Video Article

Use of a Filter Cartridge for Filtration of Water Samples and Extraction of Environmental DNA

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Abstract

Recent studies demonstrated the use of environmental DNA (eDNA) from fishes to be appropriate as a non-invasive monitoring tool. Most of these studies employed disk fiber filters to collect eDNA from water samples, although a number of microbial studies in aquatic environments have employed filter cartridges, because the cartridge has the advantage of accommodating large water volumes and of overall ease of use. Here we provide a protocol for filtration of water samples using the filter cartridge and extraction of eDNA from the filter without having to cut open the housing. The main portions of this protocol consists of 1) filtration of water samples (water volumes ≤4 L or >4 L); (2) extraction of DNA on the filter using a roller shaker placed in a preheated incubator; and (3) purification of DNA using a commercial kit. With the use of this and previously-used protocols, we perform metabarcoding analysis of eDNA taken from a huge aquarium tank (7,500 m^3) with known species composition, and show the number of detected species per library from the two protocols as the representative results. This protocol has been developed for metabarcoding eDNA from fishes, but is also applicable to eDNA from other organisms.

Video Link

The video component of this article can be found at https://www.jove.com/video/54741/

Introduction

Environmental DNA (eDNA) in aquatic environments refers to genetic material found in the water column. Recent studies demonstrated the utility of eDNA for detecting fishes from various aquatic environments, including ponds[1-3], rivers[4-8], streams[9], and seawater[10-14]. Most of these studies focused on detection of a single or a few invasive[1,4-6,8,14] and rare or threatened species[3,9], while some recent studies attempted simultaneous detection of multiple species in local fish communities[7,9,12,13,15] and mesocosms[11,12].

The latter approach is called "metabarcoding" and eDNA metabarcoding uses one or multiple sets of PCR primers to coamplify a gene region across taxonomically diverse samples. This is followed by library preparation with indexing and adapter addition, and the indexed libraries are analyzed by a high-throughput parallel sequencing platform. Recently Miya *et al.*[12] developed universal PCR primers for metabarcoding eDNA from fishes (called "MiFish"). The MiFish primers target a hypervariable region of the mitochondrial 12S rRNA gene (163-185 bp), which contains sufficient information to identify fishes to taxonomic family, genus and species except for some closely related congeners. With the use of those primers in eDNA metabarcoding, Miya *et al.*[12] detected more than 230 subtropical marine species from aquarium tanks with known species composition and coral reefs near the aquarium.

While optimizing the metabarcoding protocol to accommodate natural seawater with varying levels of eDNA concentration from fishes, we have noticed that the MiFish primers occasionally failed to amplify the target region for subsequent library preparation. One of the more likely reasons for this unsuccessful PCR amplification is lack of adequate amounts of the template DNA contained in small volumes of water filtered (*i.e.* 1-2 L). Although eDNA concentration from a specific taxonomic group is unknowable before the amplification, filtration of large water volumes (>1-2 L) would be a simple and effective means to collect more eDNA from the aquatic environments with scarce fish abundance and biomass, such as open-ocean and deep-sea ecosystems.

Relative to disk fiber filters conventionally used in a number of fish eDNA research[16], filter cartridges have the advantage of accommodating larger water volumes before clogging[17]. Actually, a recent study showed large volume (>20 L) filtration of coastal seawater samples using filter cartridges[18]. In addition, they are individually packaged and sterile, and several steps of the experimental workflow can be performed in the filter housing, thus reducing the probability of contamination from the laboratory[19]. The latter feature is critical for eDNA metabarcoding, in which the

risk of contamination remains among the greatest experimental challenges[20,21]. Despite these technical advantages of filter cartridges, it has not been used in eDNA studies of fishes with two exceptions[8,15].

Here we provide a protocol for filtration of water samples with the filter cartridge and extraction of eDNA from its filter without having to cut open the housing. We also provide two alternative water filtration systems depending on the water volumes (≤4 L or >4 L). To compare the performance of the newly-developed protocol and a previously-used protocol using a glass-fiber filter in our research group[12,14,22,23], we perform eDNA metabarcoding analysis of seawater from a huge aquarium tank (7,500 m^3) with known species composition, and show the number of detected species derived from the two protocols as representative results. This protocol has been developed for metabarcoding eDNA from fishes, but is also applicable to eDNA from other organisms.

