Determination of a target image of freshwater fish composition in a mitigation project using eDNA metabarcoding methods

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Keywords: eDNA, Freshwater fishes, NMDS, Japan, intraspecific lineages, Shigenobu River system

Abstract

Short-term target species for mitigation should be realistically and effectively selected for restoration within a few years. Current short-term target species selection is based on spatial distribution data of past and present biota in the neighborhood based on capture surveys. However, current methods have the following problems: If historical spatial distribution data are used, the risk of including large areas, where target species cannot be dispersed, exists, as

well as the risk of including extinct species. If recent spatial distribution data are used, the survey area may be narrowed due to time constraints, thereby increasing the possibility of missing species that should be selected as the target species. We examined the possibility of using the environmental DNA (eDNA) metabarcoding method, which can obtain spatial distribution data quickly, sensitively, and extensively, as a new short-term target species selection method. The study site was a restoration mitigation area (Kaihotsu–Kasumi) in the Shigenobu River system, Ehime Prefecture, Japan, where freshwater fish target species were selected based on capture surveys and past literature information. Furthermore, eDNA surveys were conducted at 17 sites in the Kaihotsu–Kasumi and its inflow and outflow rivers in 2020, and metabarcoding analysis using MiFish primers was conducted to determine the current freshwater fish fauna at each site. The visualization of the fish community composition obtained from the eDNA survey using NMDS showed that the Kaihotsu–Kasumi and surrounding watersheds were significantly divided into three clusters: upper reaches, middle and lower reaches, and estuarine reaches. Kaihotsu–Kasumi was included in Cluster 2, comprising the middle and lower reaches of the inflow and outflow rivers. Twenty-six species (including five non-native species) were detected in Cluster 2. Twenty-one fish species, excluding non-native species, were considered suitable as short-term target species as taxa with high potential for dispersal into Kaihotsu–Kasumi. The selection of short-term target species using the eDNA metabarcoding method covered all short- and medium-term target species (14 species) selected based on the capture surveys, and seven additional species were also proposed. The study also showed that intraspecific lineages of *Misgurnus anguillicaudatus* (Clades A and B), which were missed by the capture surveys, can be identified. These results indicate that the eDNA metabarcoding method can provide more comprehensive and realistic short-term target species selection than capture surveys, providing higher resolution monitoring through intraspecific lineage detection.

Introduction

The selection of restorable organisms—hereafter referred to as “target species”—is imperative for biodiversity restoration (Morimoto and Kameyama, 2001). In restoration mitigation in rivers, aquatic organisms naturally disperse from connecting waters. However, it is difficult to objectively and realistically select which species inhabiting which range should be the target species. An approach that relies on past to present spatial distribution data of organisms in neighboring areas based on capture surveys has the following problems. If historical spatial distribution data are used, the risk of including large areas where target species cannot be dispersed exists, as well as the risk of including extinct species. If recent spatial distribution data are used, the survey area may be narrowed due to time constraints, thereby increasing the possibility of missing species that should be selected as the target species. In addition, there may be no available spatial distribution data for nearby areas. The selection of target species often involves the establishment of species that can be restored in a short time (hereafter referred to as "short-term target species") and in a long time (hereafter referred to as "long-term target species"). Especially for short-term target species that are intended to be restored in a short time, selection should be more realistic, avoiding the selection of organisms that may not be distributed.

eDNA methods can obtain information on the spatial distribution of species more sensitively and extensively at a lower cost than capture surveys (Jerde et al., 2013; Sigsgaard et al., 2015; Sakata et al., 2017; Yamamoto et al., 2017; Oka et al., 2021). Many studies have shown the usefulness of eDNA methods for determining the spatial distribution and diversity of fish species (e.g., Yamamoto et al., 2016, 2017; Nakagawa et al., 2018; Doi et al., 2020; Oka et al., 2021) and that eDNA methods can be used to monitor rare species with low densities and to provide greater sensitivity in monitoring after removing artificial instream barriers (e.g., Sakata et al., 2017; Duda et al., 2021; Muha et al., 2021). In particular, the eDNA metabarcoding method can simultaneously detect many fish species at each location

(e.g., Yamamoto et al., 2017; Nakagawa et al., 2018). Because the eDNA metabarcoding method can easily obtain spatial distribution data for current fish species, it can provide objective and realistic targets for selecting target species after restoration mitigation.

Freshwater fishes are a major target species for restoration mitigation in rivers, but the presence of cryptic non-native populations that cannot be distinguished by morphology is a problem for biodiversity conservation (Mabuchi et al., 2008; Watanabe et al., 2017). eDNA is a promising tool for detecting cryptic non-native populations (Uchii et al., 2016). MiFish primers, commonly used in eDNA metabarcoding methods (Miya et al., 2015), can detect many fishes at the species level, but the resolution of intraspecific lineages is not well discussed. The ability to distinguish between native and non-native lineages using eDNA metabarcoding methods would be useful in target species selection. Three lineages of *Misgurnus anguillicaudatus* in the Japanese archipelago, which have been difficult to morphologically identify, are now identified: two native lineages (Clades A and C sensu Koizumi et al., 2009) and a non-native lineage from China (Clade B sensu Koizumi et al., 2009). The native *M. anguillicaudatus* is categorized as *near threatened* by the Ministry of the Environment Government of Japan (2020). Native and non-native lineages of *M. anguillicaudatus* can be distinguished in the cytochrome *b* and control regions of mitochondrial DNA (Morishima et al., 2008; Koizumi et al., 2009). Therefore, MiFish for the 12S rRNA region of mitochondrial DNA may also distinguish them. In this paper, we examined whether eDNA metabarcoding methods using MiFish primers can distinguish intraspecific lineages of *M. anguillicaudatus*.

The Shigenobu River flows 36 km into the Seto Inland Sea, and wetlands with springs as their source of water exist along the banks of the middle reaches of the river. In the middle reaches of the Shigenobu River, including wetlands, good habitats have decreased or deteriorated due to urbanization and levee construction in recent years (Ministry of Land, Infrastructure, Transport and Tourism. Shikoku Regional Development Bureau, 2008).

