Use of environmental DNA to survey the distribution of the invasive mussel *Limnoperna fortunei* in farm ponds

KENJI ITO* & HIROYUKI SHIBAIKE

Institute for Agro-Environmental Sciences, National Agriculture and Food Research Organization, Biodiversity Division, 3–1–3 Kannondai, Tsukuba 305–8604, Japan

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Abstract: The golden mussel *Limnoperna fortunei* (Dunker, 1857) is an invasive freshwater bivalve species that exerts harmful effects on the environment, as well as man-made structures, such as water-treatment systems. By using conventional sampling methods, it is difficult to detect mussels under low-density conditions; however, environmental DNA (eDNA) analysis may be a rapid and efficient method for monitoring this aquatic organism. In this study, we conducted surveys based on the eDNA analysis of *L. fortunei* in 15 farm ponds in Japan and compared the results with those of two conventional survey methods, visual census and plankton larval survey, to clarify the effectiveness of eDNA analysis for field surveys of *L. fortunei*. Primers and a probe specific to *L. fortunei* were developed, and a method for analysis was established. In the laboratory experiments, the species eDNA was detected in all water tanks containing the mussels, and the concentration of eDNA was high in the experimental tank that had high density of *L. fortunei*. In the field survey, *L. fortunei* eDNA was detected in all ponds where the mussels were found by conventional survey, and low concentrations of eDNA were also detected in several ponds where no *L. fortunei* were found by traditional methods. These results suggest that eDNA analysis has greater sensitivity for the detection of *L. fortunei* in farm ponds than that of conventional methods. Environmental DNA surveys have little impact on water management and are suitable for surveys at water facilities that have not yet been damaged by the mussels.

Key words: agricultural water facilities, golden mussel, environmental DNA (eDNA), *Limnoperna fortunei*

Introduction

Non-native species introduced from their natural range into other habitats are important factors affecting natural ecosystems and biodiversity (Vitousek et al. 1996). For the control of alien species, it is important to determine their distribution quickly and accurately. However, alien species in the early stage of invasion are generally low in density, often requiring much time and effort for detection. To overcome this issue, the method of analyzing environmental DNA (eDNA), which is derived from target species released into the environment, has recently been developed. The detection of alien species using eDNA has been investigated in various aquatic species (Cowart et al. 2018, Ficetola et al. 2008, Goldberg et al. 2013).

The golden mussel *Limnoperna fortunei* (Dunker 1857) is a small freshwater bivalve species native to China and Korea (Miller & McClure 1931, Morton 1973, Tominaga & Kimura 2012), which has likely been introduced into several other Southeast Asian countries (Cambodia, Laos, Thailand, Vietnam) as a result of human displacement (Morton & Dinesen 2010). This species has an epifaunal mode of life and exerts a harmful influence on the environment and man-made structures, such as water-treatment systems and power stations. The species now inhabits many countries in Asia (Hong Kong, Korea, Japan, and Taiwan) and South America (Argentina, Bolivia, Brazil, Paraguay, and Uruguay) (Boltovskoy et al. 2006, de Oliveira et al. 2006, Morton & Dinesen 2010).

The golden mussel is usually detected in freshwater systems by performing a visual census (Darrigran & de Drago 2000, Darrigran & Pastorino 1995, Ito 2008), using artificial substrata (Bergonci et al. 2009, Boltovskoy & Cataldo 1999, Ito et al. 2018), and/or collecting planktonic larvae (Cataldo & Boltovskoy 2000). Using these methods,
however, makes it difficult to detect the mussel under low-density conditions (i.e., early stage of invasion). For example, the introduction of *L. fortunei* into a reservoir was overlooked in a previous year’s visual and artificial-trap surveys (Ito 2016). To overcome this issue, research on *L. fortunei* using eDNA analysis has been conducted in Asia and South America (Pie et al. 2017, Xia et al. 2018a, Xia et al. 2018b). These studies, however, provide little information on the relationship between *L. fortunei* abundance and field eDNA concentrations. In order to use eDNA in field surveys of organisms, it is important to compare its effectiveness to that of conventional census methods (Roussel et al. 2015, Togaki et al. 2020, Tréguier et al. 2014).