Protocol

NOTE: This protocol does not deal with water sampling and metabarcoding methods. Water may be sampled in different manners depending on study purposes[16] and see Miya *et al.*[12] for details of the metabarcoding methods using MiFish primers. Note that the sampled water should be kept very cold and filtered within a few hours to avoid degradation of eDNA. Also note that this protocol involves the use of a rotary shaker and an incubator, and the latter must be large enough to accommodate the former. In addition, a centrifuge that can accommodate both 15 ml and 50 ml conical tubes is indispensable to remove the remaining liquid from the post-filtration filter and to collect extracted DNA within the cartridge, respectively.

1. Processing a Screw Cap and a 1 L Plastic Bag

NOTE: Skip this step if the filtration volume is >4 L.

1. Drill a hole through the center of a screw cap (attached to a disposable 1 L plastic bag) with the same diameter (4.8 mm) as the tube projecting from a male luer-lock connector. Using diagonal pliers, shorten the tube of the male luer-lock connector to an appropriate length (*ca*. 3 mm) to avoid clogging of a small amount of water inside the cap after filtration.
2. Apply an adhesive glue specialized for polyethylene (PE) and polypropylene (PP) to both the bottom and surface of the male luer-lock connector and screw cap, respectively. Wait a few minutes for good adhesive bonding (refer to the manufacturer's instructions).
3. Insert the male luer-lock connector into the hole of the screw cap. Wait until complete adhesive bonding of the two parts (usually >24 hr). Sterilize the screw cap with the male luer-lock connector with 10% commercial bleach (*ca*. 0.6% sodium hypochlorite) before use.
4. Punch two holes at the two bottom corners of the 1 L plastic bag in order to hang it from a mesh panel (step 3). Ensure that the diameter of the holes is larger than that of the prongs on the mesh panel.

2. Assembly of the Filtration System

1. Attach high vacuum tubing to the input connector of an aspirator pump and attach the other end of tubing to a manifold. Be sure that the three red t-valves of the manifold are in the "off position" (*i.e.*, horizontal).
2. Wearing a clean set of gloves, insert a female luer fitting and a vacuum connector at the top and bottom ends of the vacuum rubber tubing for filtration, respectively.
3. Carefully attach the female luer fitting to an outlet port of the filter cartridge and attach the vacuum connector to a silicone stopper.

3. Filtration of Water Samples (≤4 L) using the Filter Cartridge

NOTE: Skip this step if the filtration volume is >4 L. This filtration system requires a self-standing panel for hanging the plastic bag filled with 1 L of water. A mesh panel, multiple prongs, and a stand for the panel, all available from online stores, would be useful for assembling this unit. Autoclave the inlet and outlet luer caps for the filter cartridge before use.

1. Pour the 1 L sampled water into the plastic bag and close the screw cap with the male luer-lock connector.
2. Carefully connect an inlet port of the assembled filter cartridge with the male luer-lock connector of the plastic bag. Do not over-tighten the luer-lock connector; otherwise the unit will leak once the pumping starts. Be sure that all the connections are secure before hanging the plastic bag from a mesh panel.
3. Carefully hang the plastic bag with the filter cartridge from two prongs on the mesh panel and insert a silicone stopper into an inlet port of the manifold.
4. Turn on the aspirator. Open the red t-valves for filtration. Run the manifold until the filter cartridge is dry, then turn the red t-valve to the off position.
5. Repeat 3.1-3.4 steps until the desired amount of water is filtered.
   NOTE: For 1 L of water taken from the subtropical coral reefs, it takes about three min for the filtration.
6. Carefully remove the filter cartridge from the plastic bag and the vacuum connector.
7. Cap both ends of the cartridge with the inlet and outlet luer caps.
8. Label the cartridge and inlet luer cap appropriately using a solvent-proof pen for fast-drying and non-smearing labeling.
9. Store the filter cartridge at -20 ˚C before DNA extraction.

4. Filtration of Water (>4 L) Samples using the Filter Cartridge

Note: Skip this step if the water filtration volume is ≤4 L. This filtration system requires a 10 L book bottle equipped with a valve and a disposable 10-ml pipette tip. An inner diameter of the 10 ml pipette tip (15.0 mm) and a taper of the tip end should fit to an outer diameter of the the valve

(15.0 mm) and inlet port of the filter cartridge, respectively. Both connections are retained securely during filtration in a friction fit. Sterilize the pipette tip with 10% commercial bleach (*ca.* 0.6% sodium hypochlorite) before use.

