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# Autumn dispersal and limited success of reproduction of the deepbody bitterling (*Acheilognathus longipinnis*) in terrestrialized floodplain

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**Abstract –** The terrestrialization of floodplains has become a concern to river managers and ecologists because it has degraded habitats for floodplain-dependent organisms. We examined the temporal distributions of the endangered deepbody bitterling (*Acheilognathus longipinnis*) throughout its life history, which is an autumn-spawning annual fish spending its egg and larval stages in unionid mussels and emerging in spring, to understand its population decline in the terrestrialized floodplains of the Kiso River, central Japan. We first validated our *A. longipinnis* environmental DNA (eDNA) sampling method and observed an 89.3% probability of consistency between the eDNA and the direct capture surveys of 56 floodplain waterbodies (FWBs). Subsequently, the temporal distributions with autumn dispersal (9 of 14 FWBs) were found using time-series eDNA samples collected from 14 FWBs on a floodplain with a length and width of 1.4 and 0.2 km, respectively. In the following spring, juveniles were only detected in the two FWBs connected to the river channel. Moreover, the direct capture data revealed that juveniles occurred in 52.9% (9/17) of the connected FWBs, but only in 5.1% (2/39) of the FWBs isolated from the river channel. Autumn dispersal of *A. longipinnis* would be disadvantageous for reproduction in terrestrialized floodplains with numerous isolated FWBs.

**Keywords:** Endangered species / environmental DNA / floodplain-dependent fish / larval emergence / temporal distribution

## 1 Introduction

Floodplains are biodiversity hotspots in lowland rivers (Tockner and Stanford, 2002). However, they have been terrestrialized in several regulated rivers: the floodplain surface elevation relative to the main river channel has increased and marshy environments have degraded because of anthropogenic riverbed incision and flow regulation (Marston *et al.*, 1995; Takahashi and Nakamura, 2011). Terrestrialization coincides with decreases in hydrological connectivity or inundation frequency and tree cover expansion in floodplain environments (Baptist *et al.*, 2004; Nakamura *et al.*, 2017). Therefore, terrestrialization may be associated with floodplain habitat degradation in Japan (Negishi *et al.*, 2012a, b) and other nations (Bravard *et al.*, 1997; Lewis *et al.*, 2000; Baptist *et al.*, 2004).

Deepbody bitterling (*Acheilognathus longipinnis*), also known as Itasenpara bitterling, is a temperate freshwater fish belonging to the subfamily *Acheilognathinae* of the family *Cyprinidae* and endemic to central Japan. It was formerly distributed throughout the Lake Biwa–Yodo River system and the Nobi and Toyama plains. However, its current distribution in each region is considerably limited because of habitat alteration due to intensive agricultural and urban development of floodplains. Consequently, it has been designated as an endangered species by domestic and international institutes.

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The natural habitat of *A. longipinnis* probably comprised of various waterbodies formed in the broad floodplains of lowland rivers. However, the *A. longipinnis* habitat has been reduced to inter-levee floodplain waterbodies (FWBs) and small agricultural streams (Ogawa *et al.*, 2000; Kitamura *et al.*, 2009; Nishio *et al.*, 2017a). In our study segment of the Kiso River on the Nobi Plain, *A. longipinnis* inhabits FWBs formed in the terrestrialized inter-levee floodplains.

*A. longipinnis* is an autumn-spawning annual fish. Its unique reproductive ecology involves live freshwater mussels (Unionidae) (Nishio *et al.*, 2015). *A. longipinnis* deposits its eggs in the gills of mussels. There, the eggs hatch in a few days and the larvae overwinter. Then juveniles emerge from the hosted mussels in the following spring and rapidly grow and mature until autumn (Nishio *et al.*, 2015). There have been recent advances in *in situ* studies on *A. longipinnis*, including those on its reproductive ecology, life history, and habitats (Kitamura *et al.*, 2009; Nishio *et al.*, 2012, 2015, 2017a, b). However, we have little understanding of their population decline over the last half-century, during which time floodplain terrestrialization progressed. Previous studies on the present study segment disclosed that terrestrialization has decreased the inundation (disturbance) frequency of the floodplains, and this resulted in the decline of unionid mussel populations indispensable for *A. longipinnis* reproduction (Negishi *et al.*, 2008, 2012a, b). These changes in biotic/abiotic environmental conditions would negatively affect the *A. longipinnis* populations.

Environmental DNA (eDNA) sampling is a powerful tool for aquatic organism detection (Rees *et al.*, 2014). It has typically been used to determine the distributions of rare or endangered species as it is sensitive and non-invasive (Jerde *et al.*, 2011; Sigsgaard *et al.*, 2015). It is also cost-effective for field surveys (Darling and Mahon, 2011) and supports wide and repeated distribution assessments. Hence, eDNA was deployed here to monitor the distribution of the endangered *A. longipinnis* throughout its lifetime.

