An application of PCR-RFLP species identification assay for environmental DNA detection

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Recent advancement of eDNA methods for surveying species in aquatic ecosystems has been used for various organisms and contributed to monitoring and conservation of species and environments. Amphibians are one of the promising taxa which could be monitored efficiently by using eDNA. However, the cost of the eDNA detection can be quite high and requires instruments that are not usually installed in ecology laboratories. For aiding researchers foraying into eDNA studies of amphibians, especially those not specialized in molecular biology, we developed a cost efficient protocol using PCR-RFLP method. We attempted to detect eDNA of three Japanese *Rana* species (*R. japonica*, *R. ornativentris,* and *R. tagoi tagoi*) in various spatial scales including an area close to the Fukushima Nuclear Power Plant (FNPP) where the environment is recovering after the disaster in 2011. Our PCR-RFLP protocol was successful in detecting *Rana* species in static water in both laboratory and field; however, it could not detect *Rana* species in non-static water samples from the field. Even a more sensitive detection method (standard qPCR) was unable to detect frogs in all non-static water samples. We speculate that our new protocol is effective for frogs living in lentic habitats, but not for lotic habitats which may still require the gold standard of field observation for detection approach.

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22 Abstract

23 Recent advancement of eDNA methods for surveying species in aquatic ecosystems has been
24 used for various organisms and contributed to monitoring and conservation of species and
25 environments. Amphibians are one of the promising taxa which could be monitored efficiently
26 by using eDNA. However, the cost of the eDNA detection can be quite high and requires
27 instruments that are not usually installed in ecology laboratories. For aiding researchers foraying
28 into eDNA studies of amphibians, especially those not specialized in molecular biology, we
29 developed a cost efficient protocol using PCR-RFLP method. We attempted to detect eDNA of
30 three Japanese *Rana* species (*R. japonica, R. ornativentris,* and *R. tagoi tagoi*) in various spatial
31 scales including an area close to the Fukushima Nuclear Power Plant (FNPP) where the
32 environment is recovering after the disaster in 2011. Our PCR-RFLP protocol was successful in
33 detecting *Rana* species in static water in both laboratory and field; however, it could not detect
34 *Rana* species in non-static water samples from the field. Even a more sensitive detection method
35 (standard qPCR) was unable to detect frogs in all non-static water samples. We speculate that our
36 new protocol is effective for frogs living in lentic habitats, but not for lotic habitats which may
37 still require the gold standard of field observation for detection approach.
38

39

80 *ornativentris* are often located near areas of human development and thus could be threatened
81 with population decline due to human-related habitat loss. As a result, these two species have
82 been listed as locally threatened in some regions (e.g., Natural Environment Division, Breau of
83 Environment, Tokyo Metropolitan Government, 2010; Natural Environment Division, Depertmet
84 of Environmental Affairs, Fukuoka Prefectural Government, 2014). In addition, these species are
85 key indicator species for assessing the degree of disturbance to the natural environment,
86 especially in Japanese traditional rural areas which are often called "Satoyama" (Kitagawa,
87 2002). For example, agencies like the Biodiversity Center of Japan, the Nature Conservation
88 Bureau, and the Ministry of the Environment (e.g., Monitoring-Site 1000 project) have
89 conducted ecological monitoring surveys and listed the occurrence of *Rana* species in numerous
90 reports.
91   For development of our new protocol in this study, we adapted the PCR-RFLP method
92 for recognizing these three *Rana* species (Igawa et al., 2015) for eDNA detection. In this method,
93 mitochondrial 16S rRNA fragments were amplified and digested with two restriction enzymes
94 (SpeI and HphI) to identify species within the samples by electrophoretic banding pattern of the
95 digested fragments. To evaluate the performance of our protocol, we attempted to detect eDNA
96 diluted in water samples and compared the results with visual observation and qPCR methods.
97 The water samples were taken from various spatial scales from regional levels to microhabitat in
98 addition to water tank experiments. In particular, we obtained regional samples covering 60 km
99 from Fukushima Nuclear Power Plant (FNPP) in spring and summer seasons and assessed
100 presence information of *R. t. tagoi* in regenerating habitats after the disaster in 2011. Finally, we
101 reported the effectivity of the protocol for distribution surveys in the field.
102
103

