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RAPID COMMUNICATION

Note on important and novel findings

Effects of water pH and proteinase K treatment on the yield of environmental DNA from water samples

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Abstract Environmental DNA (eDNA) analysis has recently been applied to the study of aquatic macroorganisms. In most studies, sample water was filtered and the extracted DNA from the residues on the filter used for the following molecular analysis to detect species of interest. This quick, new biomonitoring method has received broad attention, but some unknowns remain, such as the eDNA yield in relation to water quality. Previous studies suggest that eDNA is composed of various forms, such as the free-floating naked form and in organelles and cells. Therefore, the eDNA yield in the filtration and extraction steps might change depending on the composition of eDNA. Especially the filtration efficiency of free-floating DNA would be affected by the electrical effect of water pH. In this study, not only the free-floating naked DNA, but also all DNA fragments released from the organisms and contained in the water were defined as eDNA, including cells and organelles. We examined (1) the effect of water pH on the eDNA yield at filtration and (2) the effect of proteinase K treatment on the extraction efficiency of DNA from filter samples, with consideration of the variety of the eDNA forms in water. In a laboratory experiment using the purified DNA of common carp (*Cyprinus carpio carpio*) spiked into ultrapure water, the water pH and DNA yield showed a negative relationship within the pH range of 5–9, that is, the DNA yield was higher in acidic conditions, plausibly because of pH-dependent adsorption onto the glass fiber filter at the filtration step. In case the field water contained eDNA derived from the inhabiting common carp and the purified DNA of ayu (*Plecoglossus altivelis altivelis*) spiked in the sample as an internal standard, adjustment of the pH to 5 prior to filtration did not increase the eDNA yield of common carp, and the spiked ayu DNA was not detected at all. During the DNA extraction step, a standard protocol including proteinase K treatment marked higher DNA yield than that without proteinase K treatment. Overall, the present results indicate successful collection of eDNA using filters without any special attention to the pH of the sample water, and a conventional protocol with proteinase K treatment is appropriate for eDNA recovery.

Keywords Environmental DNA (eDNA) · eDNA yield · pH · Proteinase K treatment

Introduction

Knowledge of species distributions and abundances is essential for ecosystem conservation, especially the conservation of endangered species, and for monitoring invasive nonnative species (Smith 2006). The effective management of rare and endangered species requires the ability to detect individuals even at low population densities (Jerde et al. 2011). For the surveillance of fish, baited traps, cast nets, electrofishing, and other methods have conventionally been used; however, these methods require trained skills and lead to variation in survey results depending on the observer, habitat type, and developmental

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stage of the target species (Dejean et al. 2011; Jerde et al. 2011). In the case of rare species, larger sampling efforts are needed to achieve accurate detection rates than for surveying abundant species (McDonald 2004); therefore, research activities themselves may threaten species survival through habitat destruction and/or direct catchment.

Recently, environmental DNA (eDNA), which is shed by organisms into ambient water and soil, has been used as a marker to detect the presence of macroorganisms including amphibians (Ficetola et al. 2008; Fukumoto et al. 2015), fishes (Minamoto et al. 2012; Takahara et al. 2012; Maruyama et al. 2014; Yamanaka and Minamoto 2016), mammals (Foote et al. 2012), invertebrates (Egan et al. 2013; Machler et al. 2014), and plants (Fujiwara et al. 2016). Assessment of aquatic organisms by eDNA analysis is advantageous because all that is needed are samples of water, and it does not require the collection of the organisms themselves. Moreover, it allows more accurate fish surveillance, especially in terms of detection rate, than conventional methods (Jerde et al. 2011; Takahara et al. 2013). Environmental DNA analysis has been applied to various bodies of water, including rivers (Fukumoto et al. 2015), lakes, ponds (Ficetola et al. 2008; Takahara et al. 2013), and oceans (Thomsen et al. 2012; Miya et al. 2015; Yamamoto et al. 2016). Despite the extensive application of this method in a wide variety of habitats, there are still some uncertainties about its reliability because the water collection methods and subsequent processes are not customized for each environment. The water quality of survey fields differs drastically; for example, water pH varies significantly, e.g., 9.9 in Lake Magadi in Kenya (Wood et al. 1994), 3.4–3.8 in Lake Usoriko in Japan (Satake et al. 1995), and between 7.8 and 8.4 in the open ocean (Bates et al. 2012). Several environmental factors, such as pH, temperature, and ultraviolet light, were determined in previous studies as the factors affecting eDNA degradation in water (e.g., Barnes et al. 2014; Pilliod et al. 2014). To confirm the effectivity of the eDNA method against a variety of water qualities, those analyses were fundamental. In the area of microbial ecology, the recovery rate of eDNA from water was intensively examined in relation to the salt content, type of suspended minerals, concentration of cations, and water pH (for a review, see Lorenz and Wackernagel 1994). In contrast to the accumulation of information on collection methods for microbial eDNA, water filtration and the following proteinase K treatment for DNA extraction are generally adopted in macrobial eDNA research without careful examination of the eDNA yield in relation to water quality.