We hypothesized that *A. longipinnis* disperse to FWBs all over the inter-levee floodplain during flooding; however, their dispersal is disadvantaged in terrestrialized floodplains as more FWBs are unsuitable for their inhabitation and/or reproduction. In this study, we examined the temporal distribution and success/failure of the reproduction of *A. longipinnis* with reference to the FWB environments in the terrestrialized inter-levee floodplain. These are vital pieces of ecological information for population conservation and habitat management in *A. longipinnis* residing in terrestrialized floodplains. We first established the validity of our *A. longipinnis* eDNA sampling method adapted to fit our study site. We then examined the temporal distribution of *A. longipinnis* throughout its free-swimming period using time-series eDNA samples and their relationships with FWB environments. We also examined the relationship between juvenile emergence and FWB type using distribution data derived from eDNA and direct capture.

## 2 Materials and methods

### 2.1 Study site

The study was conducted in a lowland segment 26.0–41.0 km from the mouth of the Kiso River in central Japan (Fig. 1). Here is the present distribution range of *A. longipinnis* in the Kiso River on the Nobi Plain (Fig. 1). The drainage area was 5275 $km^2$. The riverbed slope of the study segment was ~0.02%. Over the last decade (2009–2018), the ordinary and maximum annual water discharges ranged from 136 to 221 $m^3 s^{-1}$ and 2 887 to 11 054 $m^3 s^{-1}$, respectively. Relatively high flows were observed between June and July (East Asian rainy season) and between September and October (typhoon season). From November to May, the flows were relatively low and stable. Levees were constructed along both sides of the study segment. Floodplains and FWBs occurred in the inter-levee zone. There were two types of FWBs according to their connectivity to the main river channel: one was a connected type (connected FWB), which was perennially connected to the river channel, and another was an isolated type (isolated FWB), which was intermittently connected to the river channel when the water level increased.

Until the 1970s, most of the inter-levee floodplain in the Kiso River consisted of sand bars. However, terrestrialization and subsequent tree establishment have rapidly progressed since the 1980s, resulting in most of the floodplain being covered by trees (Negishi *et al.*, 2008; Nagayama *et al.*, 2015, 2017).

### 2.2 Fish and eDNA samplings for validation

There were 152 FWBs (40 connected and 112 isolated) in the study segment. Most of the FWBs were in the five sites along both sides of the riverbanks (Fig. 1). Fifty-six FWBs (17 connected and 39 isolated) with various areas and elevations were arbitrarily selected from the five sites for the validation of the eDNA sampling method. Direct capture of *A. longipinnis* and eDNA sampling (see below) were conducted in all 56 FWBs from May 28, 2018, to June 2, 2018, when juvenile *A. longipinnis* (~10 mm body length) emerged from the hosted mussels.

The eDNA sampling was conducted as follows. First, 1-L water samples were collected from the water surfaces at five different sampling points, which were set at approximately equal intervals along the shoreline according to the perimeter of each FWB. The water samples were pooled in a bucket and a 400-mL subsample was transferred to a polypropylene bottle for the eDNA assay. Benzalkonium chloride solution (0.1% w/v) was added to the bottles to preserve the eDNA (Yamanaka *et al.*, 2017). The samples were stored in a cooler box along with a 400-mL distilled water (DW) blank, taken to a laboratory, and refrigerated. The bucket used to pool the water was decontaminated with bleach after sampling. The standard water volume for an eDNA assay is in the range of 1–2 L (Rees *et al.*, 2014). The 400-mL sample volume used here facilitated field portability and turbid water sample filtration.

Direct fish capture was conducted for each of the 56 FWBs. Juvenile *A. longipinnis* emerged from mussels and aggregated on the water surface near the FWB shore (Ogawa *et al.*, 2000). An operator with a hand net and a small transparent tank waded slowly along the shore, enclosed a fish cluster by lifting the hand-net frame off the water surface, and scooped the fish and water with a transparent tank used for *A. longipinnis* identification and enumeration. Fish were released back into the FWB water immediately after the record was obtained. The preceding steps were repeated for each FWB until all shore areas (~3 m from the bank) were searched. The direct capture survey was conducted with the permission of the Ministry of

the Environment, the Agency for Cultural Affairs, and Aichi and Gifu prefectures.

### 2.3 DNA filtration, extraction, and real-time PCR

The water samples were pulled by vacuum through a 0.7-μm membrane filter (47-mm GF/F glass fiber; GE Healthcare, Little Chalfont, UK) within 3 days of sampling. The cooler blank was simultaneously filtered. A filtered 'equipment blank' (200 mL DNA-free DW) was incorporated before and after each sample filtration day. The 'cooler blank' and 'equipment blank' were negative controls and their DNA levels were measured to identify any DNA contamination from field preparation and transportation, filter equipment, or background sources. The filters were wrapped in DNA-free aluminum foil and stored at −20 °C until eDNA extraction.

DNA extraction was performed as previously described (Yamanaka *et al.*, 2016). The final volume of each extracted DNA sample was 100 μL and all samples were stored at −20 °C until the PCR assay.

