Title

The use of environmental DNA of fishes as an efficient method of determining
habitat connectivity

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ABSTRACT

This study demonstrated the use of environmental DNA (eDNA) to determine habitat connectivity for migration of fishes between the sea and river. Environmental DNA is DNA fragments released by fishes in water, which can be used as a species-specific marker of the presence/absence of the target species. A year-round water sampling regime at 15 sites on the Yodo River, Japan, was conducted to determine whether three major man-made barriers on the river inhibited the migration of fishes using species-specific detection of DNA fragments from three target migrant species, temperate seabass, *Lateolabrax japonicus*, flathead grey mullet, *Mugil cephalus*, and ayu, *Plecoglossus altivelis altivelis*. The presence/absence of eDNA from target species was consistent with known patterns of species' seasonal migration. The detection of the DNA of temperate seabass and flathead grey mullet at sites upstream of the dam closest to the river mouth indicated successful upstream migration of these species via a fish ladder bypassing the dam. On the other hand, DNA of these two species was not detected from the upstream side of the two remaining dams, which are not equipped with fish ladders. Ayu is the only species among the three target species with a land-locked population in Lake

19 Biwa located at the headwater of Yodo River. Ayu DNA was detected at most of
20 the sites in the freshwater area during the warm months; however, in the coldest
21 month of February, eDNA was only detected in the uppermost site of Yodo River at
22 the southern tip of Lake Biwa. The eDNA we detected at this site suggests that it
23 was derived from juvenile ayu spending their winter months in the lake. These
24 results suggest that the eDNA method presented here can accurately track the
25 seasonal migration of fishes in a river, demonstrating its application as an
26 indicator of habitat connectivity for fishes in association with man-made barriers
27 in a river. The sampling of eDNA involves merely scooping a tank full of
28 water; therefore, it is a simple, rapid, and cost-effective method for long-term
29 monitoring of habitat connectivity associated with the construction of barriers in a
30 river.

31

32 *Keywords:*

33 Dam, ecological assessment, environmental DNA, fish ladder, fish migration,
34 habitat connectivity

35

36 **1. Introduction**

37 Most animals utilize multiple habitats during their lifecycles (Noss,
38 1991), and the connectivity among habitats is essential for the growth and
39 survival of the species. Known as the River Continuum Concept, river
40 ecosystems are maintained by a series of complex physical, chemical, and
41 biological interactions (Vannote et al., 1980). From an ecological point of view, the
42 transportation of nutrients from marine habitats to freshwater habitats via
43 upstream migration of fishes plays an essential role in a river ecosystem
44 (Cederholm et al., 1999). Sufficient connectivity allows animals to sustain their
45 lifecycles (Noss, 1991); however, once barriers are inserted, their life cycles and
46 the accompanying nutrient transportation can be disrupted. Therefore,
47 adequate connectivity among habitats is an important precondition for
48 maintaining diversity of fishes as well as river ecosystem functions.

49 Artificial barriers such as dams sometimes inhibit free migration of fishes
50 (Agostinho et al., 2008; Dugan et al., 2008; Katano et al., 2006). Proper and
51 sufficient environmental impact assessment before the construction
52 of barriers could reduce negative impacts on the resident fish community. While
53 the assessment of fish migration before and after barriers were constructed is
54 essential, the post-construction phase often requires relatively long-term

55 monitoring which can be difficult due to time and budget constraints. In particular,
56 monitoring is inevitable in the case of dams that are equipped with fish
57 ladders as a precondition for the construction of the dams. Therefore, the
58 adoption of quick and cost-effective methods is required to facilitate adequate
59 assessments for ecological monitoring dealing with artificial barriers in a river.