Environmental DNA exists in various forms in water, e.g., from free floating to cells (Turner et al. 2014). When the target DNA exists in its free-floating form, special attention must be paid to the water pH in the filtration process. In many cases of eDNA study targeting macroorganisms, glass fiber filters were used to collect eDNA from water (Minamoto et al. 2016; Uchii et al. 2015; Yamamoto et al. 2016). The water pH could affect the characteristics of DNA molecules, such as the extent of their hydrophobicity and capacity for aggregation (Lorenz and Wackernagel 1994; Bratby 2006; Liang and Keeley 2013). Both are important factors affecting the eDNA yield via filtration using glass fiber filters and might result in a difference in the DNA trapping efficiency. If most of the eDNA in the field water is contained in cells or organelles, the water pH might have smaller effects on the eDNA yield. In the following process after filtration, proteinase K treatment has been adopted in the conventional protocol for eDNA extraction from filter samples (Fukumoto et al. 2015; Yamanaka and Minamoto 2016). The effect of the proteinase K treatment on the recovery efficiency from the filters in the DNA extraction step had not been determined yet, although proteinase K plausibly contributes to DNA recovery by degrading the proteins associated with cells and organelles. These examinations are beneficial to enrich our understanding of the relationship between water quality and eDNA yield for the successful expansion of the application of the eDNA method to various environments.

In this study, any form of DNA, not only free-floating naked DNA, but also DNA fragments included in the cells and organelles, that had been released from the organisms and was contained in the water was defined as eDNA. The effectivity of a generally used eDNA collection method, i.e., filtration by a glass fiber filter and eDNA extraction using proteinase K, was confirmed by the following experiments. The effect of water pH on eDNA yield as the result of the trapping efficiency of filtration using glass fiber filters was examined by the following two experiments. In experiments 1 and 2-a, we determined the effect of sample water pH on the DNA yield. Ultrapure water containing purified DNA derived from the common carp (*Cyprinus carpio carpio* L.) was used for experiment 1, and field water from an outdoor pond that contained the eDNA of the inhabiting common carp and purified DNA of ayu (*Plecoglossus altivelis altivelis* Temminck and Schlegel 1846), spiked in as an internal standard, was used for experiment 2-a. Furthermore, in experiment 2-b, we compared the difference in the DNA yield due to the presence/absence of proteinase K treatment in the extraction step using field water.

Materials and methods

Water filtration and DNA collection

Water filtration and DNA extraction were conducted as follows, although there were some differences between the

experiments in processed water volume and water quality treatment prior to the filtration step. Each water sample was filtered using a Whatman GF/F glass fiber filter (GE Healthcare Life Sciences, Piscataway, NJ; diameter 47 mm; nominal pore size of 0.7 μm). Each filter disc was folded in half with tweezers, wrapped in aluminum foil, and then placed in a plastic bag and stored at −20 °C until DNA extraction. To confirm that unintended contamination of DNA had not occurred during filtration and DNA extraction, equipment blanks were obtained by filtering the same volume of ultrapure water in each experiment. The equipment blanks were treated alongside the real samples in the following experimental steps. Between water samplings, all filtration instruments used were decontaminated following Fukumoto et al. (2015).