The eDNA was analyzed with Real-Time TaqMan PCR in the PikoReal Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). Mitochondrial cytochrome b gene fragments (131 bp) were amplified and with a TaqMan probe and the following primers: Alon_CyB-forward: 5'-GTCAGCAGTACCCTACATAGGAGATG-3'; Alon_CyB-reverse: 5'-GCGGCGGCAACAACA-3'; and Alon_CyB-probe: 5'-FAM-TAACCCGATTTTTTGCCTTCCACTTCCTCT-TAMRA-3'. The species-specificity of the primers/probe set was confirmed via PCR using templates of closely related species (Appendix A).

Each TaqMan reaction mixture contained 900 nM of each primer (Alon_CyB-forward and reverse), 125 nM Alon_CyB-probe, 10 μL Master Mix (TaqMan Environmental Master Mix 2.0; Life Technologies, Carlsbad, CA, USA), 0.1 U uracil-DNA glycosylase (Thermo Fisher Scientific, Waltham, MA, USA), and 2 μL DNA solution. The total reaction mixture volume was equivalent to 20 μL of DNA-free water. The PCR conditions were as follows: 50 °C for 2 min; 95 °C for 10 min; 55 cycles of 95 °C for 15 s, 60 °C for 1 min, and 20 °C for 10 s. There were three replicates per water sample and three positive controls. Three no-template controls were included in each PCR run. A positive result for any replicate indicated the presence of *A. longipinnis* DNA. No amplification was detected for any negative control.

### 2.4 Temporal distribution survey by eDNA

Fourteen FWBs located on the Site 4 floodplain (1.4 km long and 0.2 km wide, Fig. 1) were selected to examine the temporal distribution of *A. longipinnis* in the terrestrialized floodplain throughout its free-swimming period (Tab. 1). These FWBs were included in the 56 above-mentioned FWBs and consisted of both FWB types from a range of areas and elevations. The water from these FWBs was sampled for the eDNA assays on May 28, July 18, August 13, September 25, and October 25, 2018. All samples were subjected to filtration, extraction, and real-time PCR as previously described. Water samples and direct capture data for May 28, 2018, were shared between this distribution assay and the validation of the sampling method. In addition, from May 2019 to early June 2019 (next spring), the emergence of juvenile *A. longipinnis* was assessed by direct capture surveys as previously described to evaluate the success/failure of reproduction in the 14 FWBs.

To examine the relationship between flooding and *A. longipinnis* distribution, the hourly water levels at the nearest gauging station of a national institution (Okoshi: 35°18'42" N, 136°44'17" E) were used (Fig. 1).

### 2.5 FWB characteristics

Surface area, perimeter, and type (connected or isolated) were evaluated for the 56 FWBs wherein eDNA and fish were sampled between May 28, 2018, to June 2, 2018. The shortest distances from the shore of the main river channel and connecting height, which is an elevation when FWBs connect to the water surface of the main river channel during flooding, were estimated for 14 of the 56 FWBs to examine their relationships to the temporal distribution (Tab. 1). For these estimations of FWB characteristics, aerial photographs captured in January 2018 and a digital elevation model based on airborne laser scanning data for December 2017 were applied to ArcMap (ArcEditor v. 10.0; Esri Co., Redlands, CA, USA). In the 14 FWBs, unionid mussels were also surveyed in 2018 and 2020 based on the methods of Negishi *et al.* (2012b) following standardized protocols for mussel sampling (Tab. 1) because mussels are indispensable for *A. longipinnis* oviposition and may be an important factor of FWB selection for reproduction.

### 2.6 Statistical analyses

The eDNA detection vs. non-detection was modeled as a function of juvenile *A. longipinnis* abundance with a binomial error distribution and logit link function using generalized linear models (GLMs). The data used in this model were obtained from the 56 FWBs in spring 2018. Models were built for the individual number per unit area and the individual number per unit FWB perimeter. Juvenile *A. longipinnis* are distributed near the FWB shore immediately after their emergence from mussels (Ogawa *et al.*, 2000). Hence, abundance per unit perimeter was considered. Model significance was evaluated via a likelihood ratio test against a null model.

A Kruskal–Wallis test was performed to compare environmental variables (area, distance, and connecting height) among the isolated FWBs wherein eDNA was detected (N = 7 on September 25 and N = 8 on October 25) or not detected (N = 5 on September 25 and N = 4 on October 25). This test assessed whether site selection of *A. longipinnis* during the spawning season was influenced by the FWB environments. The connected FWBs were not evaluated in this test as the sample size was only two.

A Fisher's exact test was performed to compare the difference in the presence/absence of juvenile *A. longipinnis* between connected (N = 17) and isolated (N = 39) FWBs during the emergence period of spring 2018.

All statistical analyses were performed in R v. 3.6.3 (R Core Team, 2020) at a 0.05 significance level.

