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Effects of sample processing on the detection rate of environmental DNA from the Common Carp (*Cyprinus carpio*)

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ABSTRACT

Environmental DNA methods have been used to monitor the presence of aquatic vertebrates in natural systems, although detection of DNA in the environment is sometimes a challenge. In this study, we evaluated the effect of sample processing on the detection of a species' environmental DNA in the water. Specifically, we examined whether freezing and then thawing water samples prior to analysis was an effective method of preserving them. The detection of Common Carp DNA was lower in samples that were frozen and thawed than in samples that were not, even though there was no difference in the DNA concentration, which was included with the DNA undetectable samples. In both types of samples, the DNA detection rate tended to be higher in a 2-μL volume of template DNA solution than in a 5-μL volume. DNA was detected in all non-frozen samples that were analyzed using a 2-μL template, both in three wells (three PCR replicate reactions per sample) with 40 PCR cycles and in eight wells with 55 cycles. The detection of Common Carp DNA in samples that were frozen and thawed was likely to increase through the use of the TaqMan Environmental Master Mix, which is used recently to efficiently release PCR inhibition. Our results suggest that environmental DNA detection is influenced by the processing of water samples after collection and by PCR reaction conditions. Use of non-frozen samples and a smaller DNA solution are recommended for detection of environmental DNA with quantitative PCR assays.

1. Introduction

Environmental DNA (eDNA) methods have often been used to detect freshwater fish (Barnes et al., 2014; Bronnenhuber and Wilson, 2013; Dejean et al., 2011; Jerde et al., 2011; Minamoto et al., 2012; Takahara et al., 2012, 2013; Thomsen et al., 2012b; Wilcox et al., 2013). These techniques, which analyze a water sample for the presence of DNA from target species, reduce field survey time and have little or no impact on ecosystems (Lodge et al., 2012). In addition, species identification from DNA sequences is often easier than identification by observation of external morphology, and a variety of aquatic species may be detected from a single water sample (Thomsen et al., 2012a).

Estimating the potential absence of a species from an area is an important task for programs and agencies that monitor populations and predict their distribution (Bayley and Peterson, 2001). The "absence" of target species is almost impossible to confirm, regardless of whether the traditional method or eDNA methods are used (Darling and Mahon, 2011), because the DNA of a target species may not be captured in a 15-mL to 10-L water sample, even when the organisms are known to be present. Therefore, eDNA methods may inaccurately report the absence of a species. Thomsen et al. (2012b) reported that the success rate of eDNA-based species detection in areas with known occurrence of the targeted species was not always 100%. Current eDNA methods must be enhanced in order to decrease the probability of the causes of false negatives. A more robust understanding of false negatives can assist global advances in the field of eDNA monitoring.

Real-time quantitative polymerase chain reaction (qPCR) assays have been used for the rapid detection of freshwater animals' DNA in environmental samples (e.g., Barnes et al., 2014; Pilliod et al., 2013, 2014; Takahara et al., 2012, 2013; Thomsen et al., 2012b). In eDNA monitoring methods with qPCR assay, a sample is considered positive when one of a number of PCR replicates exceeds the fluorescence threshold (e.g., Takahara et al., 2013). Alternatively, an additional independent triplicate reaction may be performed if all wells in the first triplicate reaction did not exhibit positive results (e.g., Goldberg et al., 2013; Pilliod et al., 2013). Such PCR

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methodology is based on a repeat analysis approach and is suitable for analyzing DNA that is degraded and/or present at low concentrations (Pompanon et al., 2005; Taberlet et al., 1996). In order for PCR to detect eDNA with a high rate of success, it should be used for collections of several field samples or several PCR replicates per sample (e.g., Ficetola et al., 2008; Thomsen et al., 2012b). The confirmation of PCR inhibition using an internal positive control is an effective way to reveal the potential for false negatives to occur (Hartman et al., 2005), but this approach may increase the complexity of the experimental protocol.

Our objective was to enhance a non-invasive eDNA-based method for estimating the distribution of fish in ponds, using the Common Carp, *Cyprinus carpio* L., as a model organism. First, we evaluated the distribution of Common Carp in 70 study ponds by detecting eDNA. Next, we targeted 24 ponds in which Common Carp were detected in the initial survey. We tested whether freezing and then thawing the water samples that were collected for analysis (e.g., to test quantities of inorganic nitrogen or total organic carbon; Tamamura et al., 2012) affected the results of eDNA analysis of these samples. Our expectation was that freezing and then thawing would stop nucleases from degrading DNA in the water and that it would be an effective method of preserving samples when it was not possible to perform the next step in processing, such as filtration or precipitation, immediately in the field. There have been no previous reports about the influence of freezing and then thawing on the detection rate of macro-organisms' DNA with qPCR assays. Accordingly, we tested several different PCR conditions (i.e., the volume of the template DNA solution, the number of PCR replicate reactions per sample, the number of PCR cycles). On the basis of our findings, we present an enhanced approach for fish monitoring applications using eDNA methods, including the optimal PCR approach.

