and behavior of the target organism (Takahara et al. 2012, Maruyama et al. 2014). As a consequence, eDNA concentration in water may be affected by season because larger amounts of DNA will be released when more biomass is present or when DNA is shed at higher rates (Laramie et al. 2015). Furthermore, most frog species only remain in the water during certain life-history stages (e.g., from egg stage to tadpole stage), when a dynamic shift in their biomass and, probably, in their eDNA shedding rate occurs. As breeding seasons and phenology of tadpole stages differ among frog species, it is crucial to consider the timing of water sampling for each species in question. However, the effect of season and individual life-history stage on frog eDNA detection efficiency has not been well studied.

The Amami Islands, located in Kagoshima Prefecture, southern Japan, are designated as a quasi-national park because of their valuable natural environment. The islands contain several endangered frog species and are thus an excellent location to examine the efficiency of eDNA methods. *Babina subaspera*, *Odorrana splendida*, and *O. amamiensis* inhabit the forests of the islands (lat 28°18’N, long 129°24’E; Maeda and Matsui 1999), and all 3 species are facing population declines because of habitat degradation (Environment Agency 2000) and predator introductions (Watari et al. 2013). Each of these frog species are listed as endangered on the International Union for Conservation of Nature’s red list (IUCN 2016), indicating a high priority for conservation efforts. We conducted acoustic surveys in a previous study to establish the geographical distribution of these species, but the study results showed possible limitations in detecting mating calls, particularly in *O. amamiensis* (Iwai et al. 2018). Here, we use an alternative to traditional surveying methods: an eDNA method for which we developed species-specific primer-probe sets for the 3 target species. We then compared the results with those from the previous acoustic survey. Moreover, we assessed the seasonal difference of eDNA detection efficiency to determine the optimal time for water sampling for each species. We then discuss possible factors that drive the seasonal effects on detection efficiency considering species-specific ecological characteristics.

**METHODS**

**Study animals**

The breeding season of *B. subaspera* is from late April to early October (Iwai 2013), and late-season tadpoles often overwinter. Its mating call is often described as sounding like a man coughing loudly. Metamorphosis usually occurs during the autumn of the breeding year or during the summer of the following year.

*Odorrana splendida* breeds from February to April (Oumi 2006), and tadpoles often overwinter, sometimes for 2 winters (Oumi et al. 2011). The male frog of this species typically makes a loud call through most of the night and even during daytime at the peak of the breeding season. Metamorphosis occurs from mid-June to early September (Oumi et al. 2011).

The breeding season of *O. amamiensis* extends from mid-October to early May (Maeda and Matsui 1999). This species engages in intensive breeding activity only sporadically, that is, breeding aggregation occurs a few times at 1 breeding site within an interval of a few weeks (NI, personal observation). Their mating call is very weak and sounds like chirping birds. The tadpoles of *O. amamiensis* grow within the stream and metamorphosis starts in May.

**Primer design**

We designed species-specific primers and probes for qPCR to identify eDNA from all 3 species (*B. subaspera*, *O. splendida*, and *O. amamiensis*). To ensure inter-species specificity and intra-species similarity of the DNA sequences in the region to be amplified, we sampled multiple individuals of each species (obtained from different localities...
within their typical distribution areas: *B. subaspera* \( n = 14 \), *O. splendida* \( n = 16 \), and *O. amamiensis* \( n = 16 \). We extracted DNA from ethanol-preserved toes with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). From this DNA, we amplified and sequenced a partial region of the mitochondrial genome that included the NADH dehydrogenase subunit 5 (ND5) following the method described by Igawa et al. (2015). The oligonucleotide primers we used for amplification and sequencing are provided in Table S1. We deposited the resulting sequences in the DDBJ Sequence Read Archive (*B. subaspera*: LC381218–LC381231, *O. splendida*: LC381137–LC381152, *O. amamiensis*: LC381160–LC381175). We also used ND5 sequence data (NC_022871) from *B. subaspera* downloaded from GenBank (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/genbank/). We then aligned the nucleotide sequences of ND5 with MEGA7 (Kumar et al. 2016) and conducted a sliding window analysis to investigate nucleotide diversity within the ND5 coding region. We defined the ND5 coding regions to design the primers and TaqMan® probes for the qPCR assays for each species with PrimerExpress version 3.0.1 (Thermo Fisher Scientific, Carlsbad, California). We amplified fragments that were 90, 96, or 116 bp, depending on the species (Fig. 1A–C).