To extract and purify the eDNA from the filters, spin columns with 2.0-ml collection tubes (EZ-10, BioBasic Inc., Ontario, Canada) and the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) were used. Buffers AL, AW1, AW2, AE, and proteinase K used in the following experiments were supplied by the DNeasy Blood & Tissue Kit. The EZ-10 spin columns were originally equipped with silica-gel membranes, but these were removed prior to use. Each frozen stored GF/F filter was rolled into a cylindrical shape without unfolding and put into the spin columns. The spin columns were then centrifuged for 1 min at 6000×*g* to remove any excess water remaining in the filters. Afterward, 400 μl of ultrapure water, 200 μl of buffer AL, and 20 μl of proteinase K were dispensed onto the filter in each spin column and incubated for 15 min at 56 °C. The spin columns were centrifuged for 1 min at 6000×*g* to elute the filtrate, and this elution was moved to new 1.5-ml microtubes. Then, 400 μl of TE buffer (pH 8.0) was added to each filter and incubated for 1 min at room temperature. Spin columns were centrifuged for 1 min at 6000×*g* to recover any DNA remaining on the filters. The first elutions were then returned to the 2-ml collection tubes containing the second elutions. Then, 200 μl buffer AL and 610 μl ethanol were added to each collection tube and mixed well by pipetting. eDNA in each solution was collected and purified using the DNeasy Blood & Tissue Kit by centrifuging the solution by using the provided spin columns in three stages because of the large volume of each filtrate. The silica-gel membrane was washed two times using washing buffers AW1 and AW2, according to the manufacturer's instructions. DNA was eluted from the spin columns with 100 μl of the provided AE buffer.

Quantitative real-time PCR

eDNA was quantified according to the method described by Takahara et al. (2012). Quantitative real-time TaqMan® PCR was conducted using a StepOne-Plus™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) to determine the copy numbers of the target DNA in each eDNA sample. The common carp mitochondrial cytochrome *b* gene fragments were amplified and quantified with primers CpCyB_496F (5′-GGTGGGTTCTCAGTAGACAATGC-3′), CpCyB_573R (5′-GGCGGCAATAACAAATGGTAGT-3′), and CpCyB_550p probe (5′-FAM-CACTAACACGATTCTTCGCATTCCACTTCC-TAMRA-3′) developed by Takahara et al. (2012). The ayu mitochondrial cytochrome *b* gene fragments were amplified and quantified with primers Paa-CyB-Forward (5′-CCTAGTCTCCCTGGCTTTATTCTCT-3′), Paa-CyB-Reverse (5′-GTAGAATGGCGTAGGCGAAAA-3′), and Paa-CyB-Probe (5′-FAM-ACTTCACGGCAGCCAACCCCC-TAMRA3′) developed by Yamanaka and Minamoto (2016). These primers were confirmed to specifically amplify the cytochrome *b* gene of the target species' DNA.

Real-time PCR was performed in a 20-μl reaction for each sample and was done in triplicate. The mixture of the reagents was as follows: 1 × PCR master mix (TaqMan® gene Expression Master Mix; Life Technologies, Carlsbad, CA, USA), 900 nM of each primer, 125 nM of TaqMan® probe, and 1 μl sample DNA in each PCR. PCR products of the common carp target sequence were cloned into the pGEM plasmid, and the artificially synthesized target sequences of ayu DNA (399 bp) were cloned into qTAKN-2 plasmids. These were used in standard dilution series containing 3×10^1 to 3×10^4 copies of the target sequences. For each real-time PCR analysis, triplicated PCR negative controls were used in which 1 μl ultrapure water was added to each reaction instead of the eDNA template. The PCR thermal conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, 55 cycles of 15 s at 95 °C, and 60 s at 60 °C with a sample ramp rate of 1.6 °C s^{-1}. The R^2 values of the standard curve for experiments 1 and 2 ranged from 0.992 to 0.995.