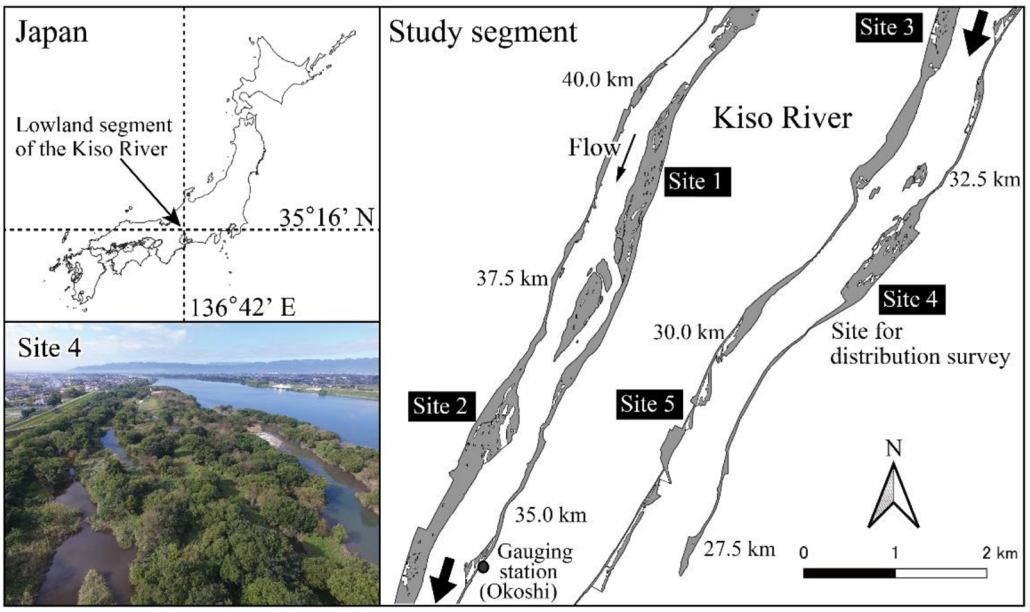


**Fig. 1.** Study segment and site locations. Distance (km) from river mouth is shown along river channel. Sites 1–5 in black boxes denote five main floodplains including most floodplain waterbodies. Site 4 was a study site for temporal distribution of *A. longipinnis*. Thin and thick arrows denote flow direction and a join in the map, respectively.

## 3 Results

### 3.1 Sampling method validation

*A. longipinnis* juveniles were captured in 11 of the 56 FWBs in spring 2018 (Fig. 2). The eDNA was detected in six of 11 FWBs where juveniles were captured and in one of 45 FWBs wherein no juvenile was captured (Fig. 2). The probability of consistency between the eDNA (detection/non-detection) and direct capture (presence/absence) data was 89.3% (50/56). The GLMs showed significant relationships between eDNA detection and juvenile abundance (Fig. 3). Logistic regression curves based on the models predicted that eDNA could be detected when juvenile *A. longipinnis* abundance was $>$ 1 per 100 $m^2$ (Fig. 3a) or $>$ 1 per 10 m perimeter (Fig. 3b).

### 3.2 Temporal distribution

No *A. longipinnis* eDNA was detected, and individuals were not captured from all 14 FWBs located on floodplain Site 4 on May 28, 2018 (Tab. 2). The eDNA was detected in only a few FWBs during the summer growing period (July 18 to August 13). During the autumn spawning period (September 25 to October 25), however, eDNA was detected in nine FWBs on each sampling day (Tab. 2). Of the eDNA-detected FWBs, seven and eight were the isolated type on September 25 and October 25, respectively.

Large floods, which connected all FWBs to the main river channel, occurred from late June to early July before eDNA sampling on July 18 (Fig. 4). They also occurred in early September and early October before eDNA sampling on September 25 and October 25, respectively (Fig. 4).

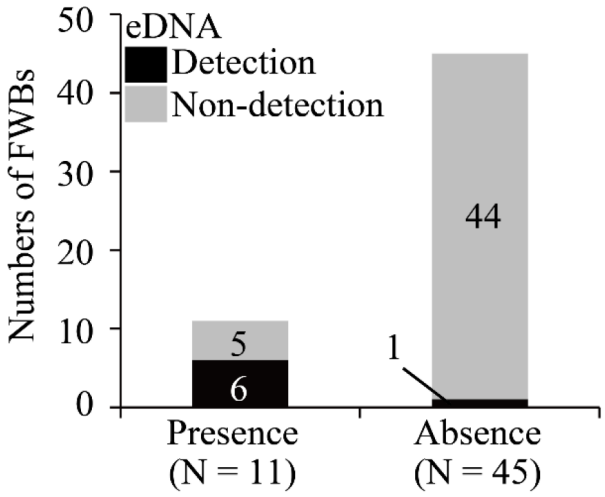


**Fig. 2.** Number of floodplain waterbodies where *A. longipinnis* individuals were captured (presence) or not captured (absence) with separation at eDNA detection and non-detection.

