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Environmental DNA reveals nonmigratory individuals of *Palaemon paucidens* overwintering in Lake Biwa shallow waters

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Abstract: In Lake Biwa, the largest freshwater lake in Japan, the lacustrine shrimp *Palaemon paucidens* has been reported to undertake seasonal migrations, living in deep waters from autumn to winter and in shallow waters from spring to summer. Some investigators have suggested that some individuals overwinter in shallow waters, but the ecology and life history of such nonmigratory populations are poorly understood. We developed a species-specific environmental DNA (eDNA) marker to detect *P. paucidens* in water samples and used it to examine the distribution and relative abundance of overwintering individuals in the shallow waters of Lake Biwa. Water samples were collected from 21 shore sites and 32 surrounding freshwater lagoons of Lake Biwa in November 2015 and February 2016. In November, 4 shore and 12 freshwater lagoon sites were positive for *P. paucidens* eDNA, and in February, 4 shore and 9 freshwater lagoon sites were positive. The relative abundance of eDNA copies was estimated by quantitative real-time polymerase chain reaction (PCR). Simultaneous sampling of *P. paucidens* and its eDNA, conducted at the inlet and outlet of 5 freshwater lagoons in December 2016, validated our method as a quantitative measure of the relative abundance of *P. paucidens* local populations. The eDNA approach used here confirmed that some *P. paucidens* individuals overwinter in the shallow waters, suggesting life-history diversity in Lake Biwa populations.
Key words: environmental DNA, eDNA, *Palaemon paucidens*, migration, Lake Biwa

Freshwater habitats, such as lakes, rivers, and wetlands, are crucial for life on Earth. Freshwater accounts for only 0.01% of the world's water and covers ~0.8% of the Earth's surface (Dudgeon et al. 2006), but provides habitat for almost 10% of known species (Balian et al. 2008). Several investigators have reported that species diversity in freshwater habitats is far lower than in terrestrial habitats because of human activities (Collen et al. 2014, Cumberlidge et al. 2009). Thus, to protect freshwater biodiversity and environments, basic information on the distributions of species, populations, and intraspecific variants is indispensably required.

Our study was focused on *Palaemon paucidens*, which belongs to the family Palaemonidae and is widely distributed over East Asia (Kim 1977, Chow and Fujio 1985) where it inhabits southeastern Siberia, Sakhalin Island, the Japanese Archipelago, Korea, and mainland China (Kim 1976). In Japan, the distribution of this shrimp species ranges from the southern limit of Yaku Island to the northern limit of Hokkaido Island (Rathbun 1902a, b, Kubo 1942, Shokita 1975). It appears in a variety of freshwater habitats, from small ponds to large deep lakes and from upstream to the river mouth (Rathbun 1902a, b, Kubo 1942, Shokita 1975). Based on migration patterns, *P. paucidens* generally is classified into diadromous and landlocked populations (Chow and Fujio 1985). The population inhabiting Lake Biwa is considered landlocked (Kawane et al. 2014), and previous investigators reported that individuals belonging to this population lived in deep waters from autumn to winter and in shallow waters from spring to summer (Harada 1966, Nishino 1983). However, some individuals have been reported as overwintering in the shallow waters of Lake Biwa. A previous reporter estimated that the proportion of such nonmigratory individuals was <1% (Biological Resource Research Team in Lake Biwa 1966). However, knowledge on nonmi-

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gratory *P. paucidens* individuals in Lake Biwa is limited, and whether they are indeed nonmigratory has not been established. Therefore, knowing the precise location of the habitat of nonmigratory *P. paucidens* individuals in winter would help our understanding of the peculiar life history of this species.

Conventional methods for shrimp surveys using traditional sampling gear (i.e., shrimp cage, fyke netting, electric bait trapping) have low sensitivity and present detection biases in the census of shrimp populations. In contrast, environmental DNA (eDNA) analysis, a method that has been developing rapidly, may provide quantitative information on the abundance of shrimp populations and on species' presence/absence because it is based on the genetic materials released by organisms into the environment through excreted mucus, feces, or other wastes (Ficetola et al. 2008). Investigators who used eDNA analysis have reported successful monitoring of the presence/absence of rare species, biodiversity, and abundance of populations in freshwater environments (Dejean et al. 2011, Thomsen et al. 2012, Fukumoto et al. 2015, Yamanaka and Minamoto 2016). Several researchers have focused on the eDNA of fish and amphibians, but studies of crustacean eDNA are still scarce (but see Deiner and Altermatt 2014, Tréguier et al. 2014, Carim et al. 2016, Ikeda et al. 2016). However, eDNA analysis may enable us to deepen our understanding on the spatiotemporal dynamics and life-history patterns of crustacean populations.