60 There are a number of methods used to examine the distribution and
61 migration of fishes, including mark and recapture, passive integrated transponder
62 (PIT) tags, acoustic telemetry, and radio telemetry (e.g., Cooke et al., 2013;
63 Cunjak et al., 2005; Pegg et al., 1997). However, the marking of a large number
64 of individuals in the mark and recapture method is labor-intensive. For the
65 application of telemetry techniques, large loss of transmitters often occurs. To
66 acquire sufficient data for statistical analysis, the large number of transmitters
67 that is needed is sometimes financially prohibitive. In recent years, techniques
68 using eDNA analysis have advanced rapidly for determining species presence
69 (Takahara et al., 2013; Thomsen et al., 2012a, b), species abundance (Pilliod et
70 al., 2013; Takahara et al., 2012), and even for estimating community
71 composition (Kelly et al., 2014; Minamoto et al., 2012) in aquatic
72 environments. The detection probability of fish using eDNA is higher than that of

73 conventional assessment methods such as direct observation and
74 capture (Dejean et al., 2012; Jerde et al., 2011; Takahara et al., 2013), and the
75 method could cut cost per sample in comparison with traditional morphological
76 identification (Jerde et al., 2011; Sigsgaard et al., 2014; Thomsen and Willerslev,
77 2014).

78 Our study is the first to determine the practicality of the eDNA method as
79 an indicator of habitat connectivity, focusing on dams on a river, and the
80 presence/absence of fish ladders. We applied the eDNA method to determine
81 habitat connectivity between the sea and a river by examining eDNA distribution
82 derived from three target species along the Yodo River and its three tributaries in
83 Japan. The three target species consisted of two marine fishes, namely
84 temperate seabass, *Lateolabrax japonicus*, and flathead grey mullet,
85 *Mugil cephalus*, and an amphidromous freshwater fish
86 ayu, *Plecoglossus altivelis altivelis*. The Yodo River has three major man-made
87 barriers on its mainstem. The objective was to determine whether the barriers
88 affected the migration of fishes between the sea and the river. We will discuss
89 the merits and possible deficiencies in the use of eDNA method in assessing
90 habitat connectivity in lotic systems.

91

92 **2. Materials and Methods**

93 2.1. Field survey

94 The current study was conducted along the mainstem of the YodoRiver,
95 draining ca. 8,240 km² of catchment area in the Kansai region, western Japan.

96 The Yodo River water system supplies water resources to a large resident
97 population of ca. 14 million people. The YodoRiver flows out from Lake Biwa and
98 connects to Osaka Bay downstream. We set 12 water sampling sites along the
99 mainstem of the YodoRiver (from river mouth to Lake Biwa; S01 to S12) and the
100 remaining three sites (ST01 to ST03) along three major tributaries (Fig. 1). The 12
101 sites along the mainstem were set 6–10 km apart. ST01 is located on a tributary,
102 OhkawaRiver, ca. 1 km downstream from a branching point from the YodoRiver.
103 ST02 and ST03 are located along the other two tributaries, Katsura and
104 Kizuri rivers, ca. 0.5 km and 0.8 km upstream from their branching points in the
105 YodoRiver, respectively.

106 We conducted water sampling at the 15 sites in one day and repeated it
107 monthly from July 2012 to June 2013. During each sampling, water collection
108 was finished within 8–9 h. We collected one full tank (2 L) of river water at each

109 site using a plastic bucket and a funnel. Concurrently, water temperature (°C), pH,
110 and electrical conductivity (EC; mS/cm) were measured on site with water quality
111 sensors (AD-5612A, A&D, Tokyo, Japan; pHep5 and DiST6, HANNA, Chiba,
112 Japan, respectively). All sampling equipment including the plastic bucket, funnel,
113 and plastic tanks were flushed thoroughly twice with river water at each site by
114 following the same protocol in the previous studies (Fukumoto *et al.* 2015;
115 Takahara *et al.* 2012; Takahara *et al.* 2013) to reduce the risk of
116 contamination. We transferred the samples in a cooler box to the Research
117 Institute for Humanity and Nature, Kyoto, Japan. The water tank used for water
118 collection was doubly sealed (i.e. with an inner cap and an outer cap), and was
119 locked tightly while in the cooler box. In all sampling, no breakage of the
120 180 water tanks or their caps was observed on arrival at the research institute.