We ensured our primers were species specific by testing them with the other 2 species included in this study, with another closely-related species that occurs in the same geographic range (*Rana kobai*), and with an in silico specificity screen. We tested species specificity of each primer-probe set to determine whether it amplified DNA extracts from the other 2 species. We then tested each primer-probe set with DNA extracts from *R. kobai*. We did all reactions in 2 technical replicates. Oligonucleotide primers used for the amplification and sequencing are available online (Table S1).

eDNA sampling and processing

We collected surface water from 26 sites at 12 headwater streams on Amami-Oshima Island in December 2014, from May to June 2015, and from August to September 2015 (Fig. 2A–B). Thus, a total of 78 (1/site/season) water samples were taken for eDNA analysis. Each water sample was taken in a new 500-mL bottle. After sampling, each bottle was immediately placed in an individual plastic bag and stored in a freezer within 8 h. Samples were frozen because the transport from the sampling site to the laboratory facilities took up to 2 d.

**Filtration and eDNA extraction of water samples** After thawing at room temperature, the 500-mL water samples were vacuum-filtered through 47-mm GF/F glass fiber filters (with a pore size of 0.7 μm; GE Healthcare, Little Chalfont, United Kingdom). Each filter was then wrapped in commercial aluminum foil and stored at −30°C until eDNA extraction. Blank controls (i.e., 500 mL of distilled water) were filtered as negative controls on each day of sample filtration.

eDNA was extracted from filters according to the methods of Uchii et al. (2016) with the DNeasy Blood and Tissue Kit for DNA purification and Salivette® tubes (Sarstedt, Nümbrecht, Germany). First, a filter was incubated by submersion in a buffer-enzyme mix (400 μL of buffer AL and 40 μL of proteinase K; Qiagen) in a Salivette tube inside a dry oven at 56°C for 30 min. The tubes containing filters were then centrifuged at 5000 g for 5 min at room temperature. Subsequently, 220 μL of a Tris-EDTA buffer (pH: 8.0; 10 mM Tris–HCl and 1 mM EDTA) was added, and the tubes were centrifuged again at 5000 g for 5 min. After this, 200 mL of buffer AL and 600 mL of ethanol (100%) were added to each tube and mixed by pipetting. The mixture was then put into a DNeasy Mini spin column and centrifuged at 6000 g for 1 min. This step was repeated until the entire volume of the mixture was processed. Further steps followed the manufacturer’s instructions, and eDNA was eluted with a final volume of 100 mL of buffer AE (Qiagen). The eDNA extract was stored in a 1.5-mL microtube (Eppendorf®, Hamburg, Germany) at −30°C until further analysis.

**Quantitative real-time PCR assays** We quantified eDNA with real-time TaqMan PCR and a StepOnePlus™ Real-Time PCR system (Thermo Fisher Scientific). Each TaqMan reaction contained 900 nM of each primer, 125 nM of the TaqMan probe in a 1-time PCR master mix (TaqMan Environmental Master Mix 2.0; Life Technologies, Carlsbad, California), and 2 μL of the respective eDNA extract, resulting in a total reaction volume of 20 μL. The PCR conditions were as follows: 2 min at 50°C, 10 min at 95°C, and 55 cycles of 15 s at 95°C then 60 s at 60°C. We conducted 8 qPCR replicates/eDNA extraction. To produce standard DNA for the qPCR, a target amplicon was inserted into a pMD20-T vector (Takara Bio, Shiga, Japan), and the vector was digested with BamHI. We amplified a dilution series of the plasmid containing 1 × 10^7 to 1 × 10^9 copies in duplicate as a standard in all qPCR assays. Eight wells of no-template negative controls were used in all qPCR assays. In addition, we used the primer for each frog species to sequence qPCR amplicons directly from a positive PCR of the respective eDNA extracts from direct sequencing of each species are available online (Table S2).