Materials & Methods

Water tank experiment

106   To characterize basic performance of our PCR-RFLP assay for environmental DNA
107 detection, we firstly conducted model experiments by preparing eight different water tank culture
108 setups with the following animals: (1) forty *R. japonica* tadpoles for two days, (2) forty *R.*
109 *japonica* tadpoles for five days, (3) nine *R. japonica* and one *R. ornativentris* adults for one day,
110 (4) one *R. japonica* and nine *R. ornativentris* adults for one day, (5) five *R. japonica* and five *R.*
111 *ornativentris* adults for one day, (6) one *R. t. tagoi* and nine *R. japonica* adults for one day, (7)
112 one *R. t. tagoi* and nine *R. ornativentris* adults for one day, (8) negative control- no animals for
113 one day (but water was used previously for other frog species). The tadpoles of *R. japonica* were
114 collected from a pond in Hiroshima University campus in Higashi-hiroshima city (Hiroshima
115 Prefecture) in April 2016 just before the experiment and were classified as growth stage 33
116 according to Tahara (1974). The adult frogs of *R. japonica, R. ornativentris*, and *R. t. tagoi* were
117 collected from Higashi-hiroshima city in 2014, Kita-hiroshima-cho (Hiroshima Prefecture) in
118 2015, and Kisa, Miyoshi city (Hiroshima Prefecture) in 2011, respectively, and reared in the
119 Amphibian Research Center, Hiroshima University, until the start of experiments. We transferred

120 and kept animals in plastic tanks (450 × 650 ×150 mm) filled with 8 L tap water (water level
121 about 30 mm which immerses half body of adults). The animals were not fed during the
122 experiment and were held at room temperature around 18°C under 12 h: 12 h light-dark cycle.
123 We collected a 500 mL water sample from each tank after the specified periods using DNA-free
124 500 mL bottles, and samples were immediately transferred to a -30°C freezer. These experiments
125 were conducted in April 2016 for tadpoles (tanks 1 and 2) and September 2016 for adults (tanks
126 3 to 8); all animals were returned to the original habitat or enclosures under healthy conditions
127 soon after the experiments.
128   All procedures were approved by the Hiroshima University Animal Research Committee
129 (Approval number: G17-4) and were carried out in accordance with the recommendations in the
130 Guide for the Care and Use of Laboratory Animals of the Hiroshima University Animal
131 Research Committee.
132
133 *Field survey and experiments*
134   To check the performance and sensitivity of PCR-RFLP assay for eDNA from the field
135 environment, we collected water samples from ponds and rivers at 28 sites (Fig. 1). For
136 exemplification of applicability in various scales of distribution monitoring studies, we collected
137 samples in three hierarchical levels: (A) regional samples covering 60 km from Fukushima
138 Nuclear Power Plant (FNPP) (sample no. 1 – 19), (B) local area samples from a single body of
139 water in Hiroshima University campus, Higashi-Hiroshima (sample no. 20 – 25), and (C)
140 microhabitat level samples from a single breeding site of *R. japonica* in Etajima Island,
141 Hiroshima Prefecture (N 34.27067, E 132.47679) (sample no. 26 and 27). We collected 500 mL
142 water samples from the water surface at each site using DNA-free 500 mL bottles. Samples from
143 Fukushima (A) were collected between 10:00 – 16:00 during two periods, 12 to 14 May 2015
144 and 11 to 13 August 2015. Samples from Hiroshima University (B) and Etajima (C) were
145 collected between 15:00 – 16:00 on 11 April and 13:00 on 17 April in 2016, respectively. To
146 prevent contamination by field sampler at each site, we carefully collected samples firstly in
147 downstream sites and moved upstream for subsequent sampling. Sampled water bottles were
148 immediately transported on ice in a cooling box to the laboratory and stored at -30°C until the
149 laboratory experiments mentioned below. In addition, we recorded the presence and non-
150 detection of frogs and/or tadpoles by visual observation, exploring the vicinity of the field
151 sampling sites. For sampling in Fukushima, permission to enter the survey area was obtained
152 from the local governments [Iitate Vilage (approval number: 2705-01 and 2707-0) and Namine
153 Town (approval number: 755 and 1959)]. Notification of the field survey was accepted by the
154 Iwaki District Forest Office, and permits were obtained from private landowners as required for
155 each site.
156
157 *DNA extraction and PCRs*
158   The water samples stored at –30 °C were thawed and vacuum-filtered through glass
159 microfiber filters (GF/F, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) with 0.7 μm

