Detection of invasive shrimp *Palaemon sinensis* (Sollaud, 1911) using environmental DNA

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Abstract.— The nonnative freshwater shrimp *Palaemon sinensis* (Sollaud, 1911) has been reported at various locations in Japan. This study compared environmental DNA (eDNA) analysis with conventional capturing and investigation as a method to detect *P. sinensis* in the Tama River. We developed species-specific primers to amplify a 225-bp fragment of the cytochrome c oxidase subunit I gene for eDNA analysis. The invasive shrimps were detected using eDNA analysis in all areas where they were observed using the capture and investigation method. Thus, eDNA analysis can be applied to detect sparsely distributed *P. sinensis* in the Tama River, making it a useful method to detect invasive shrimps and to help prevent ecological disruption.

Key words: eDNA, Real-time PCR, Species-specific primer, COI

■ Introduction

Palaemon sinensis (Sollaud, 1911) is a freshwater shrimp that is distributed in China, Myanmar, southeastern Siberia, and Sakhalin (Liu *et al.*, 1990; Bruce, 1994; Cai & Ng, 2002; Kawai & Nakata, 2011). *Palaemon sinensis* was first reported in Japan in the Shizuoka Prefecture (Oonuki *et al.*, 2010), and reports concerning *P. sinensis* distribution at various sites in Japan have been accumulating (Oonuki *et al.*, 2010; Hasegawa *et al.*, 2016; Saito *et al.*, 2016; Imai & Oonuki, 2017; Oonuki *et al.*, 2017; Chow *et al.*, 2018; Hiraoka *et al.*, 2018). Its presence in Japan is thought to originate from anglers discarding *P. sinensis* that is imported as live bait from China (Oonuki *et al.*, 2010; Saito, 2018).

Although *P. sinensis* is morphologically most similar to the native Japanese species *Palaemon paucidens* De Haan, 1844 in the structure of its thoracic legs, it can be distinguished from *P. paucidens* by some morphological differences. In *P. sinensis*, the mandibles did not have a palp, whereas a palp is present in *P. paucidens*.

Furthermore, the color pattern on the carapace of *P. sinensis* consists of three diagonal stripes, with the posterior stripe being hook-shaped, whereas *P. paucidens* has a line that lengthens in the cardiac region from the hepatic region between two diagonal lines. Lastly, the eyes of *P. sinensis* are more slender than those of *P. paucidens* (Imai & Oonuki, 2014).

Sometimes, it is difficult to distinguish these two species, particularly when specimens undergo depigmentation due to long-term storage. Furthermore, only few differential characteristics have been reported in juvenile specimens (Oonuki *et al.*, 2010; Hasegawa *et al.*, 2016; Hiraoka *et al.*, 2018). DNA barcoding has been used to identify *P. sinensis* collected in the Tama River (Hiraoka *et al.*, 2018), but this is costly and labor intensive.

Recent rapid developments in environmental DNA (eDNA) analysis have led to the use of eDNA to detect aquatic organisms (Takahara *et al.*, 2013). Although this technique is delicate and requires great care to prevent contamination, field surveys for eDNA analysis are easy and inexpensive, because only a small amount

of water needs to be collected. eDNA is analyzed using metabarcoding and quantitative (q) polymerase chain reaction (PCR) assays. Metabarcoding is applied to detect multiple species using a universal primer set and next-generation sequencing (Miya *et al.*, 2015; Ushio *et al.*, 2017; Ushio *et al.*, 2018; Komai *et al.*, 2019). qPCR is applied to detect the target species, such as invasive species with low densities, using a specific primer set (Takahara *et al.*, 2013; Furlan & Gleeson, 2016).

We employed eDNA analysis to detect the presence of invasive shrimp species *P. sinensis* in the Tama River. Our findings showed that qPCR-based eDNA analysis is a reliable method to detect *P. sinensis*.

Materials and Methods

Collection locations for water and shrimp samples

We collected water samples at six cove survey stations (Sts.) in the Tama River, Kanagawa Prefecture, Japan, on May 18, 2019 (Fig. 1). The river water was flowing at Sts. 2–4 and stagnant at Sts. 1, 5, and 6. The water in the stagnated areas was warmer and more turbid than in the areas with flowing water (Table 1

and Fig. 1). After collecting the water samples, shrimps were captured by two investigators using a hand net (50 cm × 50 cm) for 10 min. Sampling was performed once for the entire area of each St., except for St. 5, which was too large for the entire area to be sampled.