The aim of this study was to investigate the ability of eDNA analysis to detect *L. fortunei* in field surveys. For this purpose, we designed primers and a probe specific to *L. fortunei* and tested its specificity and quantitative capability. In addition, we conducted an eDNA survey of the mussel in farm ponds using the primer–probe set and compared the results to two conventional methods, visual census and plankton larval survey.

### Materials and Methods

#### Design of species specific primers and probe

To design specific primers and probes, we obtained sequences of the cytochrome c oxidase subunit I (COI) mitochondrial gene for nine bivalve species, including *L. fortunei*. Sequences of *Corbicula fluminea*, *C. japonica*,

| Species                              | Forward Primer | Reverse Primer | Probe Sequence |
|--------------------------------------|----------------|----------------|---------------|
| *Corbicula fluminea*                 | CATAGAACCCCAGCAGTTGACA | AACGAACCGCCGATTGAC | AGCTGCTTTATCTCTTC |
| *C. japonica*                        | TCTAGAAAATATTGC  | TCTCTTCGCGCCGATGAGATTTAGCTATTTTT |
| *Crassostrea nippsona*               | CATAGAAAATATTGC  | TCTCTTCGCGCCGATGAGATTTAGCTATTTTT |
| *Limnoperna fortunei*                | TCTAGAAAATATTGC  | TCTCTTCGCGCCGATGAGATTTAGCTATTTTT |
| *Musculista senhousia*               | TCTAGAAAATATTGC  | TCTCTTCGCGCCGATGAGATTTAGCTATTTTT |
| *Mytilus galloprovincialis*          | TCTAGAAAATATTGC  | TCTCTTCGCGCCGATGAGATTTAGCTATTTTT |
| *M. trossulus*                       | TCTAGAAAATATTGC  | TCTCTTCGCGCCGATGAGATTTAGCTATTTTT |
| *Perna viridis*                      | TCTAGAAAATATTGC  | TCTCTTCGCGCCGATGAGATTTAGCTATTTTT |
| *Xenostrobus securis*                | TCTAGAAAATATTGC  | TCTCTTCGCGCCGATGAGATTTAGCTATTTTT |

#### Fig. 1. Results of multiple alignment for partial COI region of nine bivalve species, *Corbicula fluminea*, *C. japonica*, *Crassostrea nippsona*, *Limnoperna fortunei*, *Musculista senhousia*, *Mytilus galloprovincialis*, *M. trossulus*, *Perna viridis*, and *Xenostrobus securis*. The underlined nucleotide sequences indicate the positions of a set of primers (Forward: CATAGAACCCCAGCAGTTGACA; Reverse: AACGAACCGCCGATTGAC) and a probe (AGCTGCTTTATCTCTTC) for TaqMan assay. A hyphen indicates a sequence gap and an asterisk indicates a common nucleotide among the sequences.
**Crassostrea nipponia, L. fortunei, Musculista senhousia, Mytilus galloprovincialis, M. trossulus, Perna viridis, and Xenostrobus securis** were downloaded from Bioinformatics and DDBJ Center (accession numbers: AB498017, AB498018, AF300616, AB498011, AB498013, AB498014, AB498016, AB498015, and AB498012, respectively). Out of these nine bivalves, *L. fortunei* and *C. fluminea* are both freshwater species. The species most closely related to *L. fortunei* is *X. securis* (Lee et al. 2019), and *C. fluminea* and *C. japonica*, from the family Corbiculidae, were found in habitats similar to those of *L. fortunei*.