1. Prepare an appropriate amount of sampled water in the book bottle. Tightly insert the 10 ml pipette tip into the valve of 10 L book bottle.
2. Tightly insert the outlet port of the filter cartridge into the pipette tip end and insert the silicone stopper into the inlet port of the manifold. Turn on the aspirator. Open the red t-valves for filtration. Run the manifold until the filter cartridge is dry, then turn the red t-valve to the off position.
   NOTE: For 10 L of seawater taken from the subtropical outer coral reefs, it takes about 30-40 min for the filtration.
3. Carefully remove the filter cartridge from the plastic bag and the vacuum connector. Cap both ends of the cartridge with the inlet and outlet luer caps. Label the cartridge and inlet luer cap appropriately using a solvent-proof pen for fast-drying and non-smearing labeling. Store the filter cartridge at -20 °C before DNA extraction.

5. Extraction of eDNA from the Filter

NOTE: In steps 4 and 5, we use a commercial kit, largely following a protocol for "nucleated blood" provided by the kit. For simplicity, we describe the procedure for processing an individual cartridge. In practice, we recommend processing 8 (or the maximum number of the centrifuge) or fewer filters at a time.

1. Preheat an incubator to 56 °C.
2. Prepare a 2.0 ml tube by cutting the hinged cap from the tube. Discard the cap.
   NOTE: This tube is used as a collection tube for the remaining liquid in the filter.
3. Remove the inlet (NOT outlet) luer cap from the filter cartridge and insert the inlet port into the collection tube. Tightly seal a connection between the cartridge and collection tube using a self-sealing film.
4. Insert the combined unit into a centrifuge adaptor for a 15-ml conical tube. Centrifuge the cartridge at 5,000 x g for 1 min to remove the remaining liquid in the filter.
5. Remove the collection tube from the filter cartridge and discard the tube. Re-cap the inlet port of the cartridge.
6. Prepare a mixture of 20 µl proteinase-K solution, 220 µl PBS (phosphate buffered saline; not provided by the kit) and 200 µl buffer AL.
   NOTE: The filter cartridge accommodates up to four times the volumes of the mixture (*ca.* 1.8 ml).
7. Remove the inlet cap and add the mixture (440 µl) into the filter cartridge using a pipette tip. Insert the pipette completely into the inlet port so that the pipette tip is visible inside the cartridge just above the membrane.
8. Re-cap the inlet port and place the filter cartridge on a rotary shaker.
9. Place the rotary shaker in the preheated incubator at 56 ˚C and turn on the shaker at a speed of 20 rpm for 20 min.
10. During the incubation, prepare a new 2.0 ml tube, label the tube appropriately using a solvent-proof pen and place it in a 50-ml conical tube.
    NOTE: The former (2.0 ml) and latter tubes (50 ml) are used for collection of the extracted DNA and for holding the 2.0 ml tube, respectively.
11. After the incubation, remove the inlet cap and insert the inlet (NOT outlet) port of the cartridge into the 2.0 ml tube within the 50 ml conical tube. Close the 50 ml conical tube with a screw cap.
12. Centrifuge the 50-ml conical tube at 5,000 x g for 1 min to collect the extracted DNA from the cartridge. Remove the filter cartridge and the 2.0 ml tube using sterilized forceps and cap the tube. Discard the filter cartridge.

6. Purification of Extracted DNA

NOTE: We elute eDNA with 100 µl buffer AE instead of 200 µl specified in the manual of the commercial kit.

1. Add 200 µl ethanol (96-100%) to the extracted DNA in the 2.0-ml tube from the above step (*ca.* 440 µl), and mix thoroughly by vortexing.
2. Pipet the mixture into a spin column placed in a 2 ml collection tube. Centrifuge at 5,000 x g for 1 min. Discard the flow-through and the collection tube.
3. Place the spin column in a new 2 ml collection tube, add 500 µl buffer AW1, and centrifuge for 1 min at 5,000 x g. Discard the flow-through and the collection tube.
4. Place the spin column in a new 2 ml collection tube, add 500-µl buffer AW2, and centrifuge for 3 min at 20,000 x g. Discard the flow-through and the collection tube.
5. Prepare a new 1.5 ml tube (not provided by the kit) and label the tube appropriately using a waterproof pen for fast-drying and non-smearing labeling. Transfer the spin column to the 1.5 ml tube.
6. Elute the DNA by adding 100 µl buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature, and then centrifuge at 6,000 x g for 1 min.
7. Discard the spin column and cap the tube. Store the purified DNA at -20 °C.