Therefore, restoration mitigation was conducted by the Ministry of Land, Infrastructure, Transport and Tourism from 2014 to 2019 for Kaihotsu–Kasumi, one of the wetlands located in the middle reaches of the Shigenobu River, to restore the spring area and improve its connectivity with the mainstream. On the Shigenobu River, there were records of capture surveys conducted every five years from 1994 to 2021 (Ministry of Land, Infrastructure, Transport and Tourism, http://www.nilim.go.jp/lab/fbg/ksnkankyo/index.html, accepted 1 April 2022), as well as information on the distribution of endangered species (Ehime Prefecture, Japan, 2014). Based on the above data on distribution, advice from experts, and discussions with local residents, short-, medium-, and long-term target species for restoration mitigation of Kaihotsu–Kasumi were selected. In addition, monitoring was conducted in 2020 using capture and eDNA metabarcoding surveys. Kaihotsu–Kasumi of the Shigenobu River is a suitable field for testing whether the application of eDNA methods provides additional benefits for target species selection by restoration mitigation. In this study, we examined the usefulness of eDNA metabarcoding methods as a short-term target species selection using eDNA metabarcoding monitoring data conducted in Kaihotsu–Kasumi in 2020.

Materials and methods

Water sampling

Water samples were collected from Kaihotsu–Kasumi (5 sites: K1–5), the outflow river Shigenobu River (9 sites: S1–9), and the rivers (Uchi and Sagawa rivers) flowing into the Kaihotsu–Kasumi (3 sites: U1–3) from October 26 to October 27, 2020 (Fig. 1; Table S1). The basic water sampling protocol followed Minamoto et al. (2021), with some modifications. Disposable plastic cups were used to collect water samples, and 500 ml of surface water was collected per site. To prevent contamination, plastic cups were replaced with new plastic cups at each water sampling site. After water sampling, 500 μl of 10% benzalkonium chloride (BAC) was added immediately to inhibit DNA degradation (Yamanaka et al., 2017). Each

water sample was filtered in the field by loading a syringe with a 0.45 μm pore size Sterivex filter. At the end of the filtration, we left the sample water containing BAC and plugged it. As a field negative control (FNC), 500 ml of ultrapure water was filtered using the same method as above after water sampling from three areas. After filtration, the samples were transported to the laboratory at room temperature, and the Sterivex was stored in a –20°C freezer on October 28, 2020.

DNA extraction and amplification

DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), basically following Miya et al. (2016), with some modifications following Hirohara et al. (2021).

PCR was performed according to Miya et al. (2015), and the partial 12S rRNA region of mitochondrial DNA was amplified by the first and second PCRs using the MiFish-6N-U-F/R primer pair. For the 1st PCR, in a 12 ul reaction volume containing 0.24 μl of KOD Fx Neo (Toyobo, Osaka, Japan), 6 μl of 2 × Buffer for KOD Fx Neo, 2.4 μl of 2 mM dNTPs, 0.7 μl each of 5 μM MiFish-6N-U-F/R, and 2 μl of 10 × diluted template DNA were mixed. The thermal cycle profile was 94°C for 2 min; 40 cycles of 98°C for 10 s, 65°C for 30 s, and 68°C for 30 s; and 68°C for 5 min. Eight PCR replications were used for each sample and a PCR non-template control (PCR–NTC) using ultrapure water. The PCR amplicons were treated with ExoSAP-IT (ThermoFisher Scientific, Waltham, USA). The PCR amplicons from each of the eight replications were aggregated by 5 μl to a total of 40 μl, and 3.6 μl of ultrapure water and 0.4 μl of ExoSAP-IT were added per sample and treated at 37°C for 15 min and 80°C for 15 min. The 2nd PCR was performed to add MiSeq adaptor sequences and eight bp index sequences to both amplicon ends. The total reaction volume of the 2nd PCR was 12 μl containing 0.24 μl of KOD Fx Neo, 6 μl of 2 × Buffer for KOD Fx Neo, 2.4 μl of 2 mM dNTP, 0.7 μl each of 5 μM MiFish-6N-U-F/R, 1 μl of ultrapure water, and 1 μl of the 1st PCR

amplicons. The thermal cycle profile was 95°C for 3 min; 10 cycles of 98°C for 20 s, 65°C for 15 s, and 72°C for 15 s; and 72°C for 5 min. A 2% agarose gel electrophoresis was performed to confirm the amplification of the 2nd PCR amplicons.

Amplicon library and Miseq sequencing

The samples were aggregated in volumes ranging from 2 or 3 μl per sample, depending on the results of the electrophoresis (Table S2). AMPure XP (Beckman Coulter, Brea CA, USA) was added to 0.8 μl per 1 μl of each sample and treated according to the manual. DNA was eluted with 60 μl of TE buffer. Then, 25 μl of the treated DNA solution was added to a 2% E-Gel Size Select (Thermo Fisher Scientific, Waltham, USA) to retrieve the target band (ca. 370 bp). The collected DNA concentration was measured using a Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Waltham, USA). Finally, a bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used to confirm that PCR amplicons of the desired size were obtained.

The libraries were sequenced using an Illumina MiSeq with a reagent kit v2 nano for 2 × 150 bp PE (Illumina, San Diego, CA, USA). Raw reads were demultiplexed into each sample by a preinstalled program in MiSeq, and the outputted fastq files were processed by an in-house bioinformatic pipeline that uses USEARCH v11.0.667 (Edgar 2010) as its core program as follows. 1) Pair-end reads were merged using the *fastq_mergepairs* function. 2) Primer sequences were removed from both ends of the reads using *stripleft* and *stripright* functions. 3) Short reads less than 100 bp and those with a Q-Score less than 30 were removed using *fastq_minlen* and *fastq_truncqual* functions, respectively. 4) Identical reads were dereplicated using the *fastx_uniques* function and treated as operational taxonomic units (OTUs) with read numbers gathered up (i.e. size) appended to the OTU information. 5) Denoising was performed using the *unoise3* function, and the OTUs with size less than 4 were removed. Moreover, the OTUs that were judged as chimera were removed. 6)

Taxonomic identity was assigned to the OTUs as follows: The sequences of each OTU were used as queries against a local sequence database at the threshold level of 0.95 using the *usearch_global* function. The database was a compilation of mitochondrial 12S sequences of fish downloaded from the National Center for Biotechnology Information website (https://www.ncbi.nlm.nih.gov/) on January 15, 2020. The hit sequences retrieved from the database for each OTU were converted into phylogenic trees and visually checked to assign a taxonomic identity for the focal OTU. The granularity of the taxonomic assignment to the OTUs was mainly at the species level, but some OTUs were identified at the genus level when a sequence cluster, including the query sequence, included more than one species. Taxa that were unlikely to be distributed around the Shigenobu River (mainland of the Japanese archipelago) based on known geographic distribution information (Hosoya, 2019) were excluded (e.g., *Acanthopagrus sivicolus* and *Luciogobius_ryukyuensis*, *Tridentiger kuroiwae*).