These sequences were aligned using Clustal W (Larkin et al. 2007), and unique regions were identified by comparing the *L. fortunei* sequence with those of the eight other bivalve species. Given that DNA in aquatic ecosystems is usually highly degraded, we designed primers with fragment lengths as short as possible by using Primer Express 3.0 (Thermo Fisher Scientific) based on suggestions from other studies (Bohmann et al. 2014, Dougherty et al. 2016). Figure 1 shows the positions of a set of primers and a probe for a TaqMan assay (Thermo Fisher Scientific) to amplify a 78-bp fragment of the COI region of *L. fortunei*.

**Specificity test for newly designed primers**

A well-designed primer pair should amplify only *L. fortunei* COI. The specificity of the primer set was evaluated by performing PCR with DNA extracted from six bivalve species, *C. fluminea*, *C. gigas*, *L. fortunei*, *M. galloprovincialis*, *P. viridis, and X. securis*, as templates. These bivalve species were fixed in 99.5% ethanol and stored at 4°C until DNA extraction. Muscle tissues were collected from each adult bivalve species, and total DNA was extracted from each tissue sample using the DNeasy Blood & Tissue Kit (Qiagen), according to the manufacturer's instructions.

The region of the mitochondrial cytochrome c oxidase subunit I (COI) gene was amplified by PCR using the following primers: 5′-CAT AGA ACC CCA GCA GTT GAC A-3′ and 5′-AAC GAA CCG CCG ATT GAC-3′ (Fig. 1). PCR was conducted in a 25 µL reaction volume containing 1× PCR buffer, 10 ng of template DNA, 0.05 mM of each dNTP, 0.4 mM of each primer, 2.0 mM MgCl2, and 0.5 unit of Taq Polymerase (TaKaRa Bio Inc.). PCR amplification was performed in an iCycler (Bio-Rad) with a thermal profile consisting of an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 40 s, with a final extension at 72°C for 7 min. PCR products were electrophoresed on a 2% agarose gel. Gels were then stained with ethidium bromide.

**Quantitative PCR analyses**

*Limnoperna fortunei* DNA was amplified using primers and probes to amplify a 78-bp fragment of the COI region. The probe was synthesized with a 6-FAM reporter dye at the 5′ end and MGB-NFQ as a quencher on the 3′ end: forward primer sequence, 5′-CAT AGA ACC CCA GCA GTT GAC A-3′; reverse primer sequence, 5′-AAC GAA CCG CCG ATT GAC-3′; and probe sequence, 5′-FAM-AGC TGC TTT ATC TCT TC-MBG-3′.

Quantitative PCR (qPCR) was performed in a final volume of 25 µL, using 3 µL of template DNA, 12.5 µL TaqMan Environmental Master Mix 2.0 (Life Technologies), 6.5 µL ddH2O, 1 µL of each primer (10 µM), and 1 µL of probe (2.5 µM) under thermal cycling at 50°C for 5 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Samples were run on a StepOnePlus Real-Time PCR System (Applied Biosystems). A dilution series of *L. fortunei* DNA from tissues, ranging from 10^2 to 10^−4 ng/µL, was used as the qPCR standard. We conducted each qPCR twice with two replicates for aquarium and field samples, and four to six wells of negative control (double distilled H2O) were used per PCR plate. The limit of detection of qPCR was identified as the lowest concentration producing at least one positive detection out of the two replicates. To avoid contamination, we conducted pre-PCR and PCR experiments in different rooms.

**Quantification standard**

To prepare a quantification standard, we amplified the 78 bp nucleotide sequence of the partial COI region of *L. fortunei* using newly designed primers. After PCR products were excised from the 1% agarose gel (NuSieve GTG; FMC), they were cloned using a Mighty TA-cloning kit (TaKaRa). Double-stranded plasmid DNA was purified from recombinant colonies using NucleoSpin Plasmid EasyPure (TaKaRa). Purified plasmid DNAs were sequenced using a Genetic Analyzer 3130xl auto sequencer (Applied Biosystems) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). We then created a calibration curve using a series of dilutions of plasmid from 10^1 to 10^6 per reaction as a quantification standard and converted the DNA concentration into the number of copies of COI.

