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

Retori Hiraoka, Hirohiko Teishima, Teruhiro Takabe, Teruaki Suzuki, Yoshito Tanaka

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 metabarcodeing 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 *Palaemon sinensis* in the Tama River. Our findings showed that qPCR-based eDNA analysis is a reliable method to detect *Palaemon 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 stagnant 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

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                       | +     | (8/8) 1 (1/0)            |
| 6       | 24.8                   | 0.5                       | +     | (8/8) 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’-TAAACTTCAGGGTGACCAAAAAATCA-3’) (Folmer *et al.*, 1994). Sequencing was performed on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems Japan, Ltd., Tokyo, Japan). 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’-CCCCCTCACTCACCCCTTC-3’) and reverse primer PS_COI_R (5’-GTTCGATCTATCGTTATTCTGGTCTTC-3’), and the probe was PS_COI_probe (5’-FAM-CTCGGCCAGGA-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.](image)

| Species                        | Accession number | Reference          |
|--------------------------------|------------------|--------------------|
| *Palaemon sinensis* _1_        | LC586830         | This study         |
| *Palaemon sinensis* _2_        | LC586831         | This study         |
| *Palaemon paucidens* _1_       | LC586832         | This study         |
| *Palaemon paucidens* _2_       | LC586833         | This study         |
| *Macrobrachium nipponense* _1_ | LC586834         | This study         |
| *Macrobrachium nipponense* _2_ | LC586835         | This study         |
| *Neocaridina* spp._1_          | LC586836         | This study         |
| *Neocaridina* spp._2_          | LC586837         | This study         |
| *Procambarus clarkii* _1_      | KT036444         | Liu *et al.*, 2016 |
| *Procambarus clarkii* _2_      | NC_016926        | Kim *et al.*, 2012 |
primer and probe sets designed for *P. sinensis* was checked by PCR assays using DNA from the decapoda species collected in the Tama River (*Palaemon paucidens*, *Macrobrachium nipponense*, *Neocaridina* spp., and *Procambarus 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 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.

**Acknowledgments**

We would like to thank Shunsuke Oku for technical support. We are also grateful to Dr. Atsunobu Hiraoka and Dr. Jun Shoji for reading the manuscript.

**Literature Cited**

Bruce, A. J., 1994. A re-examination of *Palaeomonetes sinensis* (Sollaud, 1911) (Crustacea; Decapoda; Palaemonidae). The Beagle: Records of the Museums and Art Galleries of the Northern Territory, 11: 1–7.

Cai, Y., & Ng, P. K. L., 2002. The freshwater palaemonid prawns (Crustacea: Decapoda: Caridea) of Myanmar. Hydrobiologia, 487: 59–83.

Chow, S., Imai, T., Ikeda, M., Maki, S., Oonuki, T., Muto, F., Nohara, K., Furusawa, C., Shichiri, H., Nigorikawa, N., Uragaki, N., Kawamura, A., Ichikawa, T., Ushioda, K., Higuchi, M., Tega, T., Kodama, K., Itoh, M., Ichimura, M., Matsuzaki, K., Hirasawa, K., Tokura, K., Nakahata, K., Kodama, S., Hakoyma, H., Yada, T., Niwa, K., Nagai, S., Yanagimoto, T., Saito, K., Nakaya, M., & Maruyama, T., 2018. A DNA marker to discriminate two types of freshwater shrimp *Palaemon paucidens* and the distribution of these two types in Japan. Nippon Suisan Gakkaishi, 84: 674–681. [In Japanese with English summary]

Deiner, K., & Altermatt, F., 2014. Transport distance of invertebrate environmental DNA in a natural river. PLOS ONE, 9: e88786.

Dunn, N., Priestley, V., Herrera, A., Arnold, R., & Savolainen, V., 2017. Behavior and season affect crayfish detection and density inference using environmental DNA. Ecology and Evolution, 7: 7777–7785.

Eichmiller, J. J., Bajer, P. G., & Sorensen, P. W., 2014. The relationship between the distribution of common carp and their environmental DNA in a small lake. PLOS ONE, 9: e112611.