Processing water samples for eDNA analysis

Surface water samples were collected in separate 1-L polyethylene bottles that had been bleached with a 10% sodium hypochlorite solution. The water samples were kept in a cooler along with 1 L of sterile H₂O in a 1-L polyethylene bottle as a cooler box blank (negative control). Filtration, DNA extraction, and PCR were performed in separate laboratory rooms to avoid DNA contamination. Each sample was filtered through a 47-mm GF/F glass fiber filter with a 0.7-μm pore-size (GE Healthcare Japan, Tokyo, Japan) per water sample. Then, the filters were wrapped in DNA-free aluminum foil and stored at −20°C until eDNA extraction using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA), as described previously (Yamamoto *et al.*, 2016). The extracted DNA was stored at −20°C for use in subsequent PCR assays. Filtration, DNA extraction, and

Fig. 1. Location of survey stations (Sts.) 1–6 in the Tama River, Kanagawa Prefecture, Japan.

Table 1. Sampling data and eDNA results for the detection of *Palaemon sinensis* in the Tama River, Kanagawa prefecture, Japan

Station	Water temperature (°C)	Filtered sample volume (L)	eDNA*	No. of shrimps captured**
1	22.7	1.0	– (0/8)	0 (0/0)
2	19.7	1.0	– (0/8)	0 (0/0)
3	20.3	1.0	– (0/8)	0 (0/0)
4	18.3	1.0	– (0/8)	0 (0/0)
5	24.8	0.6	+	1 (1/0)
6	24.8	0.5	+	4 (3/1)

*PCR amplification of eDNA in eight replicates (number of detected sample / replicates, 8).

–, regarded as negative if not detected at all in eight replicates.; +, others.

**Total number of shrimp (nonovigerous shrimp/ovigerous shrimp).

PCR were performed in separate laboratory rooms to avoid unexpected DNA contamination. Negative controls were similarly filtered, extracted, and applied to PCR.

Design of specific primers and probe

Two individuals each of *P. sinensis* and decapod species collected in the Tama River, including *Macrobrachium nipponense*, *Neocaridina* spp., *P. paucidens*, and *Procambarus clarkii*, were used for eDNA analysis. DNA was extracted from the pleon muscle of *M. nipponense*, *Neocaridina* spp., *P. paucidens*, and *P. sinensis*. Partial sequences of the mitochondrial cytochrome c oxidase subunit I (COI) gene were amplified by PCR using Ex Taq HS DNA polymerase (TAKARA, Tokyo, Japan) with universal primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAATCA-3') (Folmer *et al.*, 1994). Sequencing was performed on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems Japan, Ltd., Tokyo, Japan). The sequences were aligned using MEGA 7.0 software (Kumar *et al.*, 2016). The COI sequences of *P. clarkii* were derived from the GenBank database. The accession numbers of COI sequences determined in this study and those of *P. clarkii* are shown in Table 2.

We designed *P. sinensis*-specific primers to amplify a 225-bp fragment of the COI gene.

The primers were forward primer PS_COI_F (5'-CCCCCTCACTCACCCTTC-3') and reverse primer PS_COI_R (5'-GTTTCGATCTATCGTTATTCCTGGTCTTC-3'), and the probe was PS_COI_probe (5'-FAM-CTCGGCCACGCAGGA-TAMRA-3') (Fig. 2). The total volume of the reaction mixture was 25 µl, containing 12.5 µl of Premix Ex Taq (Probe qPCR) master mix (TAKARA), 0.5 µl of each 10 µM primer, 1.0 µl of the 10 µM probe, 2.0 µl of template DNA, and 8.5 µl of sterile H₂O. The thermal cycling conditions were 95.0°C for 30 s, followed by 55 cycles of 95.0°C for 5 s and 60.0°C for 30 s. Eight replicates were performed for each eDNA sample. The specificity of the

Fig. 2. The PCR primer set and probe (PS_COI_F, PS_COI_R, and PS_COI-Probe) targeting *Palaemon sinensis* using mitochondrial COI DNA and sequence information of *P. sinensis*, *Palaemon paucidens*, *Macrobrachium nipponense*, *Neocaridina* spp., and *Procambarus clarkii*. The sequence of PS_COI_R was a reverse complement of the sequence indicated in this figure.