The Kruskal–Wallis tests disclosed that during autumn (September 25 and October 25), the area, distance, and connecting height of the isolated FWBs where eDNA was detected did not differ from those of the isolated FWBs where eDNA was not detected ($P > 0.05$ in all tests). In addition, the presence/absence of mussels were not consistent with the detection/non-detection of *A. longipinnis* eDNA, as they were present in zero of seven and one of eight eDNA-detected isolated FWBs on September 25 and October 25, respectively,

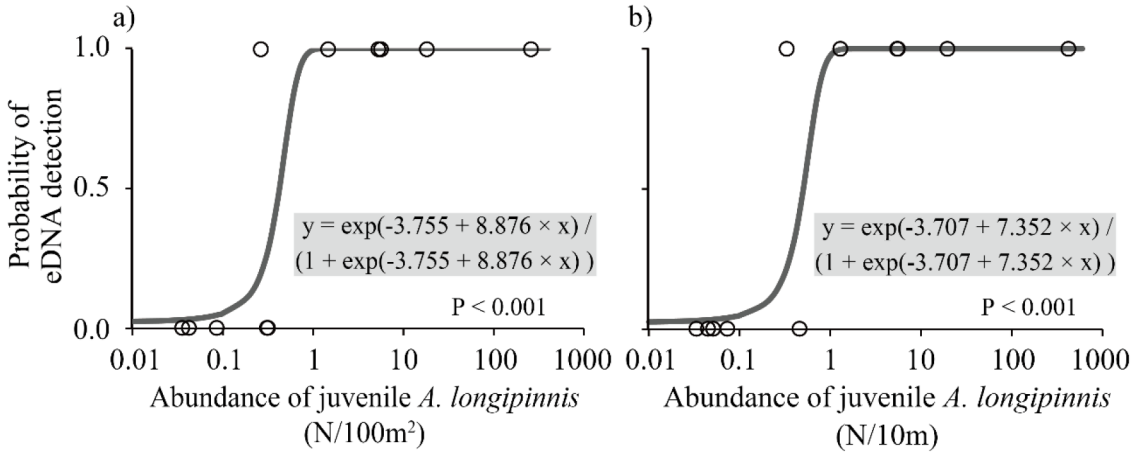


**Fig. 3.** Probability of eDNA detection for the abundance of juvenile *A. longipinnis* per unit area (a) and perimeter (b). Plots with 0 abundance are not represented, because the x-axis is displayed in logarithmic form, although 0 abundance data were used in building the models and drawing these figures. Statistically significant models are indicated by solid lines and formulae. *P*-values based on likelihood ratio test are also shown.

**Table 1.** General characteristics of 14 floodplain waterbodies (FWBs) during ordinary-flow periods.

| FWB | Type | Area ($m^2$) | Distance (m)† | Connecting height (m)‡ | Unionid mussel (CPUE)§ |
|---|---|---|---|---|---|
| 1 | Connected | 12898 | 0 | 0 | 16.9 |
| 2 | Connected | 7529 | 0 | 0 | Presence |
| 3 | Isolated | 3213 | 31 | 3.90 | 0 |
| 4 | Isolated | 2223 | 186 | 4.09 | 25.0 |
| 5 | Isolated | 1530 | 216 | 4.09 | 13.8 |
| 6 | Isolated | 468 | 97 | 4.40 | 0 |
| 7 | Isolated | 3044 | 185 | 4.59 | 1.8 |
| 8 | Isolated | 1837 | 137 | 4.59 | 0 |
| 9 | Isolated | 988 | 137 | 4.59 | 0 |
| 10 | Isolated | 659 | 244 | 4.59 | 0 |
| 11 | Isolated | 266 | 200 | 4.59 | 0 |
| 12 | Isolated | 1436 | 168 | 4.80 | 0 |
| 13 | Isolated | 744 | 223 | 4.80 | 0 |
| 14 | Isolated | 613 | 227 | 4.80 | 0 |

† Shortest distance from main river channel.
‡ Elevation (water level) connecting with main river channel based on digital elevation map derived from airborne laser scanning in December 2017.
§ Number of individual mussels caught by survey personnel in 1 h (CPUE: catch per unit effort) based on intensive survey in 2018–2020 conducted with reference to Negishi *et al*. (2012b). "Presence" was derived from our sporadic survey that did not apply the protocols of Negishi *et al*. (2012b).

and absent in two of five and two of four isolated FWBs without eDNA detection on September 25 and October 25, respectively.

### 3.3 Emergence

Between late May and early June 2019 (next spring) during the juvenile *A. longipinnis* emergence period, individuals were captured in only two connected FWBs (Tab. 2). In contrast, no individuals were caught in any of the 12 isolated FWBs regardless of eDNA detection or non-detection during the previous autumn spawning season (September 25 and October 25, 2018) (Tab. 2).