160   particle retention. Sodium hypochlorite treatment (10% for 5 min, rinsed with distilled water)
161   was performed in between samples to avoid contamination. Filtering controls (i.e., 500 mL of
162   distilled water) were filtered on each day of sample filtration. The filter papers were wrapped in
163   aluminum foil, placed in plastic bags, and stored at –30 °C. The eDNA was extracted from the
164   filters according to the methods of Uchii, Doi & Minamoto (2016), using a Salivette tube
165   (Sarstedt, Nümbrecht, Germany) and a DNeasy Blood and Tissue Kit for DNA purification
166   (Qiagen, Hilden, Germany). The filters were incubated by submersion in a mixed buffer (400 μL
167   buffer AL and 40 μL Proteinase K; Qiagen) using a Salivette tube in a dry oven at 56 °C for 30
168   min. The tubes with filters were centrifuged at 5,000 g for 5 min at room temperature. Then, 220
169   μL of TE (Tris-EDTA) buffer (pH: 8.0; 10 mM Tris–HCl and 1 mM EDTA) was added to the
170   filters, and tubes were centrifuged again at 5,000 g for 5 min. Buffer AL (200 μL) and 100%
171   ethanol (600 μL) were then added to each filtrate and mixed by pipetting. The mixture was
172   applied to a DNeasy Mini spin column and centrifuged at 6,000 g for 1 min. This step was
173   repeated until the mixture was completely processed. We followed the manufacturer's
174   instructions for further steps, and eDNA was eluted from each sample solution with a final
175   volume of 100 μL Buffer AE.
176   The eDNA samples were then used for PCR-RFLP assay to detect DNA from the three
177   target Japanese brown frog species based on Igawa et al (2015). This method utilizes species-
178   specific restriction enzyme (SpeI and HphI) digestion sites in a partial nucleotide fragment of
179   mitochondrial 16S rRNA amplified by PCR. However, the oligonucleotide primers used by
180   Igawa et al. (2015) could amplify 16S rRNA fragments originated from other vertebrate species
181   including humans and showed similar banding patterns. Therefore, we altered the primers to
182   amplify a shorter region in which only the three target frog species have restriction sites.
183   Specifically, we modified the method of Igawa et al (2015) by changing the primers: F96 5'-
184   GTCCAGCCTGCCCAGTGAYAAA -3' and R19 5'- GTTGAACAAACGAACCATTGGT -3'.
185   These new primers were designed from an internal region of the previous study and amplify
186   shorter fragments (approximately 500 bp).
187   For higher efficiency of PCR amplification, we also modified the PCR protocol described
188   by Igawa et al. (2015). In this study, PCR was conducted using KOD FX Neo (TOYOBO,
189   Osaka, Japan). The 20 μL total volume of reaction solution included 10 μL of 2× PCR Buffer for
190   KOD FX Neo, 2 μL of 2mM dNTPs, 10 pmole of each primer, 3 μL of eDNA solution and 0.4 U
191   of KOD FX Neo. Thermal cycling was performed using two-step PCR cycling: 95 ˚C for 3 min
192   followed by 35 cycles of 98 ˚C for 10 s and 65 ˚C for 30 s. Following PCR amplification,
193   independent digestion using two restrictions enzymes (SpeI and HphI) and electrophoresis were
194   conducted in the same manner as Igawa et al. (2015). Then, 5 μl of the original PCR product or
195   15 μl of each PCR product digested with the two restriction enzymes were electrophoresed on a
196   2% agarose gel for 30 min at 100 V and visualized.
197   For *R. japonica,* the amplified fragments are expected to have different digestion patterns
198   between the western (including Hiroshima) and northern part (including Fukushima) of the
199   Japanese mainland. In all *R. japonica* populations, amplicons digested with SpeI result in 255