Experiment 1: effect of water pH on eDNA collection during filtration using purified DNA and ultrapure water

The effect of the pH of the sample water on DNA yield was determined using the purified total DNA of common carp, extracted from its skeletal muscle. Five beakers containing 600 ml ultrapure water were prepared, and each was adjusted to one of five pH levels (5.0, 6.0, 7.0, 8.0, and 9.0) using 1 M HCl or 1 M NaOH. The water in each beaker was divided into six disposable cups with 100 ml of the water. Purified common carp DNA (50 ng) was added to five of the six cups at each pH level, and the rest was treated as an equipment blank.

All water samples—30 in total (five replicates and one equipment blank for each of the five pH levels)—were filtered, DNA was extracted, and the DNA concentration was determined by real-time PCR, according to the methods described above.

Experiment 2: analyzing field water samples containing eDNA from resident fish

2-a: pH control of water and its effects on eDNA collection

On 16 April 2015, 4 l surface water was sampled from an outdoor pond in Biwako Cultural Park (a public park; 34°97′20″N, 135°94′18″E, Otsu, Japan) in which dozens of large adult common carp were present. The water quality was as follows: pH 7.26, temperature 13.9 °C, and electrical conductivity of 0.06 mS/cm measured using water quality sensors (HI 98128 pHep 5, HI 98312 DiST 6, HI 98312 DiST 6, respectively; HANNA Instruments, Chiba, Japan). In the laboratory, 2 l of the water sample was adjusted to pH 5.0 using 1 M HCl because of the high DNA yield at that pH level in the previous experiment (see "Results". The pH-adjusted and unadjusted field water samples were divided into 500-ml subsamples and spiked with purified ayu DNA (50 ng) extracted from its skeletal muscle. Ayu had not lived in the pond, so ayu DNA was used as an internal standard to confirm the effect of water pH on eDNA yield when using the field water. Further, all water samples were filtered using a GF/F filter within 30 min from water sampling, according to the method described above. For each treatment, there were four replicates of the filtered samples and one equipment blank in which 500 ml ultrapure water was filtered. DNA was extracted and quantified as described above.

2-b: effects of proteinase K treatment on DNA yield

A field water sample, 2 l in total, was collected from the surface water of the same outdoor pond as in experiment 2-a on the same day. The water quality characteristics were the same as in experiment 2-a. The pH of the water sample was not adjusted in this experiment. According to the method described above, water samples of 500-ml volume were dispensed, and 50 ng of purified ayu DNA was spiked into each subsample water as an internal standard. Sample water was filtered using a GF/F filter within 30 min from water sampling. Filtration was repeated four times for sample water and one time for ultrapure water, which was used as an equipment blank. eDNA was extracted from the filters without proteinase K (code no. 9034; Takara, Shiga, Japan), 20 µl of ultrapure water was added instead, and the effect of proteinase K on eDNA yield was determined by comparing the yields from this experiment with those of the samples treated with proteinase K in experiment 2-a. Real-time PCR was conducted following the method as described above.

Statistical analysis

All data on the DNA yield were calculated as DNA copies per sample water volume and used for statistical analyses. All statistical analyses were performed using R ver. 3.0.2 software (R Core Team 2013). The dependency of pH conditions on DNA yield using ultrapure water was analyzed using simple linear regression analysis, and the effect of pH adjustment on DNA yield using field water was analyzed using a t test. The average number of DNA copies found was compared between samples treated with proteinase K and those that were untreated using a t test. The minimum level of significance was set at $p \leq 0.05$.

Results

Effect of the pH of sample water on DNA collection

In experiment 1, using the purified DNA of common carp dissolved in ultrapure water, the DNA yield (copies 100 ml^{-1}) and sample water pH levels showed a significantly negative relationship (Fig. 1, simple linear regression, $p = 0.002$, $R^2 = 0.316$). The DNA yield was higher in acidic conditions. Therefore, in experiment 2-a using the field water, the sample water was adjusted to pH 5.0 (the lowest pH treatment in this study) before filtration, and the DNA yield was compared with that of the intact field water (pH 7.26, not adjusted). However, there was no difference in common carp eDNA yield between pH 5.0 treatment and the intact water (Fig. 2, t test, $p = 0.059$). Purified ayu DNA spiked in samples was below the quantification limit in all replicates.