Our goal was to locate precisely the habitat of nonmigratory *P. paucidens* individuals in winter in Lake Biwa and surrounding freshwater lagoons. First, we designed a species-specific quantitative polymerase chain reaction (PCR) assay to detect DNA of *P. paucidens* in environmental samples. Second, to demonstrate the presence of nonmigratory populations, we used this assay to examine the distribution of overwintering populations in the shallow waters of Lake Biwa. Last, to assess whether eDNA methods can be used to estimate the density of *P. paucidens*, we compared the quantity of *P. paucidens* DNA in eDNA samples to the population densities of this species obtained through traditional sampling methods.

METHODS

Primers, probe design, and validation

We designed species-specific primers and a probe to detect *P. paucidens* but not *Macrobrachium nipponense*, which is the most closely related species to *P. paucidens* inhabiting Lake Biwa. First, we obtained mitochondrial DNA (mtDNA) sequences of the 16S rRNA region of *P. paucidens* (accession numbers: KM249043–KM249065) and *M. nipponense* (accession numbers: KU235739–KU235721) from GenBank (https://www.ncbi.nlm.nih.gov/genbank/). We used Primer Express (version 3.0; Applied Biosystems, Foster City, California) to design a set of species-specific primers and a TaqMan probe, with default settings. To verify that the designed primers would not amplify DNA from closely related species with known DNA sequences, we performed a primer basic local alignment search tool (BLAST) search with default settings. In addition, we checked the cross reactivity with 6 common freshwater shrimp species found in Japan: *Caridina multidentata*, *Neocaridina denticulata denticulata*, *Paratya compressa*, *Paratya improvisa*, *Macrobrachium nipponense*, and *Procambarus clarkia*. We extracted total DNA from tissues with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Thereafter, we performed quantitative PCR (qPCR) with the DNA of the 6 species and *P. paucidens* as the template. Amplified products were directly sequenced using a commercial sequence service (Fasmac, Atsugi, Japan).

Study area

Lake Biwa is a freshwater lake in the central part of Honshu Island, Japan (lat 35°01′ N, long 136°00′ E; altitude 85 m asl). The lake is divided into north and south basins. The north basin, which corresponds to the major part of the lake, has a maximum depth of 104 m and a water volume of 27.3 km^3 (Okamoto 1984). The south basin is small and shallow, with an average depth of 3.5 m and a water volume of 0.2 km^3 (Okuda and Kumagai 1995).

Detection of the distribution of nonmigratory individuals

To examine the seasonal distribution of *P. paucidens* in Lake Biwa, we collected eDNA samples from 21 shore sites (E1–E21), of which 5 (E1–E3, E20, and E21) were in the south and 16 in the north basin (Fig. 1, Table S1). We also collected eDNA samples from 32 freshwater lagoon sites (N1–32), of which 7 (N1–N6, and N32) were close to the south basin and 25 to the north basin (Fig. 2, Table S2). Coastal sampling was conducted on 9 November 2015 and 16 February 2016, and freshwater lagoon sampling was conducted on 16 November 2015 and 5 February 2016. Water from the freshwater lagoon sites tended to clog filters for volumes >500 mL, so we set this volume as the threshold for water samples. We measured volume of water samples with a plastic beaker, which also was used to collect samples and to pour them into disposable plastic bags. Each water sample was collected carefully from the surface water to avoid sediment resuspension that potentially influences the results of aqueous eDNA assays.

We filtered the water samples immediately on site according to the on-site water filtration system described by Yamanaka et al. (2016). The water samples were filtered using 47-mm glass-fiber filters (GF/F; GE Healthcare Japan, Tokyo, Japan; nominal pore size = 0.7 μm; cf. Minamoto et al. 2016 for selection of filter type for eDNA sampling), filters were folded inward with sterile forceps, wrapped in Al foil, and sealed in small plastic bags. Samples were kept in a cooler box filled with ice packs that had been precooled