200 and 259 bp subfragments. However, after Hph1 digestion, *R. japonica* frogs from the western
201 part have no subfragments while those from the northern part have 301 and 220 bp subfragments.
202 For *R. ornativentris* and *R. t. tagoi*, amplified fragments are commonly digested only with HphI,
203 resulting in 231, 199 and 86 bp subfragments and 313 and 200 bp subfragments, respectively. To
204 confirm the specificity of eDNA detection by our PCR-RFLP method, some of the amplified
205 fragments were directly sequenced using the same primers and BigDye Terminator ver 3.1 (Life
206 Technologies) after PEG precipitation. The obtained nucleotide sequences were annotated by
207 NCBI blastn (https://blast.ncbi.nlm.nih.gov).
208   In addition, we conducted real-time quantitative PCR (qPCR) assay for samples from
209 Fukushima to compare the sensitivity of eDNA detection for *R. t. tagoi*. At first, we defined
210 regions to design the primers and TaqMan MGB probes for the qPCR assays for this species
211 using PrimerExpress 3.0.1 (Thermo Fisher Scientific, Carlsbad, CA, USA), for amplification of
212 106 bp fragments: Rtag16SrRNA-F, 5'-AGAAGGAACTCGGCAAACCTT-3'; Rtag16SrRNA-
213 R, 5'-CCGCGGCCGTTGAAT-3'; Rtag16SrRNA-Pr, 5'-[FAM]- CCAGCCTGCCCAGTG-
214 [NFQ]-[MGB]-3'. To prove that the primers did not amplify other sympatric frog species, we
215 confirmed no amplification when the primers were applied to DNA extracted from *R. japonica*
216 and *R. ornativentris* tissues. However, in addition to *R. t. tagoi*, three closely related species (i.e.,
217 *R. sakuraii*, *R. t. okiensis* and *R. t. yakushimensis*) were also detected in an *in silico* specificity
218 screen, which was performed using Primer-BLAST, with the setting "nr" for database and
219 "vertebrates" for organisms parameters (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). *Rana*
220 *sakuraii* may inhabit sympatrically with *R. t. tagoi* in our field survey. In contrast, *Rana t.*
221 *okiensis* and *R. t. yakushimensis* never share distribution with our target species because they are
222 only found on Oki island in Shimane Prefecture and the Yakushima islands in Kagoshima
223 Prefecture, respectively. Thus, in this study, the qPCR primers we developed are specific for *R. t.*
224 *tagoi*/*R. sakuraii*.
225   Environmental DNA was quantified using a StepOnePlus™ Real-Time PCR system (Life
226 Technologies, Carlsbad, CA, USA). Each TaqMan reaction contained 10 μL of TaqMan®
227 Environmental Master Mix 2.0 (Thermo Fisher Scientific), 1 μL of the primer (900 nM)/probe
228 (125 nM) mix, 7 μL of distilled water, and 2 μL of eDNA extract. The PCR cycles were as
229 follows: 2 min at 50 °C, 10 min at 95 °C, then 55 cycles of 15 s at 95 °C, and 60 s at 60 °C. In
230 order to produce standard DNA for the qPCR, a target amplicon was inserted into a pMD20-T
231 vector (Takara Bio, Shiga, Japan), and the vector was digested with BamHI. A dilution series of
232 the plasmid containing 1×10^1 to 1×10^4 copies was amplified as standards in duplicates in all
233 qPCR assays. The qPCR for each sample was performed in 8 wells, and the mean was used as
234 the concentration of eDNA (copies/L). If any of the 8 replicates of each sample yielded a
235 positive result, the sample was designated as containing *R. t. tagoi*/*R. sakuraii* eDNA. As a
236 negative control, each qPCR assay included 8 wells that contained no template (2 μL of DNA-
237 free water) (i.e., NTC). In addition, using the primer-probe set of this species, qPCR amplicons
238 were sequenced directly from a positive PCR of the field samples (N = 6) after treatment with
239 ExoSAP-IT (USB Corporation, Cleveland, OH, USA) or DNA fragment isolation with