Representative Results

It is technically difficult to isolate and quantify only fish eDNA from the extracted bulk eDNA, because the MiFish primers coamplify the target region from some non-fish vertebrates, such as birds and mammals, with PCR products of the same size (*ca.* 170 bp)[12]. Instead of quantifying fish eDNA, we perform MiFish metabarcoding analysis of eDNA from an aquarium tank with known species composition using the two different methods of filtration and DNA extraction, and compare the numbers of detected species per library from the two protocols. This simple experiment was designed to show feasibility of the present protocol as the "representative results" and not to rigorously demonstrate its superiority over others.

We sampled a total of 30 L of seawater from a Kuroshio tank in the Okinawa Churaumi Aquarium, Okinawa, Japan (26°41'39"N, 127°52'41"E). The Kuroshio tank is designed for exhibiting marine megafauna, with dimensions (L x W x D) of 35 m x 27 m x 10 m and a total water volume of 7,500 m^3. It houses approximately 60 large-sized marine fish species characteristic to areas around the Kuroshio, one of the western boundary currents flowing northeastward along the entire length of Japan. In a previous MiFish metabarcoding study, Miya *et al.*[12] detected 61 of the 63 species (97%) contained in the tank from five 2 L seawater samples.

For filtration of the seawater, we used filter cartridges (pore size = 0.45 µm) and glass-fiber disk filters (pore size = 0.70 µm). We concurrently filtered the seawater on the same manifold using the two protocols for four water volumes (1, 2, 3, and 4 L; total, eight filters). After the filtration of seawater, we also filtered 1 L of PCR-grade water with the two filters (filter cartridge and glass-fiber filter) as the negative controls (two filter blanks). From those types of filters, we extracted eDNA using the new and previously-used protocols[12], both of which employ the same commercial kit. A total of 10 eDNA samples (including the two negative controls) were subjected to MiFish metabarcoding analysis as described in Miya *et al.*[12]

Briefly, we performed 1st-round PCR to amplify the target region (mitochondrial 12S rRNA gene; *ca*. 170 bp) and to append adapters for paired-end sequencing. For eight eDNA samples (excluding the two negative controls), we conducted 10 PCR replicates to see variations in the number of detected species within each sample (total 80 PCRs). We also added the filter and PCR blanks to each eight eDNA samples (total 16 PCRs). In total, we obtained 96 PCR products from the 1st-round PCR and they were used as templates for 2nd-round PCR for dual indexing and appending additional adapters for library preparation following Miya *et al.*[12]

The paired-end sequencing (2 x 150 bp) of the 96 libraries yielded 4,127,546 reads, with an average of 42,995 reads per library (40,539 reads in the filter cartridge and 43,121 reads in the glass-fiber filter). After demultiplexing and subsequent pre-processing of the raw data, 1,068,266 reads were retained for the BLAST searches for taxonomic assignment. Of these reads, 830,788 reads were identified as those species contained in the Kuroshio tank (**Table 1**). We detected 60 tank species from 830,788 reads and the average numbers of detected species for the 10 PCR replicates from the eight eDNA samples ranged from 29 for 1 L (glass-fiber filter) to 55 for 4 L (filter cartridge) (**Table 1**). Read numbers of the two blanks from the eight eDNA samples were minor, ranging from 0 to 12 for the tank species.

We found that the number of detected species by the filter cartridge was significantly higher than those of the glass-fiber filters (ANCOVA, F = 381.8, P <0.001; **Figure 1**) across 1-4 L seawater volumes. Number of detected species by the filter cartridge were approximately 1.5 times higher than those using the glass-fiber filters and in both methods, the number of detected species increased with the volume of water filtered (ANCOVA, F = 164.2, P <0.001).