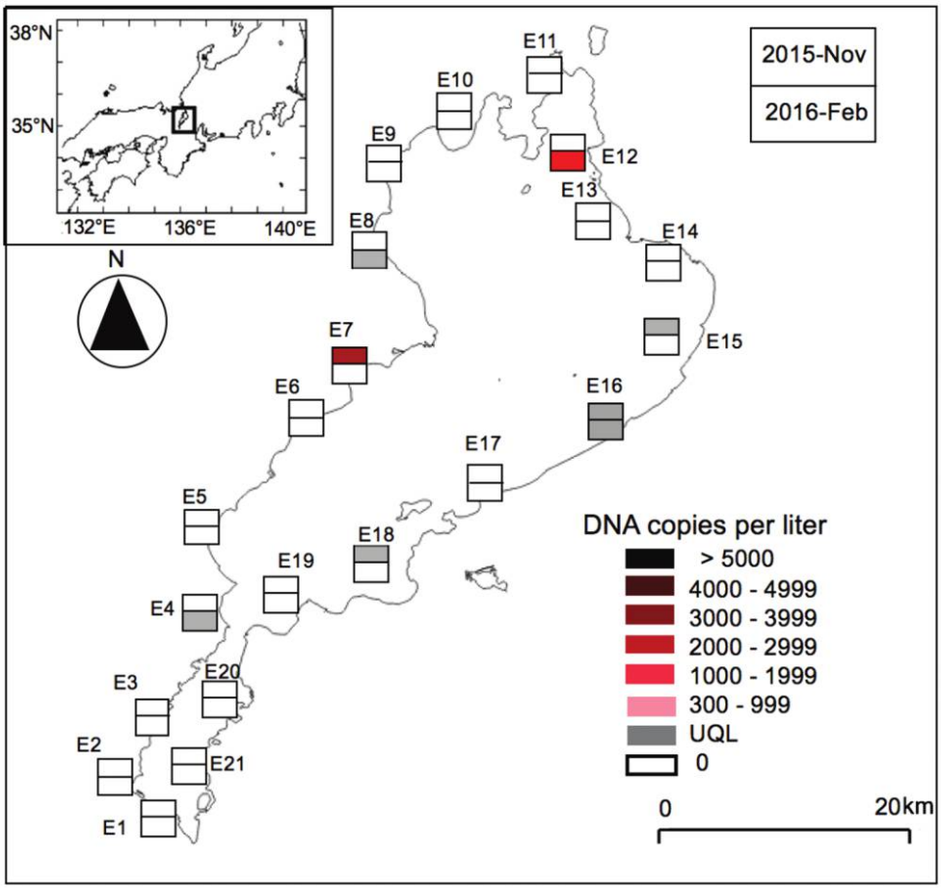

Figure 1. Locations of sampling sites along the shore of Lake Biwa and environmental DNA (eDNA) quantification for each sampling site. Boxes show the eDNA concentrations of *Palaemon paucidens* in November 2015 (upper part) and February 2016 (lower part). UQL = under quantification limit.

in ultralow freezers. The temperature in the cooler box was maintained at ~−20°C. After transportation to the laboratory, we stored the samples in a −20°C freezer. We sampled a negative control in the field every 10 sites. We brought negative control samples containing 500 mL of ultrapure water from the laboratory to the field site to check for unintended cross-contamination during sample transportation and filtration and during the following DNA analysis. All equipment used in water collection and DNA extraction was either disposable or decontaminated with bleach solution (diluted household bleach product containing ~0.1% sodium hypochlorite) for >5 min before use to remove residual DNA. The decontaminated equipment was rinsed with ultrapure water before use. Disposable gloves were used in all procedures to minimize the risk of contamination.

We extracted eDNA from filter samples with procedures published by Miya et al. (2015), with slight modifications. We removed each filter sample from the Al foil, rolled it into a cylindrical shape with sterile forceps, and placed it in a spin column (EZ-10 Spin Column and Collection Tube; Bio Basic Inc., Ontario, Canada), from which the silica membrane had been removed. Filter samples were spun dry by centrifugation at 6000*g* for 1 min and incubated in a DNA extraction buffer (200 μL ultrapure water, 100 μL Buffer AL, and 20 μL proteinase K) at 56°C for 30 min. The Buffer AL and proteinase K were available as part of a DNA extraction kit (DNeasy Blood and Tissue Kit; Qiagen, Hilden, Germany). We then centrifuged the column at 6000*g* for 1 min, after which the filtrate was recovered and temporarily stored in a microtube (1st filtrate). We added TE buffer (pH 8.0, 200 μL) to the filter, incubated it at room temperature for 1 min, and centrifuged it at 6000*g* for 1 min to recover the DNA still remaining on the filter (2nd filtrate). We mixed the 1st and 2nd filtrates in a 2-mL collection tube, to which we added 200 μL of Buffer AL and 600 μL of ethanol. We processed the mixture (final volume = ~1210 μL) in a DNeasy spin column according to the manufacturer's instructions. At the final stage, we stored DNA samples collected in 100 μL of Buffer AE at −20°C until qPCR analysis.