To avoid contamination, the PCR was conducted (including preparation and addition of the standards) in a different room than qPCR cycling. The qPCR efficiency across the entire study, calculated from the slope of the standard curves, ranged from 92.8 to 114.2% (*B. subaspera*), 94.4 to 98.3% (*O. splendida*), and 85.8 to 117.5% (*O. amamiensis*). The ranges of standard curve \( R^2 \) values were >0.96 (*B. subaspera*), >0.98 (*O. splendida*), and >0.97 (*O. amamiensis*).
We defined as the detection limit for the target species the DNA copy number detected in at least 1 well in 3 replicates. As a result, the detection limit for each species was defined as 1 copy for *Babina subaspera*, 2 copies for *Odorrana splendida*, and 3 copies for *Odorrana amamiensis*. All of the aforementioned real-time PCR procedures were done according to the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin et al. 2009).

### Frog call acoustic surveys

We assessed the breeding activity of *B. subaspera*, *O. splendida*, and *O. amamiensis* by monitoring the respective mating calls with voice recorders at 26 sites close to each water sampling site. These data were collected as part of a different study (Iwai et al. 2018). Briefly, we recorded mating calls with automated digital voice recorders (RR-XS455; Panasonic, Tokyo, Japan). The recorders were active from

![Figure 1](image-url)
April/May 2014 to May/June 2015. Recordings started every night at 2200 h and lasted for 3 min. The acoustic data during the peak of the breeding season was inspected for each species: 16 d for *B. subaspera* (14–29 July 2014), 14 d for *O. splendida* (25 March–7 April 2015), and 24 d for *O. amamiensis* (27 February–10 March, and 27 March–7 April 2015). The presumed peaks of the respective breeding season for *B. subaspera*, *O. splendida*, and *O. amamiensis* were 22 May to 7 June, 27 April to 7 May, and 28 May to 7 June, respectively.

Figure 2. Map of the survey area. North area (A); south area (B). In each panel, the small black circles near the sampling site name indicate eDNA detection, and white circles indicate non-detection. Pie charts show detection (gray) and non-detection (white) of eDNA for each season, survey site, and frog species: *B. s.* = *Babina subaspera*, *O. s.* = *Odorrana splendida*, and *O. a.* = *Odorrana amamiensis*. The upper right side, the lower side, and the upper left side within the pie chart show the results of eDNA detection in December, May to June, and August to September, respectively.
were identified based on Iwai (2014), Oumi (2006), and Iwai et al. (2018).

Statistical analysis
We used McNemar’s $\chi^2$ test to compare the proportion of sites at which each target species was detected by acoustic data analysis (1 season) and eDNA (all seasons). We report statistical significance at $\alpha = 0.05$. The statistical analyses were done with R software version 3.2.3 (R Project for Statistical Computing, Vienna, Austria).

RESULTS
We established the species specificity of each primer-probe set. Direct sequencing of the qPCR amplicons confirmed that they originated from the respective targeted species (90, 96, or 116 bp product size; Table S2). Results of direct sequencing of the qPCR amplicons originated from the respective targeted species. None of the control samples (i.e., blank controls, no-template negative controls) amplified, so contamination was unlikely.

eDNA of 1 or more target species was found at 18 of the 26 survey sites (Table 1, Fig. 2A–B). The eDNA of all 3 species was detected at 2 sites. The eDNA of 2 species was found (B. subaspera and O. amamiensis; Table 1) at 3 sites. One species was detected by eDNA at 1 (B. subaspera), 2 (O. splendida), and 10 (O. amamiensis; Table 1) sites. The number of sites with positive eDNA detection was highest from August to September for B. subaspera (December n = 0, May–June n = 2, August–September n = 5) and from May to June for both O. splendida (December n = 0, May–June n = 3, August–September n = 1) and O. amamiensis (December n = 7, May–June n = 14, August–September n = 2; Table 1).