121 We conducted further molecular analyses at the Research Institute for
122 Humanity and Nature. First, water samples were filtered using GF/F glass fiber
123 filter (nominal pore size = 0.7 µm; diameter = 47 mm; GE Healthcare Japan
124 Corporation, Tokyo, Japan). To prevent clogging, a single 2-L sample was divided
125 into three equal volumes (ca. 667 mL each), which were filtered separately. One
126 negative control sample was taken by filtering 2 L of reverse osmosis

127 ultrafiltered water to assess the occurrence of undesirable cross contamination
128 during the filtering and the DNA extraction processes. Filter funnels were flushed
129 every time with a large amount of tap water and rinsed with reverse osmosis
130 ultrafiltered water between different samples to prevent cross contamination
131 (Fukumoto *et al.* 2015; Takahara *et al.* 2012; Takahara *et al.* 2013). Filtered
132 samples were stored in a 50 mL conical tube (i.e., with the three glass filters from
133 a single sample places in one tube) and the frozen at -20°C before the
134 subsequent DNA extraction process.

135

136 2.2. DNA extraction

137 DNA was extracted from glass fiber filters and purified using the protocol
138 described by Fukumoto *et al.* (2015). Briefly, DNA was first extracted by the
139 standard phenol-chloroform method using a three-step purification, namely
140 phenol, phenol-chloroform-isoamyl alcohol, and chloroform-isoamyl alcohol. DNA
141 was collected by ethanol precipitation using a 1/10 volume of 3 M Sodium
142 Acetate (NaAc; pH 5.2) as a salt. The DNA pellet was re-dissolved into 200 µL of
143 distilled water and then further purified using the DNeasy Blood & Tissue Kit
144 (Qiagen, Hilden, Germany) following the instructions of the manufacturer, except

145 for a modification in the final step of elution of DNA from the filter column. We
146 eluted DNA using 100 µL AE buffer, though the original manual specified 200 µL.

147

148 2.3. Target species of fishes

149 We focused on species of fish that migrate from the sea to arrive to
150 monitor the successful upstream migration of fishes. We selected two species of
151 marine fishes and a freshwater fish species. Temperate seabass, and flathead
152 grey mullet (hereafter, grey mullet) are marine fish that reproduce only in the
153 sea and exhibit seasonal upstream migration to freshwater regions. These two
154 species occasionally will enter into freshwater regions for feeding during warm
155 months (Seno 2001; Kawamura 2001). Furthermore, a freshwater fish
156 species, ayu, was added to the target species. Ayu is an anadromous fish that
157 spends its early life stages in the sea and migrates up the river for further growth
158 and reproduction (Takahashi et al. 1999). Generally, ayu require a marine
159 environment for their early development; however, there are land-locked
160 populations in Lake Biwa and its tributaries (Azuma 1973).

161

162 2.4. Real-time PCR

163 We quantified DNA fragments of the mitochondrial cytochrome *b* (*Cytb*)
164 gene of the three target species in eDNA field samples using a real-time PCR
165 system (StepOne-Plus, Life Technologies, City of Carlsbad, CA, USA). Primers
166 and probes used in the current study are listed in Table 1. Each of the primer
167 pairs were species-specific to amplify *Cytb* DNA fragments of 109 bp, 105 bp,
168 and 131 bp for temperate seabass, grey mullet, and ayu, respectively. The
169 primers and probe for temperate seabass were developed by Minamoto et al.
170 (Unpublished) and the remaining primers and probes were designed for the
171 current study. DNA sequences of the target gene of each species and their
172 closely related species potentially inhabiting the Yodo River were collected from
173 GenBank. A list of candidate primers for each target species were generated
174 using Primer Express 3.0 software (Thermo Fisher Scientific, Waltham, MA,
175 USA) and a primer set was chosen for each target species, which had at least
176 three substitutions within the five bases from the 3' end of forward and reverse
177 primers, between the target species and the other related species. We
178 conducted the polymerase chain reaction (PCR) with four replicates for each
179 eDNA sample. Each reaction was conducted in a total volume of 20 μ L, which
180 consisted of 900 nM of each primer, 125 nM TaqMan probe in 1 $\times$ PCR master