Quantification of *P. paucidens* eDNA by qPCR

We quantified the copy number of *P. paucidens* 16S rRNA by qPCR on a StepOnePlus Real-Time PCR System

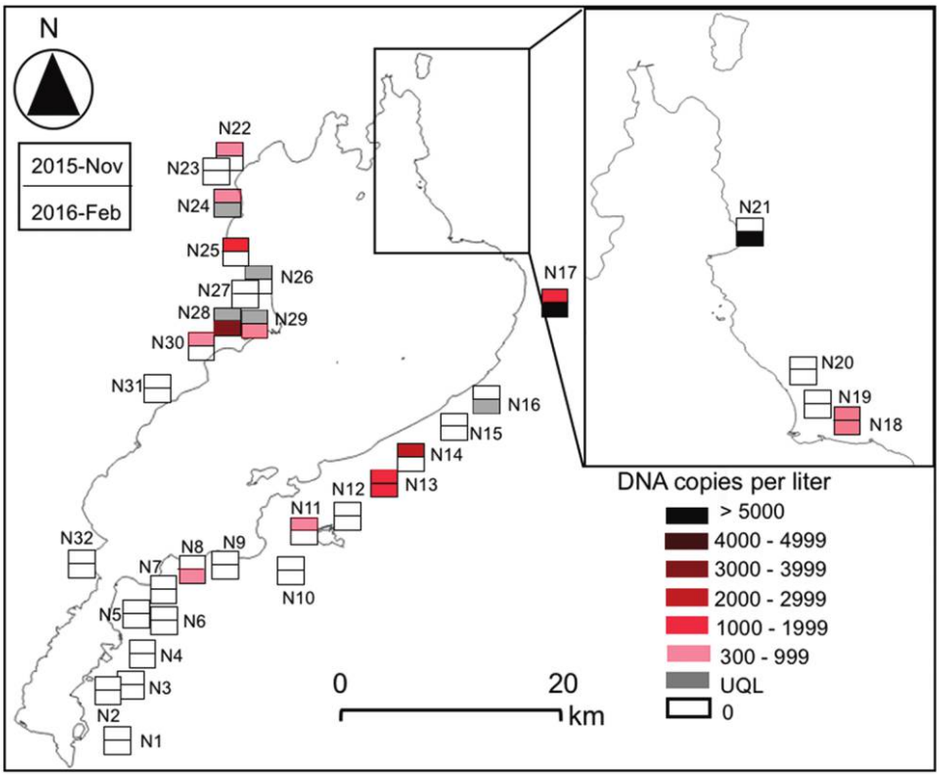

Figure 2. Location of the sampling sites within the freshwater lagoons of Lake Biwa and environmental DNA (eDNA) quantification for each sampling site. Boxes show the eDNA concentrations of *Palaemon paucidens* in November 2015 (upper part) and February 2016 (lower part). UQL = under quantification limit.

(Life Technologies, Foster City, California) based on 3 replicates/sample and with 3 replicates of quantification standards and negative controls. To construct a standard curve, we used 3.0×10^4, 3.0×10^3, 3.0×10^2, and 3.0×10^1 copies of target DNA as quantification standards. Each TaqMan reaction contained 900 nM each primer, 125 nM TaqMan probe, 1 × Gene Expression Master Mix (Life Technologies), and 2 μL template DNA, in a total volume of 20 μL. The qPCR conditions were: 2 min at 50°C followed by 10 min at 95°C, 55 cycles of 15 s at 95°C, and 1 min at 60°C. We regarded the average of the 3 replicates as the value of DNA concentration. When a negative detection was obtained for any of the replicates, we assigned the DNA concentration of that replicate to 0 (Ellison et al. 2006). To determine the limit of quantification we used 3.0×10^4, 3.0×10^3, 3.0×10^2, 3.0×10^1, 10.0, 3.0, and 1.0 copies of target DNA as templates and amplified with 3 replicates. The limit of quantification was 3 copies (see Results), so a sample with calculated concentration <3 copies/reaction was treated as 'positive but under quantification limit (UQL).'

Validation of qPCR as a quantitative method

To test whether our qPCR method was a quantitative measure of *P. paucidens* abundance, we collected individuals with seine traps (mesh: 4 × 4 mm) at 5 freshwater lagoons, Hirako-Yanagihirako, Jinjonuma, Hasuike, Kohoku-Nodanuma, and Hamabunnuma, concurrently with eDNA sampling between 6–9 December 2016. We installed the seine traps at the in- and outflow of the 5 freshwater lagoons in the afternoon and removed them the next morning to collect *P. paucidens*. We installed the seine traps at each site during the same time (i.e., within ½ d), so we regarded the number of individuals collected by each trap as catch per unit effort (CPUE), which we used as an index of the relative abundance of *P. paucidens*.

Before installing the seine traps, we collected 1-L water samples at the in- and outflow of the 5 freshwater lagoons. We collected each water sample carefully from surface water to avoid sediment resuspension. To prevent DNA degradation, we added 1 mL of 10% benzalkonium chloride solution immediately after collecting the water samples. We transported the water samples at ambient temperature and filtered them in the laboratory. We opened negative controls containing 1 L of ultrapure water in the field, added 1 mL 10% (mass/volume) benzalkonium chloride solution, and brought them back to the laboratory. We subjected these negative controls to the same experimental procedures as water samples. We stored filters at −20°C until DNA extraction.