During spring 2018, nine out of 11 FWBs where juvenile *A. longipinnis* were captured were of the connected type (Fig. 5). *A. longipinnis* juveniles were found in nine out of 17 (52.9%) of the connected FWBs but in only two out of 39 (5.1%) of the isolated FWBs (Fig. 5). The difference in the presence/absence of juveniles between connected and isolated FWBs was statistically significant (Fisher's exact test $P < 0.001$).

**Table 2.** Detection (+) or non-detection (−) of *A. longipinnis* via eDNA and direct capture. There were three replicates per water sample, and the number of replicates with eDNA detection is shown in parentheses.

| FWB† | May 28 | | July 18 | August 13 | September 25 | October 25 | Next spring (May–June) |
|---|---|---|---|---|---|---|---|
| | Direct capture | eDNA | eDNA | eDNA | eDNA | eDNA | Direct capture |
| 1, c | − | − | − | − | + (3) | − | + |
| 2, c | − | − | + (1) | − | + (2) | + (3) | + |
| 3, i | − | − | − | − | + (2) | + (2) | − |
| 4, i | − | − | − | + (1) | − | − | − |
| 5, i | − | − | − | − | − | + (1) | − |
| 6, i | − | − | − | − | + (3) | − | − |
| 7, i | − | − | − | + (1) | − | − | − |
| 8, i | − | − | − | − | + (1) | + (1) | − |
| 9, i | − | − | − | − | + (1) | + (1) | − |
| 10, i | − | − | − | − | − | + (2) | − |
| 11, i | − | − | + (1) | − | + (1) | − | − |
| 12, i | − | − | − | − | + (3) | + (1) | − |
| 13, i | − | − | − | − | − | + (1) | − |
| 14, i | − | − | + (1) | − | + (2) | + (3) | − |

† c: connected FWB; i: isolated FWB.

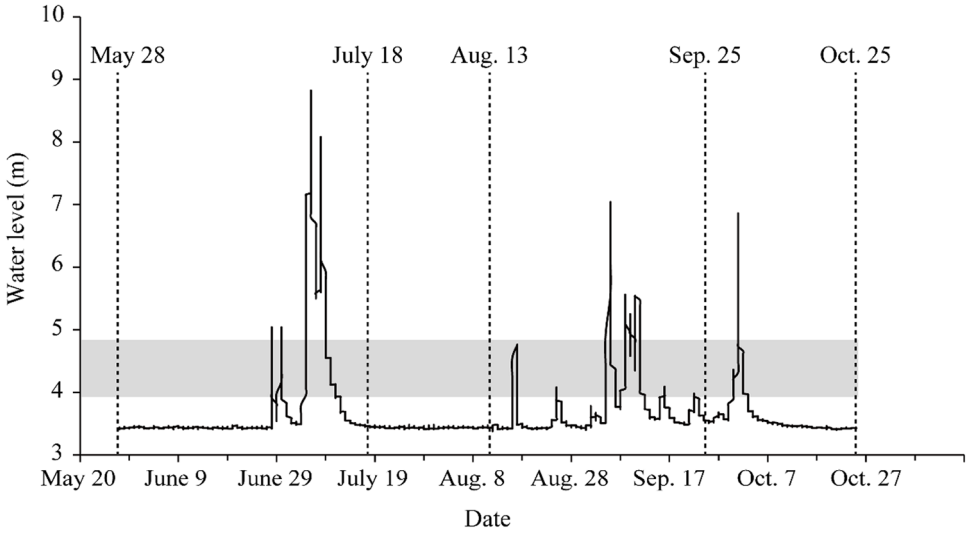


**Fig. 4.** Hourly water levels at Okoshi gauging station during survey period (May 28 to October 25) in 2018. Sampling dates for eDNA are shown. Gray belt denotes water level (elevation) range that targets FWBs connected with the main river channel. When water level gets beyond the upper belt range, all FWBs and river channel connect.

## 4 Discussion

### 4.1 eDNA sampling method validation

The eDNA sampling methods are adjusted in accordance with the ecological characteristics of the target species and its waterbody environment (Rees *et al.*, 2014; Takahara *et al.*, 2016). Here, five 1-L samples were drawn from the shorelines of each FWB and pooled before taking a 400-mL subsample for eDNA assay. This eDNA sampling method was validated by a high probability of consistency between the eDNA and direct capture and the significant regression models explaining eDNA detection probability by *A. longipinnis* abundance.