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology, 3: 294–299.

Furlan, E. M., & Gleeson, D., 2016. Environmental DNA detection of redfin perch, *Perca fluviatilis*. Conservation Genetic Resources, 8:115–118.

Hasegawa, M., Mori, A., & Fujimoto, Y., 2016. Report of the confirmation of foreign freshwater prawns *Palaeomonetes sinensis* which closely resembled the *Palaemon paucidens* fresh water prawns in Miyagi Prefecture. Izunuma-Uchinuma Wetland Researches, 10: 59–66. [In Japanese]

Hiraoka, R., Oku, S., & Teishima, H., 2018. Invasive freshwater shrimp, *Palaemon sinensis* (Sollaud, 1911) found in Tama River, Kanagawa, and identified by morphological characteristics and DNA barcoding. Natural History Report of Kanagawa, 39: 39–42. [In Japanese]

Imai, T., & Oonuki, T., 2014. Records of Chinese grass shrimp, *Palaeomonetes sinensis* (Sollaud, 1911) from western Japan and simple differentiation method with native freshwater shrimp, *Palaemon paucidens* De Haan, 1844 using eye size and carapace color pattern. BiolInvasions Records, 3: 163–168.

Imai, T., & Oonuki, T., 2017. Invasive freshwater prawn, *Palaemon sinensis* collected in the Iwamatsu River system, Uwajima City, Ehime Prefecture, Japan. The Nanki Seibut-
Iwai, N., Yasumiba, K., & Takahara, T., 2019. Efficacy of environmental DNA to detect and quantify stream tadpoles of *Odorrana splendida*. Royal Society Open Science, 6: 181798.

Kawai, T., & Nakata, K., 2011. Shrimps, Crabs and Crayfishes: Conservation and Biology of Freshwater Crustaceans. Seibutsu Kenkyusha, Tokyo, 460 pp. [In Japanese]

Kim, S., Park, M. H., Jung, J. H., Ahn, D. H., Sultana, T., Kim, S., Park, J. K., Choi, H. G., & Min, G. S., 2012. The mitochondrial genomes of *Cambaroides similis* and *Procambarus clarkii* (Decapoda: Astacidea: Cambaridae): The phylogenetic implications for Reptantia. Zoologica Scripta, 41: 281–292.

Komai, T., Gotoh, R. O., Sado, T., & Miya, M., 2019. Development of a new set of PCR primers for eDNA metabarcoding decapod crustaceans. Metabarcoding and Metagenomics, 3: 1–19.

Kumar, S., Stecher, G., & Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular Biology and Evolution, 33: 1870–1874.

Liu, R. Y., Liang, X. Q. & Yan, S. L., 1990. A study of the Palaemonidae (Crustacea: Decapoda) from China II. *Palaemon, Exopalaemon, Palaemonetes* and *Leptocarpus*. Stu- dia Marina Sinica, 31: 229–265.

Liu, Q. N., Chai, X. Y., Jiang, S. H., Zhou, C. L., Xuan, F. J., & Tang, B. P., 2016. Characterization of the complete mitochondrial genome of the red crayfish, *Procambarus clarkii* (Decapoda: Cambaridae). Mitochondrial DNA Part A, DNA Mapping, Sequencing, and Analysis, 27: 3458–3459.

Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J. Y., Sato, K., Minamoto, T., Yamamoto, S., Yamanaka, H., Araki, H., Kon- doh, M., & Iwasaki, W., 2015. MIFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: Detection of more than 230 subtropical marine species. Royal Society Open Science, 2: 150088.