2. Methods

2.1. Field survey and sample treatment procedure in 2011

Fig. 1 shows a workflow of the steps involved with sample collection and analysis of eDNA. The water samples used in this study were the same as the samples that were used in Takahara et al. (2013). We surveyed the distribution of fish in 70 ponds (34°05′–34°14′N, 132°16′–132°32′E). A 1-L water sample was collected once from the surface of each pond between 12:00 and 16:00, between October 19 and December 22, 2011. The water samples were collected near the centers of the ponds using a sampling bucket fastened to a 5-m rope, lowered from shore or from a boat, depending on the size of the pond. To prevent eDNA contamination between ponds, the water on the boat and the bucket was wiped away immediately after sampling. Upon arrival at the next pond, the bucket was thoroughly prewashed with the pond water and then used to collect a sample. In addition, we recorded whether Common Carp were visually detected from the shore. A person searched for the Common Carp in the water while walking along the whole shoreline, a procedure that lasted 10–20 min, depending on the shoreline's length.

We quantified Common Carp DNA using the method developed by Takahara et al. (2013). In brief, the water samples were stored in DNA-free 1-L bottles (Nalgene®) and transported on ice in a cooling box to the laboratory (within 4 h), where they were stored at −30 °C for 2–3 weeks. The frozen samples were then thawed under flowing tap water at 25 °C (room temperature) for about 1 h. Each thawed sample was filtered through a 3.0-μm membrane filter (cellulose acetate, 142-mm diameter, C300A142C; Advantec, Saijo, Japan) using a pressure filtration system with a stainless steel filter holder (KS-142-US; Advantec). Our previous study identified this pore-size filter as a suitable type of filter for capturing cellular or organelle materials (to which we refer as eDNA) from water samples of lentic systems (e.g., lagoons) (Takahara et al., 2012). Each filter disk containing the sample was folded inward with forceps and wrapped in DNA-free aluminum foil. The filter disk was immediately stored at −25 °C until further analysis.

To elute the eDNA from the filter surface, the filter disks were placed in autoclaved 500-mL Nalgene® bottles using forceps. The filter disks in each bottle were soaked in 10 mL autoclaved ultrapure water and stirred on a rotary shaker at maximum speed for 10 min. The suspension in the bottle was decanted into centrifugal filtration devices (Amicon Ultra-15, 30-kDa cutoff, UFC903096; Millipore, Billerica, MA, USA) and concentrated by centrifuging at

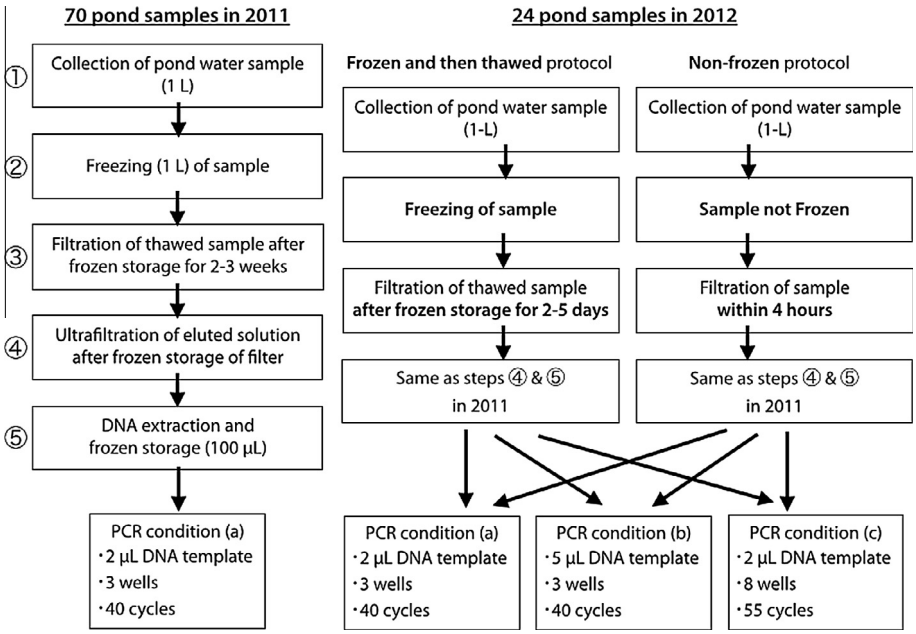

Fig. 1. The experimental design in the 2011 and 2012 surveys. The numerals indicate the same experimental procedures. The bolded words indicate processing steps that differed between experimental procedures.