Distinguishing between intraspecific lineages

For *M. anguillicaudatus*, we tried to distinguish between intraspecific lineages using the following methods. First, a 124 bp 12S rRNA (MiFish) tree was constructed using the sequence data output as *M. anguillicaudatus* OTU and *M. mizolepis* OTU. In addition, we used *Lefua echigonia*, the related species, as an outgroup (LC069488). Next, we constructed a 1015 bp cyt *b* tree, referring to Koizumi et al. (2009), in which molecular phylogenetic analyses of *M. anguillicaudatus* in Japan were conducted. For phylogenetic analysis, nucleotide sequence data of *M. anguillicaudatus* and *M. dabryanus* registered in DDBJ, EMBL, and GenBank by Koizumi et al. (2009) were used: Clade A (native lineage, AB473261, AB473312), Clade B (non-native lineage, AB473360, AB473346), Clade C (native lineage, AB473381, AB473354, AB473382), and *M. dabryanus* (AB473408, AY625701). The complete mitochondrial genome sequences included in the MiFish phylogenetic tree (AP011291, AP017654, HM856629, KC509900, KC823274, MF579257,

KF771003, KJ939363, KM186181, KM186183, MG938589, MG938590, NC027448) were also added to the cyt b phylogenetic tree. In addition, we used *L. echigonia* as an outgroup (LC079000, Ito et al., 2019). Multiple alignments of nucleotide sequences were performed using CLUSTAL W (Thompson et al., 1994). Phylogenetic analyses were conducted using the neighbor-joining (NJ) method. The NJ analyses were performed using MEGA X (Kumar et al., 2018), with the *p*-distance. The reliability of each internal branch was evaluated using a bootstrap test with 1000 replicates.

Community analysis

The differences in community composition were visualized using nonmetric multidimensional scaling (NMDS). The dissimilarity of the community in NMDS was calculated based on the Jaccard index using the vegdist function in the vegan package. The simprof function in the clustsig package was used to cluster the community, and PERMANOVA was conducted using the adonis function in the vegan package used to test whether the clusters obtained significantly differed from each other. All of the above statistics were conducted using R version 4.1.2 (R Core Team 2021).

Capture survey

Capture surveys in Kaihotsu–Kasumi were conducted on August 27 and 28, 2020, and on October 26 and 27, 2020 at the same five sites as the eDNA sampling sites. The capture survey was conducted using hand nets, net pots, and bottle traps, with permission from Ehime Prefecture. The capture survey on October 26 was conducted after the eDNA survey.

Results

Miseq sequencing

A total of 1,435,754 reads were obtained by Miseq run from 17 sampling sites, 3 of FNCs,

and 1 of PCR–NTC. After the data processing noted in the Material and Methods section, 314 OTUs were identified as fish species comprising 817,797 reads. Species assignment automatically conducted by Usearch was manually confirmed to check the independence of each OTU from the other. If the representative sequence of the focal OTU could not be identified at the species level, the OTU was identified at the genus level. Five reads of *Opsariichthys platypus* were detected from an FNC of the Shigenobu River; therefore, the equivalent read number was subtracted from the *O. platypus* reads detected from the other sites in the Shigenobu River. No read was detected from the PCR–NTC.

Fish species detected in the samples

Fish species from 38 taxa were identified in total. Common saltwater fishes for food use were excluded from the results: *Etrumeus teres*, *Trachurus japonicus*, *Pseudocaranx dentex*, *Seriola quinqueradiata*, *Pagrus major*, and *Pleurogrammus azonus*. However, peripheral freshwater fishes (*Plotosus japonicus*, *Mugil cephalus*, *Plectorhinchus cinctus*, *Acanthopagrus schlegelii*, *Omobranchus punctatus*, *Luciogobius pallidus*, *Luciogobius* spp., *Acanthogobius flavimanus*, *Tridentiger trigonocephalus*, *Favonigobius gymnauchen*, *Gymnogobius breunigii*, *Gymnogobius scrobiculatus*, *Takifugu* spp.) were included in the results. Fish species of 36, 10, and 19 taxa were identified from the Shigenobu River (nine sites), Uchi and Sagawa River (three sites), and Kaihotsu–Kasumi (five sites), respectively (Table S3).

MiFish and cyt *b* trees are shown in Fig. 2. In the MiFish tree, the sequences of *M. anguillicaudatus* OTU and *M. mizolepis* OTU differed. The *M. anguillicaudatus* OTU included three sequences, OTU 57, 259, and 283, and 1 mitogenome sequence (AP011291). The *M. mizolepis* OTU included three sequences, OTU 40, 123, and 190, and 12 mitogenome sequences. In the cyt *b* tree, three intraspecific lineages of *M. anguillicaudatus* (Clades A, B, and C), and *M. dabryanus* were distinguished with high bootstrap probability (100%), similar

to Koizumi et al. (2009) and Perdices et al. (2016). Clade C (native lineage) contained one mitogenome sequence (AP011291), and Clade B (non-native lineage) contained the other 12 mitogenome sequences. No mitogenome sequences were identified in *M. dabryanus* or *M. anguillicaudatus* Clade A.

Clustering based on community analysis

The community analysis results showed that the community was significantly differentiated into three clusters (Fig. 3; $P < 0.001$). Cluster 1 comprised three sites (S1–3) in the upper stream of the Shigenobu River (elevation 178.7–419.9 m), where five taxa were detected, including *Phoxinus oxycephalus jouyi*, *Oncorhynchus* spp, and *Rhinogobius flumineus*. Cluster 2 included five sites in the middle stream of the Shigenobu River (elevation 6.7–99.3 m), all sites in the Kaihotsu–Kasumi, and all sites in the Uchi and Sagawa River (K1–5, S4–8, U1−3), where 26 taxa were detected, including *Pseudorasbora parva*, *Gnathopogon elongatus*, *Oryzias latipes*, and *Odontobutis obscura*. Fourteen of these taxa were detected only in Cluster 2. Cluster 3 comprised only one site (S9) in the estuary of the Shigenobu River (elevation −0.8 m), where 21 taxa were detected, including *Plotosus japonicus*, *Mugil cephalus*, and *Acanthopagrus schlegelii*. Twelve of these taxa were detected only in Cluster 3 (Table 1).