Different water sampling methods were used by the 2 universities performing the surveys. Sample volume and DNA filtration and extraction protocols slightly differed between these methods, but such differences did not influence the results (see Discussion). In this experiment, DNA extraction from filter samples was carried out using the method described by Uchii et al. (2016). We placed 2 filters in a single Salivette tube (Sarstedt, Nümbrecht, Germany), and we used the DNeasy Blood and Tissue Kit. We incubated the tubes at 56°C for 30 min after adding 400 μL of Buffer AL and 40 μL of proteinase K. We recovered solutions by centrifugation, added 220 μL of TE buffer to the filters, and repeated the centrifugation procedure. We added 500 μL of ethanol to the recovered solution and followed Uchii et al. (2016) in the subsequent procedures. At the final stage, we collected DNA samples in 100 μL of Buffer AE and stored them at −25°C until qPCR. Quantification of *P. paucidens* eDNA by qPCR was carried out under the conditions described above. During water collection and DNA extraction, we took precautions to avoid contamination as described above.

We tested PCR inhibition in the eDNA samples according to Jane et al. (2015), with slight modifications. After adding 3.0 x 10^4 copies of standard DNA to the eDNA samples used in the qPCR, we performed another qPCR as described above. The threshold cycle (Ct: the number of cycles required for enough amplified PCR product to accumulate that it crosses a threshold recognized by the qPCR instrumentation) of the 3.0 x 10^4 copy standards was compared with that of eDNA samples + 3.0 x 10^4 copies of standard DNA to identify Ct shifts.

We examined the relationship between eDNA concentration and CPUE, freshwater lagoons, and sampling positions with a generalized linear model (GLM). In the model, we set CPUE, sites (lagoons), and sampling positions (inlet or outlet) as explanatory variables, and eDNA copy numbers in each PCR as the response variable. We ran the GLM analysis in R (version 3.3.2; R Project for Statistical Computing, Vienna, Austria.)

RESULTS

We designed primers and a probe specific for *P. paucidens* (Table 1). We confirmed that our assay successfully amplified the 16S rRNA sequence of *P. paucidens*. The primer-BLAST search revealed that no other freshwater shrimps in Japan would be amplified with the designed primers. In addition, the qPCR showed no cross-reactivity with 6 common freshwater shrimp species in Japan. Thus, the specificity of the primers was confirmed. Amplicon sequences were confirmed as the target sequences with BLAST on the National Center for Biotechnology Information (NCBI) web service (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

In all the runs, R^2 values of calibration curves were >0.985. The ranges of slopes, *y*-intercepts, and PCR efficiency were between −3.639 and −3.324, 44.535 and 49.891, and 89.3 and 97.4%, respectively. We calculated the copy number of gene fragments of *P. paucidens* in each PCR (copies/reaction), and eDNA concentration in the sample water (copies/L) based on the calibration curve of each run and the Ct value of each sample. The limit of quantification was determined as 3 copies, because this was the amount of template DNA copies that we could successfully quantify with 2 of 3 replicates, and no amplification was achieved with a single copy of DNA template.

Table 1. Primers and probe used in our study.

Name	Sequence (5′–3′)
Palaemon paucidens forward primer	AAAGTCTAACCTGCCCACTGAGTTA
Palaemon paucidens reverse primer	TTTAAGCCTTTTCACTTAAAGGTCA
Palaemon paucidens probe	FAM-ATGAGGGAAAAACTG-NFQ-MGB

In November 2015, eDNA of *P. paucidens* was detected in 4 of the 21 shore sites. At the sites with positive detections, DNA concentrations ranged from UQL to 2.2×10^3 copies/L. In February 2016, 4 of the 21 shore sites were positive for *P. paucidens* eDNA with concentrations ranging from UQL to 1.2×10^3 copies/L (Fig. 1). However, the eDNA positive sites were not consistent between these 2 sampling periods. In freshwater lagoons, 12 of the 32 sites were positive for *P. paucidens* eDNA in November 2015 with concentrations varying UQL to 2.2×10^3 copies/L, whereas in February 2016, 9 of the 32 sites were positive for *P. paucidens* eDNA with concentrations varying UQL to 1.2×10^5 copies/L (Fig. 2). All eDNA samples collected from shore and freshwater lagoon sites in the south basin were negative in 2015 and 2016 (Figs 1, 2). All negative controls confirmed that no contamination occurred during DNA extraction and PCR processes.

The CPUE of *P. paucidens* in the 5 freshwater lagoons varied from 3 to 87 in December 2016 (Fig. 3). All *P. paucidens* eDNA samples collected concurrently from these 5 lagoons were positive, except for the lagoon in the south basin (Fig. 3). The eDNA concentration at positive sites varied from 1.8×10^2 to 2.1×10^3 copies/L and showed great variation within a freshwater lagoon. Sampling position (inlet or outlet) and CPUE significantly affected eDNA concentration (GLM; $p < 0.01$ for both variables), but sampling sites (lagoons) did not (GLM; $p > 0.05$).