Oonuki, T., Miyajima, N., Tatsukawa, J., & Imai, T., 2017. A record of invasive freshwater shrimp, *Palaemon sinensis* (Sollaud, 1911) (Palaemonidae) from Saiki City, Oita Prefecture, Japan. Journal of Natural History of OITA—BUNGOENSIS—, 2: 63–686. [In Japanese]

Oonuki, T., Suzuki, N., & Akiyama, N., 2010. Annual reproductive cycle of the female *Palaemonetes sinensis* recorded for the first time in a pond of Hamamatsu City, Shizuoka Prefecture, Japan. Aquaculture Science, 58: 509–516. [In Japanese with English summary]

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P., 2014. Factors influencing detection of eDNA from a stream-dwelling amphibian. Molecular Ecology Resources, 14: 109–116.

Saito, H., 2018. Effect of newly established import quarantine regulations on the supply of alien palaemonid shrimp *Palaemon sinensis* in Japan. Nippon Suisan Gakkaishi, 84: 87–93. [In Japanese with English abstract]

Saito, H., Kometani, K., & A., Kodama, 2019. Seasonal occurrence of the alien freshwater shrimp *Palaemon sinensis* (Sollaud, 1911) in lower reaches of a river in western Japan. BioInvasions Records, 8: 369–378.

Saito, H., Yamasaki, A., Watanabe, J., & Kawai, K., 2016. Distribution of the invasive freshwater shrimp *Palaemon sinensis* (Sollaud, 1911) in rivers of Hiroshima Prefecture, western Japan. BioInvasions Records, 5: 93–100.

Takahara, T., Minamoto, T., & Doi, H., 2013. Using environmental DNA to estimate the distribution of an invasive fish species in ponds. PLOS ONE, 8: e56584.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H., & Kawabata, Z., 2012. Estimation of fish biomass using environmental DNA. PLOS ONE, 7: e35868.

Thomsen, P. F., Kielgast, J., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., Orlan-
do L., & Willerslev, E., 2012. Monitoring endangered freshwater biodiversity using environmental DNA. Molecular Ecology, 21: 2565–2573.

Ushio, M., Fukuda, H., Inoue, T., Makoto, K., Kishida, O., Sato, K., Murata, K., Nikaido, M., Sado, T., Sato, Y., Takeshita, M., Iwasaki, W., Yamanaka, H., Kondoh, M., & Miya, M., 2017. Environmental DNA enables detection of terrestrial mammals from forest pond water. Molecular Ecology Resources, 17: e63–e75.

Ushio, M., Murata, K., Sado, T., Nishiumi, I., Takeshita, M., Iwasaki, W., & Miya, M., 2018. Demonstration of the potential of environmental DNA as a tool for the detection of avian species. Scientific Reports, 8: 4493.

Yamamoto, S., Minami, K., Fukaya, K., Takeda, K., Sawada, H., Murakami, H., Tsuji, S., Hashizume, H., Kubonaga, S., Horiuchi, T., Hongo, M., Nishida, J., Okugawa, Y., Fujiwara, A., Fukuda, M., Hidaka, S., Suzuki, K., Miya, M., Araki, H., Yamanaka, H., Maruyama, A., Miyashita, K., Masuda R., Minamoto, T., & Kondoh, M., 2016. Environmental DNA as a ‘Snapshot’ of fish distribution: A case study of Japanese jack mackerel in Maizuru Bay, Sea of Japan. PLOS ONE, 11: e0149786.

Addresses

(RH) Marine Biological Research Institute of Japan Co. Ltd., 4–3–16 Yutaka-cho, Shinagawa-ku, Tokyo 142–0042, Japan and Graduate School of Environmental and Human Science, Meijo University, 1–501 Shiogamaguchi, Tempaku-ku, Nagoya 468–8502, Japan.

(HT) Marine Biological Research Institute of Japan Co. Ltd., 4–3–16 Yutaka-cho, Shinagawa-ku, Tokyo 142–0042, Japan.

(TT) Research Institute, Meijo University., 1–501 Shiogamaguchi, Tempaku-ku, Nagoya 468–8502, Japan

(TS) (YT) Graduate School of Environmental and Human Science, Meijo University, 1–501 Shiogamaguchi, Tempaku-ku, Nagoya 468–8502, Japan

E-mail address of corresponding author
(RH) re-hiraoka@mbrij.co.jp