Table 2. GenBank accession numbers of the nucleotide sequences used for designing eDNA assays to detect *Palaemon sinensis*. The sequences of two individuals per species were used to design specific primers

Species	Accession number	Reference
<i>Palaemon sinensis</i> _1	LC586830	This study
<i>Palaemon sinensis</i> _2	LC586831	This study
<i>Palaemon paucidens</i> _1	LC586832	This study
<i>Palaemon paucidens</i> _2	LC586833	This study
<i>Macrobrachium nipponense</i> _1	LC586834	This study
<i>Macrobrachium nipponense</i> _2	LC586835	This study
<i>Neocaridina</i> spp._1	LC586836	This study
<i>Neocaridina</i> spp._2	LC586837	This study
<i>Procambarus clarkii</i> _1	KT036444	Liu <i>et al.</i> , 2016
<i>Procambarus clarkii</i> _2	NC_016926	Kim <i>et al.</i> , 2012

Fig. 3. Species-specificity of the primer set and probe. The primer set and probe were specifically designed to amplify DNA from *Palaemon sinensis* but not from decapoda species collected in the Tama River (*Palaemon paucidens*, *Macrobrachium nipponense*, *Neocaridina* spp., and *Procambarus clarkii*). Amplification by real-time PCR was conducted using two individuals per species. These two individuals were the same as those used for COI sequencing.

primer and probe sets designed for *P. sinensis* was checked by PCR assays using DNA from the decapoda species collected in the Tama River (*M. nipponense*, *Neocaridina* spp., *P. paucidens*, and *P. clarkii*) (Fig. 3).

Results and Discussion

We detected eDNA from *P. sinensis* in two coves (St. 5 and 6) along the Tama River. No positive signal for *P. sinensis* eDNA was obtained in the blank samples (negative control). The amplified products were directly sequenced, and we confirmed that the products were indeed *P. sinensis* DNA. It is consistent with the eDNA results that *P. sinensis* was actually hand-captured in 2 coves (St. 5 and 6) but not in other areas (Table 1 and Fig. 4). eDNA analysis is certainly applicable to detect *P. sinensis*. Previous studies reported eDNA flowing distances of less than 50 m (Pilliod *et al.*, 2014) to more than 10 km (Deiner & Altermatt, 2014). Sts. 5 and 6, where *P. sinensis* was detected, were not connected. Thus, the detection of eDNA in this study was not considered to be affected by water flow. However, the distance that eDNA flows downstream should be

Fig. 4. Detection of *Palaemon sinensis*-specific eDNA at survey stations (Sts.) 1–6 in the Tama River. Amplification was carried out by qPCR, and eight replicates were performed for each eDNA sample. NC: negative control.

studied in detail in future investigations.

Dunn *et al.* (2017) investigated the impact of biomass and sex ratio on the amount of eDNA released by crayfish in aquaria and found that females released significantly higher levels of eDNA than males. Furthermore, ovigerous females significantly increased the concentration of crayfish eDNA per unit of mass, and there was a significant relationship between eDNA concentration and biomass when ovigerous females were present. Thus, eDNA analysis may be effective in detection and estimate of abundance in the spawning season. The reproductive season of *P. sinensis* in Japan is May–September, and it is thought that *P. sinensis* spawns several times during this period (Oonuki *et al.*, 2010; Hasegawa *et al.*, 2016; Saito *et al.*, 2019). *Palaemon sinensis* spawning is influenced by water temperature and daytime length (Oonuki *et al.*, 2010). In fact, we captured an ovigerous *P. sinensis* female at St. 6 (Table 1) during the spawning season, where the water temperature was higher than at other Sts. (Table 1). *Palaemon sinensis* survives the

winter; thus, further studies are necessary to confirm that eDNA of *P. sinensis* can also be detected in non-spawning seasons.

eDNA concentration is proportional to the quantity of the relevant organisms (Takahara *et al.*, 2012; Thomsen *et al.*, 2012; Eichmiller *et al.*, 2014; Yamamoto *et al.*, 2016; Iwai *et al.*, 2019). Future studies using quantitative eDNA analysis might be able to elucidate the invasion levels of *P. sinensis* in each survey area.

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