Utilizing environmental DNA for wide-range distributions of reproductive area of an invasive terrestrial toad in Ishikari river basin in Japan

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Research Article

Keywords: Environmental DNA, Terrestrial toad, Reproductive area, Watershed, Monitoring

Posted Date: April 26th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-246449/v1>

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Version of Record: A version of this preprint was published at Biological Invasions on December 15th, 2021. See the published version at <https://doi.org/10.1007/s10530-021-02709-y>.

Abstract

Understanding the distribution of invasive species and their reproductive area is crucial for their managements after invasion. While catch and observation surveys are still embraced, environmental DNA (eDNA) has been increasingly utilized as an efficient tool for identifying these species in the wild. In this study, we developed an eDNA detection system for an invasive, toxic, and terrestrial toad species *Bufo japonicus* in Hokkaido, Japan, and applied it to their reproductive area at watershed scale. We found that our system successfully detected their eDNA not only in ponds where their larvae were observed, but also in rivers downstream of the reproductive ponds. Thus, the system provided us an opportunity to estimate watersheds that include their reproductive area by collecting downstream water samples. Applying it to the Ishikari river basin, the largest river basin in Hokkaido (c.a., 14,330 km²), we detected their eDNA at 32 out of 73 river sampling sites. They are composed of eleven sites with species observation records nearby (all the sites with observation records within a 500 m radius) and 21 sites without such records. And those eDNA detections were from 14 out of 31 river systems, and they were widespread across the river basin. These results suggest that the eDNA detection system can efficiently estimate the presence of reproductive area of the terrestrial toad even from a distant downstream of the watershed, and that it provides a powerful means of detecting new reproductive area and monitoring further spread of invasive species.

Introduction

Difficulties in monitoring of current distribution of invasive species, especially at the front-line of their invasions, are one of the biggest issues in biodiversity management. To address this issue, detections of the presence of the invasive species through visual and acoustic assessments have been employed (e.g., Boback et al. 2020; Diamond and Ross 2020). However, they are often ineffective to grasp distribution and reproductive area of the invasive species because of many restrictions (large potential distributions, huge labors and money investments, difficulties in species identifications especially at their early developmental stages, etc.) (Lepczyk 2005; Ficetola et al. 2008). Therefore, it is obvious that developments of effective means of detecting them in the wild are urgently required for better understanding of the current distribution and reproductive area of invasive species and for the conservation of native fauna (Martinez et al. 2020).

Environmental DNA (eDNA) techniques have been developed rapidly since they were first applied to American bullfrog (*Lithobates catesbeianus*) in Europe in 2008 (Ficetola et al. 2008; Dejean et al. 2012; Lin et al. 2019). In particular, eDNA techniques based on river water samples are useful for screening the presence of focal species at large spatial scales because eDNA can be carried from their actual habitat to downstream by the river current (Deiner et al. 2016). In addition, large watershed systems are commonly composed of lotic and lentic waterbodies such as rivers and ponds, respectively, that are inter-connected spatiotemporally. Therefore, eDNA from pond-dwelling species can be presumably detected by collecting river water at distant downstream, although empirical evidence to support this idea is scarce at best.

Eastern-Japanese common toad (*Bufo japonicus formosus*) is one of such pond-dwelling species at its egg and larval stages. It is a subspecies of Japanese common toad (*B. japonicus japonicus*), and both subspecies are non-native to Hokkaido, the second largest island of Japan. *B. j. formosus* was introduced from mainland Japan into Hokkaido as pets several decades ago (Sarashina and Yoshida 2015; Naito & Shiga 2016). After the introduction, *B. j. formosus* were repeatedly released into Hokkaido especially in Ishikari river basin (c.a. 14,330 km² basin, length of mainstream: c.a. 268 km). This species has a neurotoxin (bufotoxin) in their body through their life cycle, and it has been reported that their embryos and hatchlings has lethal toxic effects on larvae of native amphibian species in Hokkaido (i.e., Hokkaido brown frog *Rana pirica*, Hokkaido salamander *Hynobius retardatus*) (Kazila & Kishida 2019; Oyake et al. 2020; Okamiya et al. 2021). The toxic effects (Kazila & Kishida 2019) suggest the potential threats of *B. j. formosus* as a substantial cause of declines of the native predators in invaded regions.