**Aquarium experiment**

Animals used for the aquarium experiment were collected from the shore of Lake Kasumigaura (36°02'46.19"N, 140°13'40.13"E) in June 2018. A submerged brick covered with golden mussels was collected and brought back to the laboratory in a pail with wet towels. The mussels were acclimated at room temperature (~24°C) under a 14 h : 10 h light–dark cycle in a 60 L aquarium tank for 5 days. During acclimation, animals were fed every 2 days with a commercial *Chlorella* sp. (Nama Chlorella v12; Chlorella Industry Co., Ltd, Japan).

A sample containing one mussel, and a second containing five animals, with three replicates for each treatment, and one blank control that did not include any animals were used in the aquarium experiment. The average wet weight of mussels for the two treatments were 1.46 ± 0.53 g and 6.87 ± 0.34 g per tank (mean ± SD), respectively. These animals were flushed with distilled water, taken from the ac-
climation tanks, and placed in experimental tanks filled with 9 L of distilled water. We bleached the experimental tanks before use to reduce DNA contamination. Healthy animals typically began to filter within 10 min of transfer. A 250 mL water sample was taken from each tank after 0, 2, 12, 24, and 48 h of exposure (0 h represents before exposure). Throughout the experiment, the water in the tank was well aerated and mixed. The water was filtered through a glass-fiber filter (GF/F; GE Healthcare). Filter holders (Sterilif holder, EMD Millipore Corp.) and a vacuum pump were used for filtration. Each filter disc containing the sample was folded inward with tweezers and wrapped in commercial aluminum foil. The filter disc was immediately stored at −23°C until further analysis. All filtration equipment was bleached and carefully rinsed with distilled water between filtration operations to prevent cross-contamination.

The DNeasy Blood & Tissue Kit (Qiagen) was used for DNA extraction from the filter as follows: PK-mix (10 µL of proteinase K [600 mAU/mL], 210 µL of water, and 180 µL of Buffer AL) was added to the filter paper and incubated at 56°C for 1 h. The filter paper was centrifuged at 6000 × g for 1 min, and the filtrate was squeezed out. To collect the DNA remaining on the paper, 200 µL of buffer AE was added to the filter paper and centrifuged under the same conditions. Then, 600 µL each of Buffer AL and ethanol was added to the filtrate, and the resultant solution was placed in a DNeasy column and centrifuged at 8000 rpm for 1 min. Subsequent analysis was performed per the manufacturer’s protocol. The DNA solution obtained here was stored at −23°C.

Field surveys

We investigated 15 farm ponds in Tsukuba City, Ibaraki Prefecture, Japan, from August to September 2017 (Fig. 2). All but one of these ponds were made of concrete. During the study period, most ponds were full of water, and it was difficult to explore the bottom. These ponds receive water from the downstream reach of the Sakuragawa River connected to Lake Kasumigaura, and *Limnoperna fortunei* has been found in Lake Kasumigaura since 2005 (Ito 2007, Sunoh 2006); the water intake of this channel is also inhabited by the mussel (Tsuchiura-shigai 15-choson land improvement district: personal communication).

We conducted a field survey of *L. fortunei* by visual observation, larval sampling, and the eDNA method. We searched visually for *L. fortunei* for 10 min using a hand-net from the shore of the pond to determine the presence or absence of the mussel. To calculate the density of free-swimming *L. fortunei* larvae, samples were obtained using a plankton net (diameter: 20 cm; mesh size: 72 µm, Rigusha & Co.) at each sampling site. The plankton net was hauled vertically several times (depending on the depth) from the bottom to the surface (total volume of water filtered was approximately 100–200 L). The samples were preserved in 80% ethanol. Shelled-stage larvae (straight-hinged veliger to pediveliger; Cataldo et al. 2005) were counted under a binocular microscope in the laboratory, and larval density (ind. m−3) was calculated.