Table 1. The number of positive results of eDNA detection/8 PCR replicates in 26 sites during the 3 seasons (blank cells indicate non-detection by eDNA). Twelve in December; 5 to 6 from May to June; 8 to 9 from August to September.

| Site | Site | 12 | 5–6 | 8–9 | aco<sup>a</sup> | 12 | 5–6 | 8–9 | aco | 12 | 5–6 | 8–9 | aco |
|------|------|----|-----|-----|--------|----|-----|-----|----|----|-----|-----|----|
| 1    | AGE2 | n  | 1/8 | y   | n      | 12 | 5–6 | 8–9 | aco |    |      |     |     |
| 2    | AGE5 | n  |     | n   | y      | 12 | 5–6 | 8–9 | aco |    |      |     |     |
| 3    | AK3  | n  |     | n   | 2/8    | 1/8 | y   |     | n   | 1/8 |     | n   |     |
| 4    | CA1  | n  |     | y   | 1/8    | 3/8 | n   |     |     |     |      |     |     |
| 5    | EB1  | n  |     | n   | 1/8    |     |     |      |     |     |      |     |     |
| 6    | EB3  | n  |     | n   | 8/8    | 8/8 | y   |     |     |     |      |     |     |
| 7    | EB4  | n  |     | n   | 7/8    |     | y   |     |     |     |      |     |     |
| 8    | FR1  | n  |     | n   |       |     |     |      |     |     |      |     |     |
| 9    | FR3  | n  |     | n   |       |     |     |      |     |     |      |     |     |
| 10   | OE2  | 1/8| y   | n   | 2/8    |     | y   |     |     |     |      |     |     |
| 11   | OO1  | 4/8| 3/8 | y   |       |     |     |      |     |     |      |     |     |
| 12   | OO3  | n  |     | n   | 1/8    | y   |     |      |     |     |      |     |     |
| 13   | OO5  | 1/8| n   | n   | 7/8    | 7/8 | 4/8 | n   |     |     |      |     |     |
| 14   | SEE1 | 2/8| n   | n   | 6/8    | y   |     |      |     |     |      |     |     |
| 15   | SEE2 | n  |     | 3/8 | 1/8    | y   |     |      |     |     |      |     |     |
| 16   | SEE7.1| y |     | y   | n      |     |     |      |     |     |      |     |     |
| 17   | SEW2 | 1/8| n   | 1/8 | n      | 3/8 | n   |     |     |     |      |     |     |
| 18   | SEW3 | n  |     | n   | y      |     |     |      |     |     |      |     |     |
| 19   | SN3  | n  |     | n   | y      |     |     |      |     |     |      |     |     |
| 20   | UK7  | n  |     | n   | n      |     |     |      |     |     |      |     |     |
| 21   | UK9  | n  |     | n   | n      |     |     |      |     |     |      |     |     |
| 22   | YG2  | n  |     | 3/8 | n      |     |     |      |     |     |      |     |     |
| 23   | YG4  | n  |     | 7/8 | 1/8    | y   |     |      |     |     |      |     |     |
| 24   | YG6  | n  |     | n   | 1/8    |     |     |      |     |     |      |     |     |
| 25   | YK6  | 1/8| n   | 1/8 | y      | 3/8 | 8/8 | y   |     |     |      |     |     |
| 26   | YK8  | y  | 1/8 | y   | n      |     |     |      |     |     |      |     |     |

<sup>a</sup> aco: Acoustic survey detection of mating calls: yes (y), or no (n)
Table 2. Number of sites with detection and non-detection of Babina subaspera (A), Odorrana splendida (B), and O. amamiensis (C) based on acoustic survey and environmental DNA (eDNA) surveillance.