181 mix (TaqMan gene expression master mix; Life Technologies, Carlsbad, CA,
182 USA), and 1 μ L of the DNA solution of samples. The PCR thermal cycle was as
183 follows: 2 min at 50°C, 10 min at 95°C, and 55 cycles of 15 s at 95°C and 60 s at
184 60°C. Three PCR replicates of positive and four of negative controls were
185 included in each of the PCR experiments to confirm successful amplification of
186 DNA and absence of contamination during the PCR step. Positive controls
187 contained 0.1 ng of total DNA derived from the skeletal muscle of each target
188 species. We confirmed the presence of the DNA fragments of the target species
189 in each of the eDNA samples when at least one of four PCR replicates was
190 judged as positive. We operated the PCR system according to the comparative
191 C_t method using the StepOnePlus™ Software v2.3 (Life Technologies), which
192 automatically determines the optimal baseline and threshold. While we sampled
193 eDNA every month, only samples from every second month were analyzed to
194 determine the yearly pattern in the distribution of the target species.

195 Species-specific amplification of three primer pairs was confirmed by *in*
196 *silico* PCR using Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>)
197 with default settings. The result of the *in silico* PCR showed successful
198 amplification of DNA of the three target species and in addition showed no

199 amplification of DNA of closely related species potentially living in the study area.
200 Moreover, we determined the sequence of DNA fragments amplified by real-time
201 PCR to confirm the specificity of three primer pairs using six samples for each of
202 the three species, which had tested positive, from samples obtained during August
203 2012. The DNA sequencing was conducted commercially (TAKARA Bio, Tokyo,
204 Japan). All tested samples were confirmed as a derivation of each target species
205 (see Results).

206

207 **3. Results**

208 We confirmed species-specific amplification of each of the primer sets
209 for temperate seabass, grey mullet, and ayu, by sequencing PCR amplicons
210 from six representative samples, respectively, which were tested positive
211 by real-time PCR obtained during August 2012 (S02, 03, 06, 07, ST01, and 02 for
212 seabass; S02, 03, 06, ST01, 02, and 03 for mullet; S03, 07, 10, ST01, 02, and
213 03 for ayu). All of the obtained sequences were confirmed as sequences of each
214 target species on BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

215 Water quality variables measured during the year-round survey are
216 summarized in Table 2. Based on the measured EC values, the three sites at the

217 most downstream reaches of the Yodo River (S01–S03) were estuarine,
218 subjected to the influence of tidal activity.

219 In August 2012, eDNA derived from temperate seabass was detected at
220 many freshwater sites: seven sites out of 15, including ST02 located in the
221 Katsura River tributary ca. 36 km from the sea. DNA of this species was also
222 detected at freshwater sites in October 2012 (Fig. 2a). In December 2012, the
223 water temperature dropped to an average 6.4°C among all sites, and the number
224 of PCR positive sites in the freshwater area rapidly decreased to only two sites.
225 However, positive PCR results were obtained in the three estuarine sites
226 (Fig. 2a). Water temperature was at its lowest point in February 2013,
227 averaging 6.2°C across all sites. In the coldest month, temperate seabass DNA
228 was detected at only one site located in the most downstream site (S01, Fig. 2a).
229 In April 2013, temperate seabass DNA was detected mainly at downstream sites
230 (S01, 02, 03, and ST01), and in addition, in a freshwater site in S08 (Fig. 2a). In
231 samples collected in June 2013, PCR tested positive at six sites consisting of four
232 downstream sites, that were tested positive in April, and two freshwater sites (S06,
233 ST02). Site S06 was confirmed to be a site that also tested positive during August
234 of the previous year (Fig. 2a).