In addition, we collected 1 L of surface water directly from the shore using a bottle. Before use, these bottles were bleached for over 10 min and sterilized in an autoclave to prevent contamination. The collected water was cooled with refrigerants, brought into the laboratory, and filtered in the same way as in the aquarium experiment on the day of collection. We prepared two negative controls to confirm that there was no contamination; one of distilled water, and the other of water from a rainwater tank that did not contain mussels. DNA was extracted from the filter in the same way as in the aquarium experiments.

Statistical analysis

For the aquarium experiments, we compared the eDNA concentration among sampling times (after 2, 12, 24, and 48 h) and between treatments (n=1 vs. n=5) with a generalized linear mixed model (GLMM), while assuming a gamma distribution (log link). Replicates for each treatment were used as a random effect. The effects of treatment and time were analyzed using the likelihood ratio test. For the field results, we used Fisher’s exact test to compare the proportion of ponds in which *L. fortunei* was detected based on conventional methods or the presence of eDNA. Spearman’s rank correlation was performed to examine the
correlation between larval density and eDNA concentration. Among the farm ponds where the species eDNA was detected, the eDNA concentrations of ponds where mussels were found by conventional census and ponds where mussels were not found were compared using the Mann–Whitney U-test. We conducted all statistical analyses in R version 3.4.0 (R Project for Statistical Computing). The significance of all statistical tests was set at α = 0.05.

Results

Species specificity for PCR amplification using *L. fortunei*-specific primers and probe

PCR amplification was performed for the DNA extracted from six bivalve species as templates, using newly designed *L. fortunei*-specific primers. When a PCR product using DNA derived from *L. fortunei* as a template was electrophoresed, a single DNA fragment was observed in lanes 6 and 7 (Fig. 3), whereas when DNA extracted from five other bivalve species was used as template, no DNA fragments were observed in the lanes from 1 to 5. Therefore, the newly designed primer set for quantitative PCR was considered to specifically amplify only the DNA derived from *L. fortunei*.

Conversion of DNA concentration to copy number of COI using a calibration curve

In this study, we evaluated the amount of DNA derived from *L. fortunei* contained in the samples using DNA concentration. Since we are performing quantitative PCR targeting a partial sequence of the COI region, it is valuable to know the amount of template DNA present in the samples. Therefore, we created a calibration curve to convert the DNA concentration into the copy number of COI and verified the results of the aquarium experiment and the field experiment. After confirming that the PCR products amplified with the newly designed primers were formed from the expected nucleotide sequence, a calibration curve was prepared using a dilution series of the plasmid containing the partial COI region of *L. fortunei* (Fig. 4). In the aquarium experiment, the DNA concentration varied from several ng/L to more than 10 ng/L among the samples. When these values were converted to the copy number of COI, it was suggested that 10⁶ or more copies of COI were contained in the samples. However, in the field experiment, DNA concentration varied from several pg/L to more than 100 pg/L among the samples, and it was suggested that at least several hundred copies of COI were included in the samples. Amplifications of qPCR were unstable for samples obtained from the ponds of P7, P8, and P11. Therefore, it was considered that the detection limit was approximately several hundred copies of COI in the 1 L of field water samples in our study.

Aquarium experiment

We detected the eDNA of *L. fortunei* in all the experimental tanks (Fig. 5), and found that the concentration increased 2 h after the introduction of *L. fortunei*. The average eDNA concentration in tanks containing one indi-

![Fig. 3. PCR amplification of the partial COI region in six bivalve species, using *L. fortunei* specific primer sets. Lane M: 100 bp DNA Ladder; lane 1: *Corbicula fluminea*; lane 2: *C. gigas*; lane 3: *Mytilus galloprovincialis*; lane 4: *Perna viridis*; lane 5: *Xenostrobus securis*; lanes 6 and 7: *Limnoperna fortunei* (different PCR reactions of the same individual); lane 8: distilled water. A thick band indicates 500 bp.](image1)