Higher numbers of detected species by the filter cartridge may result from one or more of the following steps in the experimental workflows: filtration of the sampled water through (1) different materials (hydrophilic PVDF [polyvinylidine difluoride] *vs.* glass microfiber) and/or (2) different nominal pore sizes (0.45 µm vs. 0.70 µm) in the filter cartridges and glass-fiber filters could retain more eDNA from fishes on the former filter; (3) use of a rotary shaker in the preheated incubator produces more DNA yield from the filter cartridges than from the folded glass-fiber filters in a spin column placed on a motionless heat block; (4) collection of the extracted DNA by centrifugation from the filter cartridge is more efficient than that from the glass-fiber filter owing to different filter materials and/or different centrifugation methods. Apparently, a more rigorously designed study is required to pinpoint the factors responsible for the consistently higher number of detected species by the filter cartridge in the present study.

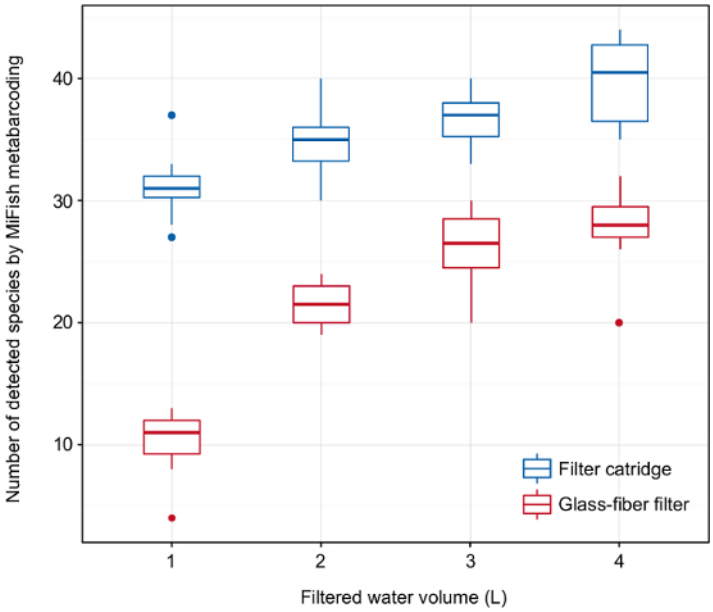

Figure 1: The Number of Detected Species Plotted against Filtered Water Volumes in MiFish Metabarcoding Analysis of eDNA from the Kuroshio Tank, Okinawa Churaumi Aquarium. The number of detected species is restricted to the species contained in the tank**.** From 30 L of seawater, we filtered 1, 2, 3, and 4 L samples using the filter cartridges and glass-fiber filters and extracted eDNA from those filters using the present and previously described protocols[12], respectively. We performed MiFish metabarcoding analysis (simultaneous detection of multiple species) for 10 PCR replicates from the four water volumes using two protocols. The horizontal bar in the box represents the median; the upper and lower edges of box correspond to inter-quartiles, the vertical bars correspond to 1.5 x quartiles, and the dots represent outliers. Please click here to view a larger version of this figure.