B. j. formosus spend most of their life in terrestrial habitats (e.g., forests and mountains) after metamorphosis. The adult individuals get close to waterbodies only in their reproductive season in spring (April to May in Hokkaido), and their egg and larval stages in May to June are their primary aquatic life stages. For controlling their further spread, identification of their reproductive area is crucial. However, identification of the actual reproductive ponds is difficult because of the following reasons. First, candidates of their reproductive ponds are too many. The adult toads are known to utilize various types and sizes of waterbodies such as natural and artificial ponds (Kusano et al. 1995), and they are located widely along rivers and their branches. Second, *B. j. formosus* breed within a few days, and they leave reproductive ponds immediately after breeding (Ishii et al. 1989; Kusano et al. 1995). Third, they are cryptic in the reproductive ponds. Adult's mating call is weak, so that one can hear it only at the peak of their reproductive season with many adults. Moreover, the adult toads deposit their egg clutches on the bottom of reproductive ponds, making it difficult to find them from the surface (Kishida O, unpublished data).

In this study, we aimed to develop a new eDNA detection system for amplifying DNA from *Bufo* species that works not only with the water samples collected directly from their reproductive ponds, but also with river water samples collected from a distant downstream of their reproductive ponds. It allows us to estimate the presence of *B. japonicus* reproductive area in the watershed without spotting each reproductive pond (hereafter, *B. japonicus* represents both species *B. j. formosus* and *B. j. japonicus*). Applying the eDNA detection system to the Ishikari river basin, we also aimed to estimate the current reproductive areas of the terrestrial toad in a wide-range of the river basin at watershed scale. To achieve these objectives, we set three goals: 1) establishing a *Bufo*-specific eDNA detection system, 2) verifying its detectability at their reproductive ponds and the downstream of these ponds during their larval period, and 3) applying it to river water samples from 31 river systems within the Ishikari river basin.

Materials And Methods

Development of the Bufo-specific eDNA detection system

There are three *Bufo* species in Japan (*B. japonicus* [*B. j. formosus* and *B. j. japonicus*], *B. gargarizans*, and *B. torrenticola*), although none of them are native to Hokkaido (Matsui 1984; Sarashina and Yoshida 2015). Because of their genetic similarities, complexities in taxonomic classification, and hybridization among species (including the three *Bufo* species in Japan and *B. bankorensis* in Taiwan, Kawamura et al. 1980; Igawa et al. 2006), we developed genus-specific eDNA primers and probe, instead of species- or subspecies-specific ones, in this study. We first collected mitochondrial DNA (mtDNA) sequence information for the 12S ribosomal RNA region in the NCBI nucleotide database. They included 174 mtDNA sequences from six *Bufo* species (*B. japonicus*, *B. gargarizans*, *B. torrenticola*, *B. bankorensis*, *B. bufo*, and *B. stejnegeri*) and 14 other amphibian genera (Supplementary Material 1, Table S1). These sequences were aligned using BIOEDIT (<https://bioedit.software.informer.com/>), and qualities of the primer candidates were evaluated using PrimerQuest (<https://sg.idtdna.com/Primerquest/Home/Index>) and OrigoAnalyzer (<https://sg.idtdna.com/calc/analyzer>). We set the following criteria for primer selection, obtaining 5'-CCTTGTGGTCAGTTATTACTTACTA-3' as a candidate of forward primer and 5'-TAGGTGTCTTGGGCGGAT-3' as a candidate of reverse primer: (1) no mismatch with *Bufo* DNA sequences in the database, (2) at least one mismatch within five base pairs at the 3'-end of each primer for the other species' DNA sequences, (3) GC content being 35-65 % for the primer binding sequences, (4) the size of primers being 17-25 bp, (5) the size of PCR amplicon being less than 150bp including the primer binding sequences, and (6) an annealing temperature being ≥ 58 °C. The probe (5'-FAM-ATTAGGGACAAGGAGCCGGTATCAGG-TAMRA-3') was then designed on the expected *B. japonicus* amplicon sequences by these primers so that the probe binding sequences should have one or more mismatches with the aligned sequences of non-*Bufo* species at the same time.