![Fig. 4. Calibration curve to convert DNA concentration to copy number of the COI. Open circles indicate the copy number of COI, and closed circles indicate the DNA concentration. Threshold cycles (vertical axis) show the number of cycles required to detect a signal from the samples. Low values indicate high amounts of target sequence (COI), whereas high values represent low amounts. The calibration curve was determined from the copy number of COI (y = -3.25 log₁₀X + 40.59, R² = 0.988).](image2)
individually ranged from 1.28 to 3.16 ng/L and in those with five individuals from 6.53 to 37.26 ng/L. The eDNA concentration in treatments with one animal showed little change until 48 h, whereas in one of the three replicates of the tank with five individuals, the concentration showed an extremely high value after 24 h (96.2 ng/L). The concentration of eDNA in treatments with five individuals was significantly higher than that in the one-individual treatments (GLMM, \( p = 0.000212 \)); however, the concentration did not vary among the times (GLMM, \( p = 0.948 \)). No positive signal was detected in the control tank throughout the experiment or in the tanks before the introduction of mussels.

**Field survey**

In our visual census, we found *L. fortunei* in P1 and P2, and the larval survey returned *L. fortunei* in P3, P4, and P10. Thus, these conventional methods detected *L. fortunei* in 5 of the 15 ponds (Table 1). The eDNA analysis detected positive signals from samples from 9 of the 15 ponds, and these nine ponds included all five ponds in which *L. fortunei* was found by conventional methods. The proportion of ponds in which *L. fortunei* was detected based on conventional methods or by the presence of eDNA was significantly different (Fisher's exact test, \( p < 0.05, n = 15 \)). The relationship between eDNA concentration and larval density was significantly positive (Spearman's rank correlation, \( p < 0.005, Fig. 6 \)). The eDNA concentration in the four ponds where *L. fortunei* was not detected by conventional census was lower than that in the five ponds where the mussels were found by traditional means (\( p < 0.05, Mann–Whitney U\)-test, Fig. 7).

**Discussion**

In laboratory experiments, *L. fortunei* eDNA was detected in all tanks containing the mussels, and the eDNA concentration was high in the experimental tanks with a high density of *L. fortunei*. In the field survey, the species eDNA was also detected in the surface waters of all ponds where the mussels were found by visual census or planktonic survey. Low concentrations of eDNA were also
detected in several ponds where no *L. fortunei* individuals were found by conventional methods. These results suggest that the eDNA survey method in this study is an effective tool for detecting *L. fortunei* in farm ponds.

There were four ponds where *L. fortunei* eDNA was detected, but larvae and adults were not found (Table 1, P7, P8, P9, and P11). *Limpoperna fortunei* has already been found in the water source of the study sites (Tsuchiura-shigai 15-choson land improvement district: personal communication), meaning it is possible that *L. fortunei* had already invaded these four ponds. There are some reports that eDNA has been detected in places where the target species has not been found by conventional surveys (Thomsen et al. 2012, Togaki et al. 2020), suggesting a high sensitivity of eDNA survey methods. Supporting this, these four ponds had lower eDNA concentrations than the sites where the presence of *L. fortunei* was confirmed by conventional surveys (Fig. 7). In these four farm ponds, the density of *L. fortunei* may be too low to allow individuals to be found visually.

In the aquarium experiment, the eDNA concentration changed little under low-density conditions, but under high-density conditions, it increased considerably after 24 h in one of three replicates (Fig. 5). One reason for this result may be the reproductive activity of the mussels used in the experiment. The experiment was conducted in June, which is the breeding season for golden mussels in Japan (Iwasaki & Uryu 1998), and the temperature of the experiment was sufficiently high (~24°C) to allow reproductive activity. In addition, during the acclimatization period, mussels spawned eggs or released sperm into the aquarium (Ito, personal observation). The eDNA concentration in the water may have changed dramatically due to the breeding activity of some individuals in the experimental tank.