Family	Species	Number of reads	Filter cartridge				Glass-fiber filter			
			1L	2L	3L	4L	1L	2L	3L	4L
		830,788	91,912 (47)	93,185 (50)	225,823 (53)	170,565 (55)	7,657 (29)	48,632 (41)	79,238 (45)	113,776 (48)
Rhincoodonti	*Nebrius ferrugineus*	273	16 (3)	60 (7)	81 (8)	75 (8)	0 (0)	19 (2)	0 (0)	22 (2)
	Rhincodon typus	1,548	201 (10)	160 (10)	320 (10)	394 (10)	27 (5)	123 (9)	135 (9)	188 (10)
	Stegostoma fasciatum	359	41 (6)	42 (6)	86 (8)	107 (9)	8 (1)	13 (3)	35 (6)	27 (4)
Carcharhinida	*Carcharhinus leucas*	151	0 (0)	0 (0)	22 (3)	62 (6)	0 (0)	19 (2)	34 (5)	14 (2)
	Carcharhinus plumbeus	159	0 (0)	24 (3)	32 (5)	53 (4)	6 (1)	0 (0)	36 (3)	8 (1)
	Carcharhinus sorrah	8	0 (0)	0 (0)	8 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Carcharodon carcharias	24	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	24 (3)
	Galeocerdo cuvier	1,753	239 (10)	178 (10)	320 (10)	431 (10)	48 (7)	135 (9)	182 (10)	220 (10)
	Negaprion acutidens	109	20 (3)	0 (0)	6 (1)	25 (3)	0 (0)	0 (0)	38 (3)	20 (3)
Sphyrnidae	*Sphyrna lewini*	107	14 (2)	0 (0)	4 (1)	55 (6)	0 (0)	12 (1)	6 (1)	16 (2)
Rhinobatidae	*Rhina ancylostoma*	172	12 (2)	20 (3)	27 (5)	77 (7)	4 (1)	4 (1)	15 (2)	13 (2)
	Rhynchobatus djiddensis	1,349	177 (10)	136 (9)	275 (10)	290 (10)	40 (5)	120 (9)	140 (10)	171 (10)
Dasyatidae	*Dasyatis* sp.	1,303	164 (10)	149 (10)	201 (10)	391 (10)	8 (2)	133 (10)	114 (9)	143 (9)
	Dasyatis ushiei	489	141 (8)	69 (7)	111 (9)	86 (8)	0 (0)	25 (3)	26 (3)	31 (4)
	Himantura uarnak	1,476	192 (10)	186 (10)	263 (10)	351 (10)	18 (3)	162 (9)	125 (10)	179 (10)
	Urogymnus asperrimus	215	47 (6)	12 (2)	64 (8)	50 (6)	0 (0)	22 (3)	20 (3)	0 (0)
Myliobatidae	*Aetobatus narinari*	1,539	193 (10)	160 (10)	249 (10)	403 (10)	57 (5)	127 (10)	161 (10)	189 (10)
	Manta birostris	1,366	164 (10)	162 (10)	228 (10)	407 (10)	20 (3)	107 (9)	138 (9)	140 (10)
	Mobula tarapacana	18	0 (0)	0 (0)	6 (1)	0 (0)	0 (0)	0 (0)	0 (0)	12 (1)
	Rhinoptera javanica	1,535	165 (10)	173 (10)	403 (10)	473 (10)	4 (1)	147 (10)	170 (10)	0 (9)
Elopidae	*Elops hawaiensis*	1,243	261 (5)	146 (6)	203 (7)	100 (3)	23 (1)	197 (3)	115 (3)	198 (4)
Megalopidae	*Megalops cyprinoides*	393	0 (0)	89 (2)	21 (1)	162 (3)	0 (0)	121 (1)	0 (0)	0 (0)
Chanidae	*Chanos chanos*	307	35 (1)	244 (9)	0 (0)	20 (1)	0 (0)	0 (0)	0 (0)	8 (1)
Serranidae	*Epinephelus bruneus*	84	0 (0)	25 (1)	0 (0)	17 (1)	0 (0)	0 (0)	42 (1)	0 (0)
	Epinephelus lanceolatus	4,913	1,376 (8)	685 (10)	1,462 (10)	702 (10)	93 (3)	200 (3)	120 (3)	275 (4)
	Epinephelus tukula	54	0 (0)	0 (0)	9 (1)	45 (2)	0 (0)	0 (0)	0 (0)	0 (0)
Coryphaenida	*Coryphaena hippurus*	2,128	146 (8)	116 (5)	297 (10)	528 (10)	23 (2)	198 (8)	102 (6)	718 (10)
Rachycentrid	*Rachycentron canadum*	893	65 (4)	155 (10)	111 (7)	460 (10)	0 (0)	16 (2)	43 (5)	43 (4)
Echeneidae	*Echeneis naucrates*	2,437	71 (2)	912 (10)	445 (6)	503 (10)	0 (0)	367 (4)	139 (3)	0 (0)
Carangidae	*Carangoides hedlandensis*	414	118 (2)	0 (0)	35 (1)	186 (6)	0 (0)	0 (0)	75 (1)	0 (0)