Results of the PCR inhibition test showed no Ct shift ($\Delta Ct < 1$), suggesting that no PCR inhibition biased the results.

DISCUSSION

Previous investigators reported that most *P. paucidens* populations in Lake Biwa migrate to deep waters in au-

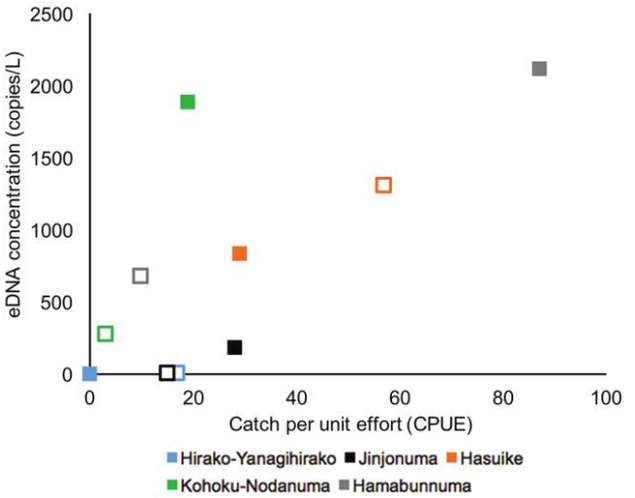

Figure 3. Relationships between the concentration of environmental DNA (eDNA) and catch per unit effort (CPUE; number of *Palaemon paucidens* captured/½ d by seine net collection) in Lake Biwa freshwater lagoons. The closed and open boxes denote out- and inflow sites, respectively. The eDNA concentration was positively correlated with CPUE values (GLM, $p < 0.01$).

tumn where they overwinter at high density (Harada 1966, Nishino 1983). Our eDNA approach revealed the existence of nonmigratory individuals that overwinter in shallow shores and freshwater lagoons of Lake Biwa. Some fish show different life-history traits within the same species (Haryu 1992, Ito et al. 2015). The Ayu-fish (*Plecoglossus altivelis*) from Lake Biwa, for example, can be classified into 2 groups based on their migration timing: one group migrates before late spring and the other migrates after late spring (Azuma 1970, 1973). However, knowledge of nonmigratory individuals of *P. paucidens* in Lake Biwa is limited. By precisely locating the habitat of nonmigratory *P. paucidens* individuals, our study might help future research on these populations.

To date, many eDNA studies of fish and amphibians have documented a positive association between eDNA quantity and abundance of an organism. However, few researchers have investigated this aspect in crustaceans (Tréguier et al. 2014, Carim et al. 2016). We surveyed a large open-water area of Lake Biwa and succeeded in detecting small *P. paucidens* individuals. Many endemic invertebrates inhabit this lake, and the further development of eDNA technology can promote understanding of their ecology and life history.

We detected *P. paucidens* eDNA extensively in shallow shore waters and freshwater lagoons of the north basin in winter, but we did not detect it in the 2 surveys conducted in the south basin. The population density of *P. paucidens* probably is very low in this basin during the winter season, as shown in the Hirako-Yanagihirako lagoon, where only a few individuals were collected by the seine traps. One possible reason for this very low abundance is predation by invasive carnivorous fishes, such as the Largemouth Bass *Micropterus salmoides* and the Bluegill *Lepomis macrochirus*, which have propagated exponentially in the south basin (Kuwamura 2001, Nakajima et al. 2001). A combination of dietary analysis and qPCR of fecal DNA revealed that Largemouth Bass have a strong feeding preference for *P. paucidens* (Sugiura and Taguchi 2012). Terashima (1980) also reported that Bluegill preferred to feed on shrimps after colonizing this lake.

We tested PCR inhibition with the 5 lagoon samples used for qPCR and found that no PCR inhibition biased the results and that negative results obtained for 3 eDNA samples were not caused by PCR inhibition. Turner et al. (2014) reported that eDNA is heterogeneously distributed in the environment or occurs in clumps. Thus, eDNA concentration might be very low in the water samples we obtained. In future studies, detection probabilities or quantified copies of eDNA may increase if larger volumes of DNA are tested.