After the designing of the primers and probe, we tested their performance using DNA extracted from tissue samples using DNeasy Blood and Tissue kits (Qiagen, Hilden, Germany) and following its extraction protocol. After DNA extraction, we conducted qPCR amplifications (See *DNA extraction and detection* for more details) with the tissue-oriented DNA from eight amphibian species that were known to be distributed in Hokkaido, *B. j. formosus*, *R. pirica*, *H. retardatus*, *Dryophytes japonica*, *Glandirana rugosa*, *Lithobates catesbeianus*, *Pelophylax nigromaculatus*, and *P. porosus* (Natural Environmental Division of Hokkaido 2015). There is one more amphibian species in Hokkaido (Siberian salamander *Salamandrella keyserlingii*), but we could not obtain the tissue sample from this species, and their DNA sequence on the database (Accession No. AY593113) had three and four mismatches in the forward and the reverse primers, respectively.

Sampling sites

Field samplings were conducted in the Ishikari river basin from 2018 to 2019. For verifying the applicability of our eDNA detection system to environmental water, we first collected water samples from eight ponds around Asahikawa on June 14th, 2018 (Naidaibu pond, Naidaibu Masuda pond, Ino experimental pond, Ino Okunoike pond, Ubun gate mountain side pond, Ubun gate river side pond, Ubun up weir pond and Ubun down weir pond). In those ponds, we observed larvae of *B. japonicus* at the time of water samplings (Supplementary Material 2, Table S2). In addition, we collected river water samples

from two branches of the Ishikari river (Ino river and Kitanosawa river), of which watersheds included reproductive ponds of *B. japonicus*. The water collection sites on the two rivers were located 2000 m and 2500 m apart from the *B. japonicus* reproductive ponds (i.e., ponds with *B. japonicus* eggs in the same year of water collection) in Ino river and Kitanosawa river, respectively, according to the observations reported by Takai K. and Kishida O. (unpublished data) and Natural Environmental Division of Hokkaido (2020). These sites were within 500 m radius from public observation records of *B. japonicus* as well (Supplementary Material 2, Table S2), indicating high frequencies of their reproductive activities in the ponds along the rivers. At the water collection sites, we conducted three temporal samplings per site from the beginning to the end of June 2019 (Ino Riv. DS and Kitanosawa Riv., see Supplementary Material 2, Table S2) in order to cover their reproductive season and egg/larval stages of their offspring. For the application on the wide-range screening of their reproductive area, we selected additional 71 sites from 29 river systems in the Ishikari river basin and collected river water samples from June 14th to 15th in 2018 and from June 5th to 26th in 2019 (Supplementary Material 2, Table S2). Including the two river sites for verification above, the number of sampling sites for the wide-range screening were 25 in 2018 and 48 in 2019 (73 from 31 river systems in total).

On-site eDNA filtering

Filtering procedures are referred from Yatsuyanagi et al. (2020) with minor modifications. In brief, all water samples were collected from the surface of water using a new plastic bag or a bleached plastic bucket, and filtered using a SterivexTM-HV filter cartridge with a pore size of 0.45 µm (Merck Millipore, Inc.) and a sterile 50 mL syringe (TERUMO, Inc., Tokyo, Japan) or a Masterflex[®] (Yamato Scientific Co., Ltd., Tokyo, Japan). Basically, we filtered 500 mL environmental water per filter, but sometimes we changed it to 250 mL because of the water turbidity. After filtration, we added 2 mL RNAlater (Thermo Fisher Scientific, Inc., Massachusetts, USA) into the Sterivex cartridge for DNA preservation. At each sampling, we collected two filter samples as replicates. After finishing the day's sampling, we prepared negative control samples by filtering 500 mL pure water with the same manner to the other samples. All sampling equipment was bleached by soaking in a 10% sodium hypochlorite solution after each sampling. All filtered samples were stored around -20 °C at field stations and at -80 °C in the lab until further analyses.

DNA extraction and detection

DNA extractions were conducted using DNeasy Blood and Tissue kits (Qiagen, Hilden, Germany) following Yatsuyanagi et al. (2020). The final volume of the extracted DNA was 100 µL/sample. In the DNA extraction step, we made extraction negative control samples using new SterivexTM-HV filters with the same extraction procedure. qPCRs were carried out in 20 µL volumes with 200 nM of *Bufo* specific primer and probe in a Brilliant III Ultra-Fast qPCR Master Mix with Low ROX (Agilent Technologies, Inc.) and 2 µL of the extracted DNA as a template sample. Triplicates PCR reactions x two filter samples per site were carried out on a Stratagene Mx3000P (Agilent Technologies, Inc.). Thermal-cycling regime was 95°C for 3 minutes, followed by 50 cycles of 95°C for 10 second and 60°C for 20 seconds. For the