240 FastGene™ Gel/PCR Extraction Kit (NIPPON Genetics, Tokyo, Japan) following agarose gel
241 electrophoresis. Products were sequenced by a commercial sequencing service (Takara Bio,
242 Shiga, Japan) or by our experiment using ABI3130xl automated sequencer (Thermo Fisher
243 Scientific).
244   The range of qPCR efficiency across the entire study, calculated from the slope of
245 standard curves, was 91.937% to 94.716%, and the R^2 value for standard curve was 0.997. Since
246 we could detect two copies of DNA in at least one of the three replicates, we defined the limit of
247 detection (LOD) for DNA from *R. t. tagoi* using qPCR assay as two copies.
248   To avoid contamination, we followed a unidirectional lab flow. That is, we performed all
249 PCR protocols, including preparation/addition of the standards and qPCR cycling, in two
250 separate rooms (rooms 1 and 2, respectively). To prevent carry-over contamination, no
251 equipment or samples were returned from room 2 to room 1.
252
253

254 **Results**
255 *Water tank experiments*
256   For the eDNA solutions from water tank experiments, we could amplify 16S rRNA
257 fragments from all samples, each showing a single band following gel electrophoresis (Fig. 2A).
258 The band patterns of digested fragments in each sample were completely matched with the
259 species that were held in each tank, except for the negative control tank without animals (Fig.
260 2A). Notably, for the samples from water tanks in which two species were cultured, we could
261 simultaneously amplify eDNA from both species whereby both band patterns specific to two
262 species were displayed.
263

264 *Field survey and experiments*
265   In the survey in Fukushima (A), field observation of *R. t. tagoi* adults were recorded in 15
266 of 19 sites (Fig 1, Supp Table 1), while tadpoles were observed in a single separate site (no. 2).
267 Among the sites where *R. t. tagoi* adults were observed, adults of *R. ornativentris* were also
268 observed sympatrically in 5 sites (Supp Table 1). However, we could not identify presence of
269 either *R. t. tagoi* or *R. ornativentris* by PCR-RFLP analyses of eDNA from any of the samples
270 from Fukushima (Supp Fig 1). Subsequently, we applied the more sensitive *R. t. tagoi*/*R.
271 sakuraii*-specific qPCR which could still only confirm presence of *R. t. tago*i in five of the fifteen
272 sites with field observations of adults of the species (Fig 1, Supp Table 1). Our direct sequences
273 of the qPCR amplicons (106 bp product size) were almost identical to haplotypes of *R. t. tagoi*
274 deposited in DDBJ. In addition, no amplification was observed in any control samples (i.e.
275 filtering controls and NTC).
276   In Fukushima site no. 1, we also reported field observation of *Bufo japonicus* tadpoles, a
277 species outside of our target species. From this sample, PCR amplification resulted in an
278 approximately 600 bp fragment; sequencing and BLAST search confirmed a match of nucleotide

279   sequence with partial sequence of 16S rRNA of *Bufo japonicus* (= *B. j. formosus*) [AB159565, a
280   haplotype from Atsumi, Yamagata Pref. (Igawa et al., 2006)].
281   From field experiment (B) and (C), field observation of *R. japonica* tadpoles was
282   recorded for three sites (no. 20, 21, and 26; Fig 1, Supp Table 1). This was consistent with our
283   PCR-RFLP which showed *R. japonica*-specific banding patterns following SpeI restriction
284   digestion in the same samples (Figure 2B). We also confirmed the sequence from site no. 20 had
285   BLAST similarity with partial sequence of 16s rRNA from *R. japonica* [LC014155, a haplotype
286   from Hiroshima University (Igawa et al., 2015)]. In addition, PCR-RFLP analyses of site no. 27
287   supports the presence of *R. japonica*, despite no field observation of tadpoles at this small
288   drainage site.
289
290