235 The overall year-round result for the presence/absence of grey mullet
236 was nearly identical with that for temperate seabass, where DNA was detected in
237 the freshwater regions during the warm season. DNA of grey mullet was detected
238 at five freshwater sites over a wide spatial range from the downstream region to
239 the upstream freshwater regions in August 2012 (Fig. 2b). In October 2012, water
240 temperature remained relatively warm (an average of 17.6°C among all
241 sites), and the number of sites that tested positive increased by one in
242 comparison with the result obtained during August (Fig. 2b). In December 2012,
243 the number of positive sites rapidly decreased to only two in association with the
244 seasonal drop in temperature (an average of 6.4°C among all sites;
245 Fig. 2b). Temperature remained low during February 2013 (an average of 6.2°C
246 among all sites) and a positive result was obtained at only one site in the
247 estuarine area (S01; Fig. 2b). During the course of water temperature rise after
248 February, the number of positive sites was increased during April and June 2013
249 (Fig. 2b). These sites included a wide range of freshwater sites.

250 Ayu DNA was detected at nearly all of the sites in August and October
251 2012 (Fig. 2c). Ayu DNA was still detected at many sites in December
252 2012; however, the number of positive sites was half of that detected in October

253 2012 (Fig. 2c). In February 2013, the coldest month of the year, there was only
254 one positive result detected at the uppermost site situated at the southern tip of
255 Lake Biwa (ST12: Fig. 2c). In April 2013, ayu DNA was detected over a wide
256 spatial range from the downstream to the upstream regions (Fig. 2c). A positive
257 result was not detected in the estuarine region during June 2013; however,
258 positive results were obtained over a wide spatial range in the freshwater region
259 (Fig. 2c).

260

261 **4. Discussion**

262 Results of the current study demonstrated that we could reveal the
263 seasonal migration of diadromous fishes between the sea and river using the
264 eDNA method. To conduct this year-round survey in the target river of ca. 70 km
265 length from the river mouth to the source of the river, Lake Biwa, only two
266 investigators were required. Each field survey consisting of water sampling at
267 15 sites was completed within 8–9 h. The eDNA-based survey provided an effort-
268 and cost-saving assessment method for determining whether artificial barriers
269 set in a river could prevent fish migration. Anthropogenic impacts on the ecology

270 of riverine species caused by these barriers could be evaluated by using eDNA of
271 target species as a biological indicator.

272 DNA derived from temperate seabass and grey mullet were detected in
273 the freshwater area during spring, summer, and autumn. Their presence in
274 freshwater regions of the river are consistent with previous studies that suggest
275 that these species migrate up the river to feed and return to the sea as the water
276 temperature drops late in the year (Fuji et al. 2010; Kawamura 2001; Secor et al.
277 1998; Seno 2001). The main interpretation of the results of the current study is
278 that the fish ladder present at the most downstream dam, which is located 12 km
279 from the sea, seemed to function adequately as a pathway for these diadromous
280 fishes (Fig. 3). The detection of their DNA at ST02 (located in the Katsura
281 River tributary) suggested their successful migration up to ca. 36 km from the sea
282 via the fish ladder. In the mainstem of the Yodo River, S08 was the most
283 upstream site where DNA of the temperate seabass and grey mullet were
284 detected, and their DNA was never detected from the upstream region of the next
285 two dams, both of which do not have associated fish ladders (Fig. 3). According to
286 a report by the Japanese Ministry of Land, Infrastructure, Transport, and
287 Tourism, there were no original natural barriers such as falls to prevent fish

288 migration between the sea and Lake Biwa before the construction of a series of
289 artificial dams on the Yodo River
290 (<http://www.kkr.mlit.go.jp/yodoto/pdf/sojoukouka/report.pdf>, in Japanese). The
291 report also noted that there were records of migration of the temperate seabass
292 and gray mullet to Lake Biwa before the construction of artificial barriers in some
293 older documents detailing biology of fishes and fisheries resources in the lake. At
294 present, habitat connectivity is conserved only between the sea and in the lower
295 portion of Yodo River facilitated by the fish ladder associated with the dam
296 leaving the remaining habitat unconnected due to additional dams without fish
297 ladders. Lake Biwa may not be indispensable for the completeness of the life
298 cycles of temperate seabass and grey mullet; however, in the case of temperate
299 seabass populations in the Ariake Sea in the Kyushu district in western Japan
300 were found to depend greatly on both the bay and the freshwater area to
301 complete their life cycle (Hibino et al., 2002, 2006; Matsumiya et al., 1982,
302 1985). Although we do not have any information regarding whether such
303 populations of temperate seabass are present currently or in the past in Osaka
304 bay, reducing the range for potential migration is not considered favorable for
305 maintaining the meta-population of temperate seabass in this area.