The sampling method was not very sensitive as the eDNA could not be detected in five FWBs wherein juvenile *A. longipinnis* abundance was very low (1–13 individuals, 0.04–0.34 N/100 $m^2$, 0.04–0.51 N/10 m). The insensitivity might be attributed to the water volume (400 mL) of a sample being less than the standard water volume (1-2 L) for an eDNA assay (Rees *et al.*, 2014) and the small number (3) of replicates per water sample in PCR. The probability of eDNA detection should decrease with decreasing eDNA concentration in the

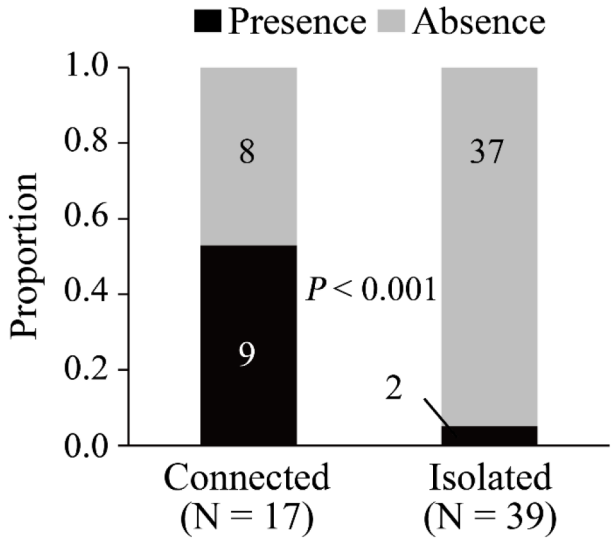


**Fig. 5.** Proportions of juvenile *A. longipinnis* presence or absence in connected and isolated FWBs. Numerical characters denote numbers of FWBs. *P*-value based on Fisher's exact test is shown in a panel.

environments (Pilliod *et al.*, 2014). The water volume and number of replicates in our method might be insufficient for detecting *A. longipinnis* in FWBs with low eDNA concentrations. However, eDNA was detected in FWBs with ≥42 individuals (0.28 N/100 $m^2$, 0.37 N/10 m), and the regression models predicted that *A. longipinnis* could almost be detected in FWBs with abundance of > 1 N/100 $m^2$ or > 1 N/10 m. Therefore, our sampling method can be used to assess the *A. longipinnis* distribution in FWBs with a certain degree of accuracy.

### 4.2 Temporal *A. longipinnis* distribution throughout its free-swimming period

No *A. longipinnis* individuals or eDNA were found in any of the 14 FWBs on May 28, 2018. However, eDNA was detected in 2–3 FWBs during the summer (July 18 and August 13) and in 9 FWBs during the autumn (September 25 and October 25). Large floods connected the FWBs to the main river channel before the summer and autumn sampling days. Thus, *A. longipinnis* could have migrated to all FWBs. Nevertheless, the *A. longipinnis* distribution was limited to a few FWBs in the summer, but spread in the autumn. These results indicate that *A. longipinnis* might move more actively during the spawning season (autumn) than during the growing season (summer). Incidentally, the changes in eDNA detection/non-detection between the summer sampling days, when no flooding occurred, might be due to the extinction of individuals moving into the FWBs or the above-mentioned insensitivity of the sampling method.

During the East Asian rainy season (June to July in central Japan) under the Asian monsoon climate system, numerous floodplain-dependent fish move to spawn preferring freshets or inundations (Saitoh *et al.*, 1988; Iwata, 2006). This might be their evolutionarily acquired habit to find preferable spawning habitats and maintain their distribution area. The typhoon season of September to October (autumn) is another rainy season in central Japan. *A. longipinnis* might adapt their spawning to the autumn rainy season and, therefore, might actively move into numerous FWBs during typhoon-fed flooding.

In autumn, there was no difference in the environmental characteristics between the isolated FWBs where eDNA was detected and not detected. This suggests that *A. longipinnis* dispersal during autumn flooding might be not greatly affected by the FWB environments. *A. longipinnis* appeared to have moved into the FWBs, regardless of whether they were suitable for reproduction or not.

### 4.3 Relationship between *A. longipinnis* emergence (reproduction) and FWB types

The eDNA analysis results showed that *A. longipinnis* spawners were distributed in the nine FWBs during each autumn sampling day. Nevertheless, juvenile emergence was found in only two connected FWBs by the following spring. These results indicate that their migration to isolated FWBs during the spawning season might not contribute to their reproduction. At our study site, potential habitats that have high inundation frequency and are suitable for unionid mussels have decreased since the 1960s because of floodplain terrestrialization with increases in floodplain height relative to the main river channel (Negishi *et al.*, 2008; Negishi *et al.*, 2012a, b). Alternatively, there was a large increase in the number of small isolated FWBs (Nagayama *et al.*, 2015, 2017) with low inundation frequency that are not conducive to unionid mussel proliferation (Negishi *et al.*, 2012a, b). This might cause the low occurrence (5.1%) of *A. longipinnis* emergence in isolated FWBs. This suggests that the inherent autumn dispersal of *A. longipinnis* might now be disadvantageous for its reproduction in terrestrialized floodplain with numerous isolated FWBs, although dispersal would be essential for population persistence.