Effects of proteinase K treatment on DNA extraction

In experiment 2-a and 2-b, DNA extraction efficiencies were significantly different between the proteinase K and control treatments (Fig. 3, t test, $p = 0.0015$). The average common carp DNA concentration was $3.70 \pm 0.50 \times 10^5$ copies 0.5 l^{-1} (mean ± standard deviation) in the proteinase K treatment and $1.87 \pm 0.35 \times 10^5$ copies 0.5 L^{-1} in the control. Purified ayu DNA, which was spiked in samples, was below the quantification limit in all replicates.

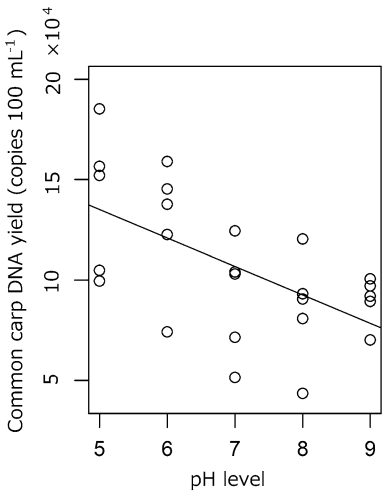

Fig. 1 Relationships between yield of purified common carp DNA (copies 100 ml^{-1}) and sample water pH. The *line* shows regression for DNA yield on water pH (simple linear regression, $p = 0.002$)

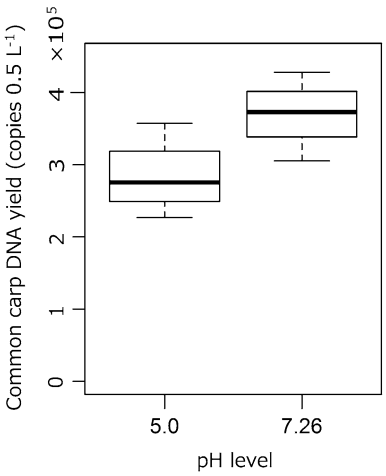

Fig. 2 The DNA yield (copies 0.5 l^{-1}) of common carp from pH-adjusted (pH 5.0) and non-adjusted (pH 7.26) field water. There was no difference in eDNA yield between the pH 5.0 treatment and original pH control (*t* test, $p = 0.059$, $N = 4$)

Discussion

The yield of eDNA was affected by the pH of the sample water when using ultrapure water containing purified DNA, and the yields were higher under acidic conditions. This was most likely due to the change in the state of DNA molecules under acidic conditions and its positive effects on the trapping efficiency of DNA during the filtration process. DNA is negatively charged in water; therefore, it was speculated that molecular size was increased by H^+, and DNA molecules were linked eith each other (Lorenz and Wackernagel 1994). This might increase the DNA trapping efficiency of the filter (Liang and Keeley 2013).

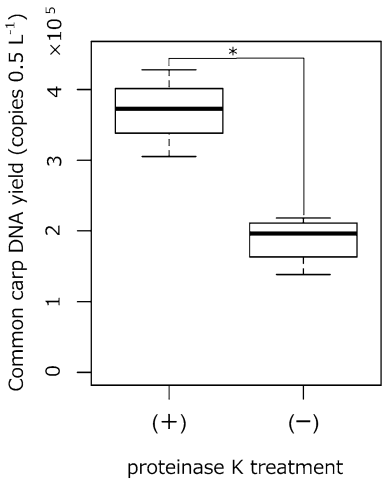

Fig. 3 The effect of proteinase K on the DNA yield of common carp from field water samples. DNA yields (copies 0.5 l^{-1}) were significantly different between the proteinase K and control treatments (*t* test, $p = 0.0015$, $N = 4$)