306 Ayu is a representative species of amphidromous fishes in the Yodo
307 River. The species migrates into the freshwater regions during spring and
308 starts feeding on benthic diatoms and cyanobacteria on the river bed (Abe et al.,
309 2007). During late autumn and early winter, they spawn in the lower reaches of
310 the river and hatched larvae drift down to the sea for feeding until they migrate
311 upstream during the following spring (Senta and Kinoshita, 1985). This seasonal
312 pattern of migration was confirmed by the results of the eDNA survey in the
313 current study. However, due to stocking activities, the eDNA results could not be
314 interpreted so simply in the case of the Yodo River system. Ayu is one of the
315 most important fishery resources in inland waters in Japan and thus, seed
316 release for stock enhancement is conducted all around Japan including within
317 the Yodo River system. The seedlings are not released to the mainstem of the
318 Yodo River but to its tributary rivers (Osaka prefectural fisheries department,
319 personal communication). Moreover, given the presence of a landlock
320 population of ayu in Lake Biwa (Azuma, 1973), we cannot rule out the possibility
321 that ayu itself or its DNA released from Lake Biwa could have affected the
322 results of the current study in the Yodo River. The eDNA method for habitat
323 connectivity assessment, therefore, should be carefully applied to a

324 species that has multiple sources of habitat in the target system. On the other
325 hand, the species that are sourced only from the sea are better suited to
326 assessment of migration up the river.

327 The existence of DNA fragments of target species in sample water does
328 not always indicate the presence of the target species in close proximity to the
329 sampling site (Deiner and Altermatt, 2014; Merkes et al., 2014). Advection and
330 diffusion of DNA in water sometimes result in false positives. This is a general
331 concern for the application of eDNA methods in lotic systems. However, in a river
332 system, the unidirectional flow gives greater confidence to the determination of
333 the upper most site of target species migration based on the detection of
334 DNA. Consequently, the eDNA method provides a powerful tool to determine
335 habitat connectivity in the river system for river dwelling migrant species that have
336 their source population only in the sea, such as temperate seabass and grey
337 mullet. Ficetola *et al.* (2014) determined the possibilities of false positives and
338 false negatives to occur in association with the ability of eDNA analysis to detect
339 species presence using numerical simulation. They simulated a variety of
340 detection probabilities (at 0.25, 0.5, and 0.75) for a target species and found that
341 by increasing the number of PCR replications per eDNA sample resulted in

342 lowering the possibility of false negatives. In the case of 4 replicates, as used in
343 the present study, a false negative can occur at a relatively high probability,
344 around 1 in 3 given a detection probability of 0.25; however, the probability of a
345 false negative will decrease to 5-6 % and <1% when detection probabilities of
346 the target species are intermediate (0.5) and high (0.75), respectively. In this
347 study, the detection probabilities of the target species in the river are unknown,
348 so we cannot estimate the possibility of false negatives associated with a
349 4-replicate in the PCR test. However, given that we have analyzed eDNA
350 samples collected at 6 separate times at each site per year, if the target species
351 were present in sufficient numbers on any single sampling date, then even a
352 4-replicate test is sufficient to detect the presence of a target species. The
353 chronological repetition of sampling would contribute to decreasing the
354 probability of false negatives for the presence of target species.