quantification of environmental samples, we applied synthetic linear DNA of the target region containing 2×10^1 , 2×10^2 , 2×10^3 , 2×10^4 and 2×10^5 copies per tube as standards in all qPCR assays ($n = 10$, average $R^2 = 0.96$, $SD = 0.02$, and average PCR efficiency = 1.10, $SD = 0.21$). In addition, sterilized distilled water samples were applied in all qPCR assays as PCR negative control samples. Based on Mizumoto et al. (2020), we accepted sites as positive detections with at least one detection in six PCR replicates per site and lowest detection limit was set at 0.01 copy per 2 $\mu\text{L} \times$ site. We had three temporal samples in Ino Riv. DS and Kitanosawa Riv., so we assumed as the positive detection sites when we had at least one positive detection samples out of three temporal samples in each site. Estimated DNA concentrations were represented as per 1000 mL for adjusting the variation in filtered water volume per sample. Overall, we had no positive detection from all negative control samples taken on the field, DNA extraction, and qPCR. Referring to Yatsuyanagi et al. (2020), each estimated eDNA concentration was calculated as per 1000 mL of environmental water for the sake of consistency in this study.

Analyses on the wide-range application

For analyzing the eDNA detection results in river water samples, we referred public observation records of *B. japonicus* reported by Natural Environmental Division of Hokkaido (2020). In this study, we defined a sampling site with observation record nearby when the site had at least one observation record within a 500 m radius. By this definition, we found seven sites with observation record nearby. In addition, we included four additional sampling sites (Ino Riv. US, Naidaibu Riv. MS-3, Naidaibu Riv. DS and Ubun Riv.) with observation record nearby because those sites were located in a 500 m radius from the ponds where we ourselves observed larvae of *B. japonicus* on the same days as the water samplings. In total, we had eleven sites with observation record nearby and 62 sites without (Supplementary Material 2, Table S2). We then tested a null hypothesis that there is no relationship between eDNA detections and observation records within a 500 m radius using Fisher's exact test. Statistical analyses were performed using R3.6.1 (R core team. 2020).

Results

Establishment of Bufo-specific eDNA detection system

In silico evaluation of the selected primers suggested that *Bufo* was the only genera from which DNA sequences had no mismatch within five base pairs at the 3'-end of both primers. Note, however, that DNA sequences of European toad (*B. bufo*) and Korean water toad (*B. stejnegeri*) had one mismatch within five base pairs at the 3'-end of either one of the primers as exceptions (Supplementary Material 3, Table S3). And the probe DNA sequence was unique to *Bufo* species according to the in silico evaluation, although one mismatch was accepted within the 26bp-long probe DNA sequence among five accessions from *B. japonicus* (Accession No. AB159468, AB159469, AB159470, AB159471 and AB303363) out of 27 *B. japonicus* Accessions in the database (Supplementary Material 3, Tables S3).

Although DNA sequence from *Anaxyrus punctatus*, formally called *Bufo punctatus* (Accession No. AY680237), had no mismatch within five base pairs at the 3'-end of forward primer, the reverse primer had two mismatches within five base pairs at the 3'-end of the *A. punctatus* DNA sequence. This species is mainly distributed to southwestern United States to northwestern Mexico, and there is no report of establishment of the species in Japan (www.nies.go.jp/biodiversity/invasive/DB/detail/40180e.html).

As the results of performance test of the eDNA detection system using DNA from tissue samples of eight co-existing amphibian species in Hokkaido (*B. j. formosus*, *D. japonica*, *G. rugosa*, *H. retardatus*, *L. catesbeianus*, *P. nigromaculatus*, *P. porosus* and *R. pirica*), we confirmed q-PCR DNA amplification only from *B. j. formosus* (Fig. 1). It is also noteworthy that none of the *Bufo* species is native to Hokkaido, and *B. j. formosus* is the only subspecies that has been identified in Hokkaido after introduction (Natural Environmental Division of Hokkaido 2015).

Verifying the eDNA detectability at reproductive pond and downstream river

In the validation of the *Bufo*-specific eDNA detection system based on pond water samples, we detected eDNA from seven out of eight pond water samples that were collected from where larvae of *B. j. formosus* were observed (Table 1). The average eDNA concentrations were in the range of 6.65×10^3 and 8.28×10^6 copies per 1000 mL of pond water sample (Table 1). One exception (site without any detection of eDNA) was Ubun gate river side pond, where many *B. japonicus* larvae were observed at the time of water collection (See Discussion for more detail).