Our study validated eDNA detection via qPCR as a quantitative measure of the relative abundance of *P. paucidens*. In many eDNA studies on fish and amphibians, the eDNA concentration reflects the relative abundance of their local populations (Takahara et al. 2012, Pilliod et al. 2014, Yamamoto et al. 2016). However, the spatial scale at which such a quantitative approach based on eDNA can be effective for estimating species' or populations' relative abundance is still controversial. In stream ecosystems, Jane et al. (2015) reported that eDNA is homogenized in a 30-m-downstream movement of the water body from its upstream source under a high-flow regime. In closed natural water bodies, Dunker et al. (2016) found that DNA of Northern Pike slowly diffused away from the source, such that the probability of detecting DNA decreased with distance. This result suggests that water sampling in rivers during high-flow or high-wind events would increase the chance of collecting eDNA resuspended from sediment beds (Evans 1994, Jamieson et al. 2005). In our study, each freshwater lagoon is >100 m, and the lake current is very weak (Biological Resource Research Team in Lake Biwa 1966, Nishino 2008). Moreover, we collected water samples carefully to avoid sediment resuspension that may influence the results of aqueous eDNA tests. Therefore, we consider that the detections were not from eDNA attached to sediment particles, homogenization of *P. paucidens* eDNA within the freshwater lagoon is unlikely, and each eDNA sample may be considered independent. In fact, eDNA concentrations in each freshwater lagoon showed great spatial variation that was accompanied by large variations in *P. paucidens* CPUE values, suggesting that the method used here can be a quantitative measure of *P. paucidens* abundance at the local scale. Our quantitative method may be applicable to other small crustacean species, increasing the potential for crustacean research and increasing our understanding of their ecology and life history in nature.

We used 2 sampling methods in which the volume of samples and the methods of DNA filtration and DNA extraction were slightly different. Yamanaka et al. (2017) re-

ported that eDNA yields between these 2 methods show nonsignificant differences and, therefore, the differences between these 2 methods should not influence the results. However, the limits of quantification were different between sample series because of varying sample volumes. Therefore, we did not compare results between the 2 sampling series.

Overall, our study successfully confirmed the existence of nonmigratory *P. paucidens* winter populations in Lake Biwa because we collected eDNA at shore and freshwater lagoon sites during winter. However, in future studies, eDNA surveys should be conducted in other seasons and sites, including offshore and lake-bottom samples, to comprehensively understand the life history polymorphism of *P. paucidens* populations in Lake Biwa.

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Author contributions: QW and TM conceived and designed the research. All authors took part in the collection of specimens. QW, ST, HY, NO, and TM wrote and edited the paper. All authors discussed the results and contributed to development of the manuscript.

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Table S1. Location details and quantitative real-time PCR (qPCR) results obtained for the water samples collected in Lake Biwa shore sites. The qPCR results show the number of positives in the 3 replicates. Environmental DNA (eDNA) concentration was assessed by qPCR. + indicates eDNA of *P. paucidens* was detected but was below quantification limit.

Site	Location	Latitude	Longitude	qPCR results		eDNA concentration (copies/L)	
				Nov 2015	Feb 2016	Nov 2015	Feb 2016
E1	Gotenhama, Otsu City	34°59'20"	135°53'54"	0	0	0	0
E2	Yanagasaki, Otsu City	35°1'50"	135°52'2	0	0	0	0
E3	Hieitsuji, Otsu City	35°4'9"	135°53'16"	0	0	0	0
E4	Mano, Otsu City	35°7'37"	135°55'17"	0	1	0	+
E5	Hachiyado, Otsu City	35°11'15"	135°55'3"	0	0	0	0
E6	Kitakomatsu, Otsu City	35°15'13"	135°58'27"	0	0	0	0
E7	Nagata, Takashima City	35°98'9"	136°1'35"	3	0	2239	0
E8	Shinasahi-cho, Takashima City	35°22'32"	136°2'44"	0	1	0	+
E9	Makino-cho, Takashima City	35°26'28"	136°2'44"	0	0	0	0
E10	Nishiazaicho, Nagahama City	35°28'38	136°6'43"	0	0	0	0
E11	Kinomoto-cho, Nagahama City	35°28'44"	136°6'47"	0	0	0	0
E12	Kohoku-cho, Nagahama City	35°27'0"	136°11'21"	0	3	0	1200
E13	Ohama-cho, Nagahama City	35°24'11"	136°12'54"	0	0	0	0
E14	Tamura-cho, Nagahama City	35°21'26"	136°16'40"	0	0	0	0
E15	Iso, Maibara City	35°18'14"	136°15'24"	2	0	+	0
E16	Hassaka-cho, Hikone City	35°15'29"	136°12'36"	1	2	+	+
E17	Shingai-cho, Hikone City	35°13'30"	136°8'59"	0	0	0	0
E18	Chomeiji-cho, Omihachiman City	35°9'32"	136°3'36"	1	0	+	0
E19	Yoshikawa, Yasu City	35°8'28"	135°59'1"	0	0	0	0
E20	Oroshimo-cho, Kusatsu City	35°4'10"	135°56'4"	0	0	0	0
E21	Kitayamada-cho, Kusatsu City	35°1'56"	135°54'46"	0	0	0	0