For Cluster 2, 23 taxa were identified from the Shigenobu River, 10 taxa from the Uchi and Sagawa River, and 19 taxa from Kaihotsu–Kasumi. Of these, *Tanakia limbata* and *Takifugu* spp. were detected only in Kaihotsu–Kasumi. Seven taxa, including *P. parva* and *Cobitis shikokuensis*, were detected only in the Shigenobu, Uchi, and Sagawa rivers (Table 1).

Fish species detected by capture surveys

The capture surveys in 2020 identified 15 taxa in Kaihotsu–Kasumi (Table 2). *C. shikokuensis* and *Lepomis macrochirus* were identified only in the capture surveys.

Discussion

All of the 38 taxa in 16 families of fishes detected by eDNA in this study were recorded in previous capture surveys in the Shigenobu River (Ministry of Land, Infrastructure, Transport and Tourism, http://www.nilim.go.jp/lab/fbg/ksnkankyo/index.html, accepted 1 April 2022). Therefore, the eDNA survey results were determined to be reasonable. The six taxa that could not be distinguished by eDNA were compared with previous capture surveys (Ministry of Land, Infrastructure, Transport and Tourism, http://www.nilim.go.jp/lab/fbg/ksnkankyo/index.html, accepted 1 April 2022), and the following taxa were identified: *Carassius* spp. as *Carassius* sp. (Silver crucian carp) or *Carassius buergeri* (Giant golden crucian carp), *Oncorhynchus* spp. as *Oncorhynchus masou ishikawae*, *Luciogobius* spp. as *Luciogobius guttatus* or *Luciogobius martellii*, *Tridintiger* spp. as *Tridintiger obscurus* or *Tridintiger brevispinis*, *Rhinogobius* spp. as *Rhinogobius nagoyae*, *Rhinogobius fluviatilis*, *Rhinogobius* sp. OR or *Rhinogobius tyoni*, and *Takifugu* spp. as *Takifugu niphobles* or *Takifugu poecilonotus*.

In this paper, we attempted to identify intraspecific lineages in loach using the eDNA metabarcoding method with MiFish primers. Based on the results of the Cyt *b* phylogenetic tree, including the mitogenome sequence, *M. anguillicaudatus* OTU is the native lineage (Clade C) and *M. mizolepis* OTU is the non-native lineage (Clade B). *M. anguillicaudatus* Clade B has also been recorded from the Shigenobu River system as a non-native lineage by capture surveys (Shimizu and Takagi, 2010). No matching sequences were found between *M. anguillicaudatus* and *M. mizolepis* OTUs; thus, both OTUs are genetically distinct. However, *M. dabryanus* (including *Paramisgurnus dabryanus* and *M. mizolepis*) and *M. anguillicaudatus* sequences were mixed in the *M. mizolepis* OTU. Molecular phylogenetic studies of mtDNA indicate that the *M. dabryanus* is not contained within *M. anguillicaudatus* Clades A and B, and shows a phylogenetic position closely related to *M. anguillicaudatus*

Clade A and *M. nikolskyi* (Koizumi et al., 2009; Perdices et al., 2016). The mtDNA of some *M. dabryanus* populations is known to be contained within *M. anguillicaudatus* Clade B, but this *M. dabryanus* mtDNA sequence is likely to have originated from hybridization with *M. anguillicaudatus* (Okada et al., 2020). Intentional exclusion of sequences derived from hybridization is difficult. Therefore, a phylogenetic tree that includes mitogenomic sequences would help identify intraspecific lineages.

Native lineage (Clade C) and non-native lineage (Clade B) of *M. anguillicaudatus* were identified from Kaihotsu–Kasumi using eDNA metabarcoding methods. The successful identification of native and non-native lineages of *M. anguillicaudatus* using eDNA is one of the advantages of introducing eDNA in monitoring. Future studies on the feasibility of identifying intraspecific lineages for a larger number of fishes will contribute to improving the resolution of monitoring using the eDNA metabarcoding method.

Community analysis showed that the fish community of the Shigenobu River was significantly divided into three clusters (Clusters 1–3). Each cluster has an upstream (Cluster 1), middle and downstream (Cluster 2), and estuarine (Cluster 3) fish community, suggesting that changes in the fish community in response to the river environment were a factor in separating the clusters. The communities in Kaihotsu–Kasumi (K1–5) were included in Cluster 2, which showed a community structure in the middle and lower reaches of the river. Thus, the fish community at Kaihotsu–Kasumi represents the community structure of the middle and lower reaches of the Shigenobu River; 26 taxa of fish were detected at the Cluster 2 sites, 17 of which were common between Kaihotsu–Kasumi and neighboring waters (S4–8 and U1–3). This indicated a high degree of commonality between the fish communities in Kaihotsu–Kasumi and neighboring waters, probably because of the natural dispersal of fishes inhabiting Kaihotsu–Kasumi from neighboring waters. Kaihotsu–Kasumi is considered to provide a good fish habitat due to its high connectivity with neighboring waters.

Nineteen taxa were detected by eDNA in Kaihotsu–Kasumi, five of which were not

detected in the capture surveys conducted during the same period. Fish surveys using eDNA tend to detect more species than capture surveys (e.g., Oka et al., 2021), and this study showed similar results. *Plecoglossus altivelis altivelis*, *C. shikokuensis*, and *L. macrochirus* were detected only in the capture surveys. This suggests that a combination of capture surveys may be effective for a more accurate understanding of the fish community.

The community structure of Kaihotsu–Kasumi resembled that of Cluster 2, which showed a community structure in the middle and lower reaches of the river. Therefore, 21 taxa could be selected as short-term target species for Kaihotsu–Kasumi, excluding non-native species (*Cyprinus carpio*, *Tanakia limbata*, *M. anguillicaudatus* non-native lineage, *L. macrochirus*, *Micropterus salmoides*; Hosoya, 2019) out of the 26 taxa included in Cluster 2. The target species not identified in Kaihotsu–Kasumi were *Pseudorasbora parva*, *Oncorhynchus* spp., and *Plectorhinchus cinctus*. *P. parva* is categorized as Near Threatened by Ehime Prefecture, Japan (2014). The slow flow of Kaihotsu–Kasumi is considered a suitable habitat and breeding environment for *P. parva*. In the future, efforts should be made to facilitate the natural dispersal of *P. parva*. The main habitats for the remaining two species are the upper reaches of rivers for *O.* spp. and estuarine and coastal areas for *P. cinctus* (Hosoya, 2019). Therefore, it is rare for the above two species to be recorded in Kaihotsu–Kasumi in the middle reaches of the river.