At the two river sites with observation records of *B. japonicus* within a 500 m radius (Supplementary Material 2, Table S2), we detected eDNA from both sites (Fig. 2). eDNA concentrations estimated in Ino Riv. DS and Kitanosawa Riv. samples were the highest at the beginning of June (1.02×10^5 and 3.04×10^4 copies/1000 mL*site, respectively), and constantly decreased toward the end of June (Fig. 2). In Ino Riv. DS, the estimated eDNA concentrations became only 5.23% of its peak on June 23rd, and those in Kitanosawa Riv. become zero on June 26th. The estimated eDNA concentrations in Ino Riv. DS samples were significantly higher than that of Kitanosawa Riv. throughout June (exact Wilcoxon-Mann-Whitney test, $p < 0.01$) (Fig. 2).

Application to the Ishikari river basin

Out of the 73 river water samples collected in the Ishikari river basin in 2018 and 2019, we had 32 positive eDNA detections (Fig. 3). Comparing with the observation records, we detected eDNA from all the eleven sites where the observation records existed within a 500 m radius (Supplementary Material 2, Table S2). In addition, we detected the eDNA from 21 out of 62 sites without any observation records nearby (Supplementary Material 2, Table 2). They belonged to twelve river systems, and two of them had no observation record throughout the river systems (Aibetsu river and Bibai river, see Supplementary Material 2, Table S2). We found a significant association between eDNA detections and observation records nearby ($p < 0.001$ by Fisher's exact test).

Discussion

In our study, we aimed to develop a *Bufo*-specific eDNA detection system and to estimate the distribution of reproductive area of the terrestrial invasive terrestrial toad at watershed scale. Our results suggest that the development of the eDNA detection system was successful (Fig. 1), and eDNA was detected from seven out of eight ponds (Table 1). In addition, we found eDNA from 32 river systems within the Ishikari river basin without spotting the reproductive ponds of *B. japonicus*.

eDNA detection systems have been applied for understanding distributions of invasive species in the world, mainly for aquatic organisms (Ficetola et al. 2008; Adrian-Kalchhauser and Burkhardt-Holm 2016; Larson et al. 2017; Crookes et al. 2020; LeBlanc et al. 2020). For terrestrial organisms, eDNA was detected from ponds and lakes that were sporadically distributed in the forests or mountains (Bista et al. 2017; Ushio et al. 2017; Tingley et al. 2019). In addition, some previous studies aimed to detect eDNA of terrestrial species from stream water samples (Cannon et al. 2016; Deiner et al. 2016). However, few studies were conducted focusing on the reproductive area of invasive terrestrial species at watershed scale presumably due to low detectability caused by low abundance at these sites. To our knowledge, this is the first study that specifically targeted detecting the presence of reproductive area of the invasive terrestrial toad at the watershed scale from a distant downstream of the riverine system.

Despite the high detectability in ponds (Table 1), we failed to detect the *Bufo* eDNA in one pond. A potential cause of the detection error is PCR inhibitors (e.g., humic acid, tannic acid). This specific pond was surrounded by silt and turbid environment, and it is known that pond water sometimes include much PCR inhibitors (Lakay et al. 2007; Fujii et al. 2019). In fact, we could recover the detection of eDNA from this pond when the sample was diluted 100 times in our additional analysis (Supplementary Material 4), although we do not recommend the 100-time dilution in the wide-scale analyses due to generally low eDNA concentration in river water samples (e.g., average eDNA concentration in the positive detections of eDNA from the river samples was only 6.18 copies/mL).

To our surprise, we found eDNA detections at all the sites with the previous observation records nearby in the river samples (11/11 sites, Table 2). It suggests that eDNA detection systems work efficiently to estimate reproductive area of terrestrial amphibians from a distance if their reproductive seasons and larval periods are well understood. The average distance between the sampling sites and the nearest observation points was 191.8 m in the eleven samples (23.6-393.6 m, one standard deviation 130.1 m, Supplementary Material 2, Table S2). Accepting observation records within 1000 m radius, on the other hand, we found seven sites with observation records without detecting eDNA (eDNA detections at 18 out of 25 sites with observation records nearby, Supplementary Material 2, Table S2). Nevertheless, it is also true that within 1000 m radius, there still were 14 cases in which eDNA was detected without observation record (Supplementary Material 2, Table S2). Obviously, no observation record does not necessarily mean no reproductive ponds, and it is likely that there are such ponds in between the sampling sites and observation points. It is also obvious that eDNA detectability depends on many conditions such as the distances from reproductive ponds, number of individuals and their developmental stages, water volume

and quality etc., and that no eDNA detection does not necessarily mean no reproductive ponds in the focal watershed. They should be carefully evaluated for better management of the invasive species during the reproductive season of the terrestrial toad in future studies, and our study provides priorities based on the eDNA detections at watershed scale.