| (A) Babina subaspera | eDNA |  |  |
|----------------------|------|---|---|
| acoustic detection   | 2    | 2 |
| non-detection        | 4    | 18 |

| (B) Odorrana splendida | eDNA |  |  |
|------------------------|------|---|---|
| acoustic detection     | 3    | 4 |
| non-detection          | 1    | 18 |

| (C) Odorrana amamiensis | eDNA |  |  |
|-------------------------|------|---|---|
| acoustic detection      | 9    | 0 |
| non-detection           | 6    | 11 |

* If any of the 8 replicates at a site yielded a positive PCR result, we considered that site positive for eDNA. We used McNemar’s χ² tests to compare the detection pattern between acoustic survey and eDNA method, which showed significant differences in O. amamiensis (p = 0.041) but not in the other 2 species.

Acoustic and eDNA detections differed among sites and species (Table 2). Out of 26 survey sites, B. subaspera was detected at 4 sites by acoustic surveys and 6 sites with eDNA (Table 2A). Both methods detected B. subaspera at 2 sites. The detection pattern did not differ significantly between the 2 methods (χ² = 0.167, df = 1, p = 0.683, n = 26). Odorrana splendida was detected at 7 sites by acoustic survey and 4 sites by eDNA (Table 2B). Both methods detected this species at 3 sites, and no significant difference was found between the 2 methods (χ² = 0.800, df = 1, p = 0.371, n = 26). Odorrana amamiensis was detected at 9 sites by acoustic survey and at 15 sites by eDNA (Table 2C). Odorrana amamiensis DNA was detected at all sites where the species was detected by acoustic survey. The detection pattern differed significantly between the 2 methods (χ² = 4.167, df = 1, p = 0.041, n = 26).

DISCUSSION

We successfully developed species-specific primer sets to detect the 3 targeted frog species. The detection results of each method did not always match for each site and species. These discrepancies might be a consequence of the inherent differences in the target of each survey method. Acoustic surveys aim to detect mating calls, which indicate the presence of male adult frogs, but an acoustic detection does not necessarily mean that the site is used for oviposition (Resetarits and Wilbur 1991). In contrast, eDNA detection indicates that individuals of either sex and at any life-history stage (i.e., egg, larva, or adult) are present in the water. Another difference between the 2 techniques is that acoustic surveys may detect calls from distant sites because frog calls can carry up to tens of m (Boatright-Horowitz et al. 2000, Schwartz et al. 2015), whereas eDNA sampled from flowing water bodies can primarily detect organisms upstream of the sampling point and sometimes only within a rather limited range (e.g., 5 m; Pilliod et al. 2013). Therefore, sites with a positive detection by acoustic surveys that do not contain adults, eggs, or tadpoles in the water will probably not produce a positive eDNA detection. In contrast, negative detection by acoustic surveys but positive eDNA detection may occur if larvae migrated from breeding sites farther than calling detection range.

The degree of disagreement between the 2 methods differed between species, probably because of different characteristics in mating calls or tadpoles. For example, in B. subaspera and O. splendida, 50% of the sites with positive acoustic detection had positive eDNA results, whereas for O. amamiensis, all sites with positive acoustic detection also had positive eDNA results. Odorrana amamiensis has low-volume calls relative to the other 2 species, and thus the acoustically-detectable distance from the breeding site should be small. Therefore, positive acoustic detection could occur only when the recorder (set up at the eDNA sampling point) was close to the breeding site (acoustic source), which might often be within eDNA detection distance. Meanwhile, calls of the other 2 species are detectable farther from the breeding site, thus the positive detection by a recorder does not necessarily mean that the places where recorders were set up (i.e., at the eDNA sampling point) were occupied by tadpoles or eggs within eDNA detectable distance. Moreover, positive eDNA detection coincided with negative acoustic detection in 66.6% of sites for B. subaspera, 25% for O. splendida, and 40% for O. amamiensis. These percentages may reflect the migration ability of tadpoles of each species from the breeding site because the smaller the distance migrated by tadpoles from the acoustic sound source, the smaller the divergence between eDNA-detectable distance and acoustically-detectable distance. A study on the migration distance of O. splendida tadpoles showed that they remain within 10 m of their hatching site and only occasionally migrate downstream as far as 90 m (Nagai et al. 2011). Our data also suggest that O. splendida tadpoles probably stay within the calling detection range because acoustic surveys were positive with high probability at the sites where eDNA was detected.
No conclusive data are available on the migration distance of *B. subaspera* and *O. amamiensis* tadpoles during their larval period. However, our results suggest that *B. subaspera* tadpoles may migrate over a longer distance than the detection range of mating calls, whereas *O. amamiensis* might have migration behaviors intermediate between the 2 other species sampled here. Thus, species with loud call volumes, long tadpole migration distances, or both may result in more pronounced discrepancies in detection efficiency between acoustic surveys and eDNA methods.

