Supplementary text

Species-specific primers and probes for Japanese anchovy

We designed species-specific primers and probes for Japanese anchovy. The sequences of the mitochondrial cytochrome *b* (CytB) gene of Japanese anchovy, and Japanese sardine (*Sardinops melanostictus*) and round herring (*Etrumeus teres*), the most closely related fish species inhabiting the Japan Sea near Japan, were collected from GenBank. Candidate primers and probes were identified using Primer Express 3.0 software (Thermo Fisher Scientific, Waltham, MA), and primers with substitutions between the target and related species within five bases from the 3′ end were identified. Finally, primer sets with more than three substitutions (total for both) within five bases from the 3′ ends were selected for each species because base-pair mismatches at the 3′ end are important for primer specificity [1] .

To confirm the specificity of the primers *in silico*, we performed primer-Blast to check the specificity of the designed primers. In addition, we performed in vitro testing. The DNA of dotted gizzard shad (*Konosirus punctatus*), the most closely related syntopic fish species in Maizuru Bay was tested. Tissue sample of the fish was obtained from the fish collection of Kyoto University (FAKU). Total DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the protocol for tissue samples, and 10 pg was applied as template. Real-time PCR (55 cycles) was performed as described in the main text.

For further confirmation of primer specificity, amplicons obtained by real-time PCR of field samples were commercially sequenced (TakaraBio, Otsu, Japan) after purification with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). Twenty-two amplicons were sequenced for each target species.

The *in-silico* specificity test showed that no syntopic fish species in the Maizuru Bay was listed by primer-Blast. Primer specificity was further assessed by real-time PCR using DNA of the related species as template. Real-time PCR with 10 pg of total DNA of the most closely related syntopic species showed no amplification in any of three replicates. To reduce the risk of false positives, amplicons from the field experiments were sequenced. The 22 amplicons were directly sequenced, and all of which matched the known sequences of the target species. Thus, the primers designed in this study have sufficient specificity to detect the target species in our survey area.

Results of sequence read processing by MiFish pipeline
The numbers of sequence reads remaining in data processing steps are shown in Table S1.

The sequence reads of standard DNA varied among eDNA samples, although the same amount (i.e., copy number) of the standard DNA was added, suggesting that influences (or concentrations) of PCR inhibitors vary among eDNA samples. The numbers of sequence reads from negative controls, excluding standard DNAs, were negligible (ranging from 0 to 130), suggesting that cross-contaminations during water sampling, filtration, DNA extraction, and library preparation were minimal in the study. Only one sample (sampling date = 2015/6/16) showed strong PCR inhibition (i.e., no sequences from standard DNA were obtained; see column "Standard DNA reads"), but for the rest of the eDNA samples, the eDNA metabarcoding successfully detected the internal standard DNAs.

A list of calculated copy numbers by qMiSeq

The sequence reads obtained by MiSeq sequencing were converted to the number of eDNA copies using correction equations, and the calculated MiSeq copies (copies μl^{-1}) for each fish species are listed in Table S2. As a result of MiSeq sequencing, 73 marine fish species were detected. Although several non-fish species (*Homo sapiens* [human], *Bos taurus* [ox], and *Trachemys scripta* [turtle]) and freshwater fish species (e.g., *Carrasius auratus*) were detected, those species were excluded from the list. In total, 3 non-fish species and 16 freshwater fish species were excluded (Original sequence read table is available in a data folder in GitHub [https://github.com/ong8181/eDNA-qmiseq], or it can be reproduced by processing original sequence data in DRA by MiFish pipeline [http://mitofish.aori.u-tokyo.ac.jp/mifish]). The detection of these species is not likely to be the result of false-positive detection; rather, we suggest that these eDNAs were indeed present in the collected sea surface water. First, the detected non-fish species were indeed common as a source of eDNA, and thus, the eDNAs might easily have been contained in the sea surface water samples. Second, although the sampling region is a marine ecosystem (shore region), a river is located close to the sampling area. Therefore, eDNAs of several freshwater (or brackish water) fish species might have originated from water flow from the river. Taking the results all together, we suggest that the non-fish and freshwater fish eDNAs were indeed present in the samples, but were not ecologically related to the marine ecosystem. Therefore, we excluded those species from the list to avoid confusion when we interpreted the time series.

Ecological interpretations of multispecies fish eDNA time series

The eDNA time series measured here by qMiSeq were ecologically interpretable, suggesting

that eDNA monitoring using our method would provide ecologically meaningful information on the dynamics of the natural fish community, at least in our case. For example, twice-a-month visual census detects generally high abundance and species richness in the summer, except for the highly abundant *Engraulis japonicus* in autumn [2] (Table S4); this corresponds well to the general trend of eDNA detection seen here. In addition, some species, such as Fugu species (*Takifugu* sp.1 [either species of *T. niphobles*/*T. snyderi*] and *Takifugu* sp.2 [either species of *T. pardadalis*/*T. xanthopterus*/*T. poecilonotus*]) and *Acanthopagrus schlegelii* were detected the whole year round by eDNA analysis, which is consistent with the detection in the visual census (Table S4). *Dictyosoma burgeri* was found only in winter in the visual census, and its eDNA was also most abundant in winter.

References cited in the supplementary text

1. Wilcox, T. M., McKelvey, K. S., Young, M. K., Jane, S. F., Lowe, W. H., Whiteley, A. R. & Schwartz, M. K. 2013 Robust detection of rare species using environmental DNA: the importance of primer specificity. *PLoS One* **8**, e59520.

2. Masuda, R. 2008 Seasonal and interannual variation of subtidal fish assemblages in Wakasa Bay with reference to the warming trend in the Sea of Japan. *Environ. Biol. Fishes* **82**, 387–399.