### Quantification of *L. fortunei* with eDNA

There are some reports on the detection efficiency of *L. fortunei* by eDNA analysis. Pie et al. (2017) carried out eDNA (real-time quantitative PCR) analysis in five reservoirs of an electric power plant and succeeded in detecting the presence and absence of *L. fortunei* (present at two sites, absent at three sites). Xia et al. (2018b) conducted a field survey of *L. fortunei* using eDNA (conventional PCR method) and detected mussels at 100% of sites with high mussel abundance, but only 40% of those had low abundance. Using conventional PCR instead of real-time quantitative PCR may be one of the causes of the low *L. fortunei* detection efficiency (Xia et al. 2018a). Neither previous study discussed the relationship between mussel abundance and eDNA concentration.

In our aquarium experiments, the eDNA concentration of *L. fortunei* was higher in aquaria with more individuals than in those with less individuals (Fig. 5), and the field survey also showed that eDNA concentrations increased in ponds with high larval density (Table 1, Fig. 6). These results suggest that eDNA concentrations can be used to assess mussel abundance in farm ponds. However, the relationship between larval density and eDNA concentration was ambiguous in our study (Fig. 6). This could relate to the larval density being very unstable in field studies (Nakano et al. 2017), or fluctuations in eDNA itself. The concentration of eDNA in freshwater bivalves varies significantly with time and space (Takahara et al. 2019). In some cases, eDNA was undetectable when the bivalve pop-

| Site | East longitude | North latitude | Visual census | Larval density (m−3) | qPCR results | eDNA concentration (ng/L) |
|------|----------------|----------------|--------------|----------------------|--------------|--------------------------|
| P1   | 140°06'48.5"   | 36°01'32.4"   | +            | 0                    | 2/2          | 0.0214                   |
| P2   | 140°06'18.9"   | 36°01'27.7"   | +            | 0                    | 2/2          | 0.0113                   |
| P3   | 140°06'08.3"   | 36°01'45.2"   | −            | 840                  | 2/2          | 0.0991                   |
| P4   | 140°05'53.5"   | 36°01'24.4"   | −            | 7                    | 2/2          | 0.2037                   |
| P5   | 140°06'03.1"   | 36°01'47.5"   | −            | 0                    | 0/2          | ND                       |
| P6   | 140°05'55.4"   | 36°01'58.4"   | −            | 0                    | 0/2          | ND                       |
| P7   | 140°05'58.2"   | 36°01'58.7"   | −            | 0                    | 1/2          | 0.0022                   |
| P8   | 140°05'51.2"   | 36°01'49.9"   | −            | 0                    | 1/2          | 0.0019                   |
| P9   | 140°05'33.9"   | 36°01'58.2"   | −            | 0                    | 2/2          | 0.0048                   |
| P10  | 140°06'00.8"   | 36°02'43.6"   | −            | 30                   | 2/2          | 0.0490                   |
| P11  | 140°05'50.1"   | 36°02'54.7"   | −            | 0                    | 1/2          | 0.0015                   |
| P12  | 140°05'46.4"   | 36°03'01.6"   | −            | 0                    | 0/2          | ND                       |
| P13  | 140°05'29.0"   | 36°03'15.3"   | −            | 0                    | 0/2          | ND                       |
| P14  | 140°04'52.2"   | 36°03'16.5"   | −            | 0                    | 0/2          | ND                       |
| P15  | 140°04'49.3"   | 36°03'17.6"   | −            | 0                    | 0/2          | ND                       |

ND, not detected
ulations were visually found (Cowart et al. 2018, Togaki et al. 2020, Xia et al. 2018b). In order to evaluate the abundance of *L. fortunei* using eDNA in field studies, more detailed information on spatiotemporal variations in eDNA concentration is required.

In conclusion, compared to conventional methods, eDNA analysis showed greater sensitivity for the detection of *L. fortunei* in farm ponds. In general, it is difficult to drain water from farm ponds when surveying *L. fortunei*. Environmental DNA surveys that use small amounts of water have little impact on water management and are suitable for surveys at water facilities that have not yet been damaged by the mussels.

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