The differences in detection rates between acoustic and eDNA surveys indicate that survey results should be interpreted and compared carefully to produce robust data for future conservation efforts. It may be insufficient to either only consider sites with positive acoustic detection for conserving larval stage habitats or rely exclusively on eDNA detection to identify breeding sites. The method of choice should instead be adapted based on the ecological characteristics of the target species. In this study, we showed that the combination of acoustic surveys and eDNA may provide relatively-robust information on the habitat use of *B. subaspera* (acoustic-only detection: \( n = 2 \), eDNA-only detection: \( n = 4 \)), acoustic surveys alone may be enough to identify habitats used by *O. splendida* (acoustic: \( n = 4 \), eDNA: \( n = 1 \)), and the eDNA method alone may be sufficient to identify habitats used by *O. amamiensis* (acoustic: \( n = 0 \), eDNA: \( n = 6 \)).

The optimal season for eDNA detection differed between species, probably because of differences in breeding seasons and larval characteristics. *Babina subaspera* oviposition and tadpole density peak in August (Iwai 2018), which is probably when *B. subaspera* eDNA is highest. In our study, eDNA detection efficiency was highest during this time (83%: 5 of 6 eDNA detection sites). The breeding activity of *O. splendida* peaks from February to April, tadpoles hatch from April to May, and metamorphosis starts in mid-June (Oumi et al. 2011). *Odorrana amamiensis* start spawning in October, and oviposition activity continues until April. Their metamorphosis occurs over a few months, from May until August (Sakoda et al. 2007). Thus, tadpoles of *O. splendida* and *O. amamiensis* should be most abundant from May to June, which should also be reflected in the highest efficiency of eDNA detection in this season (*O. splendida*: 75% [3 of 4 eDNA detection sites], *O. amamiensis*: 93% [14 of 15 eDNA detection sites]).

Our results on seasonal effects of eDNA detection efficiency suggest that even if tadpoles remain in the water for an entire year, the eDNA method may fail to detect them when their abundance or metabolism is low. The tadpoles of *B. subaspera* and *O. splendida* often overwinter (Maeda and Matsui 1999), but we did not detect their eDNA in December, even at sites where we detected eDNA in other seasons. This lack of winter detection could have occurred if either overwintering tadpoles were truly absent or slowed metabolism produced no to minute amounts of shed DNA.

*Odorrana amamiensis* do not overwinter, but this species’ breeding season is from October to April (Sakoda et al. 2007), which allowed the detection of this species in winter. One considerable advantage of the eDNA method is that any single eDNA sample may be used to detect multiple species by designing specific primers for each target species. However, our results showed that the use of only 1 sample (i.e., 1 season) for multiple species with different phenologies may sometimes produce misleading results. Thus, when applying eDNA-based methods to estimate the distribution of frog species, it is crucial to consider the ecological traits of the target species, such as the breeding season, and a possible effect of season on tadpole abundance.

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Author contributions: TT, NI, KY, and TI conceived the study. NI and KY conducted the field sampling. TT and TI conducted laboratory work. TT and NI analyzed the data. All authors drafted, contributed to, and approved the final version of the manuscript.

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