	Carangoides orthogrammus	60	0 (0)	14 (1)	0 (0)	46 (2)	0 (0)	0 (0)	0 (0)	0 (0)
	Caranx ignobilis	10,291	1,708 (10)	1,250 (10)	2,167 (10)	1,895 (10)	196 (4)	932 (8)	1,246 (9)	897 (10)
	Caranx melampygus	27,702	2,547 (10)	3,011 (10)	8,490 (10)	5,833 (10)	59 (3)	1,961 (9)	1,437 (9)	4,364 (10)
	Caranx papuensis	452	108 (2)	39 (1)	47 (1)	95 (2)	0 (0)	68 (1)	95 (2)	0 (0)
	Caranx sexfasciatus	11,761	1,721 (10)	1,243 (10)	1,830 (10)	2,007 (10)	112 (4)	977 (7)	1,899 (9)	1,972 (10)
	Decapterus macrosoma	1,513	103 (5)	352 (10)	389 (8)	406 (10)	0 (0)	90 (1)	147 (3)	26 (1)
	Elagatis bipinnulata	27,105	3,012 (10)	3,120 (10)	4,597 (10)	5,919 (10)	303 (7)	2,766 (10)	2,609 (10)	4,779 (10)
	Gnathanodon speciosus	3,076	330 (5)	215 (6)	472 (8)	958 (10)	63 (2)	233 (4)	294 (6)	511 (5)
	Scomberoides lysan	61	0 (0)	26 (2)	14 (1)	11 (1)	0 (0)	0 (0)	10 (1)	0 (0)
	Selar crumenophthalmus	1,260	353 (9)	648 (10)	145 (6)	25 (2)	0 (0)	0 (0)	0 (0)	89 (1)
	Selaroides leptolepis	616	62 (3)	31 (2)	136 (7)	197 (5)	0 (0)	0 (0)	0 (0)	190 (5)
	Trachinotus blochii	5,360	748 (8)	1,304 (10)	929 (8)	1,450 (10)	20 (1)	442 (2)	82 (1)	385 (4)
	Uraspis helvola	666	63 (1)	136 (4)	135 (3)	0 (0)	0 (0)	66 (1)	113 (3)	153 (3)
Caesionidae	*Caesio caerulaurea*	2,810	350 (5)	364 (5)	649 (8)	328 (4)	69 (1)	75 (1)	524 (5)	451 (3)
	Caesio cuning	449	0 (0)	92 (2)	146 (2)	122 (4)	0 (0)	0 (0)	0 (0)	89 (1)
	Caesio teres	9,431	931 (9)	903 (9)	1,947 (9)	2,047 (10)	4 (1)	705 (5)	1,018 (7)	1,876 (9)
	Pterocaesio chrysozona	3,961	378 (4)	349 (4)	863 (9)	676 (8)	56 (1)	119 (1)	81 (2)	1,439 (8)
	Pterocaesio tile	14,249	2,243 (9)	1,428 (10)	3,504 (10)	2,841 (10)	57 (2)	1,245 (7)	1,209 (8)	1,722 (9)
Lethrinidae	*Lethrinus nebulosus*	2,234	547 (9)	260 (8)	722 (10)	367 (8)	0 (0)	0 (0)	216 (4)	122 (3)
	Lethrinus olivaceus	93	0 (0)	0 (0)	0 (0)	66 (2)	0 (0)	0 (0)	0 (0)	27 (1)
	Lethrinus rubrioperculatus	42	18 (1)	24 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Acanthuridae	*Naso hexacanthus*	32	0 (0)	0 (0)	16 (1)	16 (1)	0 (0)	0 (0)	0 (0)	0 (0)
Scombridae	*Auxis thazard*	1,612	102 (9)	135 (9)	704 (10)	390 (10)	0 (0)	42 (5)	91 (9)	148 (8)
	Euthynnus affinis	12,144	1,460 (10)	1,147 (10)	6,953 (10)	1,026 (10)	122 (2)	52 (2)	556 (6)	828 (8)
	Gymnosarda unicolor	999	100 (1)	123 (4)	187 (4)	288 (7)	0 (0)	125 (1)	0 (0)	176 (2)
	Katsuwonus pelamis	116,071	4,394 (10)	4,777 (10)	84,066 (10)	7,223 (10)	1,089 (10)	1,845 (10)	4,978 (10)	7,699 (10)
	Rastrelliger kanagurta	440,575	56,888 (10)	58,219 (10)	85,612 (10)	87,270 (10)	2,920 (10)	27,684 (10)	49,930 (10)	72,052 (10)
	Sarda orientalis	66	7 (2)	7 (2)	0 (0)	47 (9)	0 (0)	0 (0)	3 (1)	2 (1)
	Thunnus albacares	84,387	6,688 (10)	7,029 (10)	10,169 (10)	37,775 (10)	1,592 (10)	5,290 (10)	7,609 (10)	8,235 (10)
	Thunnus orientalis	24,891	3,193 (10)	3,036 (10)	5,645 (10)	4,788 (10)	616 (5)	1,648 (9)	3,080 (10)	2,885 (9)