355 In the current study, we determined the habitat connectivity in the Yodo
356 River in Japan. We clarified that the fish ladder associated with the dam furthest
357 downstream facilitated free movement of the fishes, though we could not
358 quantitatively judge if the fish ladder worked well. A fish ladder is generally
359 adopted to counteract the negative impact of dams or other artificial barriers on

360 fish movement; therefore, the effectiveness of fish ladders should be monitored
361 rigorously as it depends on various factors including the spatial arrangement of
362 fish entrances, the length of the ladder, and the flow rate (Noonan et al., 2012).
363 Moreover, the swimming ability of fishes, which varies enormously among
364 species depending on body size and morphology (Fisher et al., 2003; Plaut,
365 2001; Webb, 1978), should be carefully considered. The overall ability of the fish
366 ladder as a pathway for fishes should be evaluated by collaborative work
367 between biologists and engineers.

368 Environmental DNA analyses are judged as effective means for fish
369 surveillance. In a case of invasive Asian carps, bighead
370 carp, *Hypophthalmichthys nobilis*, and silver carp, *Hypophthalmichthys molitrix*, in
371 a water system adjacent to the Great Lakes in the United States, the
372 effectiveness of eDNA analysis in terms of catch per unit effort (CPUE;
373 person-day) was estimated to be higher in comparison with electrofishing. The
374 CPUE was much higher in eDNA survey and it detected eDNA of the invasive
375 Asian carps at sites where the fish were not caught by electrofishing (Jerde et al.
376 2011). The actual presence of the carps in those sites were confirmed by
377 following intensive surveillance including a rotenone application motivated by the

378 detection of eDNA of the species (Jerdeet *al.* 2011). The higher detection ability
379 of eDNA analysis of rare species was also confirmed in a case of
380 highly-endangered European weather loach (Sigsgaard et al. 2014). Monitoring
381 effort and costs including salaries were estimated as person-hour and as stipend
382 for a Ph.D. student, respectively. In the case of a habitat where they
383 intensively surveyed, they spent 300 h of fishing, whereas only 60 h of eDNA
384 survey including lab works was needed for successful detection. They noted that
385 monitoring effort was highly variable among habitats depending on accessibility
386 to the site but the total effort for eDNA survey was lower than that of
387 conventional fishing methods such as electrofishing, fish traps and landing nets
388 (Sigsgaard et al. 2014). Moreover, the cost for eDNA survey was almost half of
389 that for conventional methods when we included investigators' salaries. In the
390 present study, we collected and analyzed 16 samples including a negative
391 control sample in each monthly survey. The cost for DNA extraction and
392 purification required \$120 USD per 16 samples and the PCR examination
393 costs \$120 USD including PCR reagents, custom made primers and a probe
394 per 16 samples for each target species. By repeating the PCR examinations for
395 each of the three target species, the cost for PCR was increased to \$360 USD

396 per month. In total, the cost for eDNA survey per month was \$480 USD, though it
397 did not include staff salaries. The time required for the analyses per month was
398 ca. 3 days which consisted of 1 day for water sampling and filtration, 1 day for
399 DNA extraction and purification, and 1 day for PCR examinations for three
400 species.

401 Conducting eDNA sampling is relatively simple and fast. Once the DNA
402 is extracted from the water, the sample could be stored almost indefinitely in a
403 freezer. To routinely collect fish specimens in a habitat of interest every year or
404 every month using traditional methods (i.e., beach seining, net casting, or electro
405 fishing, followed by intensive sorting) could not be justified without a critical
406 reason for the costly monitoring, even if it would provide a valuable data set for a
407 conservation activity in the future. The eDNA method enables long-term
408 monitoring on a regular basis with affordable effort. In the case of artificial
409 barriers in a river, the eDNA method affords easy multipoint sampling
410 and provides a cost-effective monitoring of habitat connectivity with less effort,
411 and can even be applied to determine historical connectivity using archived DNA
412 samples such as those collected before human disturbance such as the
413 construction of proposed dams. The eDNA method shows much potential as a

414 practical tool for pre- and post-construction monitoring of the effect of artificial
415 barriers in a river.