Table S2. Location details and quantitative real-time PCR (qPCR) results obtained for the water samples collected in Lake Biwa freshwater lagoons. The qPCR results show the number of positives in the 3 replicates. Environmental DNA (eDNA) concentration was assessed by qPCR. + indicates eDNA of *P. paucidens* was detected but below quantification limit.

				qPCR results		eDNA concentration (copies/L)	
Site	Location	Latitude	Longitude	Nov 2015	Feb 2016	Nov 2015	Feb 2016
N1	Tonotagawa-naiko	34°59'43"	135°54'36"	0	0	0	0
N2	Yanagidairako	35°02'58"	135°55'34"	0	0	0	0
N3	Hirako	35°03'00"	135°55'12"	0	0	0	0
N4	Shinanaka-naiko	35°03'31"	135°56'51"	0	0	0	0
N5	Erinohama	35°05'02"	135°56'52"	0	0	0	0
N6	Kinohama-naiko	35°05'33"	135°56'23"	0	0	0	0
N7	Yasu-gawa River	35°07'08"	135°57'50"	0	0	0	0
N8	Yasu-gawa River	35°07'29"	135°59'30"	0	1	0	466
N9	Yasu-gawa River	35°08'33"	135°03'13"	0	0	0	0
N10	Kitazawanuma	35°08'28"	136°00'20"	0	0	0	0
N11	Kitanoshosawa	35°08'54"	136°05'34"	1	0	476	0
N12	Nishinoko	35°09'48"	136°06'20"	0	0	0	0
N13	Iba-naiko	35°11'17"	136°08'11"	1	2	1180	1363
N14	Jinjonuma	35°13'44"	136°09'28"	3	0	2212	0
N15	Sonenuma	35°14'36"	136°11'35"	0	0	0	0
N16	Hikone-Nodanuma	35°14'58"	136°12'36"	0	1	0	+
N17	Hasuike	35°19'09"	136°16'09"	3	3	1479	5920
N18	Hosoe-naiko	35°23'16"	136°14'36"	1	1	482	306
N19	Minamiura-naiko	35°24'41"	136°12'33"	0	0	0	0
N20	Hayasaki-naiko	35°25'03"	136°12'14"	0	0	0	0
N21	Kohoku-Nodanuma	35°27'05"	136°11'48"	0	3	0	172248
N22	Nukigawa-naiko(north)	35°25'56"	136°02'21"	1	0	892	0
N23	Nukigawa-naiko(south)	35°25'46"	136°02'25"	0	0	0	0
N24	Hamabunnuma	35°25'22"	136°02'42"	1	1	544	+
N25	Harieokawa	35°21'54"	136°03'16"	2	0	1025	0
N26	Suganuma	35°20'56"	136°04'07"	1	0	+	0
N27	Ekainuma	35°19'16"	136°03'32"	0	0	0	0
N28	Gotandanuma	35°19'09"	136°02'45"	2	3	+	2467
N29	Matsunoki-naiko	35°18'47"	136°03'14"	2	2	+	583

N30	Otomegaike	35°17'31"	136°00'52"	3	0	834	0
N31	Oumimaikonuma	35°14'23"	135°57'57"	0	0	0	0
N32	Katata-naiko	35°06'53"	135°55'21"	0	0	0	0

Table S3. Number of captured *Palaemon paucidens* individuals and the results of quantitative real-time PCR (qPCR) at Lake Biwa freshwater lagoons. The qPCR results show the number of positives in 3 qPCR replicates. Environmental DNA (eDNA) concentration was assessed by qPCR. CPUE = catch per unit effort.

Site	Location	Latitude	Longitude	qPCR results (positive/replicates)	eDNA concentration (copies/L)	CPUE
N3	Hirako-Yanagihirako (inlet)	35°02'56"	135°55'31"	0	0	0
N3	Hirako-Yanagihirako (outlet)	35°02'59"	135°55'13"	0	0	17
N14	Jinjonuma (inlet)	35°13'36"	136°09'40"	1	181	28
N14	Jinjonuma (outlet)	35°13'45"	136°09'30"	0	0	15
N17	Hasuike (inlet)	35°19'14"	136°16'15"	3	835	29
N17	Hasuike (outlet)	35°19'14"	136°16'10"	3	1307	57
N21	Kohoku-Nodanuma (inlet)	35°26'59"	136°11'54"	3	1888	19
N21	Kohoku-Nodanuma (outlet)	35°27'00"	136°11'46"	2	275	3
N25	Hamabunnuma (inlet)	35°25'17"	136°02'39"	3	2117	87
N25	Hamabunnuma (outlet)	35°25'22"	136°02'44"	3	674	10