Table 1: Taxonomic Composition and Read Numbers of the 60 Species Detected in MiSeq Analyses of eDNA Samples from the Kuroshio Tank. Only those species contained in the tank with reference sequences in the custom database are shown. Please click here to view a larger version of this table.

Discussion

In many metabarcoding studies using environmental samples such as water and soil, post-filtration treatment of the filter cartridge is generally as follows[24,25]: 1) cutting open or cracking the housing with hand tools (tubing cutter or pliers); 2) removal of the filter from the cartridge; and 3) cutting the filter into small pieces with a razor blade for DNA extraction. To avoid such cumbersome and time-consuming procedures that are prone to contamination in the laboratory, we have attempted several DNA extraction methods within the housing of the filter cartridge using a widely-used, inexpensive commercial kit, and successfully developed the present protocol with favorable results from eDNA metabarcoding analysis (**Figure 1**).

One of the more critical steps in the present protocol is the use of a rotary shaker in a preheated incubator, that is capable of providing good DNA yield for subsequent library preparation in the eDNA metabarcoding analysis. Constant stirring of the proteinase-K solution within the housing at optimal temperature probably facilitates DNA extraction from particles trapped on the filter. Note, however, that this protocol involves the use of both a rotary shaker and an incubator, and the latter must be able to accommodate the former. In addition, a centrifuge that can accommodate both 15 ml and 50 ml conical tubes is indispensable for removing the remaining liquid from the post-filtration filter and collecting extracted DNA within the cartridge, respectively.

There is another option to achieve such DNA extraction without having to cut open the cartridge (see Table of Materials/Reagents). The kit does not use any enzymes for DNA extraction; instead, it uses a special lysis buffer in combination with additional mechanical lysis with bead beating. In preliminary experiments based on natural seawater from the temperate coastal Pacific, we attempted DNA extractions using this kit along with a prototype of the present protocol using the commercial kit. With the use of the latter prototype protocol, we consistently recognized distinct amplification products in gel electrophoresis for the 1st-round PCR. In contrast, we failed to obtain any PCR products through the use of a different kit (see **Materials/Reagents Table**) despite following two alternative protocols provided by the manufacturer. Considering that PCR inhibitor removal steps are included in the two protocols of the second kit (see **Materials/Reagents Table**), this observation suggests a lack of adequate amounts of DNA in the template. Such relatively low DNA yields from environmental samples using the second kit are reported in recent studies[26,27].

In the present protocol, we have chosen the filter cartridge with large nominal pore size (0.45 µm), while aquatic microbial studies conventionally use the smaller one (0.22 µm) after prefiltration through larger pore-size filters (1 to 3 µm)[28]. The reasons of our choice are simple and straightforward, and are not based on theoretical arguments: 1) the pore size is much closer to that of the glass-fiber filters (0.70 µm) we have conventionally used in previous eDNA studies[12,14,22,23]; 2) it is expected to allow larger volumes of water to be filtered than the smaller one. The latter feature is particularly significant for our current research projects undergoing in the open-ocean and deep-sea ecosystems with scarce fish abundance and biomass. In fact, we confirmed successful filtration of an independently sampled 10-L of seawater from the Kuroshio tank with no noticeable clogging (results not shown). Recently, however, Turner *et al.*[29] demonstrated that 0.2 µm filtration maximized carp eDNA capture (85% ± 6%) while minimizing total (*i.e.* non-target) eDNA capture (48% ± 3%), but filter clogging limited this pore size to a sample volume of less than 250 ml. Additional studies are needed to determine the optimal filter pore size and water filtration volume of the filter cartridge for eDNA metabarcoding in various types of aquatic environments with varying levels of fish abundance and biomass.

One of the limitations of this protocol is that the water filtration should be performed in the laboratory where a power supply is available. Because of limited chemical stability of DNA, eDNA begins to degrade as soon as it is shed from an organism and is detectable only for a short period of time in aquatic environments (hours to days)[30]. Therefore it is ideal to filter the water sample immediately after collection to avoid significant degradation of eDNA. In this regard, developments of the on-site filtration methods using the filter cartridge would be a promising next step. The portability and sterility of the filter cartridge enable development of the on-site filtration methods, which will provide more intact eDNA that can reflect species composition of the fish community more accurately in eDNA metabarcoding. The collected eDNA on the filter cartridges may be stabilized by the injection of a commercial reagent[31] (see **Materials/Reagents Table**) or Longmire buffer[32] into the cartridge before bringing it back to the laboratory.

Disclosures

The authors have nothing to disclose.

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