In this paper, we attempted to select short-term target species after restoration mitigation using the eDNA metabarcoding method. As a result, 21 short-term target species were selected. For target species based on historical capture surveys in Kaihotsu–Kasumi, 9, 5, and 2 species were short-term (within 3 years), medium-term (within 20 years), and long-term (>20 years), respectively (Table S4). Of these, 14 of the short- and medium-term target species were among the short-term target species selected by eDNA. Seven new species were included in the short-term target species established by eDNA. Therefore, the selection of short-term target species using the eDNA metabarcoding method is more effective than

capture surveys in selecting fish species that are expected to recover in the short term in a more comprehensive manner, probably because eDNA is more sensitive to the current fish community than capture surveys. In addition, fish such as *O.* spp. and *P. cinctus*, which are not considered to inhabit the Kaihotsu–Kasumi habitat, were also selected as short-term target species by eDNA. Future studies of selection methods, including fish preference by habitat, will bring us closer to a more realistic selection of short-term target species.

Funding

This work was supported by Environment Research and Technology Development Fund (JPMEERF20204004) of the Environmental Restoration and Conservation Agency, Japan.

Credit author statement

Gen Ito: Data analysis, Writing – original draft, Writing – review and editing. Hiroshi Yamauchi: Planning and conducting field surveys, Writing – review and editing. Miwa Shigeyoshi, Kousuke Ashino and Chie Yonashiro: Planning and conducting field surveys. Maki Asami and Yuko Goto: Experiments in the laboratory. Hiroki Yamanaka: Planning and conducting field surveys, Data analysis, Writing – review and editing, Conceptualization, Funding acquisition.

Declaration of Competing Interest

The authors declare no competing financial interests.

Acknowledgements

We express sincere thanks to members of the Civil Engineering Division1, Matsuyama river National Highway Office, Shikoku regional development bureau for their help with the field surveys.

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Figure legends

Fig. 1 Water sampling sites in the Shigenobu River system. Each circle symbol indicates sampling sites. This altitude map was used with permission from the Geospatial Information Authority of Japan (https://maps.gsi.go.jp/).

Fig. 2 Neighbor-joining trees of 124 bp 12S rRNA (MiFish) gene (left) and 1015 bp cytochrome *b* gene (right) sequences of *M. anguillicaudatus*. The numbers at the nodes indicate bootstrap probabilities. The complete mitochondrial genome sequences are indicated by open boxes.

Fig. 3 NMDS plot of fish species community compositions at different eDNA sampling sites. The community was significantly differentiated into three clusters (PERMANOVA: $P < 0.001$). Green, red, and blue indicate Clusters 1, 2, and 3, respectively.

Table 1 Fish species in each cluster.

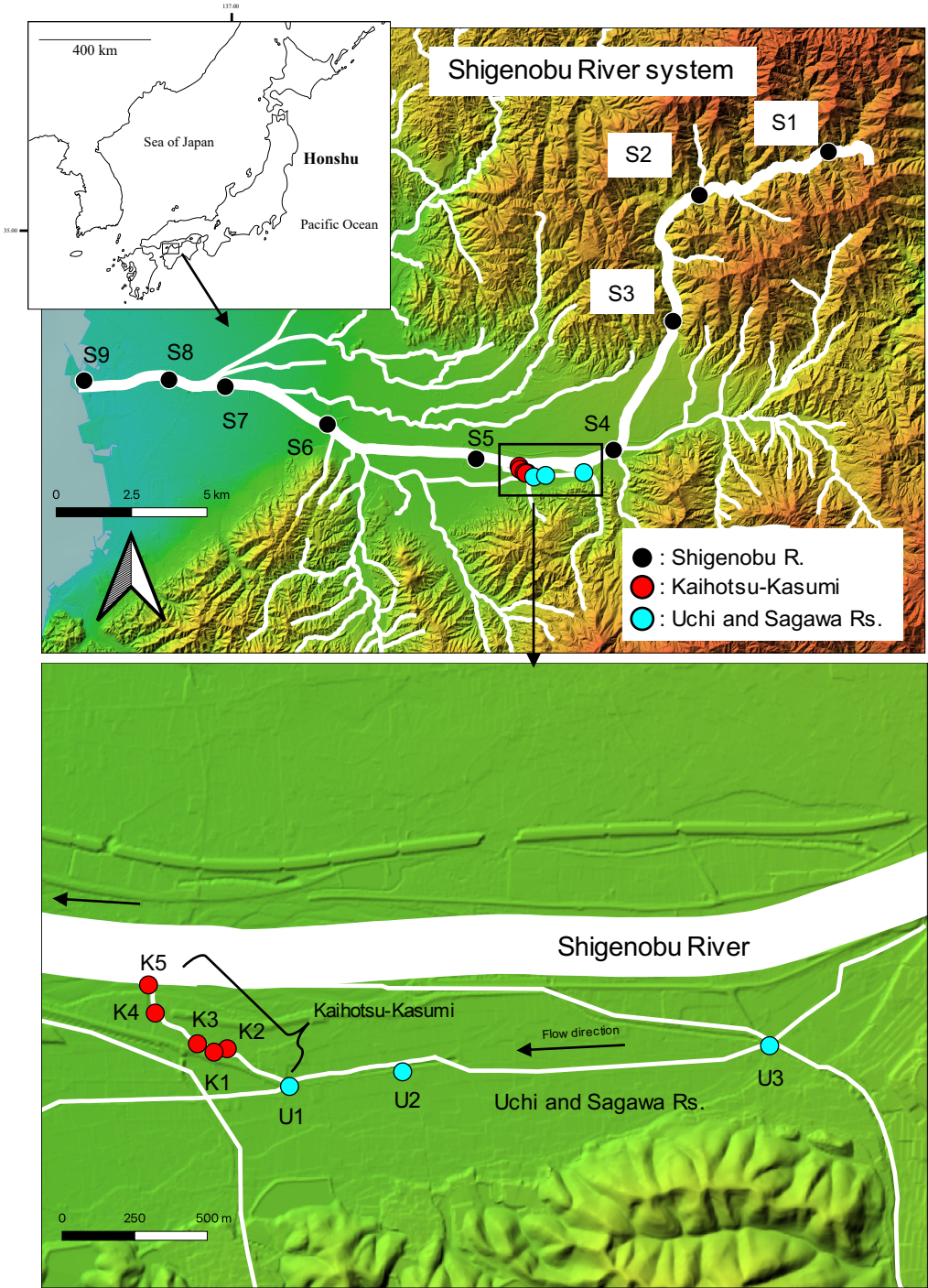
Table 2 Fish species confirmed by eDNA and capture surveys in Kaihotsu–Kasumi.