416

417 **Acknowledgements**

418 We express our gratitude to Prof. Lei, Ryukoku University, for his constructive
419 comments on the final version of the manuscript. This study was funded by
420 Water Resources Environment Center(No. 2012-02)and the Environment
421 Research and Technology Development Fund of the Ministry of the
422 Environment(No. 4RF-1302). This study was partially supported by the CREST
423 program from the Japan Science and Technology Agency and by Ryukoku
424 University Technology Fund.None of these funders played any role in study
425 design, data collection and analysis, the decision to publish, or preparation of the
426 manuscript.

427

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556

557 **Tables**

558

559 Table 1. List of primers and probes used in the current study including the set of
 560 primers and probe for temperate seabass, which are identical to the ones listed
 561 by Minamoto *et al.* (Unpublished)

562

Target species	Name	Sequence (5'→3')
Temperate seabass (<i>Lateolabrax japonicus</i>)	Lja-CytB-Forward	TCCTGTCCGCTGTTCCATATG
	Lja-CytB-Reverse	AACAGAAAGTGGAAAGCGAAAAAG
	Lja-CytB-Probe	FAM- AGGCAACACCCTGGTCCAATGAATCTG-TAMRA
Flathead grey mullet (<i>Mugilcephalus cephalus</i>)	Mce-CytB-Forward	AACAACCCCTCGGATTACC
	Mce-CytB-Reverse	GTGCGAGGGAGATGAGTGTAAG
	Mce-CytB-Probe	FAM-CAACTCAGACAAAATCCCCTTTACCCC-TAMRA
Ayu (<i>Plecoglossus altivelis altivelis</i>)	Paa-CytB-Forward	CCTAGTCTCCCTGGCTTTATTCTCT
	Paa-CytB-Reverse	GTAGAATGGCGTAGGCGAAAA
	Paa-CytB-Probe	FAM- ACTTCACGGCAGCCAACCCCC-TAMRA

563

564

565

566 Table 2. Summary of water quality data at each sampling site. Shown as ranges
 567 observed in a year-round survey (minimum–maximum).
 568

Site	Temperature (°C)	pH	Electrical conductivity (mS/cm)
S01	5.7–28.5	7.2–8.4	5.7–>20
S02	4.9–28.3	7.3–8.2	1.5–>20
S03	5.9–28.6	7.0–8.0	0.2–>20
S04	4.7–28.4	6.8–8.0	0.1–0.4
S05	6.0–29.4	7.2–8.1	0.2–0.2
S06	5.9–28.0	7.5–8.4	0.1–0.2
S07	7.0–28.7	7.2–8.0	0.1–0.2
S08	6.9–29.8	7.1–8.0	0.1–0.2
S09	6.2–29.9	7.2–8.1	0.1–0.2
S10	6.8–29.9	7.2–8.3	0.1–0.2
S11	4.8–30.1	7.3–8.4	0.1–0.2
S12	4.6–30.3	7.3–9.2	0.1–0.2
ST01	5.3–28.8	7.1–8.3	0.2–0.6
ST02	8.9–27.7	6.8–7.7	0.1–0.3
ST03	7.1–28.9	6.9–8.4	0.1–0.2

569

570

571 **Figure Legends**

572

573 **Fig. 1.**The spatial arrangement of sampling sites, located on the Yodo River
574 system in the Kansai region, Japan.

575

576 **Fig. 2.**Year-round results of eDNA detection for three target species shown on
577 aerial view maps over Osaka Bay toward Lake Biwa. The six sampling dates at
578 each sampling site is represented by a 6-portioned pie. Detection of presence in
579 the warm months of April to August is shown in red and that in the cold months of
580 October to February is shown in blue. The base terrain map was generated using
581 50 m mesh altitude data provided by the Geospatial Information Authority of
582 Japan.

583

584 **Fig. 3.**Overall result of this study interpreted from eDNA analysis showing
585 the year-round distribution of the three target species in relation to the location of
586 three dams.

Figures

Figure 1

Figure 2

Figure 3