Discussion

292   As shown in our water tank experiment, eDNA of *Rana* species was successfully
293   amplified and we could even simultaneously identify each pair of the three species using PCR-
294   RFLP method. Following success of this newly developed protocol, we applied it to a variety of
295   field collected water samples. In field experiment (A) at Fukushima, despite frogs (adults or
296   tadpoles) being identified in 16 of 19 sites by field observation, we could not detect eDNA of *R.*
297   *t. tagoi* or *R. ornativentris* using our PCR-RFLP method. Even when we applied the more
298   sensitive qPCR approach for detecting *R. t. tagoi*, this method only confirmed presence of the
299   species in five of the sites. There are differences in detection sensitivity across PCR-RFLP and
300   qPCR methods: qPCR is more sensitive than conventional PCR as shown in a study in guinea
301   pigs (Zemtsova, Montgomery & Levin, 2015). In addition, the more expensive approaches like
302   digital PCR and NGS of eDNA could prove to be more sensitive, despite the hurdles associated
303   with cost.
304   The lack of detection by PCR-RFLP and limited sensitivity by qPCR could be attributed
305   to the water system of the environment. Such factors like water volume, water discharge and
306   flow rate could markedly impact the concentration of eDNA. It seems likely that flowing waters
307   of lotic habitats could have lower eDNA concentrations due to dilution from fresh water
308   upstream. In addition, *R. t. tagoi* adults and their spawned tadpoles utilize small torrents or
309   subsoil water systems only in the breeding season. According to Matsu and Maeda (1999), *R. t.*
310   *tagoi* breed during late June in colder regions of Japan like Fukushima; our sample sessions
311   occurred at both before and after this breeding period and thus could result in low level of eDNA
312   in the water. Overall, the detection of *Rana* species from river water samples could potentially be
313   challenging compared to static water samples, and we need to select the appropriate time of
314   sampling considering ecology and behavior of the target species.
315   We then applied PCR-RFLP to static water samples collected in the field. From the eight
316   sites in field experiments (B) and (C), PCR-RFLP could successfully detect eDNA in all three
317   sites where *R. japonica* tadpoles were visually observed. An additional site, site no. 27, had no
318   tadpoles observed in field study, but showed some evidence of presence from PCR-RFLP

319 analyses. This site is a small drainage site with a small volume and water flowing upstream from
320 a paddy field. Thus, it seems possible that PCR-RFLP can detect presence of frogs that may
321 inhabit upstream or in close proximity, even in the absence of visual confirmation. When we
322 compare this site of flowing water to that of sites in Fukushima, the water volumes and flow rate
323 are much smaller and slower, respectively.

324
325

Conclusion

327 We have successfully developed a cost-effective method for detecting three common
328 *Rana* frog species from Japan. This approach works well in field-collected samples that originate
329 from static or slow-flowing water systems. However, our approach cannot be applicable to lotic
330 water systems. Further exploration of alternative cost-effective methods are required, and could
331 include collection of larger volumes of water and experimental assessment of the impact of
332 various factors like water volume and flow rate on eDNA concentration. Nevertheless, this
333 method has potential to be used to monitor frog populations that are facing decline due to
334 urbanization, especially *R. japonica* which inhabits static water bodies like rice paddy fields.

335
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408

Figure 1(on next page)

Sampling localities of field experiments.