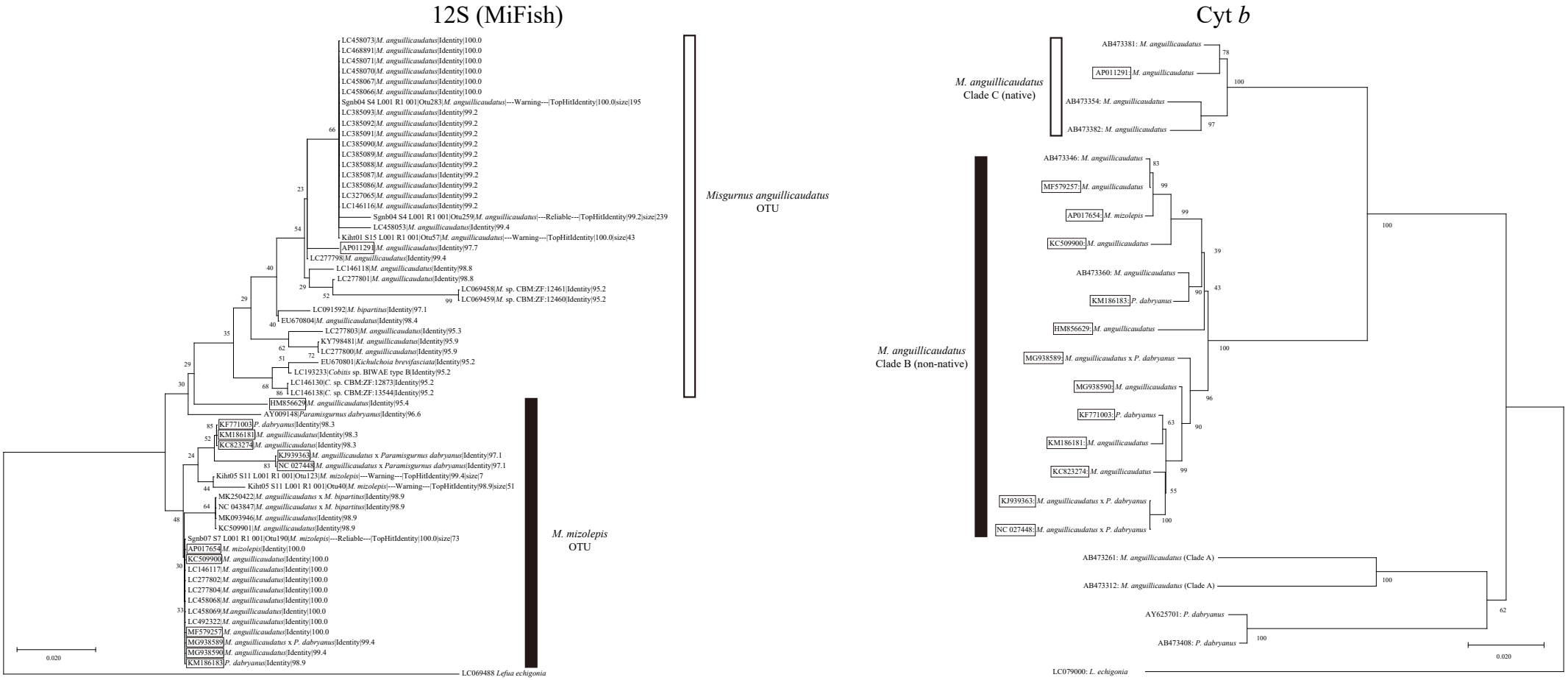
Table S1 Latitude, longitude, and elevation of each sampling site.