Unlike isolated FWBs, connected FWBs relatively functioned as *A. longipinnis* reproductive sites. Connected FWBs are also conducive to unionid mussel proliferation: low benthic organic matter levels and infrequent hypoxia contributed to mussel survival and growth (Negishi *et al.*, 2012a, b, 2014; Nagayama *et al.*, 2016). Hypoxia is also a critical limiting factor for bitterling embryo survival in mussel gills (Smith *et al.*, 2000; Kitamura, 2005). In our preliminary investigation on May 11-12, 2017, low dissolved oxygen levels (0.33-2.77 mg/L) were recorded in an isolated FWB without mussels, and relatively high dissolved oxygen levels (3.35-10.68 mg/L) were recorded in a connected FWB with mussels and *A. longipinnis* emergence, although the dissolved oxygen levels were high (>7 mg/L) in both FWBs on February 6-7, 2018. Connected FWBs can provide suitable habitats for mussels and foster bitterling embryo development and emergence from mussels.

No *A. longipinnis* emergence was observed in certain isolated FWBs containing both *A. longipinnis* spawners and mussels, indicating that *A. longipinnis* reproduction might not necessarily succeed even in the presence of mussels. The reproduction failure may be related to hypoxia inhibiting bitterling embryo development (Smith *et al.*, 2000; Kitamura, 2005) because hypoxia occurs more frequently in isolated than connected FWBs (Negishi *et al.*, 2012a).

## 5 Conclusions

*A. longipinnis* may find preferable spawning habitats and maintain their distribution in pristine broad floodplains via dispersal to various types of waterbodies, such as ponds and creeks. In the inter-levee terrestrialized floodplain of our study site, however, many of the isolated FWBs did not function as *A. longipinnis* reproduction sites and might have caused abortive autumn dispersal for their reproduction. Its autumn dispersal might be detrimental under the current conditions of the study river site. This finding raises important concerns on the persistence of the *A. longipinnis* population there. Current hydrogeomorphic conditions facilitate inter-levee floodplain terrestrialization. Therefore, future research should explore ways of increasing functioning reproduction sites, thereby enhancing the reproductive efficiency of *A. longipinnis*.

Migration and dispersal during spawning are life-history strategies in various floodplain-dependent fish (Saitoh *et al.*, 1988; Tonkin *et al.*, 2008). Regardless of how river environments change, their habits would be necessary for searching preferable spawning sites and maintaining their distribution. However, they may be disadvantageous in terrestrialized floodplains because of increases in isolated waterbodies with reduced hydrological connectivity (Marston *et al.*, 1995; Tockner and Bretschko, 1996; Negishi *et al.*, 2012a, b). Low hydrological connectivity or infrequent inundation alter physicochemical environments of floodplain waterbodies and thereby affect the habitat quality (Van den Brink *et al.*, 1993; Lewis *et al.*, 2000; Negishi *et al.*, 2014). Evolutionarily acquired habits of aquatic organisms cannot adapt to the rapidly altered riverine conditions. Attention needs to be paid to detriments of their habits in terrestrialized floodplains. Moreover, restoration efforts that increase the number of connected FWBs and the hydrological connectivity of isolated FWBs are necessary to conserve floodplain-dependent fish in terrestrialized floodplains.

## Conflict of interest

Toshifumi Minamoto and Hiroki Yamanaka are inventors holding a patent for the use of benzalkonium chloride in eDNA preservation.

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### Appendix A: Checking the specificity of the real-time PCR assay

Materials and methods

We newly developed a primer/probe set for specifically amplifying *Acheilognathus longipinnis*. The mitochondrial DNA cytochrome b sequences of *A. longipinnis*. and potential sympatric *Acheilognathus* species (*A. cyanostigma, A. rhombeus*, and *A. tabira tabira*) were obtained from the National Center for Biotechnology Information (NCBI) database. Using these sequences, we designed species-specific primer/probe sets using Primer Express 3.0 (Thermo Fisher Scientific, Waltham, MA, USA). The specificity of the primer/probe set was then checked by real-time PCR using DNA of bitterling species that may inhabit the study area. In addition to the above-mentioned *Acheilognathus* species, the total DNA of *Tanakia lanceolata*, *Tanakia limbata*, and *Rhodeus ocellatus ocellatus* were used. Real-time PCR was performed using 100 pg of extracted DNA from the target and related species as templates under the same conditions as those described in the main text. All PCRs were performed with three replicates.

Results

Alon_CyB-forward: 5'- GTCAGCAGTACCCTACATAG-GAGATG-3', Alon_CyB-reverse: 5'-GCGGCGGCAA-CAACA-3', and Alon_CyB-probe: 5'-FAM-TAACCCGATTTTTTGCCTTCCACTTCCTCT-TAMRA-3' were designed as the species-specific primers and probe. All three PCR replicates using the extracted DNA of *A. longipinnis* showed successful amplification by the established assay. The averaged Ct value was 27.42. The PCRs for the other species, excluding A. rhombeus, showed no amplification. One of three replicates for A. rhombeus showed amplification; however, the Ct value was very high (51.99) and considered negligible. Therefore, the designed assay was confirmed to be specific to *A. longipinnis*.

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