Moreover, the hydrophobicity of DNA was increased via deionization (Bratby 2006), which might promote the adherence of DNA to the GF/F filter. By contrast, DNA yields were low in alkaline conditions. This was most likely because of the low binding rate of H^+ to DNA due to the decrement of the hydrophobicity of the DNA molecules under alkaline conditions. The dissolved DNA (dDNA), which is a constituent of eDNA and has been well studied in the area of microbial ecology, is mainly composed of soluble or particle-binding forms of naked DNA (Jiang and Paul 1995), and they have been reported to be adsorbed on suspended particles or colloids contained in the field water (Lorenz and Wackernagel 1994; Siuda and Chrost 2000). The adsorption to the surfaces of suspended particles could be promoted by a decrease in the water pH due to the reduction of the electric repulsion force of DNA molecules (Lorenz and Wackernagel 1994). The increased eDNA yield in experiment 1 in the present study could be well explained by these findings in the previous studies about kinetic interactions between naked DNA and sorbed substrate.

On the other hand, the pH control, i.e., adjustment to pH 5.0, did not cause any difference in eDNA yield in the examination of the field water samples. This result implies that a large part of macrobial eDNA in the field water captured by the GF/F filter was not free floating; it was rather within the cells, organelles, or any complex with components that were less affected by pH than purified DNA. Purified common carp DNA that dissolved in ultrapure water in the laboratory experiment (experiment 1) was recovered by GF/F filters, although purified ayu DNA, which was spiked in the field water, was not detected regardless of pH conditions (experiments 2-a and

2-b). Sample water was filtered within 30 min after the water sampling and spiking in of the purified DNA of ayu, but this internal standard was not detected. This suggests that free-floating DNA is prone to fast decomposition in field water. It is also possible that the purified DNA was adsorbed on colloids in field water that were smaller than the pore size of the filter and passed through the filter, as suggested in a previous study focusing on microbial dDNA (Siuda and Chrost 2000). Environmental DNA shed by aquatic animals as a cellular form or as organelles could remain in the water for some time (Pilliod et al. 2014); however, once DNA molecules have been released from the particles, they would be prone to decompose and disappear immediately because of being susceptible to DNase released from the microorganisms (Pedersen et al. 2015).

A substantial increase in DNA yield was observed in the proteinase K treatment in which the yield was 1.97 times larger than in the untreated control when field water samples were used. Turner et al. (2014) suggested most macrobial eDNA exists in cells or organelles, and the present result was consistent with their suggestion. Since proteinase K is an enzyme that digests proteins, the extraction efficiency of DNA from filter samples could be improved by the digestion of the proteins of the cells, organelles, or tissue fragments. However, even the untreated sample, which was not treated with proteinase K, showed some DNA yield. This implies that DNA was possibly eluted from cells or tissue fragments without the aid of proteinase K, because the DNA extraction method used in this study includes some buffers and centrifuge steps that might cause chemical or physical disruption of cells, organelles, or tissue fragments. Moreover, it is possible that the addition of proteinase K during the DNA extraction step in experiment 2-a might slow the decomposition of free-floating DNA by inactivating the DNase contained in the sample solution and intensified the yield of DNA. However, the spiked ayu DNA was not detected at all, regardless of the proteinase K treatment. This implies that the effect of proteinase K as a DNase inhibitor might be limited and the main effect of proteinase K on the increment of eDNA yield was the digestion of the cell and organelle membranes.

To the best of our knowledge, the pH of water samples prior to filtration was not a concern in previous macrobial eDNA studies, although there were some studies that clarified the effect of pH on the degradation rate of DNA (Barnes et al. 2014; Strickler et al. 2015). Currently, filtration is one of the primary methods used to collect eDNA from water samples, and it has been used in many studies (e.g., Deiner et al. 2015; Wilcox et al. 2016; Yamanaka and Minamoto 2016). This study indicated that strict control of the pH of sample water is not required when sampling eDNA in the field by filtration. The methods relating to eDNA sampling and its processing are still in the form of generalizations, and all researchers are now accumulating basic methodological information. This study will contribute to further eDNA studies conducted in various areas with diverse water qualities.

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