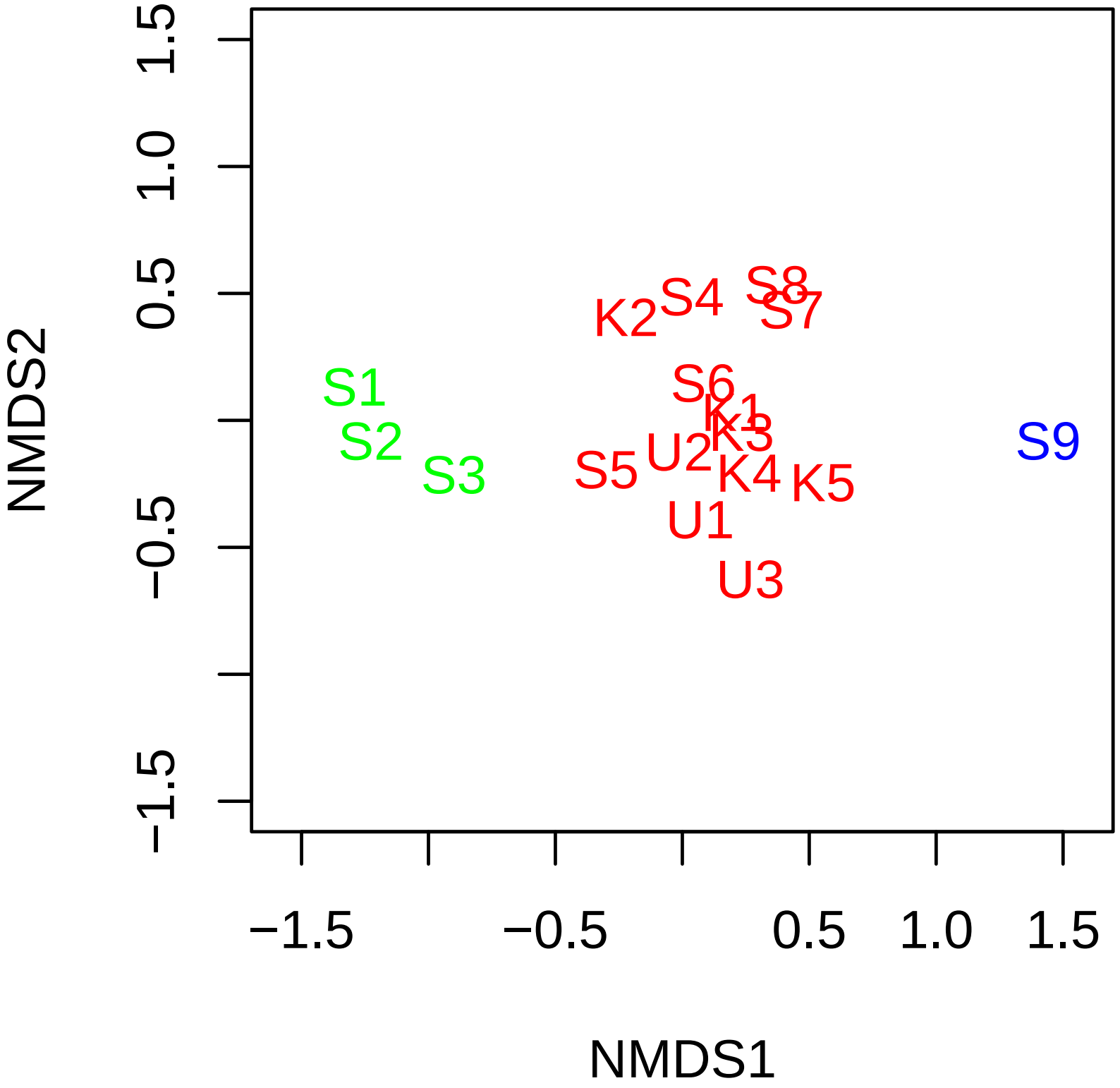
Table S2 The volume of aggregated 2nd PCR amplicons in each sample.

Table S3 Number of reads of detected species by MiFish metabarcoding.

Table S4 Target species by eDNA and capture surveys.


400 km
Sea of Japan
Honshu
Pacific Ocean
Shigenobu River system
S1
S2
S3
S4
S5
S6
S7
S8
S9
0
2.5
5 km
: Shigenobu R.
: Kaihotsu-Kasumi
: Uchi and Sagawa Rs.
Shigenobu River
K5
K4
K3
K2
K1
Kaihotsu-Kasumi
Flow direction
U1
U2
U3
Uchi and Sagawa Rs.
0
250
500 m


12S (MiFish)
LC458073|M. anguillicaudatus|Identity|100.0
LC468891|M. anguillicaudatus|Identity|100.0
LC458071|M. anguillicaudatus|Identity|100.0
LC458070|M. anguillicaudatus|Identity|100.0
LC458067|M. anguillicaudatus|Identity|100.0
LC458066|M. anguillicaudatus|Identity|100.0
Sgnb04 S4 L001 R1 001|Otu283|M. anguillicaudatus|---Warning---|TopHitIdentity|100.0|size|195
LC385093|M. anguillicaudatus|Identity|99.2
LC385092|M. anguillicaudatus|Identity|99.2
LC385091|M. anguillicaudatus|Identity|99.2
LC385090|M. anguillicaudatus|Identity|99.2
LC385089|M. anguillicaudatus|Identity|99.2
LC385088|M. anguillicaudatus|Identity|99.2
LC385087|M. anguillicaudatus|Identity|99.2
LC385086|M. anguillicaudatus|Identity|99.2
LC327065|M. anguillicaudatus|Identity|99.2
LC146116|M. anguillicaudatus|Identity|99.2
Sgnb04 S4 L001 R1 001|Otu259|M. anguillicaudatus|---Reliable---|TopHitIdentity|99.2|size|239
LC458053|M. anguillicaudatus|Identity|99.4
Kiht01 S15 L001 R1 001|Otu57|M. anguillicaudatus|---Warning---|TopHitIdentity|100.0|size|43
AP011291|M. anguillicaudatus|Identity|97.7
LC277798|M. anguillicaudatus|Identity|99.4
LC146118|M. anguillicaudatus|Identity|98.8
LC277801|M. anguillicaudatus|Identity|98.8
LC069458|M. sp. CBM:ZF:12461|Identity|95.2
LC069459|M. sp. CBM:ZF:12460|Identity|95.2
LC091592|M. bipartitus|Identity|97.1
EU670804|M. anguillicaudatus|Identity|98.4
LC277803|M. anguillicaudatus|Identity|95.3
KY798481|M. anguillicaudatus|Identity|95.9
LC277800|M. anguillicaudatus|Identity|95.9
EU670801|Kichulchoia brevifasciata|Identity|95.2
LC193233|Cobitis sp. BIWAE type B|Identity|95.2
LC146130|C. sp. CBM:ZF:12873|Identity|95.2
LC146138|C. sp. CBM:ZF:13544|Identity|95.2
HM856629|M. anguillicaudatus|Identity|95.4
AY009148|Paramisgurnus dabryanus|Identity|96.6
KF771003|P. dabryanus|Identity|98.3
KM186181|M. anguillicaudatus|Identity|98.3
KC823274|M. anguillicaudatus|Identity|98.3
KJ939363|M. anguillicaudatus x Paramisgurnus dabryanus|Identity|97.1
NC 027448|M. anguillicaudatus x Paramisgurnus dabryanus|Identity|97.1
Kiht05 S11 L001 R1 001|Otu123|M. mizolepis|---Warning---|TopHitIdentity|99.4|size|7
Kiht05 S11 L001 R1 001|Otu40|M. mizolepis|---Warning---|TopHitIdentity|98.9|size|51
MK250422|M. anguillicaudatus x M. bipartitus|Identity|98.9
NC 043847|M. anguillicaudatus x M. bipartitus|Identity|98.9
MK093946|M. anguillicaudatus|Identity|98.9
KC509901|M. anguillicaudatus|Identity|98.9
Sgnb07 S7 L001 R1 001|Otu190|M. mizolepis|---Reliable---|TopHitIdentity|100.0|size|73
AP017654|M. mizolepis|Identity|100.0
KC509900|M. anguillicaudatus|Identity|100.0
LC146117|M. anguillicaudatus|Identity|100.0
LC277802|M. anguillicaudatus|Identity|100.0
LC277804|M. anguillicaudatus|Identity|100.0
LC458068|M. anguillicaudatus|Identity|100.0
LC458069|M.anguillicaudatus|Identity|100.0
LC492322|M. anguillicaudatus|Identity|100.0
MF579257|M. anguillicaudatus|Identity|100.0
MG938589|M. anguillicaudatus x P. dabryanus|Identity|99.4
MG938590|M. anguillicaudatus|Identity|99.4
KM186183|P. dabryanus|Identity|98.9
LC069488 Lefua echigonia
0.020
Misgurnus anguillicaudatus
OTU
M. mizolepis
OTU
Cyt b
M. anguillicaudatus
Clade C (native)
AB473381: M. anguillicaudatus
AP011291: M. anguillicaudatus
AB473354: M. anguillicaudatus
AB473382: M. anguillicaudatus
M. anguillicaudatus
Clade B (non-native)
AB473346: M. anguillicaudatus
MF579257: M. anguillicaudatus
AP017654: M. mizolepis
KC509900: M. anguillicaudatus
AB473360: M. anguillicaudatus
KM186183: P. dabryanus
HM856629: M. anguillicaudatus
MG938589: M. anguillicaudatus x P. dabryanus
MG938590: M. anguillicaudatus
KF771003: P. dabryanus
KM186181: M. anguillicaudatus
KC823274: M. anguillicaudatus
KJ939363: M. anguillicaudatus x P. dabryanus
NC 027448: M. anguillicaudatus x P. dabryanus
AB473261: M. anguillicaudatus (Clade A)
AB473312: M. anguillicaudatus (Clade A)
AY625701: P. dabryanus
AB473408: P. dabryanus
LC079000: L. echigonia
0.020