For nailing the spread of invasive species, it is essential to understand hotspots of their reproductions. As the results of stationary monitoring in Ino Riv. DS and Kitanosawa Riv. aimed to verify the detectability of their reproductive seasons and/or larval stage, estimated eDNA concentrations were gradually decreased from the start to the end of our sampling period (Fig. 2). Reproductive season of *B. j. formosus* is quite limited in a few days and they leave their reproductive ponds immediately after their reproduction (Ishii et al. 1989; Kusano et al. 1995). According to a local researcher, the number of observed larvae of *B. j. formosus* in ponds decreased during our sampling period, and no larva was observed after June 18th, 2019 around the Kitanosawa (Tokuda T., personal communication). Furthermore, Villacorta-Rath et al. (2020) reported that cane toad eDNA drastically decreased within a few days after its last presence in aquaria. Thus, estimated eDNA seems to be mostly originated from larvae. It suggests that positive detections reflected the presence of their reproductive ponds.

In addition, many ponds along the river are persistent by inflow water from spring, and the pond water overflow into the adjacent river in our survey area (Kishida O. & Takai K., unpublished data), which allowed us to estimate the presence of their reproductive ponds from a distance. The high consistency between the eDNA detections and previous observation records suggests that they provide similar quality of information regarding their distribution, and that the distribution does not change rapidly once they invaded in each watershed (The two kinds of information [eDNA and observation] had 0-8 years of time lag, Supplementary Material 2, Table S2). Furthermore, the eDNA detections at the sites without observation records nearby suggest relatively new reproductive ponds in the watersheds, which might include hubs of their further spread within the river basin. Given that *B. j. formosus* invaded round Asahikawa City several decade ago, the eDNA detection patterns in the Ishikari river basin (Fig. 3) represents a stable distribution of *B. j. formosus* reproductive ponds around the original invasion sites, as well as further spreads of them along the Ishikari river. It indicates the importance of wide-scale monitoring of their reproductive ponds along with the mainstem and branches of the Ishikari river with and without direct observation of *B. j. formosus*.

In conclusion, we detected *B. japonicus* eDNA not only from pond water samples but also from river water samples in the Ishikari river basin in this study. It suggests that our eDNA detection system can be used for screening their presence in watershed, and that information seemingly reflects their reproductive activities in the watershed. The watershed-scale screening of their reproductive area based on eDNA is a practical approach for grasping spread of frontline of the invasive species because it has a potential to save huge labor costs for the pond-scale inspection by the direct observation methods. With more technical advances and follow-up studies, eDNA detection systems can be a standard tool for estimating reproductive areas of amphibian species in general, with which biodiversity managements become much more efficient.

Declarations

Funding: This study was funded by the Mitsui & Co. Environment Fund (R16-0008: OK) and the Environment Research and Technology Development Fund of the Environmental Restoration and Conservation Agency of Japan (JPMEERF20204004: HA).

Conflicts of interest: The authors declare that they have no conflict of interest.

Availability of data and material: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability: Not applicable.

Authors' contributions: OK and HA conceived the original idea and acquired the funding. HM, KT, OK and HA collected samples and revised the manuscript, after HM analyzed data and HM and HA wrote the original draft. OK and HA administrated the project and gave final approval for publication.

Acknowledgements: We thank the members of staff and the members of Tomakomai Experimental Forest of the Field Science Center for Northern Biosphere Hokkaido University, and staff and the members of Animal Ecology Lab in Research Faculty of Agriculture, Hokkaido University. We also thank T. Aota and T. Tokuda for sharing local information. This study was funded by the Mitsui & Co. Environment Fund (R16-0008), Asahi glass foundation to OK and the Environment Research and Technology Development Fund of the Environmental Restoration and Conservation Agency of Japan (JPMEERF20204004: HA). We declare that the experiments complied strictly with the current laws in Japan.

Data availability: All data was shared in Tables (Table 1, S1-S2) and Supplementary Materials.

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