Results of field observation, qRCR and PCR-RFLP detection were indicated respectively (X/X/X). NA: qPCR not conducted.
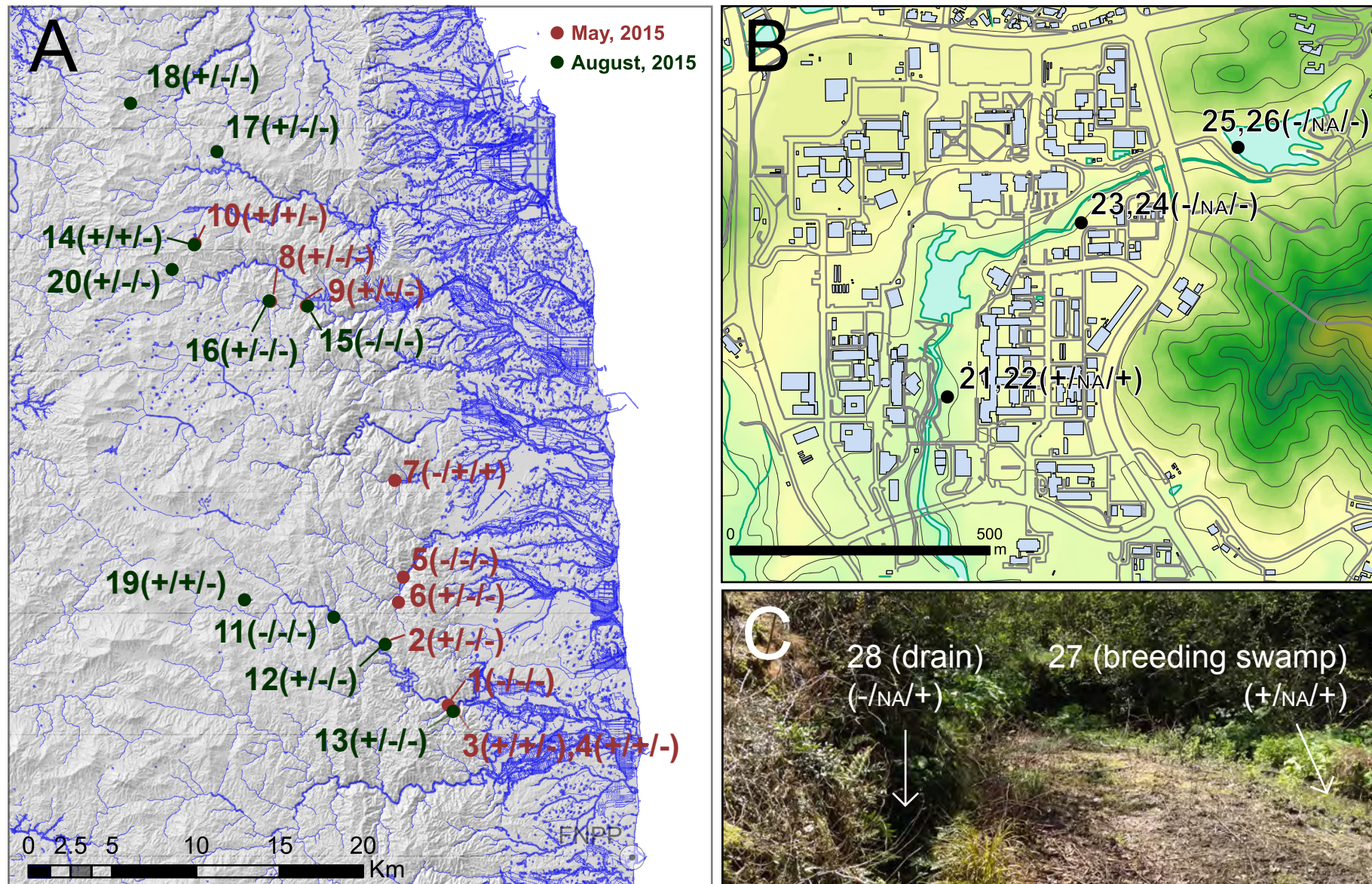
Figure 1: Sampling localities of field experiments.
Results of field observation, qRCR and PCR-RFLP detection were indicated respectively (X/X/X). NA: qPCR not conducted.

Figure 2(on next page)

PCR-RFLP and gel electrophoresis of water tank experiment and field experiment (B).

Figure 2: PCR-RFLP and gel electrophoresis of water tank experiment and field experiment (B).