NMDS2
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−1.5
−1.5
−0.5
0.5
1.0
1.5
S1
S2
S3
S4
S5
S6
S7
S8
S9
K1
K2
K3
K4
K5
U1
U2
U3

Table 1 Fish species in each cluster

		Cluster 1	Cluster 2			Cluster 3
Family	Species	S1 − 3	S4−8	U1−3	K1−5	S9
Anguillidae	*Anguilla japonica*		+		+	+
Cyprinidae	*Cyprinus carpio**		+	+	+	+
	Carassius spp.		+		+	
	*Tanakia limbata**				+	
	Opsariichthys platypus	+	+	+	+	+
	Candidia temminckii	+	+	+	+	+
	Phoxinus oxycephalus jouyi	+	+	+	+	
	Pseudorasbora parva		+			
	Gnathopogon elongatus elongatus		+		+	
	Pseudogobio esocinus		+	+	+	+
Cobitidae	*Misgurnus anguillicaudatus* (native)		+		+	
	Misgurnus anguillicaudatus (non-native)*		+		+	
	Cobitis shikokuensis		+			
	Cobitis sp. BIWAE type A		+		+	
Siluridae	*Silurus asotus*		+		+	
Plotosidae	*Plotosus japonicus*					+
Plecoglossidae	*Plecoglossus altivelis altivelis*		+			+
Salmonidae	*Oncorhynchus* spp.	+	+			
Mugilidae	*Mugil cephalus*					+
Adrianichthyidae	*Oryzias latipes*		+		+	
Centrarchidae	*Lepomis macrochirus**		+			
	*Micropterus salmoides salmoides**			+		
Haemulidae	*Plectorhinchus cinctus*		+			
Sparidae	*Acanthopagrus schlegelii*					+
Blenniidae	*Omobranchus punctatus*					+
Odontobutidae	*Odontobutis obscura*		+	+	+	
Gobiidae	*Luciogobius pallidus*					+
	Luciogobius spp.					+
	Acanthogobius flavimanus					+
	Tridentiger spp.		+	+	+	+
	Tridentiger trigonocephalus					+
	Rhinogobius flumineus	+	+	+	+	
	Rhinogobius similis					+
	Rhinogobius spp.		+	+	+	+
	Favonigobius gymnauchen					+
	Gymnogobius breunigii					+
	Gymnogobius scrobiculatus					+
Tetraodontidae	*Takifugu* spp.				+	+
	Total	5	23	10	19	21

* non-native species

Table 2 Fish species confirmed by eDNA and capture surveys in the Kaihotsu-Kasumi

Family	Species	eDNA	Capture
Anguillidae	*Anguilla japonica*	+	
Cyprinidae	*Cyprinus carpio**	+	+
	Carassius spp.	+	+
	*Tanakia limbata**	+	
	Opsariichthys platypus	+	+
	Candidia temminckii	+	+
	Phoxinus oxycephalus jouyi	+	+
	Pseudorasbora parva		
	Gnathopogon elongatus elongatus	+	
	Pseudogobio esocinus	+	+
Cobitidae	*Misgurnus anguillicaudatus* (native)	+	+
	Misgurnus anguillicaudatus (non-native)*	+	
	Cobitis shikokuensis		+
	Cobitis sp. BIWAE type A	+	+
Siluridae	*Silurus asotus*	+	
Plotosidae	*Plotosus japonicus*		
Plecoglossidae	*Plecoglossus altivelis altivelis*		+
Salmonidae	*Oncorhynchus* spp.		
Mugilidae	*Mugil cephalus*		
Adrianichthyida	*Oryzias latipes*	+	+
Centrarchidae	*Lepomis macrochirus**		+
	*Micropterus salmoides salmoides**		
Haemulidae	*Plectorhinchus cinctus*		
Sparidae	*Acanthopagrus schlegelii*		
Blenniidae	*Omobranchus punctatus*		
Odontobutidae	*Odontobutis obscura*	+	+
Gobiidae	*Luciogobius pallidus*		
	Luciogobius spp.		
	Acanthogobius flavimanus		
	Tridentiger spp.	+	+
	Tridentiger trigonocephalus		
	Rhinogobius flumineus	+	+
	Rhinogobius similis		
	Rhinogobius nagoyae	+	+
	Rhinogobius sp. OR		+
	Favonigobius gymnauchen		
	Gymnogobius breunigii		
	Gymnogobius scrobiculatus		
Tetraodontidae	*Takifugu* spp.	+	
	Total	19	17

* non-native species